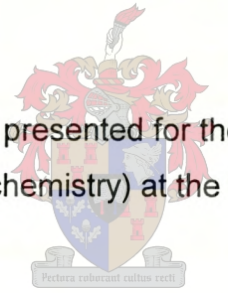
CLONING AND IDENTIFICATION OF GENES INVOLVED IN THE INTERACTION BETWEEN THE BACTERIAL STONE FRUIT PATHOGEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAIN NV AND PLUM TREES

Maryke Appel



Dissertation presented for the degree of
Doctor of Philosophy (Biochemistry) at the University of Stellenbosch

Promoter: Prof. Dirk U. Bellstedt
Co-promoter: Prof. E. Lucienne Mansvelt

Department of Biochemistry
University of Stellenbosch

March 2001

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Bacterial canker of stone fruit, caused by *Pseudomonas syringae* pv. *syringae*, is one of the most destructive crop diseases in South Africa. Chemical control has failed completely and effective long-term management strategies will have to rely on the breeding of resistant host trees. To assist in such breeding programmes, investigations into the molecular basis of the interaction between *P. s.* pv. *syringae* and stone fruit trees have been undertaken in collaboration with the ARC-Fruit, Wine and Vine Research Institute in Stellenbosch.

The aim of this dissertation was to clone and identify genes that are involved in interaction between the bacterial canker pathogen and stone fruit trees. In the first part of the study, the harpin encoding gene of a local strain of the pathogen, *P. s.* pv. *syringae* NV, was amplified in a polymerase chain reaction (PCR) strategy with primers based on the *hrpAZB* sequences of the bean pathogen, *P. s.* pv. *syringae* 61. Sequencing of this *hrpZ*$_{PssNV}$ gene revealed a high degree of homology (96%) between the harpin encoding genes and harpin proteins of the two strains. The *hrpZ*$_{PssNV}$ gene was subsequently cloned into the pMAL-c2 expression vector and expressed in *Escherichia coli*. This system was used for the production of purified, biologically active, recombinant HrpZ$_{PssNV}$ protein.

In the second part of the study, differential display (DD) technology was used to identify genes that are induced in stone fruit trees in response to *P. s.* pv. *syringae* and/or its harpin elicitor. For this purpose, actively growing shoots of two *Prunus salicina* cultivars, the moderately resistant cv. 'Laetitia' and the highly susceptible cv. 'Songold' were treated with recombinant harpin$_{PssNV}$ protein or live *P. s.* pv. *syringae* NV bacteria. An untreated control and wounding control was included in the experiment. Total RNA was isolated for comparative mRNA analysis 24 hours after treatment. DD profiles were generated with fifteen primer combinations. Eight candidate bands were re-amplified, cloned and sequenced. Reverse transcription PCR was employed to verify the expression patterns of the cloned bands in the original RNA sample set. Two bands, DDc and DD4 were shown to be differentially expressed between treatments and/or cultivars, while no differences in the expression levels of the remaining six bands (DDa, DDe, DD3, DD5, DD6 and DD7) were observed. BLAST similarity searches yielded significant matches for DDe, DD4 and DD7 with plant defense-related genes.

OPSOMMING

Bakteriese kanker van steenvrugte, wat deur *Pseudomonas syringae* pv. *syringae* veroorsaak word, is een van die mees verwoestende siektes van landbougewasse in Suid-Afrika. Chemiese beheermaatreëls het geheel en al misluk en effektiewe langtermyn beheerstrategieë sal op die teling van weerstandbiedende gasheerbome moet staatmaak. Ondersoeke na die molekulêre basis van die interaksie tussen *P. s.* pv. *syringae* en steenvrugbome is in samewerking met die LNR-Vrugte-, Wyn- en Wingerdnavorsingsinstituut in Stellenbosch van stapel gestuur om tot sulke telingsprogramme by te dra.

Die doelwit van hierdie proefskrif was om gene wat betrokke is in die interaksie tussen die bakteriese kanker patogeen en steenvrugbome te kloneer en te identifiseer. In die eerste gedeelte van die studie is die harpien-koderende geen van 'n plaaslike ras van die patogeen, *P. s.* pv. *syringae* NV, geamplifiseer in 'n polimerase kettingreaksie (PKR)-strategie met peilers wat op die *hrpAZB*-geenopeenvolgings van die boontjiepatogeen, *P. s.* pv. *syringae* 61, gebaseer is. Volgordebepaling van hierdie $hrpZ_{PssNV}$-geen het 'n hoë vlak van homologie (96%) tussen die harpien-koderende gene en harpien proteïene van die twee rasse getoon. Die $hrpZ_{PssNV}$-geen is vervolgens in die uitdrukkingsvektor pMAL-c2 gekloneer en uitgedruk in *Escherichia coli*. Hierdie sisteem is vir die produksie van suiwer, biologies-aktiewe, rekombinante $HrpZ_{PssNV}$-protein gebruik.

In die tweede gedeelte van die studie is die differensiaalvertoon (DD) tegniek gebruik om gene te identifiseer wat deur *P. s.* pv. *syringae* en/of sy harpien elisitor in steenvrugbome geïnduseer word. Vir hierdie doel is aktief-groeiende lote van twee *Prunus salicina* kultivars, die matig weerstandbiedende kv. 'Laetitia' en die hoogs vatbare kv. 'Songold', met rekombinante $harpien_{PssNV}$ proteïen of lewende *P. s.* pv. *syringae* NV bakterieë behandel. 'n Onbehandelde- en verwondingskontrole is in die eksperiment ingesluit. Totale RNA is 24 uur na behandeling vir vergelykende mRNA-analise geïsoleer. DD-profiele is met vyftien peilerkombinasies gegenereer. Agt kandidaatbande is geheramplifiseer en gekloneer, waarna hul DNA-opeenvolgings bepaal is. Trutranskriptase-PKR is gebruik om die ekspressiepatrone van die gekloneerde bande in die oorspronklike RNA monsters na te gaan. Daar is vasgestel dat twee van die bande, DDc en DD4, differensieel tussen kultivars en/of behandelings uitgedruk is, terwyl geen verskille in die ekspressievlakke van die oorblywende ses bande (DDa, DDe, DD3, DD5, DD6 en DD7) waargeneem is nie. BLAST-soektogte het betekenisvolle ooreenkomste vir DDe, DD4 en DD7 met plant weerstandsgeassosieerde gene opgelewer.

For Fritz

ACKNOWLEDGEMENTS

I express my sincere gratitude and appreciation to everyone who has contributed to this study in some way or another, and who has assisted and supported me in the course thereof. In particular, I thank:

My promoter, **Prof. Dirk U. Bellstedt**, and co-promoter, **Prof. E. Lucienne Mansvelt**

Past and present members of the Bellstedt laboratory: **Coral de Villiers**, **Erika Engelbrecht**, **Jolanda Engelbrecht**, **Annelise Blignaut**, **Johann Strauss**, **Hano Maree**, **Sonja Vorwerk** and **Dr. Heidi de Wet**

Prof. Peter M. Gresshoff and past members of the PMG laboratory at the University of Tennessee in Knoxville

Prof. Janet Hapgood and her students

Mathias Hampf, **Dr. Johan Burger**, **Anita Burger**, **Carel van Heerden**, **Dr. Thinus van der Merwe**, **Dr. Winston Hide** and **Helena Opperman**

Benno Rehder, **Welma Maart**, **George Damonse**, **Kerneels Botha** and **Anita Februarie**

Researchers, technical staff and students at the ARC-Fruit, Wine and Vine Research Institute in Stellenbosch

My mom **Marieta**, dad **André**, sisters **Lise** and **Anneri**, brother **André**, brothers-in-law **Herman** and **Heinrich**, and friends **Francois**, **Ann**, **Nadia** and **Ruan Venter**

Above all, my husband **Fritz**

Financial support from the National Research Foundation (NRF), University of Stellenbosch and Harry Crossley Foundation is gratefully acknowledged.

ABBREVIATIONS

2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
acd	accelerated cell death (mutation)
ANT	adenine nucleotide transporter
AOS	active oxygen species
ARC	Agricultural Research Council
avr, Avr	avirulence
CAD	cinnamyl-alcohol dehydrogenase
CC	coiled-coiled (motif)
CDPK	calcium-dependent protein kinase
CEL	conserved effector locus
CHI	chalcone isomerase
CHS	chalcone synthase
cv.	cultivar
DAF	DNA amplification fingerprinting
DD	differential display
ECF	extracytoplasmic function
EEL	exchangeable effector locus
EPS	extracellular polysaccharides
EREBP	ethylene-responsive element-binding protein
ERF	ethylene-responsive transcription factor
ES-MS	electrospray mass spectrometry
FLS	flagellin-sensitive (locus)
GAPDH	glyceraldehyde phosphate dehydrogenase
GST	glutathione-S-transferase
$harpin_{Ea}$	harpin produced by *Erwinia amylovora* Ea321
$harpin_{Pss}$ ($HrpZ_{Pss}$)	harpin produced by *Pseudomonas syringae* pv. *syringae*
$harpin_{Pss61}$ ($HrpZ_{Pss61}$)	harpin produced by *Pseudomonas syringae* pv. *syringae* 61
$harpin_{PssNV}$ ($HrpZ_{PssNV}$)	harpin produced by *Pseudomonas syringae* pv. *syringae* NV
hin	harpin-induced (gene)
Hop	Hrp-dependent outer protein
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HR	hypersensitive response
hrc	HR and conserved (gene)
HRGP	hydroxyproline-rich glycoprotein
hrp	hypersensitive response and pathogenicity (gene)
$hrpZ_{PssNV}$	harpin encoding gene of *Pseudomonas syringae* pv. *syringae* NV
ITS	internal transcribed spacer
IL-1R	interleukin-1 receptor
IPTG	isopropyl-β-D-thiogalactopyranoside
ISR	induced systemic resistance
JA	jasmonic acid
lacZα	α-peptide of β-galactosidase
LAR	localized acquired resistance
lls	lethal leaf spot (mutation)
lsd	lesions simulating disease (mutation)
MAPK	mitogen-activated protein kinase
MBP	maltose-binding protein
MCR	multiple cloning region
MeSA	methyl salicylic acid
nim	non-immunity (mutation)
NO	nitric oxide
npr	non-expresser of PR genes (mutation)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PARP	poly(ADP-ribose) polymerase
PCD	programmed cell death
PCR	polymerase chain reaction
PGIP	polygalacturonase-inhibiting protein
PIN	proteinase inhibitor

PR	pathogenesis-related
PT	permeability transition (pore)
pv.	pathovar
R	resistance (gene)
RAP-PCR	RNA fingerprinting by arbitrarily-primed PCR
RFLP	restriction fragment length polymorphism
RIP	ribosome-inactivating protein
ROI	reactive oxygen intermediate
RT	reverse transcription
RTase	reverse transcriptase
RT PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SOD	superoxide dismutase
S-RNase	self-incompatibility associated ribonuclease
TAL	tyrosine ammonia lyase
TIR	Toll/Interleukin-1 receptor like (domain)
T_m	melting temperature
TMV	tobacco mosaic virus
VDAC	voltage-dependent anion channel
win	wound-induced (protein)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Yop	*Yersinia* outer protein

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 HISTORICAL OVERVIEW

Bacterial canker of stone fruit is an economically important crop disease in all temperate and Mediterranean fruit-producing regions of the world (Hattingh et al. 1989). The disease has been reported in South Africa since the early 1900's, when apricot trees in the South Western Cape region were found to be the most severely affected (Heyns 1960). The disease has subsequently spread to other stone fruit crops (plum, peach, nectarine and almond) produced in this region, as well as to the sweet cherry orchards of the Eastern Free State. During the 1980's, bacterial canker was reported to be one of the most destructive crop diseases in South Africa (Roos and Hattingh 1983a, Hattingh et al. 1989). Two pathovars of the bacterium *Pseudomonas syringae*, namely pv. *syringae* and pv. *morspronorum*, as well as intermediate forms, have been identified as the causative agents of the disease in South Africa. *P. s.* pv. *morspronorum* was found to be mainly restricted to the destruction of cherry orchards in the summer rainfall region of the Eastern Free State, whereas pv. *syringae* caused widespread devastation in the winter rainfall region of the Western Cape (Roos and Hattingh 1983a, 1983b).

During the 1980's, the Department of Plant Pathology at the University of Stellenbosch (Stellenbosch, South Africa) and the Agricultural Research Council (ARC) Fruit and Fruit Technology Research Institute in Stellenbosch* conducted extensive research on the epidemiology of the disease, the life cycle of the causative pathogen and possible control measures. These investigations revealed that bacterial canker appeared to be more destructive in South Africa than elsewhere in the world. Reasons for this were obscure, but a number of predisposing factors, including climatic and soil conditions and stress-inducing horticultural practices, were implicated (Hattingh et al. 1989).

In 1989, the South African stone fruit industry was estimated to forfeit US$ 10 million annually due to the destruction of trees in nurseries and orchards, and subsequent production losses. By that time, chemical control of bacterial canker had failed completely

* Formerly known as Infruitec, now the ARC-Fruit, Wine and Vine Research Institute.

in this country and it was suggested that long-term disease management strategies could only be effective if a strong emphasis was placed on the breeding of resistant host trees. A thorough knowledge of the genetics of the pathogen was concomitantly identified as a future research priority (Hattingh et al. 1989).

The Department of Biochemistry at the University of Stellenbosch was approached by Infruitec in 1994 to collaborate on investigations of the bacterial canker pathogen and its interactions with stone fruit trees, in order to provide a molecular basis for future resistance breeding programmes. Such investigations have subsequently been performed by the author of this dissertation, under the supervision of Prof. Dirk U. Bellstedt of the Department of Biochemistry, and in collaboration with Prof. E. Lucienne Mansvelt of Infruitec. Research to date has been conducted in two phases. A preliminary study was completed at the end of 1995 and comprised the M. Sc. thesis of the author (Appel 1996). The second phase, which was completed during the period 1996-2000, will be presented in this Ph. D. dissertation.

1.2 MOLECULAR CONSIDERATIONS IN THE STUDY OF BACTERIAL HOST-PATHOGEN INTERACTIONS

1.2.1 In general

Plants evolved in an environment already colonized by bacteria, yet very few bacterial species have evolved the ability to cause disease in plants (Bonas 1994). Those that have, belong to only six major taxonomic groups, namely the Gram-negative genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Ralstonia* (previously part of the genus *Pseudomonas*; Yabuuchi et al. 1992, 1995) and *Agrobacterium,* and the Gram-positive "coryneform bacteria" (*Arthrobacter*, *Clavibacter*, *Curtobacterium*, *Nocardia* and *Rhodococcus*) (Sigee 1993). Each group has evolved specialized gene systems that determine pathogenicity, host-range and virulence and consequently, the nature of the disease caused (Sigee 1993, Collmer 1998). The products of these genes interact directly or indirectly with the products of a vast array of constitutive and inducible plant genes that determine whether contact between a phytopathogenic bacterium and a plant leads to compatibility (disease) or incompatibility (resistance) (Heath 2000). To provide the necessary background for the experimental strategies described and results presented in this dissertation, the salient features of the gene systems involved in bacterial host-

pathogen interactions, as well as the interplay of their products, are reviewed in **Chapter 2**.

1.2.2 In the interaction between *P. syringae* and its hosts

P. syringae is generally regarded as a weak, but opportunistic plant pathogen with a complex life cycle that is characterized by an epiphytic phase on non-hosts or symptomless host plants (Hattingh et al. 1989, Roos et al. 1993). Studies involving this pathogen are complicated by (i) a poor understanding of the factors controlling transitions between the epiphytic and pathogenic phases, and (ii) the huge genotypic and phenotypic heterogeneity with regard to host-range (Schroth et al. 1992), biochemical characteristics (Hirano and Upper 1990), and the production of virulence factors (Hirano and Upper 1990) that exists within the fifty odd recognized pathovars of *P. syringae*.

The application of molecular techniques in the study of plant-pathogen interactions has led to the discovery that the fundamental ability of *P. syringae* and other Gram-negative phytopathogenic bacteria (*Pseudomonas, Xanthomonas*, *Erwinia* and *Ralstonia* species) to cause disease in host plants and the so-called hypersensitive response (HR; Klement 1963, Klement et al. 1964) in non-hosts or resistant hosts, is determined by homologous gene clusters called *hrp* genes (Lindgren et al. 1986, Lindgren 1997). *Hrp* gene clusters are structurally and functionally conserved and encode three groups of proteins: components of a type III protein secretion system, regulatory proteins, and secreted proteins (He 1996, Collmer 1998)*.

Harpin, the first secreted Hrp protein to be identified, was chosen as the focus of our molecular investigations into the genetics of *P. s.* pv. *syringae* and its interactions with stone fruit trees, for the following reasons:

a. At the time when this project was initiated, *hrp* genes and avirulence (a*vr*) genes* were the foci of molecular research into the interactions between Gram-negative phytopathogenic bacteria and plants. *Hrp* genes were shown to be a fundamental determinant of pathogenicity in all of the Gram-negative bacteria studied, and harpins were proposed to be the only *hrp*-encoded factors required by these bacteria to interact with plants (Wei et al. 1992, He et al. 1993). *Avr* genes on the other hand,

---

* Discussed in detail in Chapter 2.

were not considered to be essential for pathogenicity (Sigee 1993). Instead, they were regarded as determinants of host-range and, in some cases, as virulence factors (Dangl 1994).

b. When infiltrated in its purified form, the harpin isolated from the model organism *P. s.* pv. *syringae* strain 61 ($harpin_{Pss}$) was demonstrated to be an elicitor of the HR (a localized defense response; He et al. 1993), as well as a general inducer of systemic acquired resistance (SAR) in plants (Strobel et al. 1996). As such, it promised to be a valuable tool in the assessment of plant responses to *P. s.* pv. *syringae* in the second phase of this study.

1.3 OBJECTIVES AND OUTPUTS OF RESEARCH PRESENTED IN THIS DISSERTATION

The long-term objective of our collaborative research with Infruitec was to clone the harpin encoding gene of a local *Pseudomonas syringae* pv. *syringae* strain* for the production of recombinant harpin that may be used in molecular investigations into the interaction between the bacterial canker pathogen and stone fruit hosts. During the preliminary study, *P. s.* pv. *syringae* strain NV was shown to produce a protein similar in size, biochemical properties and antigenic characteristics to $harpin_{Pss}$. The *P. s.* pv. *syringae* NV harpin could, however, not be purified successfully from its native bacterium using classical protein purification techniques (Appel 1996).

The study presented in this dissertation therefore had the following objectives:

a. To amplify, clone and sequence the harpin encoding gene ($hrpZ_{PssNV}$) of the nectarine fruit pathogen.

b. To determine the extent of homology between this gene and its protein product ($HrpZ_{PssNV}$ or $harpin_{PssNV}$) with known nucleotide and amino acid sequences of other *P. syringae* harpin encoding genes and their proteins.

c. To establish the $hrpZ_{PssNV}$ gene in a protein expression system that would enable the production of biologically active, recombinant harpin.

---

* The experimental strain used in this study, *P. s.* pv. *syringae* strain NV, was isolated by Prof. E. Lucienne Mansvelt of Infruitec in 1983 from the fruit of a nectarine tree. It is one of the most virulent strains of the pathogen isolated in South Africa, and also the first local strain to be isolated from the **fruit** of a diseased tree.

d. To identify genes that are induced in commercial stone fruit cultivars* in response to challenge with recombinant harpin$_{PssNV}$ protein and/or the bacterium from which it was derived, and may be involved in resistance against the pathogen.

The first three of these objectives were met by the end of 1997. A paper entitled "Amplification, sequence and expression of the harpin encoding gene of the bacterial canker pathogen, *Pseudomonas syringae* pv. *syringae* strain NV", was subsequently published in the *Journal of Plant Physiology* (Appel, M., Hampf, M., Mansvelt, L., Hapgood, J. and Bellstedt, D. (1999). *J. Plant Physiol.* **154**: 489-497). The paper describes (i) the amplification and sequencing of the *hrpZ*$_{PssNV}$ gene, (ii) comparison of the *hrpZ*$_{PssNV}$ nucleotide and deduced amino acid sequences with that of other *P. syringae* *hrpZ* genes and HrpZ proteins, (iii) cloning and expression of the *hrpZ*$_{PssNV}$ gene in the pMAL-c2 expression system and (iv) the purification of biologically active, recombinant harpin$_{PssNV}$. **Chapter 3** is dedicated to these aspects of the study and contains the paper in its published form. The chapter is introduced with a brief overview of the experimental approach. It also includes discussions of certain aspects of the experimental design, as well as a description of the contributions of the co-authors of the paper.

To meet the final objective of this study, a technique for the analysis of differential gene expression patterns in plants had to be employed. The differential display (DD) technique (Liang and Pardee 1992) was identified as the most suitable for these assessments. Although this powerful technique was first described in 1992, it was still poorly established in South Africa by 1997. A generous award to the author from the former South African Foundation for Research Development (currently the National Research Foundation) in the form of the Sir Aaron Klug Scholarship for Molecular Biology, secured an opportunity for her to visit the Plant Molecular Biology laboratory of Prof. Peter M. Gresshoff† at the University of Tennessee (Knoxville, Tennessee, USA) from November 1997 to May 1998 to gain expertise in DD technology.

During this period, the author researched the principles of DD, criticisms against the technique, as well as modifications of the original protocols extensively, and subsequently wrote a review paper entitled "Differential Display of eukaryotic mRNA: meeting the

---

* Two cultivars of *Prunus salicina* (the Japanese plum), the moderately resistant cv. 'Laetitia' the highly susceptible cv. 'Songold' were chosen for this purpose.

† Currently Head of the School of Life Sciences and Director of the Center for Molecular Plant Sciences, University of Queensland, Brisbane, Australia.

demands of the new millennium?", which was published in the *Journal of Plant Physiology* (Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1999). *J. Plant Physiol.* **154**: 561-570). This paper is included in its published form in **Chapter 4**, as a background for the experimental work presented in the following chapter. The paper is prefaced by a brief outline of the reasons why DD was considered to be the most suitable technique for the purpose of this study. A description of the contribution of each of the co-authors is also included.

DD technology was established by the author in the laboratory of Prof. Dirk Bellstedt (Department of Biochemistry, University of Stellenbosch) during the latter part of 1998 and the first part of 1999. Efforts were also made to transfer this technology to other research institutions in South Africa. This included the presentation of a lecture (Appel et al. 1998a) and a poster (Appel et al. 1998b) on DD at the joint 15th congress of the South African Society for Biochemistry and Molecular Biology and 2nd congress of the Federation of African Societies for Biochemistry and Molecular Biology (held in Potchefstroom, South Africa, from 29 September to 3 October 1998), as well as a hands-on Differential Display Workshop (presented in the above laboratory from 21 to 25 June 1999; attended by ten delegates from seven research institutions across South Africa).

During 1999 and 2000, DD and the recombinant harpin$_{PssNV}$ protein were used to identify plant genes that are involved in the interaction between *P. s.* pv. *syringae* NV and plum trees. In accordance with the format followed in preceding chapters, this work is presented in **Chapter 5** in the form of an independent manuscript, complete with an introduction, discussion and reference list. Background information on certain aspects of the experimental design, and a description of the contribution of each of the co-authors precede the manuscript. This final part of the author's Ph. D. work has not yet been published. Certain aspects thereof (the experimental strategy and preliminary results) were, however, presented as a poster (Appel et al. 2000) at the BioY2K Combined Millennium Meeting (held in Grahamstown, South Africa, from 23 to 28 January 2000).

The work presented in this dissertation comprises the first molecular investigation into the interaction between the bacterial canker pathogen, *P. s.* pv. *syringae*, and a stone fruit host. It has laid a sound foundation for continued collaborative studies of this nature between the Department of Biochemistry, University of Stellenbosch and the ARC-Fruit, Wine and Vine Research Institute. To conclude the dissertation, the achievements and

pitfalls of the project are evaluated, and suggestions for future remedies and research prospects are discussed briefly in **Chapter 6**.

CHAPTER 2

THE MOLECULAR BASIS OF INTERACTIONS BETWEEN PLANTS AND BACTERIAL PHYTOPATHOGENS

The aim of this chapter is to provide a concise theoretical background for the experimental work described in this dissertation, namely the cloning of the harpin encoding gene of *Pseudomonas syringae* pv. *syringae* NV (Chapter 3) and the identification of plant genes that are involved in the interaction between this bacterium and plum tree hosts (Chapter 5). For this purpose, the molecular basis of pathogenicity in bacterial phytopathogens and the molecular basis of resistance in plants are reviewed in sections 2.2 and 2.3, respectively. To introduce these themes, the types of interactions between plants and bacterial phytopathogens, as well as the pressures that directed the evolution of these interactions, are briefly discussed in section 2.1.

2.1 INTRODUCTION: INTERACTIONS BETWEEN PLANTS AND BACTERIAL PHYTOPATHOGENS

The interaction between a plant and a bacterial phytopathogen is characterized as either *compatible* or *incompatible*, and depends primarily on the type of bacterium and plant, rather than on other factors such as the environment, developmental or physiological features (Sigee 1993).

A compatible interaction is the result of association between a bacterial plant pathogen and a plant which is a **susceptible host** to that specific bacterium. It is characterized by a delayed host response, prolonged bacterial multiplication and spread to surrounding tissues, and eventually leads to the production of macroscopic disease symptoms. Incompatible interactions result from the association of bacterial phytopathogens with plants that are either **non-hosts** or **resistant hosts** to these bacteria. In such interactions, bacterial multiplication is limited and spread of the pathogen is prevented by a rapid induction of host resistance mechanisms. This is typically (but not always) manifested in the localized death of plant cells at the site of infection (the hypersensitive response or

HR*; reviewed in Klement 1982). The specific ability of a bacterium to cause disease or induce a resistance response in plants has a common molecular basis (see next section), and the outcome depends on whether the plant involved in a particular interaction can recognize the pathogen as "non-self" and express its genetic information for resistance quickly enough and to a sufficient magnitude to counteract the pathogen's advances (Kuc 1982, Dean and Kuc 1985).

Plants have evolved in an environment already colonized by bacteria. The ability to grow in plant tissue (and cause disease) has, however, been acquired by a relatively small number of bacterial species (discussed in section 1.2.1), and has evolved under an intense pressure for *compatibility*. This has been achieved with regard to particular plants or groups of related plants (the host-range)† through the development and adaptation of specialized gene systems which relate specifically to (the genes of) these plants (Sigee 1993). These gene systems are discussed in more detail in section 2.2.

Plant survival, on the other hand, has evolved under the pressure for *incompatibility*, and has involved the development of diverse and broad mechanisms to isolate and restrict pathogen growth, and afford protection against future attack (Paul et al. 2000). These mechanisms include several constitutive defenses and an array of inducible responses that are triggered by non-specific or pathogen-specific elicitors produced by the pathogen (Heath 2000). Section 2.3 is dedicated to the components of these resistance mechanisms and the signalling pathways that connect them.

2.2 THE MOLECULAR BASIS OF PATHOGENICITY IN BACTERIAL PHYTOPATHOGENS

2.2.1 "Pathogenicity genes"

Pathogenicity refers to the genetic predisposition of a bacterium to cause disease (Shaner et al. 1992). For most of the extracellular plant pathogens, comprised mainly of the Gram-

* The HR is not elicited by non-phytopathogens, and provides a useful test for pathogenicity (Klement et al. 1964).

† Host-ranges vary considerably between different phytopathogenic bacteria. Some pathogens are restricted to a specific plant species, whereas others have the ability to cause disease in a number of species from related or different genera (Sigee 1993).

negative necrogens* belonging to the genera *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia*, pathogenicity is based on the fundamental ability to perturb the plasma membrane of host cells (without killing the cells) to cause the leakage of water, ions and organic nutrients into the intracellular space where bacterial growth is sustained (Sigee 1993). This ability, as well as the capacity to elicit a hypersensitive response in non-hosts or resistant hosts, is determined by a specialized "hypersensitive response and pathogenicity" (*hrp*; Lindgren et al. 1986) gene system, which is physically and functionally conserved among these bacteria. *Hrp* genes encode a type III secretion system that is capable of delivering the primary effectors of pathogenesis - the protein products of a variable array of avirulence (*avr*) genes - into plant cells (Collmer 1998).

Bacterial pathogens have further evolved through the acquisition and development of *virulence factors* which promote colonization of plant tissue and lead to more pronounced disease symptoms (see Table 2-1). Virulence factors are expressed and secreted independently of *hrp* systems, and the genes that encode them are regarded as a subset of the genes required for bacterial pathogenicity (Panopoulos and Peet 1985, Daniels et al. 1988, Lindgren 1997)†.

The remainder of this section is dedicated to a current model for the molecular basis of bacterial pathogenicity, based on the Hrp-dependent delivery of *avr* gene products. To provide background for the work presented in Chapter 3, emphasis is placed on the *hrp* genes and proteins of *Pseudomonas syringae* pv. *syringae* strain 61, which served as a model for experiments with the local strain, *P. s.* pv. *syringae* NV, in this study.

---

* Bacterial phytopathogens which cause necrotic diseases and wilts are collectively referred to as **necrogens** and are mostly *biotrophic* (i.e. obtain their nutrients from living host cells). A second class of extracellular, Gram-negative phytopathogens (including *Erwinia carotovora* and *E. chysanthemi*) are the **macergens** (see Table 2-1). They cause soft-rot diseases and, although they are *necrotrophic* (feed off dead host cells), also possess *hrp* genes as a basic determinant of pathogenicity (Collmer and Bauer 1994, Alfano and Collmer 1996).

In the **oncogenic** (tumour disease-inducing) *Agrobacterium* species, pathogenicity is based on the ability to alter the nutrient level of the internal plant environment through the transformation of plant cells with bacterial DNA, and the subsequent synthesis and secretion of specific microbial nutrients (opines) (Hooykans and Beijersbergen 1994). The molecular basis of pathogenicity of the oncogens falls outside the scope of the current study and will not be discussed in this chapter.

† The complete *hrp* clusters of *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *phaseolicola*, *Erwinia amylovora* and *E. chysanthemi* have been cloned. When transferred to non-pathogenic bacteria (such as *Pseudomonas fluorescens* or *Escherichia coli*), these genes confer the ability to elicit the HR in non-host plants, but not the capacity to induce disease in host plants. This underlines the requirement of non-*hrp* "pathogenicity genes" for the pathogenic phenotype (Huang et al. 1988, Beer et al. 1991, Puri et al. 1997, Ham et al. 1998).

Table 2-1. Major virulence factors associated with bacterial plant diseases (adapted from Sigee 1993).

Virulence factors are characteristics of the pathogen which are important in the determination of disease severity (Sigee 1993), and are included under the broad term "determinants of pathogenicity" (Daniels et al. 1988). The major type of virulence factor associated with the symptoms of each of the four distinct classes of bacterial plant diseases are listed below. Although phytopathogenic bacteria that induce similar diseases produce the same *type* of major virulence factor, the genetics and biochemistry of specific factor production vary significantly between species, strains and pathovars (reviewed in Daniels et al. 1988, Sigee 1993). Phytopathogenic bacteria produce numerous other virulence factors which are not listed here. Some have been linked to pathogen fitness (e.g. attachment to plant surfaces, infection-associated differentiation, nutrient assimilation, stress tolerance, competition for resources and ice nucleation) whilst many others are as yet uncharacterized (Panopoulos and Peet 1985, Lamb et al. 1989, Beattie and Lindow 1994) .

Disease description	Symptoms	Causative bacteria	Major virulence factor	General function of virulence factor
NECROTIC	Necrotic leaf spots, stem cankers and blossom blight	*Pseudomonas*, *Xanthomonas* and some *Erwinia* species	Toxins[a]	Non-enzymatic, non host-specific chemical injury to host cells
VASCULAR	Vascular wilts and yellows	Coryneform bacteria, some *Erwinia*, *Pseudomonas* and *Xanthomonas* species	EPS[b]	Induction and maintenance of watersoaking, prevention of bacterium/ plant recognition, occlusion of plant vessels
SOFT ROT	Soft rots	*Xanthomonas*, *Pseudomonas* and *Clavibacter* species *Erwinia carotovora* and *E. chrysanthemi*	Extra-cellular enzymes[c]	Enzymatic degradation of plant cell walls and other cellular components
TUMOUR	Galls and hyperplasias	*Agrobacterium* spp., *P. syringae* pv. *savastanoi*, *E. herbicola* pv. *gypsophilae*	Plant hormones[d]	Induces uncontrolled plant cell division

[a] Best characterized in pathovars of *Pseudomonas syringae*. Specific examples of toxins produced by these bacteria are coronatine, tagetitoxin, phaseolotoxin, syringotoxin, syringomycin, tolaasin and tabtoxin (reviewed in Gross 1991).

[b] EPS = extracellular polysaccharides; forms a "capsule" around bacteria that secrete it.

[c] Mainly pectinases, cellulases, proteases, amylases and phospholipases.

[d] Auxins (mainly indoleacetic acid) and cytokinins (zeatin, zeatin riboside and other derivatives).

2.2.2 Current model for the molecular basis of pathogenicity in phytopathogenic *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia* species

A current model for bacterial plant-pathogen interaction and co-evolution based on the Hrp delivery of Avr proteins into plant cells is outlined in Fig. 2-1. This model proposes that:

a. The possession of a conserved Hrp type III protein secretion pathway and a variable collection of genes encoding Avr (effector) proteins is a fundamental characteristic of phytopathogenic *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia* species.

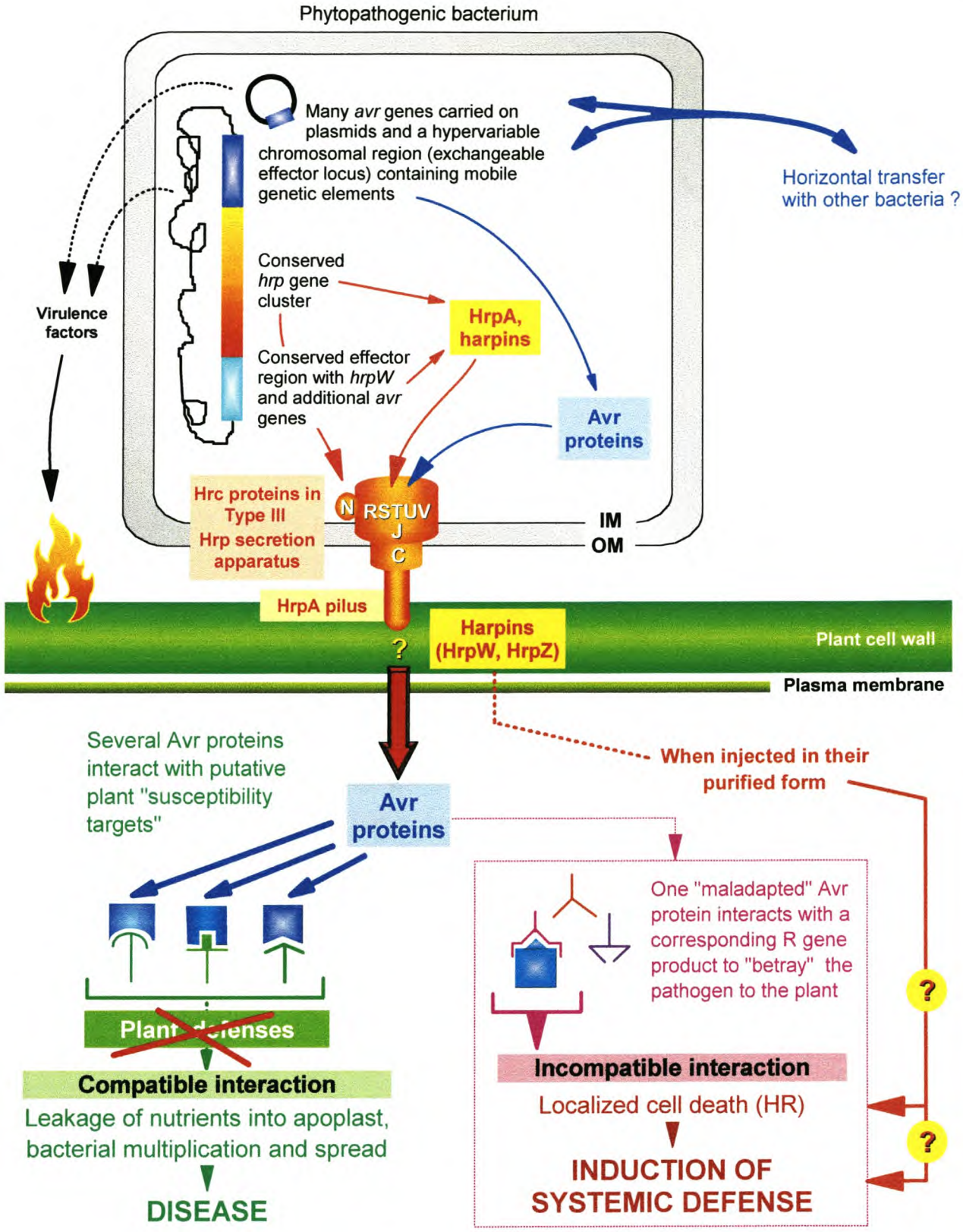


Fig. 2-1. Current model for the molecular basis of pathogenicity in bacterial phytopathogens, depicted in a typical *Pseudomonas syringae* pathovar (adapted from Alfano and Collmer 1996, Collmer 1998, Alfano et al. 2000).

Abbreviations: IM = inner membrane, OM = outer membrane. Caption continues on next page.

b. Conserved Hrp type III secretion systems are capable of delivering many, diverse Avr proteins into plant cells in a manner that is dependent upon contact between the pathogen and plant cell.

c. The protein products of *avr* genes are the primary effectors of pathogenesis. As such they should have two general functions: (i) to defeat host defenses, and (ii) to locally modify the apoplast for bacterial colonization through nutrient release, water-soaking and pH increase.

d. Genetic changes in host plant populations that reduce the pathogenic benefit of an effector protein, or allow its recognition by the plant resistance (*R*) gene surveillance system, will lead to a proliferation of complex arsenals of *avr* genes in co-evolving bacteria. Such pools of diverse and interchangeable genes, whose products may either promote or betray pathogens, may account for the enormous diversity in

---

Fig. 2-1. Continued.

According to this model, the ability of the pathogen to cause disease in host plants is based on the injection of avirulence (Avr) proteins into plant cells via the Hrp type III secretion system. Broadly conserved Hrc proteins that form the core of this type III secretion apparatus are indicated by their last letters. Other Hrc/Hrp proteins proposed to be involved in secretion are not indicated. Two classes of Hrp proteins are secreted via the type III system: pilins and harpins. The *hrpA* encoded pilin is a subunit of the Hrp pilus which is required for pathogenicity. The role of the HrpW and HrpZ harpins in the transfer of Avr proteins across the plant cell membrane and the mediation of the compatible interaction is not yet understood.

Several Avr proteins are proposed to interact with putative plant "susceptibility targets" to effect pathogenesis. The mechanisms by which these interactions defeat host defenses and change plant metabolism to alter the nutrient status and favour bacterial growth in the apoplast, are still unknown. *Avr* genes are carried on plasmids, or on hypervariable chromosomal regions with mobile genetic elements flanking the conserved *hrp* gene cluster (as part of a "pathogenicity island"). It is suggested that Hrp^+ pathogens have the ability to acquire *avr* genes from co-evolving bacteria to retain their pathogenic advantage within adapting plant populations.

Secreted virulence factors (such as toxins and extracellular enzymes, see Table 2-1) are encoded by genes located on the bacterial chromosome or on plasmids, and add to the severity of disease symptoms.

In certain plant-pathogen interactions, incompatibility is "accidentally" mediated by a "maladapted" *avr* gene acquired horizontally from another bacterium. Such a gene encodes an Avr protein for which a corresponding resistance (*R*) gene product is expressed in the infected plant. Interaction between the *R* and *avr* gene products "betrays" the bacterium to the plant and leads to the induction of localized defense responses (including the HR) and systemic acquired resistance (SAR) (see section 2.3). In this context, *avr* genes perform the secondary, negative function of determining host-range, and the *avr* gene product is referred to as a "(pathogen) specific elicitor" (Ji et al. 1998). Interactions between *avr* and *R* gene products (in both the compatible and incompatible situation) are depicted as being direct (according to the "elicitor-receptor model"), but may also be indirect (Lamb et al. 1989, Parker and Coleman 1997).

Harpins induce the HR and SAR when injected into plants in their purified form. It is not known whether or how this capability relates to the "natural" role of harpins in the mediation of disease development or in the elicitation of an *avr-R* dependent HR by Hrp^+ bacteria.

host-range and other pathogenic attributes among closely related pathovars or strains (Alfano and Collmer 1996, 1997; Collmer 1998).

Hrp and *avr* gene systems are discussed in more detail in the context of this model in the subsections that follow.

2.2.2.1 *Hrp genes encode a conserved type III protein secretion system, secreted and regulatory proteins*

Hrp genes have been identified as a major determinant of pathogenicity in many phytopathogenic *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia* species and pathovars. They appear to be universally conserved among plant pathogens of these genera and control a variety of interactions between these bacteria and plants, including the elicitation of the HR (reviewed in Willis et al. 1991, Bonas 1994, Lindgren 1997).

Hrp genes have been characterized extensively in four representative pathogens: *Pseudomonas syringae* pv. *syringae* 61, *Erwinia amylovora* Ea321, *Ralstonia* (previously *Pseudomonas*) *solanacearum* GMI1000 and *Xanthomonas campestris* pv. *vesicatoria* 85-10 (Alfano et al. 2000). Most of the *hrp* genes of these strains are contained in large, polycistronic, chromosomal clusters (pathogenicity islands*) of about 25 kb, with the exception of the *R. solanacearum* cluster, which is carried on a megaplasmid (Alfano and Collmer 1997, Collmer 1998, Alfano et al. 2000). The transcriptional organization of the *P. s.* pv. *syringae* 61 *hrp* cluster, which has served as a model for numerous investigations, is illustrated in Fig. 2-2.

Key features of *hrp* gene clusters that have been revealed by cloning, sequencing, mutation, complementation and functional analysis, are the following:

a. Based on gene similarity, operon structure and regulatory systems, the four model *hrp* clusters can be divided into two groups. Group I contains the clusters of *P. s.* pv. *syringae* and *E. amylovora*, and group II the clusters of *R. solanacearum* and *X. campestris* (reviewed in Alfano and Collmer 1997).

---

* "Pathogenicity islands" are gene clusters that (i) include many virulence genes, (ii) are selectively present in pathogenic strains, (iii) occupy large chromosomal regions, (iv) are often flanked by direct repeats, (v) are bordered by tRNA genes and/or cryptic mobile genetic elements, and (vi) are unstable. Differences in codon usage and G+C content between genes in such islands and those in the rest of the genome provide evidence that these islands may be horizontally transferred between bacteria. See Lawrence and Roth 1993, Groisman and Ochman 1996 and Hacker et al. 1997 for reviews of this topic.

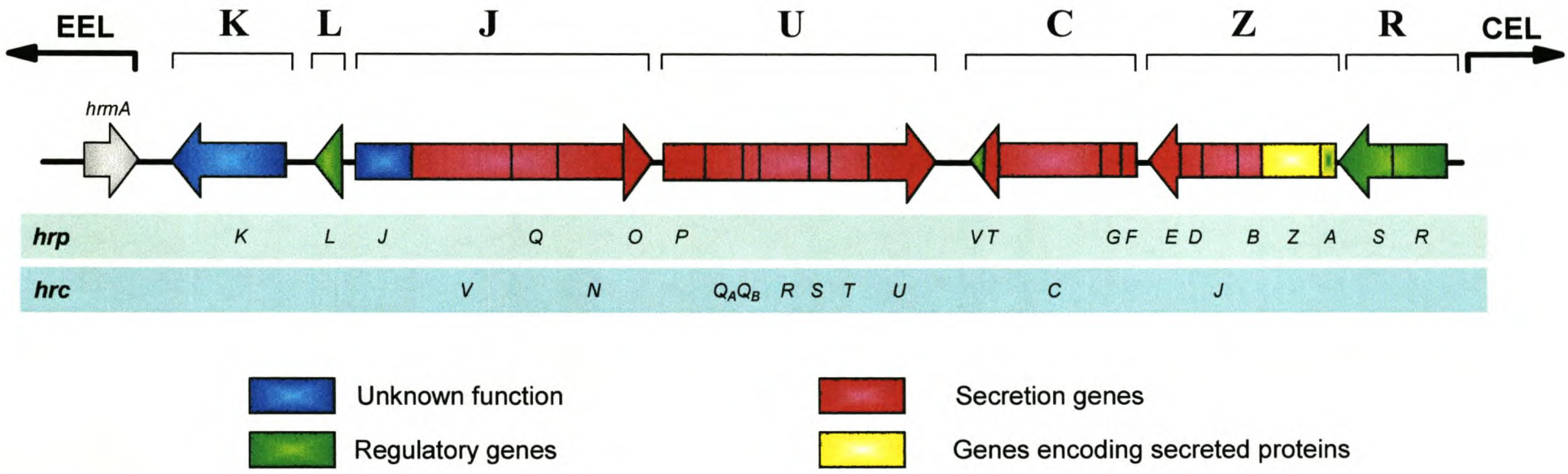


Fig. 2-2. Transcriptional organization and function of genes in the *Hrp* cluster of *Pseudomonas syringae* pv. *syringae* 61 (compiled from He 1996, Alfano and Collmer 1997, Alfano et al. 2000).

The conserved *hrp*/*hrc* cluster of *P. syringae* is delineated by *hrpK* and *hrpR*, and consists of seven operons. The designation of each operon is indicated at the top, and direction of transcription with arrowheads. The last letter designation of each *hrp* or *hrc* open reading frame is given below. *hrmA* (recently redesignated as *hopPsyA*) was originally characterized as a "hypersensitive reaction modulating" gene (Huang et al. 1991, Heu and Hutcheson 1993), but was later demonstrated to have *avr* gene function, and is currently placed in the "exchangeable effector locus" (EEL) found to the left of the *hrp*/*hrc* cluster in the *P. syringae* pathogenicity island. Harpin elicitors are encoded by *hrpZ* and *HrpW*. *HrpW* is not situated inside the conserved *hrp* cluster, but falls within the "conserved effector locus" (CEL) found to the right of *hrpR* in the *P. syringae* pathogenicity island (not shown).

b. A number of *hrp* genes show homology with components of the virulence protein secretion system of animal pathogens such as *Yersinia*, *Salmonella* and *Shigella*, as well as with the *fliIflh* genes of *Escerichia coli* and other bacteria, which encode components of a system involved in the secretion of flagellar proteins. This provided evidence of the existence of a conserved "type III" virulence protein secretion pathway in Gram- negative pathogens of animals and plants (reviewed in Van Gijsegem et al. 1993, Collmer and Bauer 1994, Salmond 1994, Alfano and Collmer 1997, Baker et al. 1997).

   Nine *hrp* genes have thus far been identified as universal type III secretion system components. They have been redesignated *hrc* (HR and conserved)* and all but *hrcV* have been given the last-letter designations of their *Yersinia ysc* homologs (Bogdanove et al. 1996). These genes encode one outer- and five inner-membrane proteins, an outer-membrane associated lipoprotein and two cytoplasmic proteins (including a putative ATPase) which form the core of the Hrp type III secretion apparatus. Additional evidence exists that at least four more Hrc/Hrp proteins are involved in this assembly (Alfano and Collmer 1997, Lindgren 1997). Other Hrp proteins are proposed to be peripheral components of this secretion system and to perform extracellular functions which are specific to protein transfer across the plant cell wall (Collmer 1998).

c. Apart from the components of a type III secretion system, *hrp* genes also encode proteins with **regulatory** functions and at least two classes of **secreted** proteins.

   The two groups of *hrp* clusters have different regulatory systems. In the group I clusters, transcription of *hrp* operons is activated by an extracytoplasmic function (ECF) sigma factor (HrpL), whereas an AraC homolog (HrpX in *Xanthomonas* and HrpB in *Ralstonia*) activates transcription in group II clusters. Different upstream activators of these factors have also been characterized (HrpR and HrpS in *P. s.* pv. *syringae*, HrpG in *X. campestris* pv. *vesicatoria* and PrhA in *R. solanacearum*). A putative negative regulator of *hrp* gene expression, which is encoded by *hrpV* and acts upstream of HrpR and HrpS, has also been identified in *P. syringae* (Preston et al. 1998). The current understanding of *hrp* gene regulation is reviewed in Bonas 1994, Lindgren 1997 and Collmer 1998, but is still fundamentally incomplete, especially with regard to regulation *in planta*.

---

* References to "*hrp* genes" normally encompass the *hrc* subset.

Two classes of extracellular (secreted) Hrp proteins have been identified: **harpins** and **pilins**. Pilins are represented by the *P. syringae* HrpA protein. HrpA is an integral component of a Hrp pilus that is 6-8 nm in diameter, formed on bacteria in a Hrp-dependent manner and required for pathogenicity and HR elicitation (Roine et al. 1997). HrpA has recently been demonstrated to also have a regulatory function in the expression of *hrp* and *avr* genes, which may be independent of its role in the secretion of the proteins encoded by these genes. In this context, HrpA is proposed to be involved in the sensing of host plant cells (Wei et al. 2000).

Harpins ("*hrp*-encoded HR elicitors") were the first secreted Hrp proteins to be identified and are of particular importance to this study. They are discussed in more detail below.

2.2.2.2 *Harpins: secreted Hrp proteins of unknown biological function that induce systemic acquired resistance in plants*

The first harpin ($harpin_{Ea}$) was discovered in culture fluids of *E. coli* carrying a highly expressed *hrp* gene cluster of the apple and pear pathogen, *E. amylovora* Ea321. Mutations in the *E. amylovora* harpin encoding gene (*hrpN*) strongly diminished HR elicitation in tobacco and pathogenicity in susceptible, immature pear fruits. It was therefore concluded that harpin was the primary virulence factor travelling the Hrp pathway (Wei et al. 1992). The harpin encoding gene (*hrpZ*) of the bean pathogen *P. s.* pv. *syringae* 61 was identified and cloned soon thereafter, and similar importance was assigned to its product ($harpin_{Pss}$) (He et al. 1993). Evidence from subsequent studies has, however, pointed to Avr proteins as the primary effectors of virulence and genotype-specific HR elicitation (Alfano et al. 1996, Alfano and Collmer 1997, Collmer 1998; see section 2.2.2.3), and has left the biological functions of harpins in HR elicitation and pathogenesis largely unexplained.

The principal findings of studies on harpin encoding genes, harpin structure and function are summarized below:

a. Homologues of the harpin encoding *hrpN* gene of *E. amylovora* have been isolated from a number of other *Erwinia* species, and homologues of the *P. s.* pv. syringae *hrpZ* gene from several other *P. syringae* pathovars. A harpin elicitor encoding gene (*popA*) has also been isolated from *R. solanacearum*. Although *hrp* genes have been

characterized in *X. campestris* and *X. oryzae*, harpin encoding genes have not been identified in these bacteria (Lindgren 1997, Charkowski et al. 1998). More recently, *E. amylovora* and *P. syringae* were shown to possess and express a second harpin encoding gene, designated *hrpW* in both bacteria (Gaudriault et al. 1998, Kim and Beer 1998, Charkowski et al. 1998).

b. The harpin encoding genes of *E. amylovora* (*hrpN* and *hrpW*), the *P. syringae hrpW* gene and the *popA* gene of *R. solanacearum* are located adjacent or near to their respective *hrp* clusters, whereas the *P. syringae hrpZ* gene resides within the *hrpZ* operon (see Fig. 2-2) (Alfano and Collmer 1997, Gaudriault et al. 1998, Kim and Beer 1998, Charkowski et al. 1998).

c. The HrpN, HrpZ and PopA1 proteins share a number of biochemical characteristics. They are heat-stable, glycine-rich proteins that lack cysteine and appear to be highly susceptible to proteolysis. They also lack an N-terminal signal peptide, which is consistent with their secretion in culture and *in planta* in a Hrp-dependent manner (Preston et al. 1995, Lindgren 1997). These characteristics are shared by the N-terminal halves of the HrpW proteins (Charkowski et al. 1998, Kim and Beer 1998).

d. The primary structures of HrpZ, HrpN, PopA1 and the N-terminal harpin-like domain of HrpW show no significant homology to each other or to any other proteins of known function, and do not contain any motifs of known biological function (Lindgren 1997, Charkowski et al. 1998). However, the C-terminal half of HrpW shows a high degree of similarity with a newly defined class of pectate lyases found in bacterial and fungal pathogens (Gaudriault et al. 1998, Kim and Beer 1998, Charkowski et al. 1998).

e. Harpins were originally identified on the basis of their ability to elicit the HR when infiltrated at high concentrations (> 0.1 μM) into the leaves of tobacco and several other plants (Alfano and Collmer 1996, Gaudriault et al. 1998). Subsequent studies, in which harpins were delivered by *hrp* mutants or heterologous bacteria, have shown the contribution of harpins to pathogenicity and *avr-R* dependent HR elicitation to be variable among the different bacteria that express them naturally.

   According to these studies, HrpN appears to play an important role in HR elicitation in *E. amylovora*, *E. chrysanthemi*, *E. carotovora* and *E.* (*Pantoea*) *stewartii*. It seems to be required for pathogenicity and virulence in the former two species, but not in the latter. Results obtained with *R. solanacearum popA* mutants suggested that PopA1 is neither essential for HR elicitation nor for pathogenesis, but may play a role in the

determination of host-range. Similar results have been reported for the *P. syringae* HrpZ* protein, which has additionally been implicated in virulence (reviewed in Alfano and Collmer 1997, Lindgren 1997).

The ability of the HrpW harpins to elicit an HR (in a similar fashion as the HrpN and HrpZ harpins) has been shown to reside in their harpin-like N-terminal domains. *P. syringae* and *E. amylovora hrpW* mutants were able to induce disease to the same extent as wild type strains, indicating that HrpW is not required for pathogenicity. However, the ability to elicit the HR was variably affected by *HrpW* mutations in the two bacteria (no effect in *E. amylovora* Ea321, an enhanced HR eliciting ability in *E. amylovora* CFBP1430, and a reduced ability in *P. syringae* pv. *tomato* DC3000; Gaudriault et al. 1998, Kim and Beer 1998, Charkowski et al. 1998).

f. The biological function of harpins (i.e. the way in which they contribute to Hrp phenotypes when secreted by bacteria in compatible and incompatible interactions), as well as the molecular basis of their ability to elicit the HR when infiltrated into the apoplast of cells in a purified form, remain unknown (Collmer 1998).

Harpin activity is not likely to be enzymatic, as different fragments retain activity (Arlat et al. 1994, Barny 1995, Alfano et al. 1996). The heterologous delivery of *avr* gene signals by the *P. s.* pv. *syringae* 61 *hrp* gene cluster (carried on cosmid pHIR11) depends on the endogenous co-expression of HrpZ with the AvrB protein. This has led to the suggestion that HrpZ may act as an extracellular chaperone for the secretion of some Avr proteins by Hrp$^+$ bacteria (in a similar fashion as the Syc chaperones required for the delivery of some *Yersinia* outer proteins (Yops); Cornelis 1994, Wattiau et al. 1994, Alfano et al. 1996, Gopolan et al. 1996a).

Several earlier observations have led to the hypothesis that harpins are involved in establishing pathogenesis by raising the pH of apoplastic fluids, which leads to the release of nutrients into the apoplast and bacterial multiplication (reviewed in Collmer and Bauer 1994). Two lines of evidence currently suggest that harpin activity may involve interactions with the plant cell wall. Firstly, purified HrpZ protein binds to the walls of intact cells but not to protoplasts, and fails to trigger HR-associated responses

---

* Earlier reports assigned a central role for HrpZ in HR elicitation (He et al. 1993, Yuan and He 1996). The results from these investigations were reinterpreted when the Avr-like protein HrmA was shown to be the actual elicitor of the HR by bacteria carrying the *P. s.* pv. *syringae* 61 cluster (on cosmid pHIR11), and *P. syringae* was shown to express another harpin elicitor, HrpW (Alfano et al. 1996, 1997; Alfano and Collmer 1997, Charkowski et al. 1998, Collmer 1998).

in protoplasts (Hoyos et al. 1996). Secondly, the HrpW harpins have been shown to bind specifically to pectate through their C-terminal pectate lyase-like domains (which lack enzymatic activity, and are not required for HR elicitation). It has thus been suggested that harpins may loosen the plant cell wall to facilitate penetration of Hrp secretion apparatus for the delivery of Avr and other proteins (Charkowski et al. 1998, Kim and Beer 1998).

The induction of several defense-related plant responses (see Fig. 2-3 in section 2.3) has been described in conjunction with the HR eliciting ability of harpins (when applied in their purified form, or delivered by Hrp^+ bacteria into intact plants or to plant cell cultures). These include: (i) membrane depolarization, accompanied by Ca^{2+} and H^+ influx/K^+ efflux (Wei et al. 1992, Baker et al. 1993, Popham et al. 1995, Hoyos et al. 1996), (ii) the production of active oxygen species (Baker et al. 1993, Desikan et al. 1996, Chandra et al. 1997, Xie and Chen 2000), (iii) the activation of mitogen-activated protein (MAP) and MAP-like kinases (Ádám et al. 1997, Desikan et al. 1999), (iv) an increase in the levels of salicylic acid (Wang and Liu 1999), (v) lignin accumulation (Wang and Liu 1999), (vi) the inhibition of mitochondrial cytochrome pathway electron transport, with a concomitant inhibition of ATP synthesis (Xie and Chen 2000), and (vii) the activation of defense-related genes. The latter includes *hin1* (a harpin-induced tomato gene that is also activated by the *avrPto-Pto* mediated signal; Gopolan et al. 1996b), genes encoding the phenylpropanoid pathway enzyme phenylalanine ammonia lyase (PAL), the phytoalexin biosynthetic enzyme anthranilate synthase, and the anti-oxidant protective enzyme glutathione-S-transferase (GST) in *Arabidopsis* (Desikan et al. 1998), and genes encoding several tobacco peroxidase isozymes (Wang and Liu 1999). Although these studies have elucidated some aspects of signal transduction that may be involved in the activation of harpin-induced defense responses, they have not revealed much about the exact role of harpin in these processes.

g. A characteristic linked to the ability of purified harpins to elicit the HR in non-hosts or resistant host plants, is their ability to induce systemic acquired resistance (SAR, see section 2.3.4) in plants (Strobel et al. 1996, Wei and Beer 1996, Qui et al. 1997). Purified $HrpZ_{Pss}$ was demonstrated to induce SAR in cucumber plants in an identical fashion to wild-type *P. s.* pv. *syringae* 61 bacteria. The results from that study suggested that (i) the induction of SAR by *P. s.* pv. *syringae* is dependent on the production of HR elicitors that traverse the cell membrane via the Hrp type III secretion

system, and (ii) that $HrpZ_{Pss}$ was either the major inducer of SAR in *P. s.* pv. *syringae* or representative of the SAR inducers produced by this bacterium (Strobel et al. 1996)*. Purified *E. amylovora* harpin has been shown to induce SAR in *Arabidopsis*. In that study, the induction of SAR was correlated with the expression of *PR-1* and *PR-2* genes and was shown to be dependent on the accumulation of salicylic acid, as well as on a functional *NIM1/NPR1*† gene product, but not to require jasmonic acid or ethylene (Dong et al. 1999).

2.2.2.3 Avr *genes encode the primary effectors of pathogenesis and may be horizontally transferred between co-evolving bacteria*

"Avirulence genes" in plant pathogens were initially defined by Flor in his studies of fungal host-pathogen interactions. His "gene-for-gene" hypothesis proposed that the nature of the interaction between a pathogen and plant is related to interactions between individual pathogen and host genes. According to this theory, incompatibility was the result of active interaction between a dominant pathogen "avirulence" (*avr*) gene and a complementary or reciprocal dominant plant "resistance" (*R*) gene. Likewise, compatibility resulted when dominance was eliminated experimentally, or when either gene was recessive (Flor 1955, 1971). On the molecular level, *avr* gene products ("race-specific elicitors") are hypothesized to be specifically recognized by *R* gene products (which are receptors), hereby provoking a resistance response (incompatibility) in the host plant (Keen 1982, Gabriel and Rolfe 1990, De Wit 1995, Bent 1996, Parker and Coleman 1997)‡.

Avr genes have subsequently also been identified in plant pathogenic viruses, bacteria, fungi, nematodes and insects as determinants of host range in relation to their corresponding plant *R* genes. The question as to why pathogens retain these genes despite the negative selection pressure of complementary *R* genes in host plant populations has led to the notion that *avr* genes must have a positive function in virulence

---

* In this study and others, some plant defense responses, as well as defense-related ultrastructural changes to plants, were induced by Hrp mutants in the absence of HR elicitation. These results emphasize that not all defense responses are induced by signals derived from harpin and/or Avr proteins, but that some pathogen-derived signals are produced independently of their *hrp* systems (reviewed in Collmer and Bauer 1994, Lindgren 1997). The induction of plant defense responses by such "non-specific" elicitors are discussed in section 2.3.2.

† Non-immunity/Non-expresser of PR genes; see section 2.3.3.4 for more details.

‡ Physical contact between matching *avr* and *R* gene products has only been demonstrated for one such a pair (*AvrPto* and *Pto*) (Scofield et al. 1996, Tang et al. 1996), which suggests that indirect interactions between *avr* and *R* gene products may also occur (Lamb 1989, Dangl 1994).

that outweighs their negative function of restricting host range (Dangl 1994, White et al. 2000).

A growing body of direct and indirect evidence*, has led to the current model which suggests that Avr proteins are the primary effectors of pathogenesis, and are acquired by Hrp$^+$ bacteria from diverse sources during their co-evolution with changing plant populations (as illustrated in Fig. 2-1). The "betrayal" of pathogens by some of their *avr* genes to the *R* gene system of plants (i.e. the restriction of host range) is hence a secondary consequence of the horizontal transfer of *avr* genes between co-evolving bacterial populations (or put from another perspective: some pathogenicity factors **become** avirulence factors once they are recognized by the surveillance system of the plant; De Wit 1997, Collmer 1998).

The salient findings on which this model was based, were the following:

a. Diverse virulence or virulence-associated functions/properties have been described for several of the more than 30 bacterial *avr* genes that have been cloned†. Some of these functions/properties are associated with pathogen fitness, whilst others directly affect the expression of plant (defense) genes‡ (reviewed in Dangl 1994, Leach and White 1996, White et al. 2000). The exact mechanisms by which plant metabolism is altered to increase the nutrient status of the apoplast for bacterial growth are, however, still unknown (Collmer 1998).

b. Elicitation of the HR by a specific Avr protein in plants carrying a corresponding *R* gene is dependent on functional *hrp* gene expression, which suggests that Avr proteins are secreted via the Hrp type III secretion system (He 1996, Bonas and Van den Ackerveken 1997, De Wit 1997). The finding that the functional *P. s.* pv. *syringae* 61 *hrp* cluster (carried on the cosmid pHIR11) is sufficient to deliver heterologous *avr*

---

* The understanding of *avr* gene function has been advanced greatly by molecular studies of the type III Hrp secretion pathway in plant pathogenic bacteria. The information that follows is limited to analyses of the role of **bacterial** *avr* genes, although conclusions/hypotheses may be applicable to interactions between plants and non-bacterial pathogens.

† Details of the properties and functions of all of the cloned *avr* genes fall beyond the scope of this study. See Dangl 1994, Leach and White 1996 and White et al. 2000 for extensive reviews in this regard.

‡ One family of Avr proteins (the AvrB3 family encoded by *Xanthomonas* spp.) contains functional nuclear localization signals which target them to the plant nucleus, where their structural features suggest they function as plant transcription factors (Yang and Gabriel 1996, White et al. 2000). Other Avr proteins appear to interfere with the signalling and structural pathways of general plant defense responses, in an analogous fashion to factors secreted via a type III pathway by bacterial animal pathogens such as *Yersinia* (White et al. 2000).

signals strongly supported this idea (Gopolan et al. 1996a, Pirhonen et al. 1996). More indirect evidence of the Hrp-dependency of Avr phenotypes lies in the fact that all characterized Avr proteins lack N-terminal signal peptides or other recognizable secretion signals; properties which are consistent with secretion via a type III pathway (Salmond and Reeves 1993, Alfano and Collmer 1997). *Hrp*-dependent secretion has been demonstrated unambiguously for at least one Avr protein (DspE; Bogdanove et al. 1998a)*.

c. Bacterial transfer of Avr proteins into plant cells (via the Hrp type III system) has not been observed directly. However, all of the cloned *avr* genes are predicted to encode soluble, hydrophilic proteins, which suggest a cytoplasmic location. By expressing avr genes *in planta*, four Avr proteins (AvrB, AvrBs3, AvrPto and AvrRpt2) have been demonstrated unambiguously to be recognized and to function inside the plant cell (reviewed in Bonas and Van den Ackerveken 1997, White et al. 2000).

d. Unlike *hrp* genes, *avr* genes are not clustered, but scattered throughout the genomes of bacteria (Lamb 1989). Some *avr* genes are, however, linked to *hrp* gene clusters in "pathogenicity islands" (Heu and Hutcheson 1993, Mansfield et al. 1994, Lorang and Keen 1995, Alfano et al. 2000). Certain *hrp*/*avr* ensembles are functional in that they enable non-pathogens to elicit the HR in appropriate test plants (i.e. plants carrying an *R* gene which corresponds to the particular *avr* gene) (reviewed in Collmer 1998).

e. The regulation of *avr* gene expression is linked to the regulation of *hrp* gene expression. *Hrp* genes and several *avr* genes are transcriptionally induced *in planta* and in environments representative of plant intracellular spaces (e.g. minimal culture media containing sucrose, fructose or mannitol). Furthermore, a HrpL-dependent conserved consensus sequence (the "harp or avr box") has been identified in the promoters of *P. syringae avr* and *hrp* genes, and the *hrp* regulatory system seems to be a universal requirement for the transcription of all *P. syringae avr* genes (reviewed in Bonas 1994, Dangl 1994, Lindgren 1997).

f. *Avr* genes are potentially mobile due to their presence on plasmids (Leach and White 1996, Vivian and Gibbon 1997), or their linkage on chromosomes with transposable elements or phage sequences (Hanekamp et al. 1997). Additionally, the conserved *hrp*

---

* One example of a Hrp-independent *avr* gene exists. AvrD of *P. syringae* pv. *glycinea* encodes an enzyme involved in the synthesis of syringolide elicitors. These elicitors induce the HR in a *hrp*-independent manner when injected into appropriate soybean cultivars (Keen et al. 1990, Keith et al. 1997).

clusters of different *P. syringae* strains are bordered by a hypervariable region which is enriched in *avr* genes and mobile DNA elements (Collmer 1998, Alfano et al. 2000).

g. *P. syringae avr* genes have been demonstrated to function heterologously in *E. amylovora* and *E. chrysanthemi* to support pathogenesis and *avr*-R dependent HR elicitation (Bogdanove 1998b).

In the light of the amassing evidence that the proteins encoded by *avr* genes primarily have a function in pathogen virulence rather than avirulence, a more appropriate designation for these proteins has been proposed. In analogy with the Yops secreted by the prototypical *Yersinia* type III secretion system (Cornelis 1994), it has been suggested that members of this class be redesignated "Hop" proteins (Hrp-dependent outer proteins) in future (Alfano and Collmer 1997).

2.3 THE MOLECULAR BASIS OF RESISTANCE IN PLANTS

2.3.1 Perspectives and concepts

Plants are continually under threat from a variety of biotic and abiotic stresses. These include parasitism by fungal, bacterial and viral pathogens, wounding (caused by animal or insect herbivory, or by other means), and other stress conditions such as drought and exposure to UV, heavy metals and ozone (Baron and Zambryski 1995, Schneider et al. 1996). Current knowledge of plant responses to these threats has accumulated from a variety of ecological and molecular studies, and has greatly been advanced through the analysis of *Arabidopsis* mutants (e.g. Bell 1981, Baker et al. 1997, Glazebrook et al. 1997) and, more recently, transgene expression *in planta* (see Glazebrook et al. 1997, Melchers and Stuiver 2000 for references). The literature dealing with plant resistance is indeed voluminous, but has left us with a less than thorough understanding of many aspects of this complex phenomenon.

Ecologists attempt to analyze the induction of plant resistance in response to pathogen attack in the broad context of the plant's overall strategy to cope with multiple enemies. To this end, "civilian responses" are distinguished from "defense responses" (Paul et al. 2000). "Civilian responses" encompass physiological and metabolic adjustments made to buffer the effects of wounding or disease (i.e. to "tolerate" damage), whereas "defense responses" refer to the pathways that have been dissected by molecular biologists and

geneticists for years in attempts to identify the components of localized and systemic resistance, and the signals that elicit and connect them (Paul et al. 2000).

Although there is a growing demand for the integration of ecological and molecular perspectives to advance the understanding of plant responses to multiple enemies, this review does not constitute such an attempt. Instead, it is aimed at providing a concise background for the experimental work presented in Chapter 5*, which deals with the identification of plant genes that may be induced as a result of infiltration with purified $harpin_{PssNV}$ elicitor protein and/or *Pseudomonas syringae* pv. *syringae* bacteria in a resistant and a susceptible plum cultivar. As such, it will concentrate on the mechanisms of plant resistance induced by the biotrophic, necrogenic, Gram-negative, Hrp^+ bacterial phytopathogens discussed in the previous section†.

To integrate the information in this section with that of sections 2.1 and 2.2, the following concepts must be considered:

a. Localized and systemic resistance

(Disease) resistance refers to the ability of a plant to prevent, restrict or retard pathogen growth and multiplication, i.e. disease development (Bell 1981, Benhamou 1996), and is characterized by the activation of defense mechanisms in response to pathogen attack (Schneider et al. 1996).

All pathogens induce plant defense responses. When the plant is a susceptible host to the attacking bacterium (*compatible* interaction), plant defense genes are induced more slowly and transcripts accumulate at much lower levels than when the plant is a non-host or resistant host (Dixon et al. 1994, Baron and Zambryski 1995, Glazebrook et al. 1997). This delayed response allows the pathogen to multiply, spread and cause widespread necrotic lesions throughout the plant (disease). In the *incompatible* situation, plant defense mechanisms are rapidly induced to accomplish **localized induced** or **acquired resistance** (LAR; Ross 1961a). LAR is typically (but not always) characterized by hyper-

---

* The experiment presented in Chapter 5 included a control for responses induced as a result of the **mechanical wounding** which accompanies the infiltration of experimental plants. Much of the published information about wounding responses in plants were derived from the effects of insect feeding, but are also applicable to abiotic wounding (Baron and Zambryski 1995). Where applicable, references to wounding responses are therefore included in this section.

† Unless stated otherwise, all subsequent references to "pathogen(s)" apply to this group of bacterial phytopathogens only.

sensitive cell death (the HR), and prevents multiplication and spread of the attacking pathogen (Lamb et al. 1989, Dangl et al. 1996, Heath 2000).

Both forms of necrotic cell death lead to the production of systemic signals which induce defence mechanisms in tissues untouched by the initial infection, resulting in **systemic acquired resistance** (SAR; Ross 1961b)*. Unlike localized resistance, SAR confers long-term protection against a broad range of bacterial, fungal and viral pathogens (Schneider et al. 1996, Maleck and Dietrich 1999).

b. Non-specific and pathogen-specific resistance

Localized resistance is divided into non-host resistance and host resistance. **Non-host resistance** (also termed "general resistance" or "race-general resistance") is regarded as the most common form of disease resistance exhibited by plants. It is defined as resistance shown by an entire plant species to a specific pathogen and is expressed by every plant towards the majority of potentially pathogenic micro-organisms. This basic incompatibility is the consequence of a pathogen's inability to recognize and infect a plant, or the ability of the plant to activate its defense mechanisms rapidly and effectively (Kombrink and Somssich 1995, Heath 2000). In contrast, **host resistance** (or "race-specific resistance") is defined to be expressed by certain plant genotypes ("resistant hosts") within an otherwise susceptible host species against specific pathogen genotypes. Variation in host resistance is ascribed to the segregation of resistance (*R*) genes, the products of which interact directly or indirectly with the products of pathogen avirulence (*avr*) genes (Bell 1981, Heath 2000).

The above distinction may be useful to accommodate genotype-specific incompatibility attributed to "gene-for-gene" resistance, but it has become confusing in terms of the current model for bacterial pathogenicity discussed in section 2.2. According to this model, all Hrp$^+$ phytopathogens secrete Avr proteins as the primary effectors of pathogenesis. These proteins are proposed to interact with "susceptibility targets" in *susceptible host plants* to mediate disease development, and with *R* gene products in *resistant hosts* to

---

* SAR is triggered by infection with necrogenic phytopathogens. It is distinguished from **induced systemic resistance** (ISR), where protection against future pathogen attack is triggered by colonization of the rhizosphere with non-necrotizing, mutualistic, "biocontrol" bacteria such as *Pseudomonas fluorescens* and *Serratia marcescens*, or by cell wall-derived elicitors from these bacteria (Pieterse et al. 1996, Van Wees et al. 1997).

elicit the HR and defense responses. What then is the molecular basis of HR elicitation and subsequent defense induction in *non-hosts*?

One view that unifies the traditional role of *avr* genes as determinants of host-range with the current model argues that non-host and host resistance have a similar genetic basis: what traditionally has been termed "non-host resistance" may be the additive effect of many, concomitant gene-for-gene interactions within a specific plant. This view is supported by studies that demonstrated (i) the expression of multiple *avr* genes by a single pathogen, (ii) the presence of a *R* gene or genes in different plants that all interact with the same *avr* gene cloned from a specific pathogen, and (iii) the cloning of more than one *R* gene from a particular plant species (Dixon and Lamb 1990, Dangl 1994, Bent 1996).

In the light of the above it seems more appropriate to use the terms **non-specific** and **pathogen-specific** to distinguish between localized resistance mediated by Hrp-dependent avr-R interaction and those that are not (see Lindgren 1997 for a review of the latter). Localized resistance in any incompatible plant-pathogen interaction is the product of both non-specific and pathogen-specific defense induction, and both triggers produce signals for the induction of SAR.

c. Constitutive and inducible defenses

Plants possess a vast and diverse arsenal of constitutive and inducible defense mechanisms to protect them against pathogen attack. **Constitutive** mechanisms exist independently of pathogen presence and are regarded as non-specific, broad-spectrum defenses that have evolved as a result of the collective pressure of pathogens, herbivores and abiotic stresses. These include physical barriers such as thick cell walls, seed coats, thorns and hairs, as well as preformed peptides, proteins, proteinase inhibitors and non-proteinaceous secondary metabolites (Bell 1981, Bennet and Wallsgrove 1994, Peumans and Van Damme 1995, Osbourn 1996, Heath 2000).

Inducible defense mechanisms (outlined in Fig. 2-3) have been studied far more extensively as the principal effectors of plant resistance, and are the focus of this review. It is important to note that:

a. Inducible defense mechanisms include the generation of active oxygen species (AOS), the synthesis of low-molecular weight antimicrobial compounds (phytoalexins), cell wall

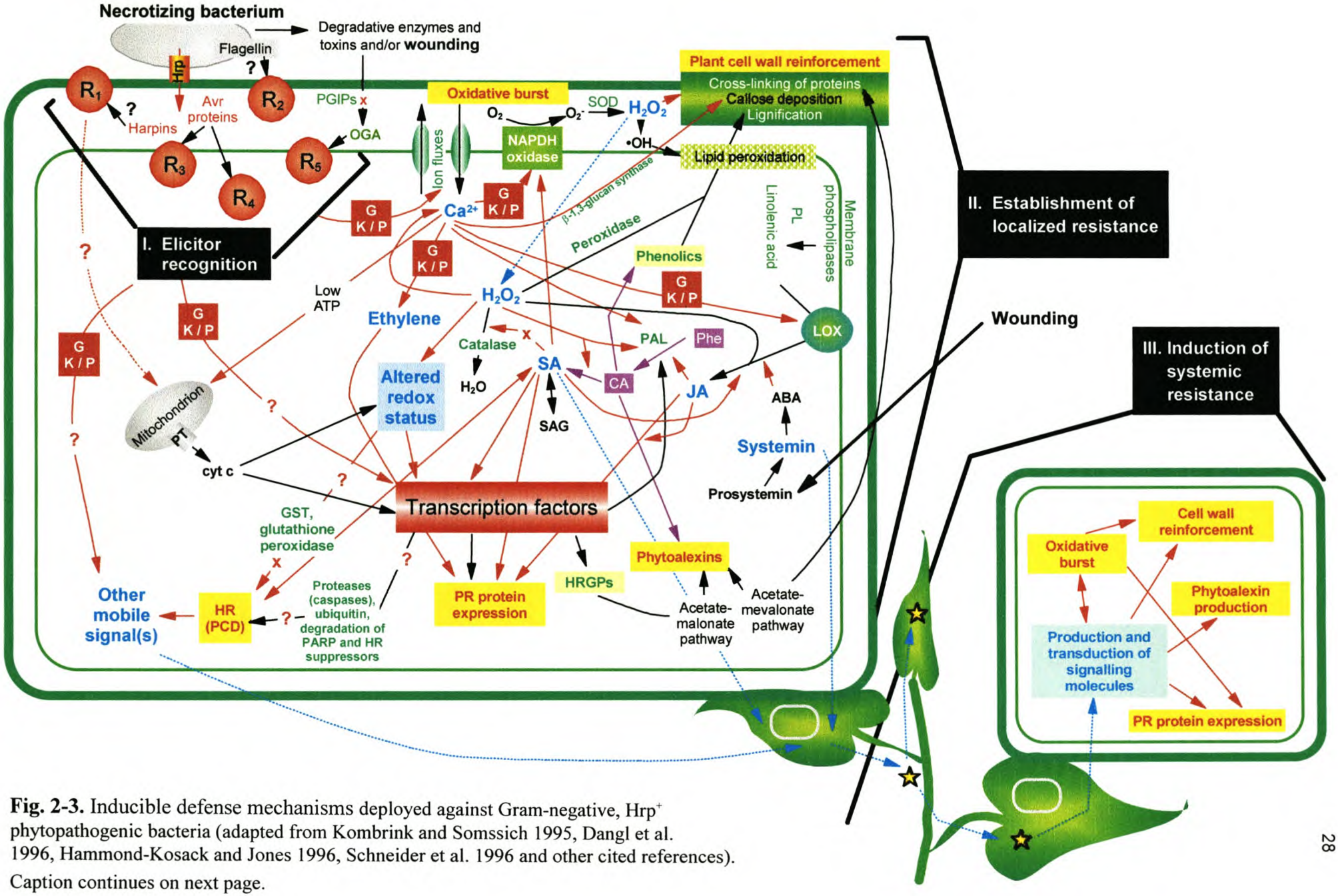


Fig. 2-3. Inducible defense mechanisms deployed against Gram-negative, Hrp$^+$ phytopathogenic bacteria (adapted from Kombrink and Somssich 1995, Dangl et al. 1996, Hammond-Kosack and Jones 1996, Schneider et al. 1996 and other cited references).

Caption continues on next page.

Fig. 2-3. Continued.

Exogenous non-specific elicitors (flagellin, harpins, and others?), pathogen-specific elicitors (Avr proteins), and oligogalacturonides (OGA; produced as a result of plant cell wall degradation by pathogen enzymes and toxins) are perceived through a variety of specific plant receptors (R_N), some of which are *R* gene products (R_3 and R_4). *R* gene encoded receptors may also occur and recognize corresponding Avr proteins inside the nucleus (not shown). The perception of these signals is followed by a complex series of events which leads to the establishment of localized defense (LAR) through the induction of multiple defense mechanisms (indicated in yellow blocks, discussed in more detail in section 2.3.3). Characterized components of these early signal transduction pathways include (i) Ca^{2+} (the free intracellular concentration of Ca^{2+} is elevated through membrane depolarization, concomitantly leading to the influx of H^+ and efflux of K^+ and Cl^-), (ii) G-proteins (G), and (iii) phosphorylation cascades consisting of various phosphatases (P) and kinases (K), including mitogen-activated protein kinases (MAPKs; Ligterink et al. 1997) and calcium-dependent protein kinases (CDPKs; Sheen 1996, Romeis et al. 2000). The involvement of Ca^{2+}-calmodulin complexes, cAMP, phospholipase C and inositol phosphates has also been postulated.

The oxidative burst is one of the first defense mechanisms to be activated, and leads to the production of active oxygen species, including H_2O_2 that plays an important role in subsequent induction events. The phenylpropanoid pathway (represented in purple by Phe = phenylalanine, PAL = phenylalanine ammonia lyase and CA = cinnamic acid, see Fig. 2-6 for more detail) also plays a central role in defense, as it leads to the production of the antimicrobial phytoalexins, the synthesis of phenolics (incorporated in lignin and suberin to reinforce cell walls) and the production of the mobile endogenous elicitor, salicylic acid (SA). Other mobile secondary elicitors that are produced as a result of elicitor recognition or wounding are systemin, the jasmonates (JA) and ethylene. Ca^{2+}, H_2O_2, SA, JA and ethylene all contribute to the transcriptional activation of defense-related genes. Many aspects of the pathways by which these signalling compounds are transduced intracellularly have not yet been elucidated, but are certain to involve phosphorylation cascades. "Cross-talk" exists between pathways, with one signal influencing the production and actions of the other(s).

The establishment of LAR is typically associated with hypersensitive cell death (HR). The relationship between the activation of defense-related genes and localized cell death is, however, not entirely clear. The HR is accepted to be a form of programmed cell death (PCD), and is mediated by the release of cytochrome c (cyt c) from the mitochondria.

The establishment of localized resistance leads to the activation of systemic acquired resistance (SAR) through the translocation of mobile signals to tissues unaffected by the initial infection. Systemin and SA have been shown to be translocated, but the induction of SAR is believed to require at least one other, as yet unknown, mobile signal. Defence mechanisms induced to achieve LAR are also employed in the expression of SAR. Intracellular signalling pathways in remote tissues are proposed to resemble those in the originally infected tissue, and definitely rely on phosphorylation cascades.

This overview is a generalized and simplified one. It was compiled from investigations of a wide variety of plant-pathogen interactions, using bacterial, viral and fungal pathogens and/or chemical defense elicitors, intact plants and/or cell cultures, as well as mutant and transgenic plants. Not all of the interactions and relationships between the components of inducible plant defense depicted here are applicable to every plant-pathogen combination.

Other abbreviations: ABA = abscisic acid, Hrp = Hrp type III secretion system, LOX = lipoxygenase, GST = glutathione-S-transferase, PARP = poly(ADP-ribose) polymerase, PGIPs = polygalacturonidase inhibiting proteins, PL = phospohlipase, PT = permeability transition pore, SAG = salicylic acid glucosides, SOD = superoxide dismutase, X = inhibitory action.

re-inforcement, the expression of a diverse range of pathogenesis-related (PR) proteins, and hypersensitive cell death (the HR) (Lamb et al. 1989, Schneider et al. 1996). Each of these responses involves changes in the expression of numerous genes, the products of which effect the biochemical changes associated with each response.

b. Diverse plants deploy the same inducible defense mechanisms in response to pathogen-specific and/or non-specific signals and/or wounding (Kombrink et al. 1993, Heath 2000). This "non-specificity of effect" is the result of evolutionary pressures on plants to achieve incompatibility (see section 2.1; Paul et al. 2000).

c. Not all inducible defenses are mobilized in every response, and different plant-pathogen interactions induce different expression patterns of the genes associated with a particular response (Heath 2000). This "specificity of response" suggests that plants have the capacity to distinguish between different threats (i.e. attack by different pathogens or wounding) and may "fine-tune" different mechanisms through "cross-talking" signalling pathways to deal with each challenge (Reymond and Farmer 1998, McDowell and Dangl 2000, Paul et al. 2000).

d. Defense mechanisms induced to achieve localized resistance are also employed in the expression of SAR. Again, the expression patterns of the genes associated with each mechanism differ from one plant-pathogen interaction to another. As a result, the time required to establish SAR, the level and extent of protection, and the range of pathogens to which protection is afforded vary considerably between different combinations of plants and pathogens involved in the initial infection (Schneider et al. 1996).

e. Differences in the spatial and temporal patterns of gene expression during compatible and incompatible interactions, localized and systemic responses, and/or responses induced by pathogens or wounding are determined by signalling molecules and pathways. The magnitude, nature and extent of a response depends on the perception of the primary signal and the subsequent ability to induce secondary signalling within the infected tissue and throughout the rest of the plant (Lamb et al. 1989, Ebel and Cosio 1994, McDowell and Dangl 2000). Differential regulation of varying subsets of defense-related genes by signalling molecules might be explained by differences in regulatory elements within their promoters (Maleck and Dietrich 1999).

f. The expression of a number of inducible defense-related genes (e.g. those encoding enzymes of the phenylpropanoid pathway, peroxidases, chitinases and glucanases and hydroxyproline-rich glycoproteins; HRGPs) are also developmentally regulated in healthy plants. This may be correlated with a build-up of general, non-specific resistance as the plant matures (Bowles 1990, Dixon and Lamb 1990).

Three stages in the establishment of plant resistance during an incompatible interaction may be identified: (i) the perception of primary signals (elicitors), (ii) the establishment of localized resistance (through the expression of defense molecules and/or hypersensitive cell death), and (iii) the induction of systemic acquired resistance (Kombrink and Somssich 1995; see Fig. 2-3). Each of these stages is briefly discussed in the subsections that follow.

The generation and transduction of inter- and intracellular signals that connect elicitor perception, LAR and SAR are infinitely complex and the aspect of plant defense that is probably least understood. Extensive efforts, many of which involved mutant and/or transgenic plants, have been made in recent years to integrate knowledge on plant defense-associated signal transduction. An overview of this topic falls outside the scope of this study, and the reader is referred to reviews by Lamb et al. 1989, Dixon and Lamb 1990, Scheel and Parker 1990, Dixon et al. 1994, Ebel and Cosio 1994, Boller 1995, Benhamou 1996, Baker et al. 1997, Blumwald et al. 1998, Reymond and Farmer 1998, Maleck and Dietrich 1999, Pieterse and Van Loon 1999, McDowell and Dangl 2000). Key aspects of signal generation and transduction during the establishment of plant resistance are, however, highlighted in Fig. 2-3 and in some of the subsections that follow.

Current knowledge of plant defense phenomena has been accumulated through the analysis of plant responses to a whole range of threats. The discussions that follow are, however, limited to information relevant to the interaction between plants and the Gram-negative, Hrp^+ bacterial phytopathogens as far as possible.

2.3.2 Plant perception of primary signals (elicitors)

"Elicitors" are broadly defined as signals capable of inducing structural and/or biochemical responses associated with the expression of plant disease resistance. Plant resistance may therefore be seen as the result of localized and systemic effects of the parallel and/or

sequential induction of a whole array of defense responses by elicitors* (Ebel and Cosio 1994, Benhamou 1996).

Elicitors of plant defense responses are classified as exogenous or endogenous. **Exogenous elicitors** originate in the pathogen, appear to have a limited mobility within plant tissues, and evoke a response in the immediate vicinity of the infected cells. As such, they are considered the primary signals in plant defense induction. **Endogenous elicitors**, on the other hand, originate in plant cells as a result of pathogen attack and determine the extent and nature of the response in the surrounding tissue (Ebel and Cosio 1994).

2.3.2.1 Exogenous elicitors

Exogenous elicitors vary widely in their chemical nature. All of them are, however, either structural components of pathogen surfaces or secreted pathogen metabolites which are released during normal pathogen growth or during interactions with plants (Dixon et al. 1994, Ebel and Cosio 1994). According to the discussion in section 2.1, exogenous elicitors are further subdivided into non-specific and pathogen-specific elicitors.

Non-specific exogenous elicitors

Per definition, non-specific elicitors elicit non-specific or non avr-R mediated plant defense responses. A number of non-specific exogenous fungal elicitors, most of which are proteins, glycoproteins, glucans, and chitosan- and chitin-derived oligosaccharides, have been characterized (reviewed in Yoshikawa et al. 1993, Ebel and Cosio 1994). To date, the only non-specific elicitors isolated from bacteria are the **harpins** and **flagellin** (Heath 2000).

Harpins play an as yet unknown role in the pathogen-specific, avr-R mediated elicitation of the HR in incompatible interactions when secreted by Hrp$^+$ bacteria. However, when harpins are injected into plants in their purified form, they elicit the HR (i.e. localized defense) in a non-specific (avr-R independent) manner. The mechanism by which this is accomplished, is also still unknown†.

---

* Elicitors are regarded as "signals", rather than direct physiological effectors (Ebel and Cosio 1994). "Abiotic elicitors" such as UV damage, toxic compounds and chemicals (see Bell 1981, Dixon 1986) are excluded from this definition, and from subsequent discussions.

† See section 2.2.2.2 for detailed discussions and references.

Flagellin subunits make up the filament of eubacterial flagella. Synthetic peptides comprising 15 to 22 amino acids of the most highly conserved domain within the N-terminal of flagellin were recently shown to elicit defense responses in cell cultures of several plant species. This constituted the first report of a universal, non-specific bacterial elicitor (Felix et al. 1999).

The term "non-specific elicitor" does not imply a non-specificity in the plant's **perception** of these signals (Ebel and Cosio 1994). Very specific receptor-like binding sites for non-specific elicitors from fungi and yeast have been identified on plant plasma membranes (reviewed in Scheel and Parker 1990, Boller 1995), and predict that non-specific bacterial elicitors may also be recognized by specific receptors*. Although plant receptors for harpins and flagellin have not yet been identified, some progress towards elucidating the manner in which plants perceive these signals has been made:

a. Two lines of evidence suggest that harpin function is cell-wall associated (discussed in section 2.2.2.1).

b. A dominant flagellin-sensitive locus (*FLS-1*) in *Arabidopsis* has been mapped to a region of chromosome 5 that contains a cluster of *R* genes. This has raised the question whether non-specific elicitors may be perceived in a fashion similar to the avr-R interaction that triggers pathogen-specific resistance (Gómez-Gómez et al. 1999).

Pathogen-specific exogenous elicitors

Pathogen-specific exogenous elicitors are, per definition, the products of bacterial *avr* genes. The first bacterial *avr* gene (*avrA* or *avrPgyA*) was cloned in 1984 through a shotgun approach. This was based on the conversion of a virulent race of *P. s.* pv. *glycinea* to an avirulent one† through the transconjugation of cosmid clones containing the DNA of the avirulent race. Clones that carried the *avr* locus were screened for by

---

* A recent study involving elicitors from yeast and commercial enzyme preparations used to make plant protoplasts suggested that these elicitors interacted directly with lipid bilayers to form conductive pores. Some non-specific elicitors may therefore not function via a receptor-based mechanism (Klüsener and Weiler 1999).

† The terms "virulent" and "avirulent" bacteria are used throughout the literature (in a somewhat confusing manner) in the context of the gene-for-gene hypothesis. "Virulent" refers to the **absence** of an *avr* gene that may confer incompatibility. The **presence** of such a gene in a pathogen interacting with a plant that expresses a corresponding *R* gene, renders the pathogen "avirulent" on that particular plant. It was in this context that "avirulence" genes were originally defined as negative virulence factors through their ability to diminish or abolish the induction of disease symptoms by a pathogen (refer to section 2.1; Sigee 1993, Dangl 1994).

incompatibility with soybean leaves carrying a corresponding resistance gene (Staskawicz et al. 1984). Since then, over 30 bacterial *avr* genes have been cloned. The reader is referred to references in section 2.2.2.3 for reviews of origin and biochemical characteristics of these genes and their protein products. For the discussion that follows, it is important to note that two types of *avr*-related elicitors exist (Bent 1996, Hammond-Kosack and Jones 1997):

a. *"Indirect" avr gene products*. This group is represented by the syringolide elicitors produced by *avrD*-encoded enzymes. The syringolides, and not the AvrD protein, induce plant defense responses in plants carrying the *Rpg4* resistance gene (see Dangl 1994 for references).

b. *The* avr *gene product itself*. Known examples of these are proteins encoded by the *avrPto*, *avrB* and *avrBs3* families. The latter family is of special interest as they encode proteins with a variable number (13.5 to 17.5) of serial repeats of highly similar 34-amino acid motifs, which have been identified as nuclear localization signals (Leach and White 1996, White et al. 2000).

Avr proteins have been proposed to be perceived by plants through their interaction with the products of (disease) resistance (*R*) genes. The cloning of *R* genes was substantially retarded by the fact that no functional assays for *R* gene function existed. Efforts in this regard had to rely on restriction fragment length polymorphism (RFLP) and other marker analysis, positional cloning and transposon tagging (Lamb et al. 1989, Lamb 1994, Bent 1996), and led to the cloning of the first *avr* gene-specific *R* gene (*Pto*) from tomato in 1993 (Martin et al. 1993).

Endeavours to clone plant *R* genes were accompanied by much speculation about the nature of their products and how they interact with pathogen-specific elicitors to elicit plant defense responses. The most popular model proposed that *R* genes encode components of signalling pathways, which were most likely receptors (Keen 1982, Gabriel and Rolfe 1990).

R genes have now been cloned from a variety of plants and have been shown to confer resistance against a diverse range of phytopathogenic fungi, bacteria, viruses and nematodes (reviewed in Dangl 1995, Bent 1996, Baker et al. 1997, De Wit 1995, 1997; Gebhardt 1997, Hammond-Kosack and Jones 1997, Parker and Coleman 1997, Ellis et al. 2000, Young 2000). The salient findings from these studies are the following:

a. Sequence analysis of predicted *R* gene products revealed a low degree of overall sequence similarity, but the presence of a common structural framework that is represented by a limited number of motifs in R proteins from diverse origins and pathogen specificity*. These motifs are: leucine rich repeats, a nucleotide binding site (P loop), a serine/threonine protein kinase domain, a transmembrane domain, a leucine zipper†, and a sequence similar to the cytoplasmic domains of the *Drosophila* Toll and mammalian interleukin-1 receptor (IL-1R) proteins. The organization of these motifs has provided some clues to the possible location of different R proteins (Fig. 2-4) and suggest that they may function as receptors and/or components of signal transduction pathways.

b. Several *R* genes have been shown to function heterologously in plant species. This suggests that Avr-R triggered signalling cascades through which plant defense responses are induced, are conserved between plant species.

c. Low abundance transcripts of some *R* genes have been observed in unchallenged plants. A rapid response to pathogen attack predicts the expression of *R* genes in healthy plants in the absence of a corresponding *avr* gene-expressing pathogen. It is uncertain whether the levels of *R* gene expression increase at the site of infection.

d. *R* genes are often clustered on chromosomes in regions that may be highly recombogenic. This predicts a capacity to evolve rapidly and correlates with the role of R proteins as receptors for *avr* gene products, which may be horizontally transferred between co-evolving populations of plant pathogens (see section 2.2.2.3).

e. Direct interaction between a bacterial avr gene product and a plant *R* gene product has only been demonstrated for the *avrPto-Pto* pair (Tang et al. 1996, Scofield et al. 1996). A number of other proteins involved in this interaction have subsequently been

---

* The predicted proteins of two cloned *R* genes do not share this common structural framework:

(i) The product of the maize *hm1* gene, which confers resistance to the necrotrophic fungus, *Cochliobolus carbonum*. *Hm1* encodes a NADPH reductase, which is involved in detoxification of the toxin produced by this fungus (Johal and Briggs 1992). As such, *hm1* is functionally distinct from the classical *avr* gene-dependent *R* genes, and its unique character may be related to the fact that plants employ different defense strategies against necrotrophic and biotrophic pathogens (Hammond-Kosack and Jones 1997).

(ii) The product of the barley *Mlo* gene. This gene confers broad spectrum resistance against the powdery mildew fungus, *Erysiphe graminis*. The Mlo protein is predicted to be membrane-anchored by at least six membrane-spanning helices (Büschges et al. 1997).

† More recently identified as members of a broader group of structural elements, the coiled-coiled (CC) structures (Young 2000).

R gene(s)	Isolated from	Associated pathogen	(Type)
Cf-2*, *Cf-4*, *Cf-5*, *Cf-9	Tomato	*Cladosporium fulvum*	(Fungus)
Xa-21	Rice	*Xanthomonas oryzae* pv. *oryzae*	(Bacterium)
HS1^{pro1}	Sugar beet	*Heterodera schachtii*	(Nematode)
Pto	Tomato	*Pseudomonas syringae* pv. *tomato*	(Bacterium)
Prf	Tomato	"	"
RPM1	*Arabidopsis*	*P. s.* pv. *maculicola*	(Bacteria)
RPS2		*P. s.* pv. *tomato*	
N	Tobacco	Tobacco mosaic virus	(Virus)
L6, M	Flax	*Melampsora lini*	(Fungus)
RPP5	*Arabidopsis*	*Peronospora parasitica*	(Fungus)
***IC2-1* (I_2)**	Tomato	*Fusarium* spp.	(Fungi)

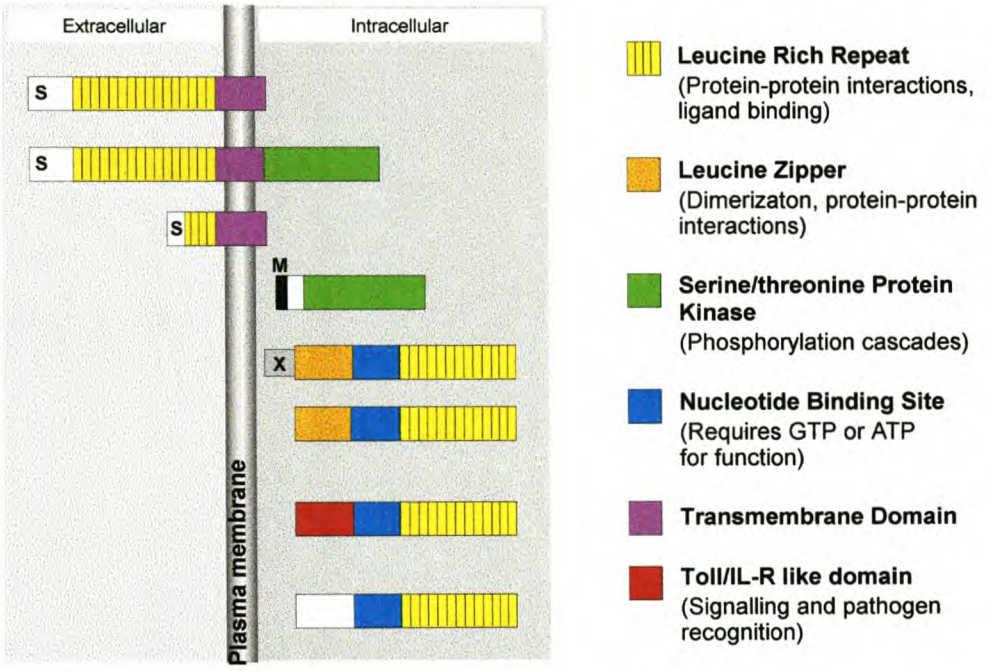


Fig. 2-4. Structural organization of the predicted proteins encoded by cloned plant *R* genes (compiled from Gebhardt 1997, Parker and Coleman 1997, Hammond-Kosack and Jones 1997).

The name, plant of origin and pathogen associated with each of the cloned *R* genes are given in the table on the left. *Prf* is not a *R* gene, but encodes a protein shown to be required for Pto function. Predicted R proteins are shown in their N → C orientation (left to right) relative to the plasma membrane, and are not drawn to scale (see Hammond-Kosack and Jones 1997 for precise lengths of proteins and stretches of homology). The location of each protein has been deduced from common structural motifs. Each of these motifs is indicated in a different colour and the role/function that it probably confers to the R protein in the recognition of elicitors and/or signal transduction is as indicated. **M** = a possible myristoylation site (membrane anchor) in Pto (not required for resistance to *P. syringae*). **X** = a 720 amino acid N-terminal extension of Prf that shows no homology with any known protein. **S** = putative signal peptides, indicating transmembrane transport. The L6 N-terminus contains a putative signal anchor sequence, which suggests that the protein may enter, but not pass, the secretory pathway.

identified. This has led to the model that Avr-R recognition* leads to the transcriptional activation of defense-related genes via the activation of transcription factors through a short phosphorylation cascade (Zhou et al. 1997, De Wit 1997, Parker and Coleman 1997).

2.3.2.2 Endogenous elicitors

Endogenous elicitors have a secondary function in the elicitation of plant defense responses. They are components of the intra- and intercellular (systemic) signal transduction system of plants which are formed in response to primary elicitor recognition (Ebel and Cosio 1994, Fritig et al. 1998).

Pectic oligosaccharides derived from the enzymatic degradation of plant cell walls by invading bacteria and fungi were the first group of endogenous elicitors to be characterized (Nothnagel et al. 1983, Davis and Hahlbrock 1987). Oligogalacturonides† were initially recognized for their ability to elicit the synthesis of phytoalexins, but later shown to also induce the production of lignin-like compounds and proteinase inhibitors (reviewed in Ebel and Cosio 1994). Despite their apparent versatility in the elicitation of plant defense responses, the limited mobility of these elicitors within plant tissues (Baydoun and Fry 1985) suggested that they act as second messengers in the perception of exogenous elicitor signals to mediate localized resistance, rather than to induce systemic responses (Lamb et al. 1989, Yoshikawa et al. 1993, Boller 1995).

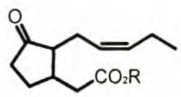
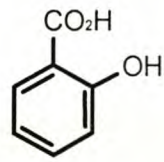
Research has subsequently focused on the identification of transmissible signal compounds, that are synthesized by plants, increase systemically in response to pathogen attack, move throughout the plant, and induce defense mechanisms in remote tissues at low concentrations. Four such compounds have been identified to date: **salicylic acid**, **systemin**, **jasmonic acid** (and its volatile derivative methyl-jasmonate) and **ethylene** (Table 2-2).

---

* The results from other Avr-R studies have been interpreted to indicate that initial recognition of Avr elicitors may utilize proteins other than the product of *R* genes, and that the latter serves another role in the transduction of the recognition signal (Ji et al. 1998).

† Longer oligogalacturonides are better inducers of defense responses. Plant polygalacturonase-inhibiting proteins (PGIPs) have been shown to retard the hydrolysis of polypectate by pathogen enzymes. This results in the prolonged existence of longer oligomers, whereby the potential activity of endogenous elicitors released during cell wall degradation is increased. PGIPs accumulate in response to wounding, abiotic elicitors and pathogen infection, suggesting that they should be included among the known classes of inducible defense-related cell wall proteins (reviewed in Dixon and Lamb 1990, Benhamou 1996).

Table 2-2. The structure, origin and function of mobile endogenous elicitors (compiled mainly from Taiz and Zeiger 1991, Ebel and Cosio 1994, Baron and Zambryski 1995, Benhamou 1996, Hunt and Ryals 1996, Schneider et al. 1996, Reymond and Farmer 1998, Maleck and Dietrich 1999, Pieterse and Van Loon 1999, Paul 2000).

Elicitor	Structure	Origin / Biosynthetic pathway	Associated with[a]			
			Wounding	Pathogen attack	LAR	SAR
Systemin	18 amino acid polypeptide: NH_3-**AVQSK PPSKRDPPK MQTD**-COOH	Post-translationally processed from the C-terminal of a 200 residue prosystemin precursor in response to wounding	**yes**	**no**	yes	**yes**
Jasmonic acid[b] (JA)		Wounding induces a lipid based signalling pathway through which linolenic acid is released from plasma membrane lipids. JA is synthesized from linolenic acid, through β-oxidation involving a lipoxygenase	**yes**	yes	**yes**	yes
Ethylene	$CH_2=CH_2$	A gaseous plant hormone derived from methionine via the action of 1-aminocyclopropane-1-carboxylate (ACC) synthase. Synthesis is promoted by fruit ripening, flowering, wounding, infection and other stresses	**yes**	yes	yes[c]	yes[c]
Salicylic acid[d] (SA)		Derived from the general shikimate-phenylpropanoid pathway. Phenylalanine is converted to cinnamic acid, then to *ortho*-coumaric acid or benzoic acid. Either of these may be converted to SA, but the latter appears to be the most important route[e] *(see Fig 2-6)*	**no**	**yes**	**yes[f]**	**yes[f]**

[a] **Boldface** entries designate consensus in the literature, whereas non-boldface entries indicate weak, conflicting and/or new evidence or notions. Increasing evidence suggest that there is considerable cross-talk between signalling pathways mediated by ethylene, JA and SA. Antagonistic interactions include inhibition of the synthesis of one signal by another, and suppression of the responses elicited by one signal by another. In contrast, evidence of synergism between signals and the co-regulation of signalling pathways also exist.

[b] Methyl-jasmonate (a volatile derivative of JA), as well as oxo-phytodienoic acid (the octadecanoid precursor of JA) and dinor-oxo-phytodienoic acid (a hexadecanoid regulator) are also implicated in signal transduction.

[c] Current evidence strongly suggests that ethylene modulates the expression of LAR and SAR, but that it is not a systemic signal for SAR.

[d] The volatile SA derivative methyl salicylic acid (MeSA) may act as an airborne signal for SAR.

[e] The intracellular concentration of free SA is controlled by conversion to inactive β-O-D-glucosyl conjugates. This pool of conjugates may be able to rapidly provide free SA.

[f] A large body of evidence (much of which has been derived from studies with mutant and transgenic plants overexpressing the bacterial *nahG* gene, which converts SA to the non-signalling compound catechol; Gaffney et al. 1993, Delaney et al. 1994, Ryals et al. 1995) indicates that SA is required for the establishment of both LAR and SAR. Despite the fact that a substantial fraction of SA present in SAR-expressing leaves appears to be transported from inoculated leaves, whereas the rest is synthesized *de novo*, the collective results from many studies suggest that SA is not the primary translocated signal between infected and remote tissues. The as yet unknown systemic signal seems to be released without the accumulation of SA, but SA accumulation appears to be required for its transduction in remote tissues.

2.3.2.3 Elicitation of plant defenses by wounding

Wounding of plants by herbivores or abiotic stresses induces responses that strongly resemble those induced by pathogen attack. Observed responses include the synthesis of proteinase inhibitors (PINs), the production of phytoalexins and phenolic compounds through the induction of the phenylpropanoid biosynthetic enzymes, the expression of chitinases, HRGPs, peroxidases and lipoxygenases. These apparent similarities between plant responses to wounding and pathogen attack suggest that one or more common signal transduction pathways may be active in both phenomena (Baron and Zambryski 1995).

Jasmonic acid and ethylene are proposed to act synergistically to induce wounding response genes, and to stimulate the biosynthesis of each other. Systemin appears to play an important role upstream of JA and ethylene in wounding response signalling, as it seems to control the endogenous levels of both compounds (Schneider et al. 1996, Maleck and Dietrich 1999). Abscisic acid (ABA), electrical and hydraulic signals have also been implicated in the wounding response (Peña-Cortés et al. 1995, Sticher et al. 1997). One important difference between the elicitation of plant defense responses by wounding and pathogen attack is that the former does not induce hypersensitive cell death (Baron and Zambryski 1995).

2.3.3 Inducible defense mechanisms involved in localized acquired resistance

Plant recognition of exogenous elicitors (and wounding) triggers a series of metabolic events which lead to the induction of defense mechanisms, first locally and then systemically. This involves the rapid transcriptional activation of defense-related genes as part of a massive switch in the pattern of host gene expression (Lamb et al. 1989)*. Five prominent inducible mechanisms have been identified through investigations of plant responses to elicitors, pathogens and wounding, using intact wild-type, mutant and transgenic plants, as well as plant cell cultures. The salient features of each of these mechanisms are outlined in the following subsections.

---

* Two examples of inducible plant defenses that are **not** associated with altered gene expression are known. Both are associated with cell wall reinforcement (see section 2.3.3.3): (i) the production of callose, which involves the stimulation of pre-existing callose synthase, and (ii) the deposition of a proline-rich cell wall protein, which appears to involve elicitor-stimulated insolubilization of pre-existing soluble precursors (Dixon et al. 1994).

2.3.3.1 Generation of active oxygen species (AOS): the "oxidative burst"*

The generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) was reported in 1983 by Doke as a novel plant defense response, distinct from other classical transcription-dependent responses (Doke 1983). The rapid generation of active oxygen species (AOS) has since been described as a characteristic feature of the HR in many plant-pathogen interactions. This "oxidative burst" involves a massive activation of oxidative metabolism, reminiscent of that following neutrophil activation in the mammalian immune system, and is currently regarded as one of the earliest responses to pathogen attack (Dixon et al. 1994, Lamb and Dixon 1997). Research on the oxidative burst has been reviewed extensively in Sutherland 1991, Dixon et al. 1994, Baker and Orlandi 1995, Tenhaken et al. 1995, and Lamb and Dixon 1997. The key features and functions of this mechanism in plant defense are the following:

a. The accumulation of AOS in plants inoculated with bacterial pathogens exhibits two-phase kinetics (Fig. 2-5). Phase I comprises a weak, transient oxidative burst that is observed in both compatible and incompatible interactions, and is regarded as a non-specific response. Phase II is a massive and prolonged burst which is only observed in incompatible interactions. It is a pathogen-specific response, which induces hyper- sensitive cell death in a manner dependent on Hrp-mediated avr-R interaction.

b. A plasma membrane NADPH-dependent oxidase appears to be the main producer of AOS during the oxidative burst†. The oxidase‡ converts O_2 to O_2^-, which is released extracellularly. This is followed by the dismutation to H_2O_2, spontaneously at neutral/slightly acidic pH, or catalyzed rapidly by the enzyme superoxide dismutase (SOD). Hydroxyl radicals (•OH) are believed to be generated by Fe^{2+}-containing enzymes (via the Haber-Weiss reaction). The reactivity and stability of these AOS decrease in the order •OH > O_2^- > H_2O_2. H_2O_2 is the most diffusable and major accumulating AOS. The involvement of the hydroxyl radical in plant-pathogen interactions is difficult to monitor, as it is highly reactive and has a half-life in the range of milliseconds.

---

* Also referred to as **reactive oxygen intermediates** (ROIs).

† Some evidence points to a contribution of other H_2O_2-producing enzymes, such as peroxidases and lipoxygenases, particularly in the maintenance of high levels of AOS during phase II of the oxidative burst.

‡ Reaction: $2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$. O_2^- exists in the plant in equilibrium with its conjugated acid, the hydroperoxyl radical (•O_2H).

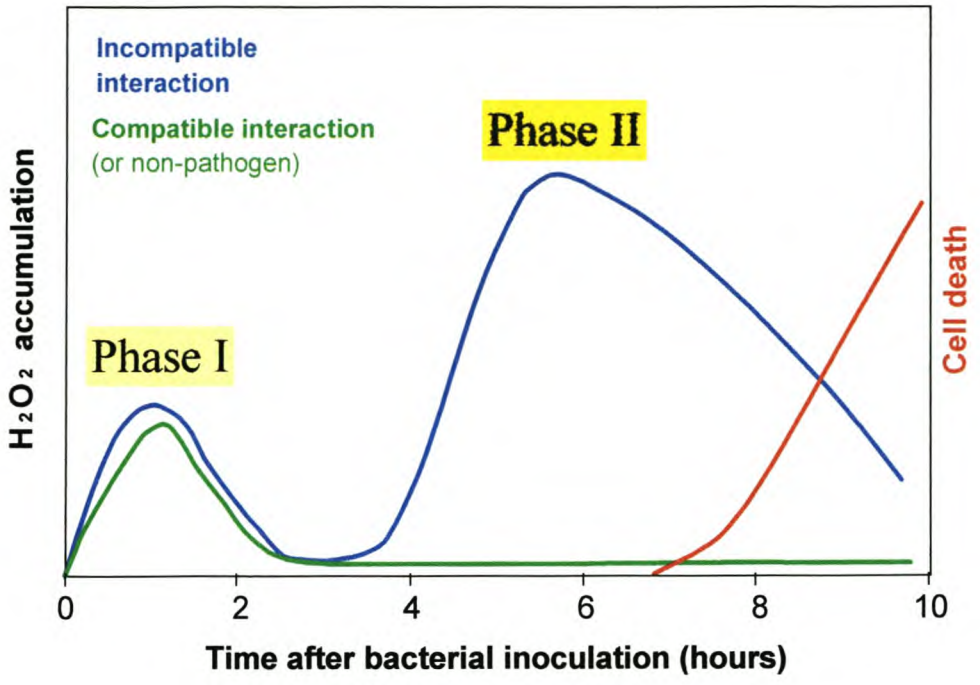


Fig. 2-5. Kinetics of the accumulation of H_2O_2 and induction of cell death in plant cells following bacterial inoculation (from Lamb and Dixon 1997).

The events which follow elicitor recognition and lead to the activation of the NADPH-dependent oxidase are not yet fully understood. Ca^{2+} influx and H^+ influx/K^+ efflux have been shown to be important in the activation of the oxidative burst, and signalling is proposed to be mediated by G-proteins and phosphorylation cascades involving a number of protein kinases.

c. AOS generated during the oxidative burst is proposed to have three functions in plant defense: (i) direct antimicrobial activity, (ii) cell wall reinforcement (directly through the cross-linking of pre-existing tyrosine- and (hydroxy)proline-rich cell wall proteins and indirectly through the role of AOS in lignin formation; see section 2.3.3.3), and (iii) involvement in the transcriptional activation of some defense-related genes (only H_2O_2).

The role of H_2O_2 as a second messenger in the transcriptional activation of defense-related genes remains controversial*. It has been implicated in the indirect activation of phytoalexin production, the production of JA from linolenic acid (by acting as a substrate for lipoxygenase), the production of SA (through stimulation of the

* H_2O_2 is proposed to effect transcriptional activation through the modulation of redox-sensitive transcription factors, akin to the activation of NF-κB in the human immune and inflammatory responses, but this remains to be demonstrated.

benzoic acid 2-hydrolase enzyme), and the Ca^{2+}-dependent mediation of hypersensitive (programmed) cell death*. Additionally, it has been implicated in the induction of SAR through a network of oxidative microbursts (Alvarez et al. 1998). In both the HR and SAR, H_2O_2 is proposed to function upstream of SA.

H_2O_2 also been shown to activate cellular protectant enzymes such as glutathione-S-transferase (GST), glutathione peroxidase and polyubiquitin. These enzymes destroy AOS, block oxidant-mediated programmed cell death, and detoxify lipid hydroxyperoxides generated by AOS (GST only). H_2O_2 generated during the oxidative burst may thus act both as a local trigger for cell death in infected cells, and also as a diffusable signal for the induction of cellular protectants to block oxidant-mediated programmed cell death in surrounding cells. As such, it would promote the strict spatial limitation of the HR, and help to maintain the capacity of surrounding cells to deploy transcription-dependent responses.

d. H_2O_2 is detoxified by catalase† and various peroxidases, including ascorbate and glutathione peroxidase. Peroxidative reactions that utilize phenolic compounds play an important role in cell wall reinforcement through formation of lignin (see section 2.3.3.3). Recently, the suppression of detoxification enzymes during HR development was demonstrated to play an important role in pathogen-induced programmed cell death (Mittler et al. 1999).

2.3.3.2 Phytoalexin production

The accumulation of phytoalexins was the first defense-related response to be identified and assayed in plants (Muller 1961 and references therein). Since then it has been applied in numerous studies as a simple assay for defense gene activation, by measuring the activity of the first enzyme in the phenylpropanoid phytoalexin biosynthetic pathway‡, phenylalanine ammonia lyase (PAL) (Scheel and Parker 1990).

---

* Increasing evidence points to the HR as a form of programmed cell death, analogous to animal cell apoptosis. This notion is discussed in more detail in subsection 2.3.3.5 . O_2^- has also been implicated in hypersensitive cell death, through its peroxidation of membrane lipids.

† A salicylic acid binding catalase has been identified. SA inhibits the H_2O_2 scavenging actions of this catalase in tissues immediately surrounding the site of pathogen infection. The subsequent increase in H_2O_2 accumulation (which again stimulates SA production) is proposed to provide a positive feedback loop for the amplification of H_2O_2 action in LAR, but not in SAR (Neuenschwander et al. 1995, Hunt and Ryals 1996).

‡ The phenylpropanoid pathway also leads to the formation of lignin (see next section).

More than 350 phytoalexins have been characterized from a variety of organs and tissues of approximately 30 plant families. The structure, biosynthesis, distribution and function in plant defense of these "low molecular weight, antimicrobial compounds produced by plants in response to infection or stress" has been the focus of plant resistance studies for many years, and the topic of regular reviews over the past 40 years (e.g. Cruickshank 1963, Kuc 1972, 1995; Bell 1981, Dixon 1986, Dixon and Harrison 1990, Paxton et al. 1994). The most important aspects of phytoalexin production as an inducible plant defense response are the following:

a. Although phytoalexin accumulation has been reported as a consequence of plant infection by viral, fungal and bacterial pathogens and nematodes, it is not a unique feature of pathogen infection. More than 200 compounds (of diverse biological origin, organic and inorganic nature, including some fungicides), micro-organisms and physiological stresses (such as low temperatures, UV radiation and wounding) have been reported to elicit phytoalexin accumulation. This has led to the notion that any cause of metabolic perturbation generally results in the accumulation of phytoalexins.

b. Phytoalexins are lipophilic compounds of very diverse structure. Some consist only of carbon, hydrogen and oxygen, whereas others contain nitrogen and sulphur as well. As a result of this structural diversity, no obvious relationship between structure and antimicrobial activity exists. The distribution of different classes of phytoalexins between plant families, and within plants of the same family is as diverse.

c. Phytoalexin precursors common to all plants are derived from one (or a combination) of three major biosynthetic pathways responsible for the synthesis of vital housekeeping compounds in all plants*: the shikimate-phenylpropanoid, acetate-malonate and acetate-mevalonate pathways. An overview of these pathways are shown in Fig. 2-6.

d. Phytoalexins rapidly accumulate in the immediate vicinity of pathogen infection sites. Substantial evidence exist that this is associated with *de novo* synthesis of mRNA and biosynthetic enzymes. Transcriptional activation of genes encoding some phenylpropanoid biosynthetic enzymes, including PAL, chalcone synthase (CHS), chalcone isomerase (CHI) and cinnamyl-alcohol dehydrogenase (CAD) has been observed

---

* All of the phytoalexins characterized from the Brassicaceae family (which includes the model plant *Arabidopsis thaliana*) are indole derivatives, which are derived from the shikimate-tryptophan biosynthetic pathway (see Fig. 2-6). The best characterized example of this indole group of phytoalexins is camalexin, the major phytoalexin produced by *Arabidopsis* (Tsuji et al. 1992, Zook and Hammerschmidt 1997).

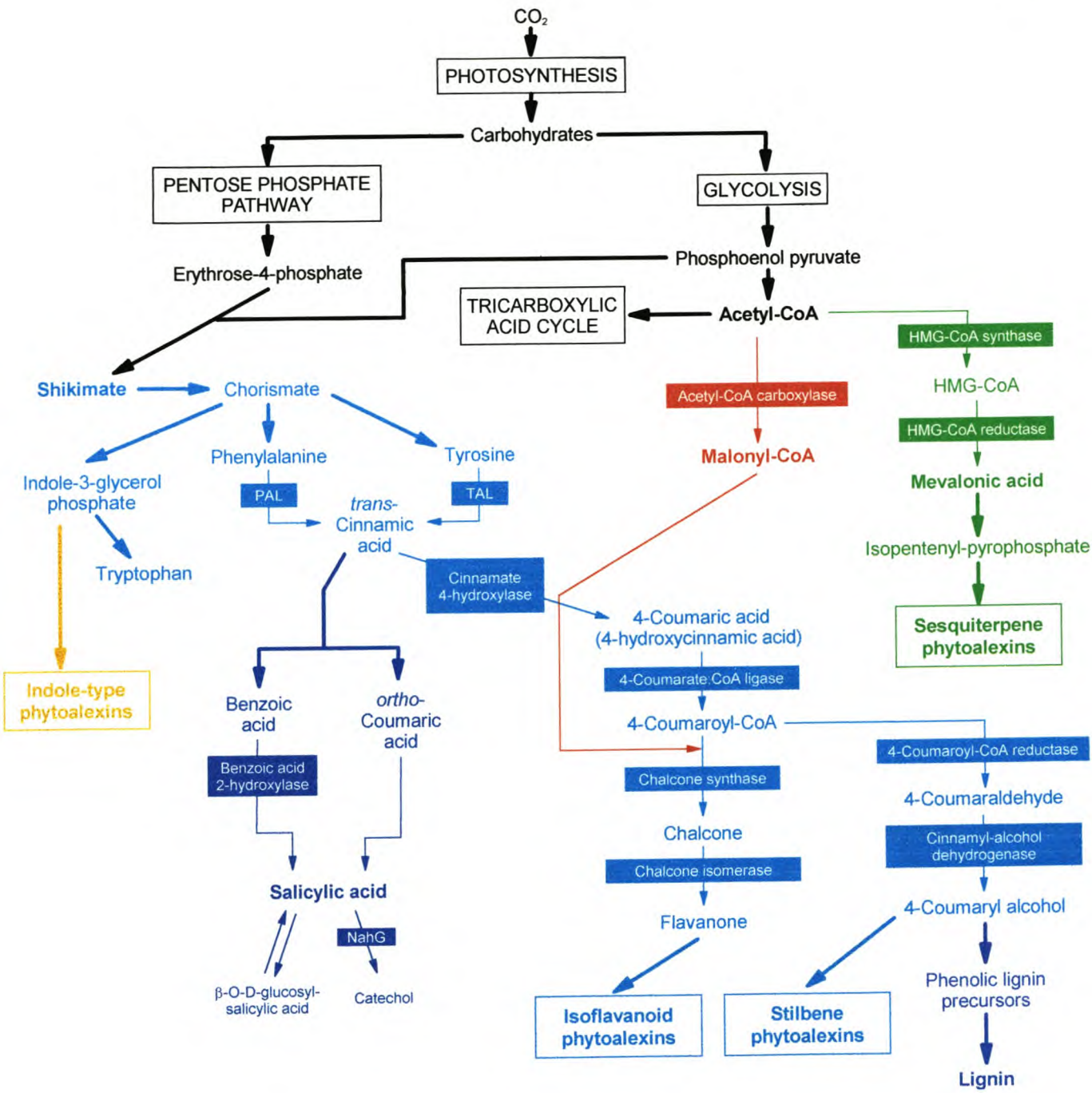


Fig. 2-6. Overview of phytoalexin biosynthetic pathways (compiled from Hahlbrock 1981, Hahlbrock and Scheel 1989, Lamb et al. 1989, Taiz and Zeiger 1991, Zhao and Last 1996).

The three major phytoalexin biosynthetic pathways: the shikimate-phenylpropanoid pathway (blue), acetate-malonate pathway (red) and acetate-mevalonate pathway (green), are shown in relation to some primary metabolic pathways (black). Biosynthesis of the *Brassica* indole group of phytoalexins is indicated in yellow. Only key intermediates and enzymes of each phytoalexin biosynthetic pathway are shown. Thin arrows indicate single reactions, whereas thick arrows represent multiple steps. The biosynthesis of other key products of the phenylpropanoid pathway involved in plant defense (lignin and salicylic acid) are also shown.

Abbreviations: CoA = coenzyme A, HMG = β-hydroxy-β-methyl glutaryl, NahG = salicylic acid hydroxylase, PAL = phenylalanine ammonia lyase, TAL = tyrosine ammonia lyase.

within 2-3 min after elicitor treatment, and represents one of the most rapid gene activation systems in plant cells in response to exogenous signals.

Some evidence, however, points to a different perspective on the rapid accumulation of phytoalexins after pathogen infection. Conjugates of some phytoalexin precursors have been shown to be constitutively expressed in large quantities in plants, and to be localized in vacuoles in some cases. The rapid accumulation of phytoalexins after pathogen infection is accordingly ascribed to the release of these precursors, obviating the need to induce the early enzymes of the biosynthetic pathways. *De novo* synthesis of phytoalexins is hence proposed to replace the loss of conjugates (Graham et al. 1990, Barz and Welle 1992)*.

e. The rapid accumulation of high levels of phytoalexins appears to be a prerequisite for a contribution to plant resistance. No obvious differentiation with regard to the rate and levels of phytoalexin accumulation can be made between resistance induced by non-specific and pathogen-specific signals. In compatible interactions, pathogens either avoid the elicitation of phytoalexin production, suppress phytoalexin accumulation, tolerate the accumulated phytoalexins or detoxify them.

2.3.3.3 Cell wall reinforcement

The plant cell wall may be seen as the first line of defense against pathogens. The cuticle, which is comprised of cutin and waxes, is a hydrophobic surface that prevents water from accumulating as a film on exposed cell surfaces and also restricts the flow of nutrients to the cell surface. Evidence that the cuticle constitutes an effective physical barrier against pathogens comes from the isolation of cutinases from several phytopathogens (Bell 1981).

Cell wall-associated defenses form an important aspect of non-specific resistance expressed by non-host plants and resistant hosts in incompatible reactions (Heath 2000). Collectively, these defenses result in reinforcement of the plant cell wall. Such reinforcement may contribute to resistance in a variety of ways. The diffusion of pathogen-produced toxins and lytic enzymes may be retarded and their efficiency reduced. Additionally, sealing of the cell wall may slow pathogen ingress prior to the deployment of

---

* VanEtten et al. (1994) proposed that low-molecular weight, antimicrobial compounds that are present in plants before challenge by micro-organisms, or are produced after infection solely from pre-existing constituents (i.e. that are not synthesized *de novo* in response to microbial infection or elicitors) be referred to as "phytoanticipins" rather than "phytoalexins".

transcriptionally dependent defenses, and trap pathogens in host cells destined to die (Tenhaken et al. 1995, Hammond-Kosack and Jones 1996, Lamb and Dixon 1997).

Cell-wall reinforcement is achieved in the following ways:

a. The cell wall is thickened by the rapid deposition of carbohydrates, particularly callose, as well as other substances such as silica, to form papillae between the cell wall and plasma membrane in and around infection sites (Bell 1981, Heath et al. 1992). Callose (a β-1,3-glucan polymer) is synthesized by the constitutively-expressed plasma-membrane-associated enzyme callose synthase, which is activated by an influx of Ca^{2+}. Because it does not require transcriptional activation, callose deposition is probably one of the earliest events following elicitor recognition (Köhle et al. 1985, Benhamou 1996, Blumwald et al. 1998). Callose deposition is particularly important in resistance to viruses and fungi (reviewed in Bell 1981).

b. Pre-existing tyrosine- and (hydroxy)proline-rich glycoproteins, as well as phenolics attached to cell wall polysaccharides, are rapidly cross-linked by AOS via peroxidase action. Hydroxyproline-rich glycoproteins (HRGPs; of which extensin is the best-known example), proline-rich and glycine-rich proteins are important structural components of plant cell walls (Cassab and Varner 1988). Increased expression of these proteins is typically associated with plant defense (reviewed in Collinge and Slusarenko 1987, Linthorst 1991, Cassab and Varner 1998, Raggi 1998). The accumulation of an mRNA corresponding to a glycine-rich *Petunia* cell wall protein has been observed within 5 min after wounding (Condit and Meagher 1987). Transcriptional activation of cell wall-associated HRGP encoding genes has been observed within 1-2 hours of elicitor treatment of bean cells, but transcripts were shown to accumulate much more slowly (Showalter et al. 1985, Lawton and Lamb 1987). Fungal elicitors and wounding have been shown to inhibit the expression of genes encoding structural cell wall proteins with low tyrosine contents and stimulate those encoding hydroxyproline-, proline- and tyrosine-rich versions (Corbin et al. 1987, Sauer et al. 1990, Sheng et al. 1991, Kawalleck et al. 1995). This is believed to increase the capacity of the wall for subsequent oxidative reinforcement.

c. Lignin and suberin are deposited around infection sites. Lignin is a complex, phenolic polymer formed mostly by the free radical condensation of hydroxycinnamyl alcohols, derived from phenylalanine and tyrosine via phenylalanine ammonia lyase (PAL) and

tyrosine ammonia lyase (TAL), respectively, through the phenylpropanoid pathway (see Fig. 2-6). It forms covalent bonds with cellulose, pectates and structural proteins when synthesized in the presence of these compounds. Lignin also forms ester linkages with fatty acid polyesters to yield suberin (Bell 1981). Both lignin and suberin are integral components of plant cell walls (Cassab and Varner 1988).

Lignin and suberin have been shown to accumulate in response to pathogen attack in plant cell walls during incompatible interactions (reviewed in Vance et al. 1980, Collinge and Slusarenko 1987). This accumulation has been observed within a day of infection (Hahlbrock and Scheel 1989). It is associated with the transcriptional activation of PAL, TAL, the phenylpropanoid biosynthetic enzymes (which produce lignin precursors) as well as anionic peroxidases (which are responsible for the H_2O_2-dependent polymerization of these precursors) (reviewed in Cassab and Varner 1988, Linthorst 1991, Kolattukudy et al. 1992).

2.3.3.4 Expression of pathogenesis-related (PR) proteins

The term "pathogenesis-related or PR protein" was first used to describe the numerous extracellular proteins that accumulated in response to tobacco mosaic virus (TMV) infection in susceptible tobacco genotypes (reviewed in Bol and Linthorst 1990). Differential PR gene induction has subsequently been associated with incompatibility in a variety of plant-pathogen interactions, and the definition of a PR protein has been extended to include intra- and extracellular proteins that are expressed *de novo* during pathological or related situations in intact plant tissue or cultured plant cells (Hammond-Kosack and Jones 1996).

The salient features of characterized PR proteins are the following:

a. PR proteins share a number of characteristic physical and chemical properties which aid in their detection and isolation. They are (i) monomers of relatively low molecular mass (8-50 kDa), (ii) selectively extractable at low pH, where they remain soluble (in contrast to the majority of plant proteins), and (iii) resistant to endogenous and exogenous proteinases. Some PR proteins are targeted extracellularly to the plasma membrane, cell wall or apoplast, whereas others are localized in compartments such as the vacuole. Several characterized PR proteins are glycosylated (Van Loon 1985, Bowles 1990, Stintzi et al. 1993).

b. PR proteins characterized from different plant families have been classified into eleven families, based mainly on molecular mass, sequence similarities and serological properties (Linthorst 1991, Van Loon et al. 1994; Table 2-3*). Members of each family have been characterized in diverse plant species.

c. Basic and acidic protein isoforms with relatively high sequence similarities have been identified within many PR protein families. The exon-intron organization of the genes encoding the different isoforms is conserved, suggesting a common ancestry. Acidic PR proteins appear to be secreted into the apoplast, whereas basic isoforms accumulate intracellularly in vacuoles. An extra C-terminal domain present in basic isoforms may be consistent with targeting to the vacuole.

   Genes encoding cytoplasmic PR proteins are rapidly activated after elicitor treatment, and it has been suggested that these PR proteins may present a first line of defense against pathogen attack. In contrast, vacuolar and extracelluar PR proteins appear to play an important role after cellular decompartmentalization has occurred, and particularly against biotrophic pathogens. Basic PR proteins show similar tissue-specific expression in roots and respond similarly to ethylene and wounding, whereas acidic PR proteins respond weakly to ethylene, are not induced by wounding and are not constitutively expressed in the roots of healthy plants (Bowles 1990, Linthorst 1991, Hammond-Kosack and Jones 1996).

d. Although some PR proteins are known to be expressed during development and in the storage organs and seeds of healthy plants, the accumulation of PR proteins is generally associated with pathogen infection, wounding or abiotic stress. Both LAR and SAR are characterized by the co-ordinated expression of several PR proteins. In the former case, PR protein accumulation is associated with hypersensitive cell death and represents the major quantitative change in protein composition that occurs during the HR. The highest concentrations of PR proteins are found at the margins of necrotizing lesions during the HR, while some PR proteins appear between lesions in inoculated leaves (Van Loon 1985, Bowles 1990, Fritig et al. 1998).

e. PR protein families and isoforms are differentially expressed during LAR and SAR. Not all families or the same members of each family are induced in each plant-pathogen

---

* A number of proteins that have been observed to accumulate in response to wounding, pathogen attack or stress, and are presumed to play a role in plant defense, have not yet been included in the formal PR protein classification. Several of these proteins are listed in Table 2-3 under "unclassified".

Table 2-3. Plant pathogenesis-related protein families (compiled from Bowles 1990, Linthorst 1991, Van Loon et al. 1994, Schneider et al. 1996, Fritig et al. 1998, Maleck and Lawton 1998).

Family	Designation	Biochemical properties/functions in plant defense
PR-1	None	Unknown (exhibits anti-fungal activity)
PR-2	β-1,3-glucanases	Endohydrolytic[a] enzymes which degrade the β-1,3-glucan component of fungal cell walls
PR-3	Chitinases	Endohydrolytic enzymes which degrade the chitin component of microbial cell walls and insect cytoskeletons[b]
PR-4	Hevein-like[c] proteins	Unknown (similar to potato wound-induced (*win*) proteins)
PR-5	Thaumatin[d]-like proteins (permatins)	Creates transmembrane pores ??
PR-6	Proteinase inhibitors[e]	Inhibits microbial serine proteinases
PR-7	Proteinases	Degrades pathogen proteins
PR-8	Chitinases	Endohydrolytic enzymes which degrade the chitin component of microbial cell walls and insect cytoskeletons
PR-9	Peroxidases	Involved in generation of AOS with direct antimicrobial activity, as well as in protecting the host through the oxidative cross-linking of cell wall proteins and the formation of lignin and suberin
PR-10	RNAses ?	??
PR-11	Chitinases	Endohydrolytic enzymes which degrade the chitin component of microbial cell walls and insect cytoskeletons
Unclassified		
Thionins		Small cysteine-rich proteins with cytotoxic activity against micro-organisms
Lectins		High-affinity carbohydrate-binding proteins with toxic activity against viruses, bacteria, fungi and insects
Polygalacturonase-inhibiting proteins (PGIPs)		Retards degradation of plant cell wall oligosaccharides by fungal enzymes, to yield larger oligogalacturonides that may act more efficiently as endogenous elicitors.
Ribosome-inactivating proteins (RIPs)		Cleaves the N-glycosidic bond of adenine in a specific ribosomal RNA sequence, thereby rendering them incapable of protein synthesis. Presumed to inhibit viral replication in this way.
α-Amylases		Hydrolytic enzymes which degrade α-amylose component of microbial cell walls
Antimicrobial peptides, e.g. defensins		Targeted to pathogen plasma membranes

[a] The hydrolysis of pathogen cell walls is implicated in the release of non-specific elicitors, especially in fungi.

[b] Some chitinases have lysozyme activity, which allows them to degrade bacterial cell wall peptidoglycans.

[c] Hevein is a small wheat germ-agglutinin-like protein from *Hevea brasiliensis*.

[d] PR-5 proteins show similarity to thaumatins, sweet-tasting proteins from the African shrub *Thaumatococcus daniellii*, and to an α-amylase/trypsin inhibitor of maize. An osmotic stress-induced plant protein, osmotin, also exhibits similarity to PR-5 proteins.

[d] Best characterized in the context of local and systemic responses to wounding caused by insect feeding.

interaction. These differential temporal and spatial expression patterns are determined by intra- and intercellular signals generated in the host plant, such as ethylene, salicylic acid and the jasmonates. PR proteins involved in SAR are not transported from infected cells, but are synthesized *de novo* in remote tissues in response to systemic signals (Van Loon 1985, Bowles 1990, Linthorst 1991).

f. A number of regulatory elements involved in the transcriptional activation of PR protein encoding genes have been identified. These include a salicylic acid responsive element in the promoters of acidic *PR-1* genes (Linthorst 1991) and the MYB sequence found in the promoter of the tobacco defense gene *PR1* (which is recognized by a MYB transcription factor associated with the regulation of survival and death in many cell types; Yang and Klessig 1996).

An ethylene responsive element (GCCGCC, the GCC-box or PR-box) has been found in the promoters of many basic *PR* genes. This conserved *cis*-acting element is recognized by two functionally homologous groups of transcription factors: the tobacco ethylene-responsive element-binding proteins (EREBPs, recently renamed as ethylene-responsive transcription factors or ERFs; Suzuki et al. 1998), and the tomato transcription factors *Pti4*, *Pti5* and *Pti6*, which have been shown to interact with the *Pto* encoded serine/threonine protein kinase (Zhou et al. 1997). Protein phosphatases and protein kinases have been shown to be involved in the elicitor-responsive, ethylene-independent signal transduction pathway that leads to the expression of some ERFs, and the subsequent GCC-box mediated activation of *PR* genes (Yamamoto et al. 1999).

More recently, the novel bZIP transcription factor G/HBF-1 was shown to be involved in the transcriptional activation of phenylpropanoid biosynthetic genes with *cis*-acting promoter elements called G- and H-boxes. G/HBF-1 is rapidly and specifically phosphorylated by a cytosolic serine protein kinase in response to elicitor treatment. This provided more evidence for the involvement of protein phosphorylation cascades in the transcriptional activation of plant defense genes (Dröge-Laser et al. 1997). Another elicitor-responsive element has been identified in the promoters of genes encoding PR-10 proteins, which are activated by a non-specific fungal elicitor in parsley. This element, which is comprised of a novel arrangement of palindromically positioned W-boxes [(T)TGAC(C)], is also present in the promoter of the nuclear zinc-finger WRKY transcription factors. WRKY1 is proposed to be activated by phosphorylation,

after which it binds to the W-boxes in its own promoter, as well as in the promoters of *PR* genes (Eulgem et al. 1999).

Some plant mutants that fail to develop SAR also fail to express *PR* genes associated with SAR (see section 2.3.4). Analysis of mutants carrying the *nim1/npr1* (non-immunity/non-expresser of PR genes) mutation has shown that the NIM1/NPR1 protein acts in the SAR signal transduction pathway downstream of SA. Cloning and sequencing of *NPR1/NIM1* have revealed that it probably encodes a cytoplasmic protein containing ankyrin repeat motifs, which are commonly involved in protein-protein interactions. Limited sequence similarity of *NPR1/NIM1* with the human transcription factor inhibitor IκB has led to the suggestion that NPR1/NIM1 may act in the transcriptional activation of *PR* genes by inhibiting the action of a transcriptional repressor of these genes (Cao et al. 1997, Glazebrook et al. 1997, Ryals et al. 1997).

2.3.3.5 Hypersensitive cell death

The hypersensitive response (HR), or the rapid death of cells limited to the site of pathogen infection, is characteristic of the induction of plant defenses by viral, fungal and bacterial pathogens and nematodes (Hammond-Kosack and Jones 1997, Heath 1998), and is associated with the induction of both non-specific and pathogen-specific localized resistance (Dangl et al. 1996, Baker et al. 1997, Heath 2000).

HR phenotypes induced by fungi and viruses are more conspicuous in nature, and were described much earlier than the bacterially-induced HR (Stakman 1915, Holmes 1929). Visible or confluent necrosis is only observed with levels of incompatible bacteria that are much higher than that present in natural inocula, and the bacterially-induced HR only became a focus of study when Klement *et al.* demonstrated a technique for the artificial infiltration of such high inocula (>10^6 to 10^9 cells/mL) into plant tissues (Klement 1963, Klement et al. 1964). The HR typically appears as a desiccated lesion within 6-24 hours of inoculation, with symptom development being influenced by environmental factors (e.g. temperature, light and humidity), the age of the infiltrated tissue and inoculum levels (reviewed in Klement 1982, Sigee 1993).

Biochemically, the HR is associated with a number of processes associated with plant defense, such as a rapid oxidative burst, ion fluxes (influx of H^+ and Ca^{2+} and efflux of K^+ and Cl^-), phosphorylation and dephosphorylation events, plant cell wall reinforcement,

phytoalexin production, *PR* gene expression, and cellular decompartmentalization (Bell 1981, Klement 1982, Dixon and Lamb 1990, Baron and Zambryski 1995, Kombrink and Somssich 1995, Mittler et al. 1999). The relationship between defense gene activation and the HR in the establishment of resistance is, however, not clear. Several studies have suggested that, although cross-talk between signal transduction pathways exists, the pathways that lead to defense gene activation become separated from those leading to cell death*. Accordingly, cell death itself may be the primary defense against biotrophic pathogens (by depriving them from further access to nutrients), and the activation of defense genes may be a "back-up" mechanism to protect the plant against subsequent invaders. In contrast, resistance against necrotrophic pathogens (which grow in dead tissue) may primarily be effected by the induction of defense genes, and hypersensitive cell death may contribute to defense through the release of antimicrobial substances (like hydrolytic enzymes) stored in the vacuole. In both cases, the HR as a whole may act as an endogenous signal that re-inforces the induction of localized defenses and releases signals that trigger SAR (Greenberg et al. 1994, Dangl et al. 1996, Hammond-Kosack and Jones 1996, Heath 1998).

Investigations of the signals and mechanisms that induce the HR have culminated in the notion that the HR is not caused by pathogen toxicity, but that it constitutes a form of genetically programmed cell death (PCD) (Greenberg et al. 1994, Dangl et al. 1996, Greenberg 1997, Heath 1998). The best studied form of PCD is apoptosis in mammalian cells, which is typically (but not always) associated with a characteristic set of morphological features, including membrane blebbing, chromatin condensation, marginalization of DNA to the nuclear envelope, DNA fragmentation, and shrinkage of the cytosol with retention of the integrity of internal organelles. This leads to the development of electron opaque apoptotic bodies in a shrunken corpse, which is removed by

---

* Evidence to this extent include the following:

(i) Many defense responses are activated by pathogens in the absence of cell death (see Heath 1998, 2000 for references).

(ii) Cell death induced by physical damage and abiotic stress also triggers localized and systemic defense responses (see Heath 1998 for references).

(iii) After interaction with its corresponding *avr* gene product (avrPto), the *R* gene-encoded Pto kinase interacts with a family of transcription factors that bind to a *cis*-element in certain PR protein encoding genes. Pto also interacts with a different kinase that is involved in the triggering of the HR (Zhou et al. 1995, 1997).

(iv) Some transgenic "lesions simulating disease" (*lsd*) *Arabidopsis* mutants unable to accumulate salicylic acid (through incorporation of the *nahG* transgene) exhibited cell death, but reduced PR gene expression and resistance (Hunt et al. 1997).

macrophages. Biochemical markers for apoptosis include the cleavage of DNA into oligonucleosomal fragments, degradation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), and the activation of a specific family of cysteine proteases called caspases (Steller 1995, Chinnaiyan and Dixit 1996, Jones 2000). The primary function of mammalian apoptosis is to kill unwanted, damaged or pathogen-invaded cells in such a manner that they are phagocytosed and do not cause an inflammatory response (Hale et al. 1996). It has been suggested that apoptosis is the "default" state in mammalian cells, and that even healthy cells will die if they do not receive signals to survive (Raff 1992, Roy and Nicholson 2000).

Various examples of PCD that occur during the life cycle of vascular plants and are triggered by internal signals are known. These include the differentiation of xylem tracheary elements, the death of various tissues during different stages of sexual reproduction and the controlled senescence of specific plant parts such as petals and leaves (see Heath 1998 for references). Although exceptions exist, there is no strong evidence of a consistent "apoptotic" morphology in plants, and attempts to identify plant homologues for genes known to be involved in animal PCD have generally failed (reviewed in Heath 1998). The activation of cysteine (and other) proteases does, however, provide a fundamental link between some of the abovementioned forms of plant PCD and animal apoptosis (Buchanan-Wollaston 1997, Fukuda 1997).

Several lines of evidence have indicated that the defense-related HR exhibited by plants may also constitute a form of PCD:

a. The HR is dependent on active plant metabolism (Sigee 1993).

b. Plants have multiple signal transduction pathways, which may include a distinct and programmed pathway leading to cell death. This is strongly supported by the identification of "lesion mimic" plant mutants (such as the lesions stimulating disease (*lsd*) and accelerated cell death (*acd*) mutants), which exhibit spontaneous lesion formation (i.e. constitutive initiation of HR-like cell death) in the absence of pathogens, and express LAR- and SAR-associated defense mechanisms (reviewed in Dangl et al. 1996, Hunt et al. 1997, Glazebrook et al. 1997, Heath 1998).

c. At least two characterized alleles associated with lesion mimic mutants have been proposed to encode negative regulators of hypersensitive cell death. The wild-type allele *lls1* of the "lethal leaf spot" maize mutant is predicted to encode an aromatic

ring-hydroxylating dioxygenase, which is proposed to suppress the HR in uninfected cells by degrading a phenolic mediator of cell death (Gray et al. 1997). The wild-type *Arabidopsis LSD1* gene has been shown to encode a novel zinc finger protein, which is proposed to limit the initiation of HR-associated defense responses and the subsequent extent of the HR in response to a superoxide-dependent signal (Dietrich et al. 1997). These findings imply that the HR is conceptually different to other known forms of plant PCD in that it represents the default plant cell state with respect to pathogen penetration (akin to apoptosis in animal cells, Raff 1992) (Baker et al. 1997, Heath 1998). If this is the case, pathogens capable of establishing compatible interactions with plants must be adapted not to trigger (or to suppress) the HR in susceptible host species, and *R* genes must form part of a signalling system leading to PCD in incompatible interactions (Greenberg et al. 1994, Heath 1998).

d. The activation of cysteine proteases (caspases) during the HR has been demonstrated in some cases (D'Silva et al. 1998, Del Pozo and Lam 1999).

e. The Toll/Interleukin-1 receptor-like (TIR) domain common to some plant *R* gene products (see Fig. 2.4) is shared by animal cell death regulators (Van der Biezen and Jones 1998).

In contrast, the HR exhibits a number of characteristics fundamentally different to that of mammalian apoptosis:

a. Necrotic, HR-associated cell death does not display the morphological changes characteristic of apoptosis, or any morphological features that distinguish it from death caused by any other means. It involves swelling of the cytoplasm and organelles, DNA degradation in the absence of discrete processing intermediates, rapid disintegration of internal cell structures and leakage of cellular contents (including the hydrolytic enzymes sequestered in the vacuole) into the surroundings without subsequent corpse removal (Lamb and Dixon 1997, Heath 1998, Jones 2000)*.

b. Unlike the role of apoptosis in limiting inflammatory responses in surrounding tissues, the HR is associated with the activation of LAR and SAR (Heath 1998).

---

* Morphological features characteristic of apoptosis have, however, been reported in several host-pathogen systems (reviewed in Dangl et al. 1996).

The morphological features of HR-associated cell death have recently led to the notion that the plant HR constitutes a form of "programmed oncosis"*, in which cell death is regulated by the mitochondrion. According to this model, oxidative stress and nitric oxide (NO)† trigger PCD by the release of cytochrome c from the mitochondrion into the cytoplasm. This is achieved through the formation of a pore into the mitochondrial matrix. This so-called "permeability transition (PT) pore" is a transient complex of the outer membrane voltage-dependent anion channel (VDAC), the inner membrane adenine nucleotide transporter (ANT), and cyclophilin D in the matrix. Formation of the PT pore results in the rapid movement of water into the mitochondrion, resulting in its swelling and rupture, with the subsequent release of cytochrome c. Alternatively, cytochrome c may be released directly through the VDAC. In both cases, the release is activated by Ca^{2+} at low ATP levels. Cytochrome c is proposed to activate cell death in plants either through its interaction with proteases (in a similar fashion to its activation of apoptosis in animal cells through the recruitment of caspases), or by generating lethal levels of AOS by blocking electron transport (reviewed in Jones 2000, Roy and Nicholson 2000).

More evidence for the universal conservation of this fundamental mitochondrion-mediated PCD pathway comes from the functional expression of apoptosis-related animal genes in plants. Expression of the anti-apoptotic gene *Bcl-x_L* (which encodes a member of the Bcl-2 family‡ of cytochrome c release suppressors) suppressed cell death induced by UV, paraquat or TMV (all known inducers of the oxidative burst; Mitsuhara et al. 1999). Likewise, mitochondrion-targeted expression of the apoptotic protein Bax‡ (which counteracts the action of Bcl-2) via TMV in TMV-resistant plants led to the development of lesions in a HR-characteristic fashion (Lacomme and Santa Cruz 1999).

2.3.4 Systemic acquired resistance

The phenomenon that the inoculation of plants with necrotizing pathogens affords varying degrees of non-specific, long-term, systemic protection (in the form of reduced disease severity) against future attack by a broad range of pathogenic microbes, was reported as

---

* Oncosis is a type of cell death which is generally considered to be unprogrammed. It has been associated with animal cell death that occurs in the absence of caspase function, and has been proposed to be an "primordial" cell death mechanism which underlies apoptosis and is universal in eukaryotes (Jones 2000).

† The role of NO in plant defense responses is reviewed in Hausladen and Stamler 1998, Bolwell 1999 and McDowell and Dangl 2000.

‡ The most recent models for Bcl-2 and Bax action are discussed in Roy and Nicholson 2000.

early as the beginning of the twentieth century (Chester 1933). It was initially referred to as the "immunization" of plants, and was compared to the immunization of animals against disease (Kuc 1982). Although the molecular basis of this phenomenon, later termed "systemic acquired resistance" (SAR; Ross 1961b), was not investigated until the 1990's, earlier studies correctly predicted that induced immunity was based on the local production and systemic transduction of a mobile, endogenous chemical signal in response to the metabolic stress and host cell death caused by the initial infection. Processing of such a signal, which may or may not be mediated by signal receptor molecules, was proposed to result in biochemical changes in the host (some of which were also associated with localized defense in incompatible interactions) through which protection was effected (Kuc 1982, Dean and Kuc 1985). SAR therefore constituted an active host defense response, distinct from induced systemic resistance (ISR) or "biocontrol" (see footnote on p. 26; Schneider et al. 1996).

Although LAR expressed against necrotizing pathogens generally (but not always) leads to the induction of SAR (Hunt and Ryals 1996), SAR must also be regarded in a broader context as a distinct plant defense mechanism as: (i) it affords long-term, "non-specific" future protection against a wide range of pathogens, which may be widely different from the initial infecting organism, (ii) it may also be caused by normosensitive (as opposed to hypersensitive) necrotic cell death resulting from compatible interactions, and (iii) its activation during compatible interactions potentiates plants to express future resistance against pathogens to which they would normally be susceptible. SAR therefore contributes to the overall resistance displayed by plants and may provide a selective advantage for survival (Ryals et al. 1996, Schneider et al. 1996, Sticher et al. 1997, Maleck and Dietrich 1999)

Molecular investigations into the induction and maintenance of SAR, in which transgenic plants have been employed extensively, have confirmed the basic aspects of the earlier SAR models and have provided clues to the molecular basis of SAR. Salient findings from these studies are reviewed comprehensively in Ryals et al. 1994, 1996; Hunt and Ryals 1996, Hunt et al. 1996, Schneider et al. 1996, Sticher et al. 1997 and Heil 1999, and are the following:

a. SAR is induced by necrotizing fungal, bacterial and viral pathogens and insects (i.e. wounding), as well as by a range of natural and synthetic inorganic and organic

chemicals. Many of these compounds contain fatty acid moieties or derivatives of nicotinamide (see Kessman et al. 1994 for a review of SAR inducers).

b. The time required for the establishment of SAR may vary from a few hours after the initial infection to several weeks. This time, the range of pathogens against which protection is effective, as well as the level and duration of protection, depend on the plant and the nature of the pathogen causing the initial infection.

c. Depending on the particular plant-pathogen interaction, a set of "SAR genes" is co-ordinately expressed during SAR. A gene is classified as an SAR gene if its expression is correlated with the onset of SAR or the maintenance of resistance*. The expression of several of these genes may target a particular pathogen and different, overlapping sets of SAR genes may (synergistically) affect different pathogens (see also Uknes et al. 1993).

d. Apart from the induction of SAR genes, a number of biochemical and cytological changes have been observed in cells undergoing SAR. These include (i) an elevation of soluble carbohydrate levels, (ii) enhanced superoxide dismutase and peroxidase activities, (iii) the systemic accumulation of cell wall HRGPs, (iv) an increase in lipoxygenase activity, (v) cell wall fortification by the deposition of callose- and silicon-containing papillae and lignification, (vi) the alteration of cuticle structure to make it a poorer substrate for fungal cutinases, (vii) an accumulation of cyclophilins (proteins that catalyze rotation of X-Pro peptide bonds (where X is any amino acid) and facilitate the folding of proteins), and (viii) a huge increase in the concentrations of β-ionone and its 3-hydroxy esters (compounds that inhibit fungal spore germination).

e. The endogenous signalling molecules, ethylene, jasmonic acid, systemin and particularly salicylic acid, have been implicated in the establishment of SAR. Key aspects of their involvement in SAR are highlighted in Table 2-2 and Fig. 2-3.

---

* Although this definition of SAR genes should theoretically include any subset of the genes involved in the defense mechanisms described in section 2.3.3, it is generally used in the literature as a synonym for the PR protein encoding gene families listed in Table 2-3 (Sticher et al. 1997).

CHAPTER 3

AMPLIFICATION, SEQUENCE AND EXPRESSION OF THE HARPIN ENCODING GENE OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAIN NV

3.1 INTRODUCTION: PREVIOUS STUDIES OF *PSEUDOMONAS SYRINGAE* HARPIN ENCODING (*hrpZ*) GENES AND THE HARPIN ELICITOR OF *P. S.* PV. *SYRINGAE* NV

Hrp genes were identified in 1986 (Lindgren et al. 1986, Lindgren 1997). In the following years, molecular genetic techniques such as transposon mutagenesis, complementation analysis and DNA hybridization were employed extensively to elucidate the organization of *hrp* clusters and their apparent structural and functional conservation amongst phytopathogenic *Xanthomonas*, *Erwinia* and *Pseudomonas* species (including *Pseudomonas solanacearum*, which has been reclassified as *Ralstonia solanacearum*; Yabuuchi et al. 1992, 1995). Over-expression of subclones generated from the *hrp* clusters of *Erwinia amylovora* strain Ea321 and *Pseudomonas syringae* pv. *syringae* strain 61 has led to the identification and cloning of *hrp* elicitor encoding genes in these bacteria during 1992 (Wei et al. 1992) and 1993 (He et al. 1993), respectively. Purified preparations of the "harpin" elicitor proteins encoded by these genes shared the ability to elicit the hypersensitive response (HR; Klement 1963, Klement et al. 1964) in non-host plants.

One of the long-term objectives of our collaborative research with the ARC-Fruit, Wine and Vine Research Institute into the molecular basis of the interaction between the bacterial canker pathogen and stone fruit trees was to clone the harpin encoding gene of a local strain of *P. s.* pv. *syringae* isolated from a stone fruit host (strain NV)*. When a preliminary project was initiated in our laboratory in 1994, three factors predisposed us not to follow the strategy employed by the abovementioned groups, but to adopt the approach outlined in Fig. 3-1. These factors were:

* See Chapter 1 for more detail.

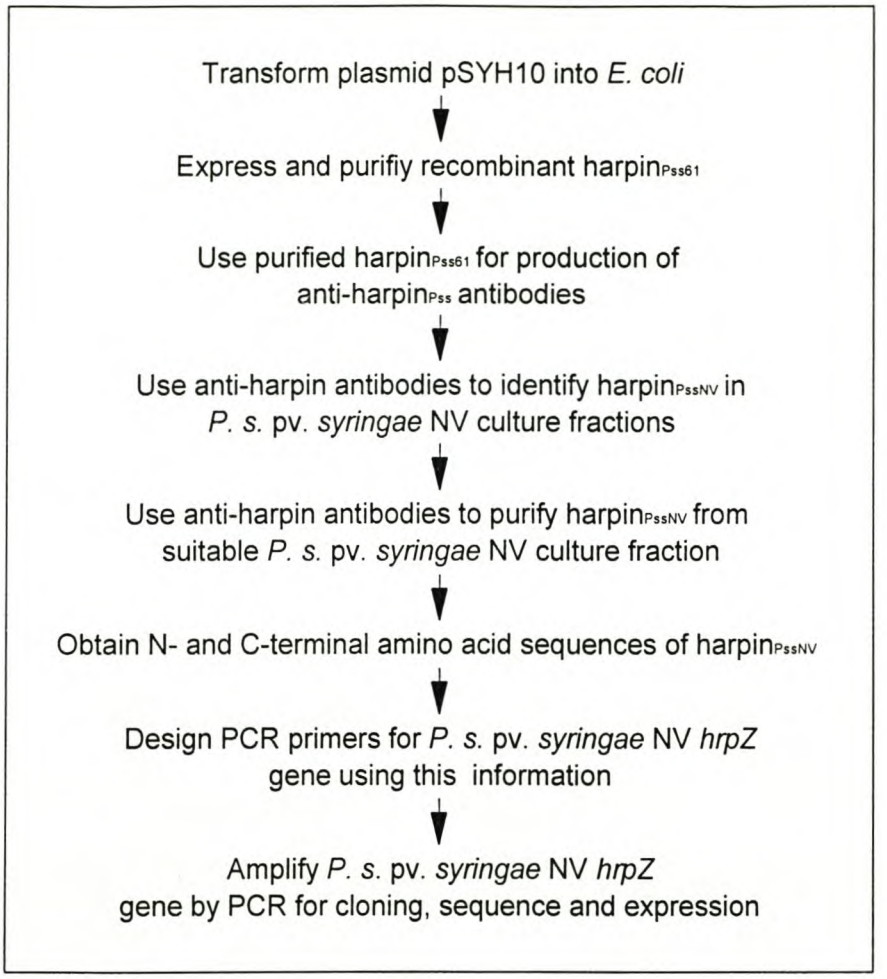


Fig. 3-1. Envisaged strategy for cloning the *P. s.* pv. *syringae* NV harpin encoding gene ($hrpZ_{PssNV}$) in the preliminary study (Appel 1996).

a. A separate project had been initiated by Infruitec with the aim of constructing a genomic library of *P. s.* pv. *syringae* NV for gene identification and cloning purposes. This library was, however, not available at the time.

b. Plasmid pSYH10, containing the *hrpZ* open reading frame (ORF) of *P. s.* pv. *syringae* 61 ($hrpZ_{Pss61}$), had been obtained from Alan Collmer (Cornell University, Ithaca, New York, USA). This presented a means of purifying *P. s.* pv. *syringae* 61 harpin ($harpin_{Pss61}$) protein for the production of anti-$harpin_{Pss}$ antibodies.

c. At the time when these studies were initiated, our research team was well-versed in protein isolation and purification, as well as applied immunological techniques, but lacked advanced expertise in molecular biology and gene cloning technologies.

The approached outlined in Fig. 3-1 worked well up to the stage where a polyclonal anti-harpin$_{Pss}$ antiserum was raised in rabbits against harpin$_{Pss61}$ purified from *Escerichia coli* DH5α (pSYH10). This antiserum was used successfully in Western blot analyses to identify a protein band similar in size to harpin$_{Pss61}$ in cellular fractions and culture supernatants of *P. s.* pv. *syringae* NV that were shown to contain an extracellular, heat-stable and protease sensitive elicitor of the HR in tobacco. The sensitivity and specificity of this antiserum for harpin$_{Pss}$ protein was, however, too low for it to be used in an affinity chromatography system for the purification of harpin$_{PssNV}$ from any of these fractions (Appel 1996).

Despite the technical failure of our initial approach, the information obtained in the process confirmed that *P. s.* pv. *syringae* NV produced a protein similar in size, biochemical characteristics and antigenic properties to harpin$_{Pss61}$ (Appel 1996). Towards the end of the preliminary project, more comprehensive nucleotide sequences of the *hrpZ* operon* of *P. s.* pv. *syringae* 61 and two other *P. syringae* pathovars (*P. s.* pv. *glycinea*, which causes bacterial blight of soybean, and *P. s.* pv. *tomato*, the causative agent of bacterial speck of tomato) were published (Preston et al. 1995). The sequence data demonstrated that significant similarity existed between the *hrpZ* ORFs of the three pathovars. Together with our preliminary results, this indicated that a more direct approach was merited in continued efforts to clone the stone fruit pathogen's harpin encoding gene.

The preliminary project was concluded in 1995 with a first attempt to amplify the *hrpZ*$_{PssNV}$ ORF from genomic DNA using primers based on the published *hrpZ* sequences of other *P. syringae* pathovars. Primers MAPf1 and MAPr1, based on nucleotides 1-27 and 1007-1026 of the *hrpZ*$_{Pss61}$ ORF, respectively (see Fig. 3-4 in section 3.2.2), were used in a polymerase chain reaction (PCR) with *P. s.* pv. *syringae* NV genomic DNA as template. A 1.0 kb DNA fragment was amplified in this way. Partial sequencing and hybridization to the *Bst*X1 fragment of *hrpZ*$_{Pss61}$ were used to show that this fragment probably constituted the harpin encoding gene of the stone fruit pathogen (Appel 1996).

3.2 CURRENT STUDY: BACKGROUND INFORMATION

Results obtained in the preliminary study served as a basis for the current study. The paper included in section 3.3.3 comprises the first three objectives of this study (as

---

* The ORFs of *hrpA*, *hrpZ*, *hrpB*, *hrpC*, *hrpD* and *hrpE* are included in the *hrpZ* operon. See Fig. 2-2 in Chapter 2 for more details.

outlined in Chapter 1). It fully describes how the putative *hrpZ* gene amplified from *P. s.* pv. *syringae* NV genomic DNA was cloned, sequenced, characterized in terms of its similarity with other *P. syringae hrpZ* sequences, and expressed in *E. coli* TB1 (pMNV1) and (pMNV2) from which biologically active, recombinant $harpin_{PssNV}$ was purified. To assist the reader, background on the following aspects of the experimental design is included in this section:

3.2.1 The strategy for cloning the putative *P. s.* pv. *syringae* NV *hrpZ* gene amplified with primers MAPf1 and MAPr1, in order to sequence it fully and confirm its identity as the harpin encoding gene of the stone fruit pathogen.

3.2.2 The design of primers MAPf2 and MAPr2 with which areas flanking $hrpZ_{PssNV}$ could be amplified and sequenced to ascertain whether the 5'- and 3'-ends of $hrpZ_{PssNV}$ were different to that of $hrpZ_{Pss61}$ (on which primers MAPf1 and MAPr1 were based).

3.2.3 The expression system used for the production and purification of biologically active, recombinant $harpin_{PssNV}$.

3.2.1 Cloning of the putative *P. s. pv. syringae* NV *hrpZ* gene

3.2.1.1 Initial approach

Primers MAPf1 and MAPr1 were originally designed to permit cloning of PCR products obtained with these primers by means of the *Eco*RI and *Xba*I restriction sites included in MAPf1 and MAPr1 respectively (Appel 1996). The choice of restriction sites was based on the polylinker configuration of the pMAL™-c2 expression vector (New England Biolabs; see section 3.2.3). Numerous attempts to clone the 1.0 kb *P. s.* pv. *syringae* NV PCR product into pMAL™-c2 in this way were unsuccessful*. Direct cloning of the PCR product into the fairly large (6.6 kb) pMAL™-c2 expression vector with its very limited polylinker was also a weak strategy, as this vector did not afford the flexibility needed for subsequent DNA manipulations such as cycle sequencing.

---

* Reasons for the consistent failure of this procedure were not investigated extensively. Some results suggested that it was attributable to inefficient digestion of the *Xba*I site in the MAPr1 region of the PCR product, despite the 5'-terminal random trinucleotide (TAA) included in the primer sequence.

3.2.1.2 Cloning of the 1.0 kb P. s. *pv.* syringae *NV PCR product into the pGEM®-T vector*

A new generation of DNA vectors, the so-called "T vectors" or "T-A cloning vectors" were introduced in 1991 for the convenient cloning of PCR products. These vectors contain 3'-deoxythymidine overhangs at both ends of the insertion site, which provide compatible overhangs for the single deoxyadenosines added by the majority of DNA polymerases in a template-independent fashion to the 3'-ends of amplification products generated by PCR (Clark 1988, Mezei and Storts 1994). Such a cloning vector, pGEM®-T (Promega Corporation; see Fig. 3-2), was made available to us during the course of the current study.

The pGEM®-T vector offered several advantages for the cloning of the putative *P. s.* pv. *syringae* NV *hrpZ* gene and subsequent manipulations thereof:

a. The PCR product obtained with MAPf1 and MAPr1 could be ligated without any prior restriction digestion, as the vector is supplied in its linearized form (Promega Corporation 1996a).

b. Recombinant plasmids could be transformed into *E. coli* and transformants selected by means of ampicillin resistance and blue-white screening (Promega Corporation 1996a).

c. The isolation, analysis and manipulation of recombinant plasmid DNA would be facilitated by the small vector size (3000 bp, exclusive of the 3'-T overhangs at the insertion site) and high copy number (300-700 copies per *E. coli* cell) (Promega Corporation 1996a, 1996b).

d. The entire cloned 1.0 kb *P. s.* pv. *syringae* NV PCR product could be sequenced with available, universal primers Uf and Ur (see p. 491 of the paper included in section 3.3.3 for sequences). The annealing sites of these primers in pGEM®-T (positions 2934-2957 for Uf and 173-196 for Ur) are respectively 94 and 121 nucleotides away from the insertion site (position 51).

e. Configuration of the multiple cloning site (which flanks the insertion site on both sides) would still permit directional subcloning of *the P. s.* pv. *syringae* NV *hrpZ* ORF into the pMAL™-c2 expression vector (see section 3.2.3 for more details).

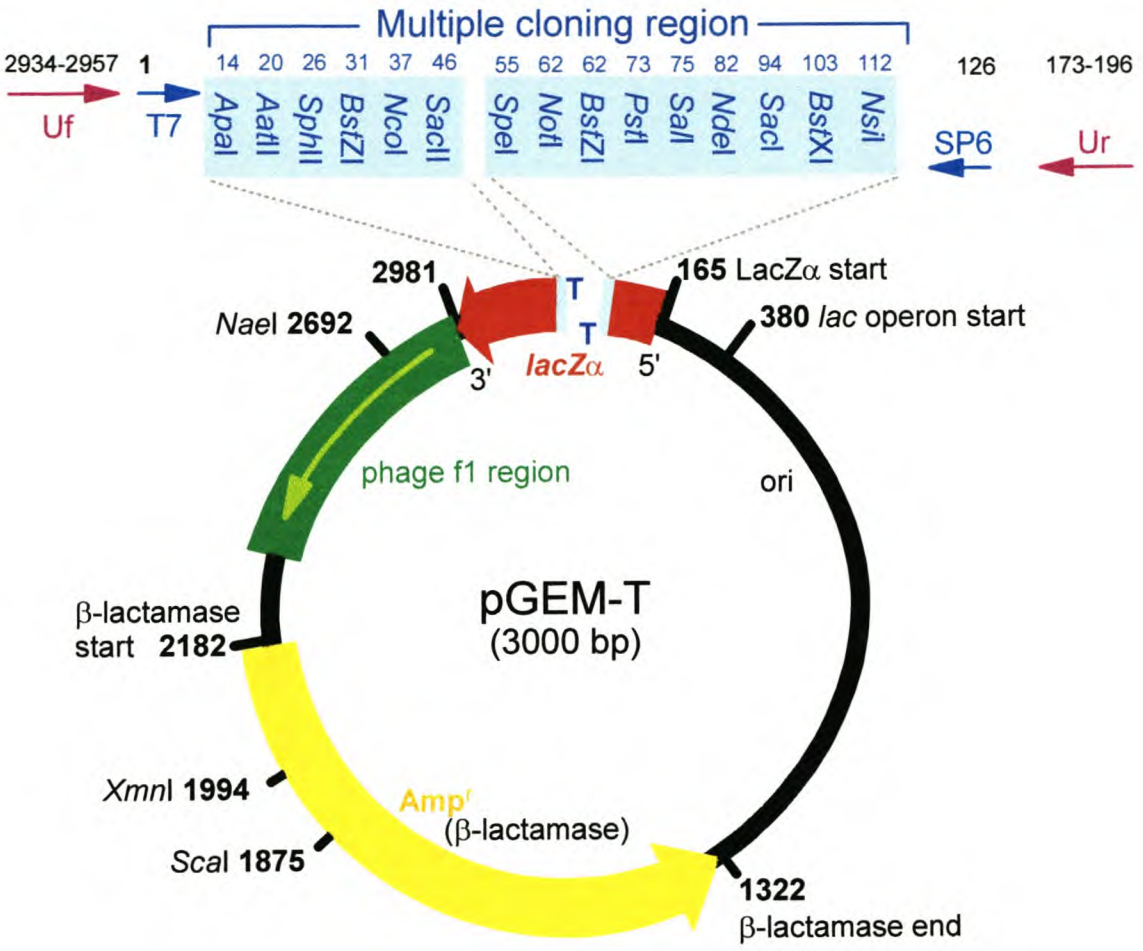


Fig. 3-2. Salient features of the pGEM®-T cloning vector (compiled from Promega Corporation 1996a).

pGEM®-T is derived from Promega's pGEM®-5Zf(+) vector, and is prepared by cutting this vector with *Eco*RV and adding a 3'-terminal deoxythymidine to both ends. The vector contains promotor sequences for two different bacteriophage RNA polymerases (T7 and SP6). These sequences flank the multiple cloning region (MCR) within the α-peptide coding region of the enzyme β-galactosidase (*lacZ*α). Successful ligation of a PCR product into the T-insertion site within the MCR leads to the inactivation of lacZα. Recombinant *E. coli* clones transformed with such ligation products will appear as white or light blue colonies on indicator plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The β-lactamase gene (Amp^r) confers ampicillin resistance to clones harbouring the plasmid.

The vector is numbered from the T7 transcription start site (no. 1), with the numbers running in the 5'→3' direction of the RNA synthesized by this polymerase. The RNA synthesized by SP6 polymerase starts at position 126 and has the opposite 5'→3'direction. The 5'→3'direction of the *lac* operon and β-lactamase gene corresponds to that of the SP6 RNA. The number indicated above each of the restriction enzymes that have recognition sequences in the MCR denotes the nucleotide on the 5'-side of the cut site in each recognition sequence. The T-overhangs added after linearization of pGEM®-5Zf(+) with *Eco*RV at position 51 are **excluded** from the numbering of pGEM®-T. The binding sites within the *lac* operon of primers Uf and Ur, used for sequencing of the 1.0 kb *P. s.* pv. *syringae* NV PCR product cloned into pGEM®-T, are as indicated.

The 1.0 kb *P. s.* pv. *syringae* NV PCR product obtained with primers MAPf1 and MAPr1, was cloned into pGEM®-T as described in section 3.3.3, to create the recombinant plasmid series pGNV 1-15. The two possible orientations of the PCR product in these recombinant plasmids are illustrated in Fig. 3-3. Those recombinant plasmids shown by restriction digestion analysis to contain an insert of the correct size were sequenced to obtain a consensus sequence for the *P. s.* pv. *syringae* NV *hrpZ* gene, as described in section 3.3.3.

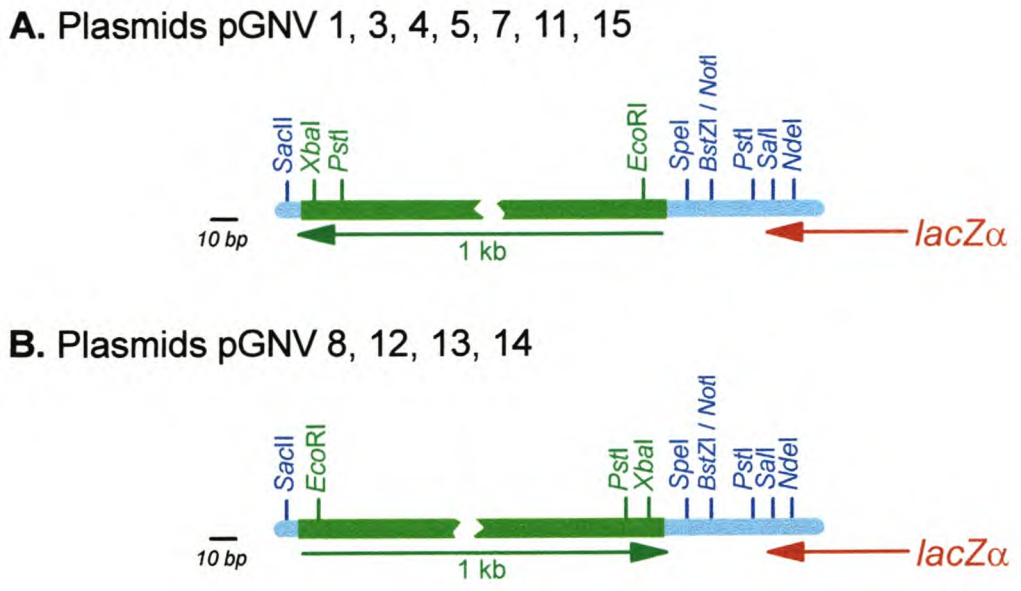


Fig. 3-3. Partial restriction map of the recombinant plasmid pGNV series, showing the two possible orientations of the cloned 1.0 kb *P. s.* pv. *syringae* NV PCR product in pGEM®-T.

The green bar corresponds to the PCR product obtained with primers MAPf1 and MAPr1 and the blue line to nucleotides 45 to 85 of the multiple cloning (MCR) region of pGEM®-T. The 5'→3' directions of the PCR product and the *lacZα* gene in which the MCR is situated, are indicated by arrows.

The enzymes that are indicated have no other recognition sites in the recombinant plasmids than those shown here. Only the restriction sites of interest in the PCR product are shown. The *Eco*RI and *Xba*I sites form part of sequences of primers MAPf1 and MAPr1 (see section 3.2.1.1).

All of the recombinant plasmids, except pGNV 2, 6, 9 and 10 yielded a single, linear 4.0 kb product after digestion with *Eco*RI, which indicated that they contained the PCR product. *Pst*I digestion yielded two bands of 1.0 and 3.0 kb, respectively, for the plasmids in A, and one linear band of 4.0 kb for the plasmids in B (the smaller product of 40 bp was not visible on an ethidium bromide stained agarose gel). The plasmids in group B contained the PCR product in the correct orientation for directional subcloning of $hrpZ_{PssNV}$ into the pMAL™-c2 expression vector, using *Eco*RI and *Sal*I.

3.2.2 Alignment of *P. syringae hrpAZB* nucleotide sequences and the design of primers MAPf2 and MAPr2

The rationale behind the design of primers MAPf1 and MAPr1, that were initially used to amplify the putative *P. s.* pv. *syringae* NV *hrpZ* gene, was discussed previously and will not be repeated here (Appel 1996).

A similar strategy was followed in the design of primers MAPf2 and MAPr2. This second pair of primers was designed for the amplification of a fragment of the *P. s.* pv. *syringae* NV *hrpAZB* region that would include the entire $hrpZ_{PssNV}$ ORF, as well as flanking regions that would allow sequencing of the 5'- and 3'-ends of the ORF by direct cycle sequencing with the same primer pair. For this purpose the flanking regions had to be long enough for each primer to bind outside the borders of the *hrpZ* ORF, but short enough to permit sequencing of the entire PCR product without any additional (internal) primers.

The nucleotide sequences of the *hrpAZB* region of the *hrpZ* operons of *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 were published previously (He et al. 1993, Preston et al. 1995) and were obtained from the GenBank database (see p. 491 of the paper in section 3.3.3 for individual accession numbers). The sequences were aligned with the DNA and protein sequence analysis programme, DAPSA (version 4.61; E.H. Harley, University of Cape Town, South Africa, 1999) and are presented in Fig. 3-4.

The alignment was similar to the one published previously by Preston et al. (1995) and displayed the following features that were considered in the final design of the primer pair:

a. A high degree of similarity over the entire *hrpAZB* region existed between the *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* sequences, but this was not shared with the *P. s.* pv. *tomato* sequence. The latter sequence was subsequently excluded from further decisions regarding the primer design.

b. The 3'-terminal region of *hrpA* was less variable than the *hrpAZ* spacer region and was chosen as the preferred site for the forward primer (MAPf2). A primer based on the sequence of a coding gene was deemed to have a better chance of annealing to the sequence of a related strain than that based on the sequence of a spacer region.

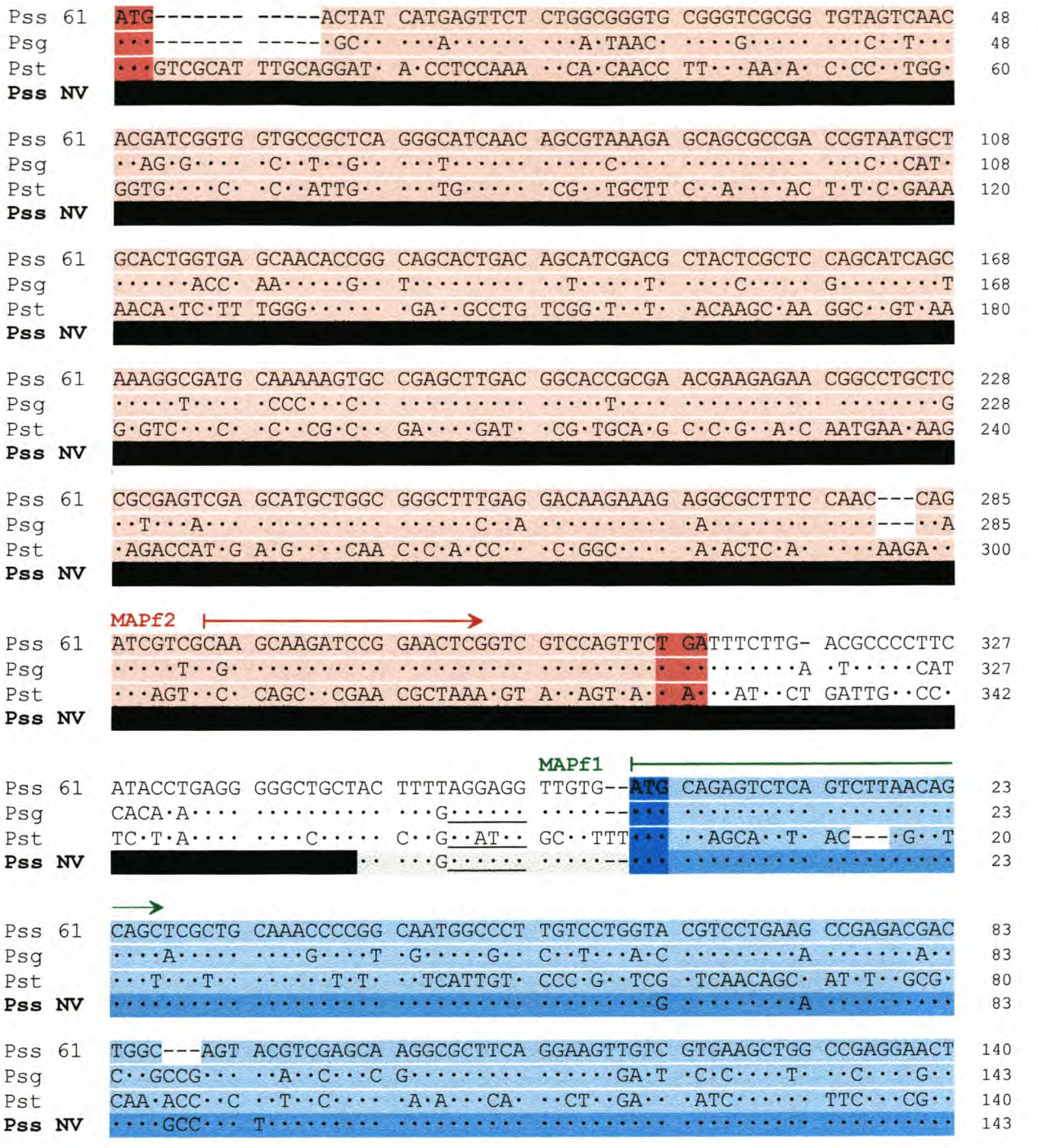


Fig 3-4. Consensus nucleotide sequence of the 1158 bp DNA fragment amplified from *P. s.* pv. *syringae* NV (**PssNV**), aligned to the *hrpAZB* sequences of *P. s.* pv. *syringae* 61 (Pss61), *P. s.* pv. *glycinea* race 4 (Psg) and *P. s.* pv. *tomato* DC3000 (Pst).

The alignment corresponds to the amino acid sequence alignment of *hrpA*, *hrpB* (not shown) and *hrpZ* (Fig. 1 of the paper included in section 3.3.3). Each group of three dashes denotes a gap of one amino acid in sequences with fewer amino acids. Nucleotides identical to the Pss61 sequence are indicated with dots in all subsequent sequences. To facilitate interpretation, the ORFs of *hrpA*, *hrpZ* and *hrpB* are indicated in different colours, with the respective initiation and termination codons highlighted. Areas where the **PssNV** sequence is incomplete are indicated in black. The putative ribosome binding site for each gene is underlined. The three ORFs are also numbered separately. Numbers run from the first nucleotide of the initiation codon to the last nucleotide of the termination codon for each ORF and do not include nucleotides in the *hrpAZ* and *hrpZB* spacer regions. The Pss61 regions on which primers MAPf1 and MAPr1 (green), and MAPf2 and MAPr2 (red) were based, are indicated with arrows.

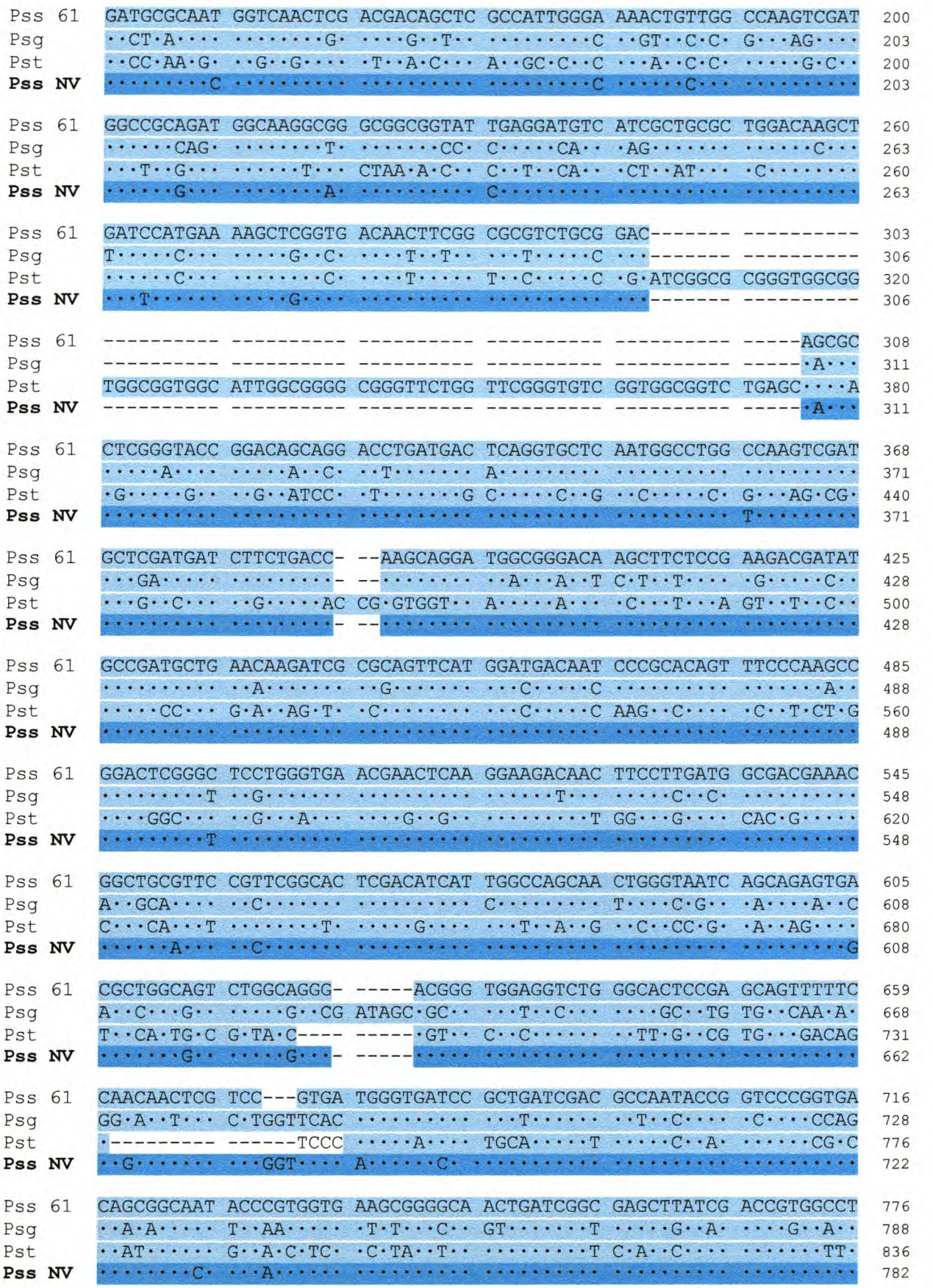


Fig 3-4 *continued.*

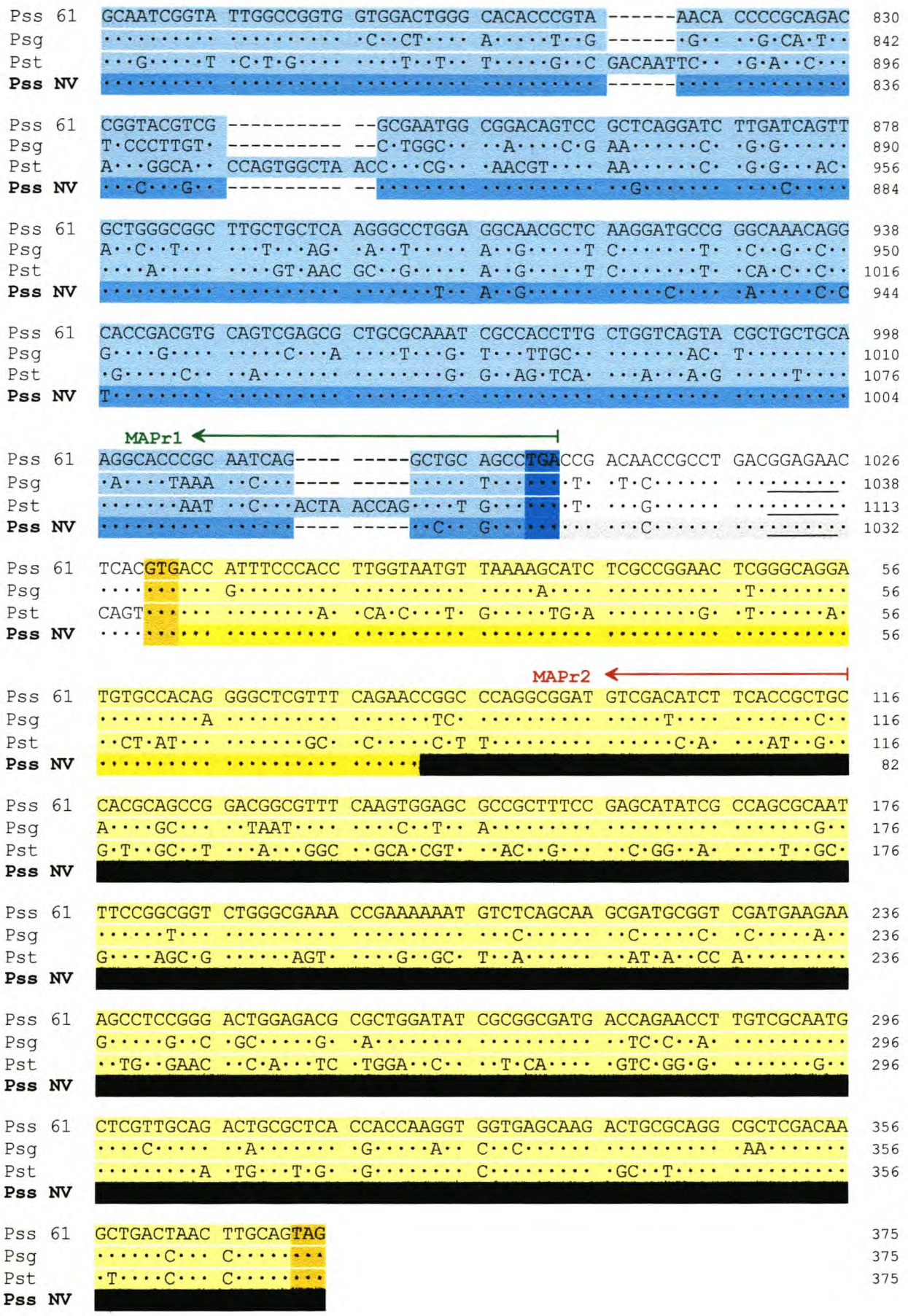


Fig 3-4 *continued.*

c. The *hrpZB* spacer region showed a very high degree of similarity in all three pathovars. It was, however, short enough to permit the placement of the reverse primer (MAPr2) in the 5'-terminal region of *hrpB* for the reason outlined in b.

Once the general areas on which the primers were to be based were selected, the relevant *P. s.* pv. *syringae* 61 sequences were used as templates in the programme Primer Designer (version 1.01; Scientific & Educational Software, 1990) to design a pair of primers, each of which conformed to the following criteria (Dieffenbach et al. 1995b):

a. Length = 20 nucleotides.

b. G+C content between 50 and 60%, with a difference of no more than 5% between the two primers.

c. Melting temperature (T_m)* between 55 and 80°C, with a difference of no more than 3°C between the two primers.

d. G or C as the most 3'-terminal nucleotide, but no more than 3 G's or C's at the 3'-end of either primer.

e. No stable secondary structure possible within either primer sequence.

f. Fewest possible interactions between the primers (in any combination), especially where 3'-nucleotides are involved, in order to minimize the formation of primer-dimers during the PCR.

The sequences that best met these criteria within the target regions corresponded to nucleotides 293-312† of the *hrpA* ORF (MAPf2) and nucleotides 97-116 of the *hrpB* ORF (MAPr2) of *P. s.* pv. *syringae* 61, respectively, and are indicated in Fig. 3-4. A number of possible GC-interactions involving 3'-ends between MAPf2 and itself, MAPr2 and itself, and MAPf2 and MAPr2 were identified by the Primer Designer software. Nevertheless, a 1.2 kb fragment was amplified successfully from *P. s.* pv. *syringae* NV genomic DNA (Fig. 3-5) and sequenced as described in section 3.3.3. The consensus sequence of 1158 nucleotides of this fragment is included in the alignment in Fig. 3-4.

---

* Best estimated by the formula: $T_m = 2°C(A + T) + 4°C(G + C)$ (Suggs et al. 1991).

† The position of MAPf2 was incorrectly published as nucleotides 193-212 of the *hrpA* ORF on pp. 490-491 of the paper included in section 3.3.3.

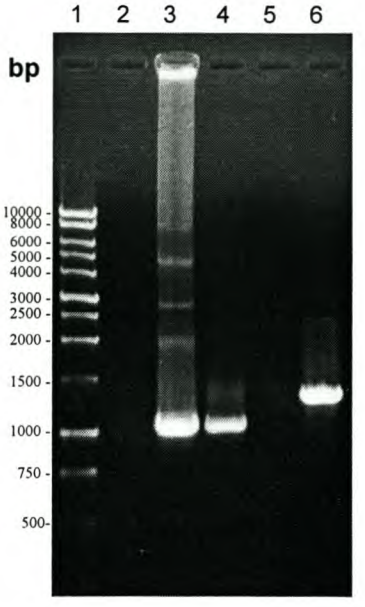


Fig. 3-5. Agarose gel electrophoresis of *P. s.* pv. *syringae* NV amplification products.

PCRs were performed as described in section 3.3.3. *P. s.* pv. *syringae* NV genomic DNA was replaced with an equal volume of double distilled water in negative controls, or pSYH10 plasmid DNA in positive controls. 15 µL of each PCR product were electrophoresed in 1 x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in a 1% agarose gel containing 0.5 µg/mL ethidium bromide. The sizes of molecular marker fragments are given on the left.

Lanes 1: 1 kb DNA ladder (Promega); ***2:*** Negative control for PCR with primers MAPf1 and MAPr1; ***3:*** Positive control for PCR with MAPf1 and MAPr1 (1026 bp *hrpZ*$_{Pss61}$ ORF); ***4:*** PCR with MAPf1 and MAPr1; ***5:*** Negative control PCR with MAPf2 and MAPr2; ***6:*** PCR with MAPf2 and MAPr2.

3.2.3 Directional subcloning of *hrpZ*$_{PssNV}$ into the pMAL™-c2 expression vector for the production and purification of recombinant harpin$_{PssNV}$

A number of heterologous protein expression systems, designed for the convenient purification of recombinant proteins over-expressed in *E. coli* by affinity chromatography of translational fusions (reviewed by Uhlén and Moks 1990), were available at the time when these experiments were performed. Most popular were those that made use of translational fusions with β-galactosidase (Shuman et al. 1980; Germino et al. 1983; Ullmann 1984, Das 1990), glutathione-S-transferase (Smith and Johnson 1988) or the maltose-binding protein (MBP; Guan et al. 1988; Maina et al. 1988). The use of N- or C-terminal poly(histidine) tags (Smith et al. 1988; Uhlén and Moks 1990) was also becoming increasingly popular.

Successful expression and purification of a biologically active recombinant protein cannot be guaranteed for all foreign genes in any of the above systems. A variety of factors may cause unstable, inactive or insoluble protein products. These include aggregation, proteolysis, improper folding and disulphide bond formation, insufficient post-translational modification and the absence of associated prosthetic groups (Marston 1986, Das 1990, Gold 1990, Rusnak et al. 1991). Due to the nature of harpins (see Chapter 2), the latter three factors would, however, not be relevant in the expression of recombinant harpin$_{PssNV}$ in *E. coli*.

The pMAL™-c2 expression system (New England Biolabs), in which foreign genes are expressed in *E. coli* as translational fusions with the MBP, was chosen for the expression and purification of recombinant harpin$_{PssNV}$, for the following reasons:

a. This technology was well-established in our Department.

b. The system had been used successfully for the production and purification of *Pseudomonas syringae* proteins *in vivo* (Peñaloza-Vázquez *et al.* 1996).

c. Cleavage of the MBP-harpin$_{PssNV}$ fusion and subsequent purification of the recombinant harpin theoretically posed no complications. The predicted amino acid sequence of harpin$_{PssNV}$, which was derived from the *hrpZ*$_{PssNV}$ sequence, did not contain any factor Xa cleavage sites. Furthermore, all of the previously characterized *P. syringae* harpins were demonstrated to be soluble, cytoplasmic proteins (He et al. 1993, Preston et al. 1995).

The salient features of the pMAL™-c2 expression vector are illustrated in Fig. 3-6. Directional subcloning of the *hrpZ*$_{PssNV}$ ORF into this vector to create recombinant plasmids pMNV1 and pMNV2, and the subsequent expression and purification of biologically-active, recombinant harpin$_{PssNV}$ from *E. coli* TB1 clones harbouring these plasmids, are described fully in the next section.

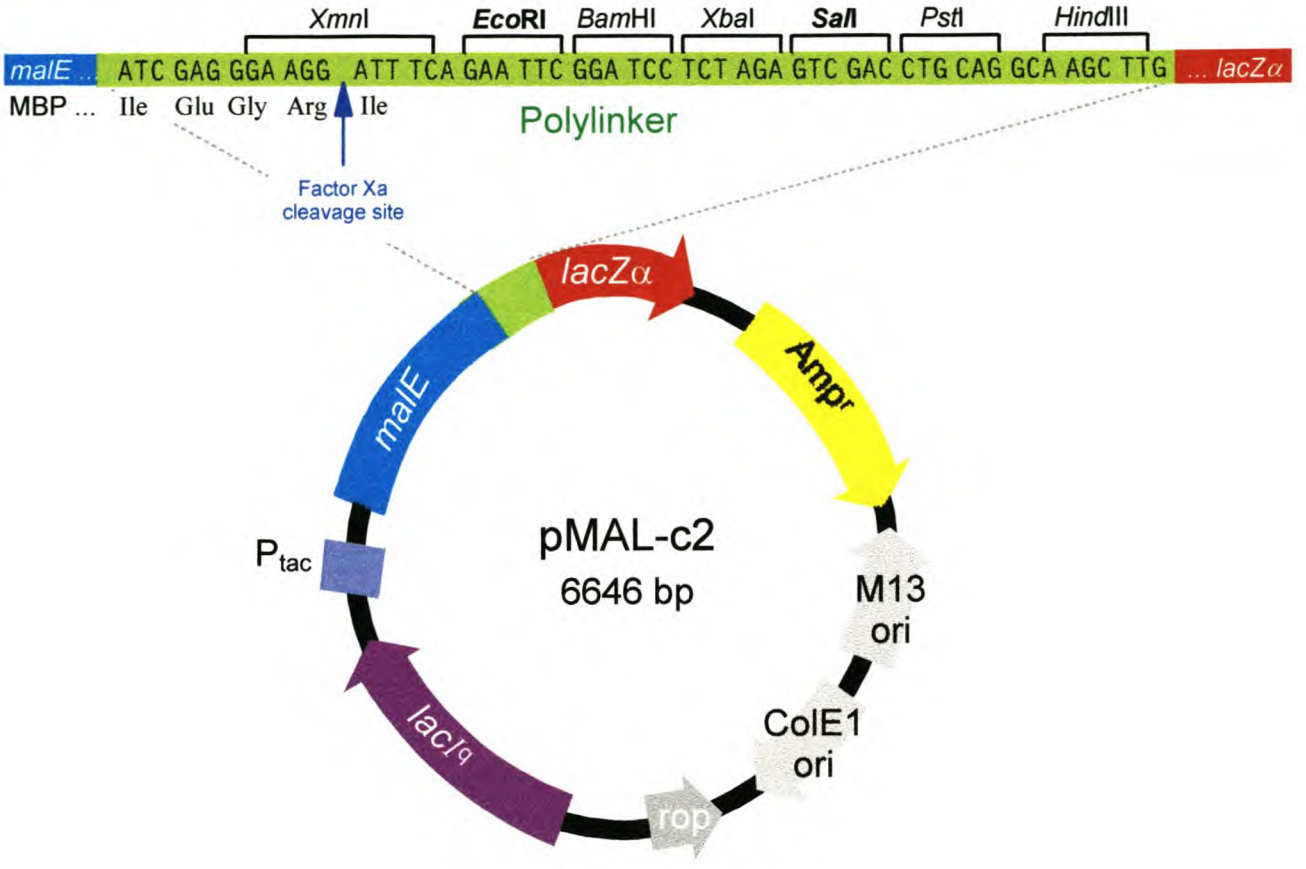


Fig. 3-6. Salient features of the pMAL™-c2 expression vector (compiled from New England Biolabs 1993).

The 5'→ 3' direction of all genes encoded in the vector are indicated with arrowheads. Genes of interest which are to be expressed in pMAL™-c2 are inserted into the polylinker using the restriction sites indicated. The polylinker is situated downstream from the *E. coli* maltose binding protein (MBP) encoding gene (*malE*), and upstream from the *lacZα* gene encoding the α-peptide of the enzyme β-galactosidase. Insertion of a gene into the polylinker in the same reading frame as *malE* results in the high-level expression of a cytoplasmic fusion protein, under the control of the strong *tac* promotor and *malE* translation initiation signal. This also inactivates the β-galactosidase activity of the *malE-lacZα* fusion, which permits the identification of recombinant clones containing successful ligation products as white colonies on indicator plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The presence of the Lac repressor encoding gene (*lacI*^{q}) ensures low-level expression from the *tac* promotor in the absence of IPTG.

Fusion proteins expressed in this way may be purified conveniently the using MBP's affinity for maltose in a column chromatography system. After purification, the protein of interest may be recovered from the fusion protein by cleavage with the specific protease, factor Xa. The recognition site of this protease in the MBP-portion of the fusion protein is indicated with an arrow.

The restriction sites in the polylinker which were used for the directional subcloning of *hrpZ*$_{PssNV}$ into pMAL™-c2 are indicated in **boldface**.

3.3 AMPLIFICATION, SEQUENCE AND EXPRESSION OF THE HARPIN ENCODING GENE OF THE BACTERIAL CANKER PATHOGEN, *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAIN NV

3.3.1 Contributions of co-authors

The paper that follows contains the original and independent work of the author of this dissertation. All experimental work, with the exception of automated sequencing and electrospray mass spectrometry (ES-MS), was performed by the author. She also compiled the manuscript, inclusive of tables and figures.

The contribution of each of the co-authors were as follows:

a. *Mr Mathias Hampf* is a doctoral student at the Max Delbrück Centre in Berlin who visited the Department of Biochemistry at Stellenbosch University for a brief period during 1995. Mr Hampf has extensive experience in automated cycle sequencing technology and assisted the author in the partial sequencing of the PCR product amplified from *P. s.* pv. *syringae* NV with primers MAPf1 and MAPr1 during his visit (Appel 1996). Since the University's Core sequencing facility was not operational at the time when this putative *P. s.* pv. *syringae* NV *hrpZ* gene was cloned and had to be sequenced, Mr Hampf offered to supply the universal primers Uf and Ur and to perform automated sequencing of the cloned fragment in the recombinant pGNV plasmids upon his return to Germany. He also supplied primer MASr1 and performed automated sequencing of the inserts in plasmids pMNV1 and pMNV2 to confirm successful subcloning of the *hrpZ*$_{PssNV}$ ORF into pMAL™-c2.

b. *Prof. E. Lucienne Mansvelt** is a senior researcher at the Plant Biotechnology and Pathology Division of the ARC-Fruit, Wine and Vine Research Institute. She has collaborated with the author and her supervisor on molecular investigations of the bacterial canker pathogen since 1994 and is the co-promoter of this dissertation. As such, she has provided logistical support and partial funding for this project. For this study, she supplied the stock cultures of *P. s.* pv. *syringae* NV, as well as tobacco plants and greenhouse facilities for HR tests. The bacterium was cultivated and HR tests were performed under her supervision.

c. *Prof. Janet P. Hapgood*, formerly from the Department of Biochemistry at the

---

* Recently appointed as special associate professor of Plant Pathology at Stellenbosch University.

University of Cape Town, joined Stellenbosch University as an associate professor of Biochemistry in 1996. She made the pGEM®-T system available for cloning of the putative *P. s.* pv. *syringae* NV gene and supervised this cloning experiment.

d. *Prof. Dirk U. Bellstedt* is an associate professor of Biochemistry at the University of Stellenbosch, and the promoter of this dissertation. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He promoted all collaborations, provided funding for the project and facilitated all logistical aspects within the Department of Biochemistry.

Proff. Bellstedt, Mansvelt and Hapgood suggested minor revisions to the original text, which were incorporated in the final manuscript.

3.3.2 Errata

The published paper contains two numerical printing errors:

a. "... primers were based on nucleotides **193-212** of ..." should read **293-312** (p. 490).

b. " ... pGEM-T vector (**3005 bp**) ..." should read **3000 bp** (p. 491).

J. Plant Physiol. Vol. 154. pp. 489–497 (1999)
http://www.urbanfischer.de/journals/jpp

JOURNAL OF
Plant Physiology

Amplification, Sequence and Expression of the Harpin Encoding Gene of the Bacterial Canker Pathogen, *Pseudomonas syringae* pv. *syringae* Strain NV

Maryke Appel[1,2], Mathias Hampf[3], Lucienne Mansvelt[4], Janet Hapgood[2], and Dirk Bellstedt[2]

[1] Plant Molecular Genetics, Institute for Agriculture and Center for Legume Research, The University of Tennessee Knoxville, P.O. Box 1071, Knoxville, TN 37901-1071, USA

[2] Department of Biochemistry, University of Stellenbosch, Stellenbosch 7602, South Africa

[3] Max Delbrück Centrum Berlin, Robert-Rössle-Straße 10, 13125 Berlin, Germany

[4] ARC-Infruitec, Private Bag X5013, Stellenbosch 7599, South Africa

Received April 2, 1998 · Accepted July 3, 1998

Summary

DNA fragments containing a putative harpin elicitor encoding (*hrpZ*) gene were amplified from genomic DNA of the South African stone fruit pathogen, *Pseudomonas syringae* pv. *syringae* strain NV, using primers based on the *hrpAZB* sequences of the wheat pathogen, *P. s.* pv. *syringae* strain 61. Sequencing of these amplification products revealed a *hrpZ* open reading frame, which showed 96.2 % identity with the *hrpZ* gene of *P. s.* pv. *syringae* 61. A similar degree of identity (96.4 %) was obtained when the predicted amino acid sequence of the *P. s.* pv. *syringae* NV harpin elicitor protein (*HrpZ*) was aligned with that of the *P. s.* pv. *syringae* 61 harpin. The *P. s.* pv. *syringae* NV *hrpZ* gene was subsequently cloned into the pMAL-c2 vector and expressed in *Escherichia coli*. This system proved useful for the production of purified, biologically active recombinant HrpZ protein.

Key words: Prunus persica (L.) Batsch, Prunus salicina Lindl., bacterial canker, elicitor, harpin, hrpZ, pMAL-c2, Pseudomonas syringae pv. syringae.

Abbreviations: ES-MS = electrospray mass spectrometry; HR = hypersensitive response; IPTG = isopropyl-β-D-thiogalactoside; *lacZα* = α-peptide of β-galactosidase enzyme; MBP = maltose binding protein; NJ = neighbour-joining; ORF = open reading frame; PCR = polymerase chain reaction; PMSF = phenylmethyl-sulphonyl fluoride; PR = pathogenesis-related; pv. = pathovar; $rHrpZ_{PsNV}$ = recombinant *P. s.* pv. *syringae* NV harpin protein; SAR = systemic acquired resistance.

Introduction

Bacterial canker of stone fruit trees (mainly *Prunus persica* (L.) Batsch and *Prunus salicina* Lindl.), caused by *Pseudomonas syringae* pathovar *syringae*, has become one of the most destructive crop diseases in the Western Cape region of South Africa. Losses due to diseased trees and reduced production are estimated at more than US$ 10 million annually. Although the disease is not unique to South Africa, a number of predisposing factors, including unfavourable environmental and agricultural conditions and poor horticultural practices, have been implicated in the exceptional severity of the disease in this country, and in the failure of chemical control measures. Effective, long-term solutions will have to rely heavily on the selection and breeding of resistant host trees (Hattingh et al., 1989). For this reason, we have launched

0176-1617/99/154/489 $ 12.00/0

investigations into the molecular basis of the interaction between *P. s.* pv. *syringae* and stone fruit trees.

In the past decade, *hrp* genes (hypersensitive response (HR) and pathogenicity; Lindgren et al., 1986) have been identified as essential pathogenicity determining factors of necrotizing phytopathogenic bacteria from the genera *Pseudomonas, Xanthomonas* and *Erwinia. Hrp* genes are found in large clusters and are physically and functionally conserved among these phytopathogens (Bonas, 1994). We have decided to focus our attention on the major secreted *hrp* gene product, harpin, which was demonstrated to be a proteanaceous elicitor of the HR in non-host plants (Wei et al., 1992; He et al., 1993).

The harpin encoding genes of *Erwinia amylovora* Ea321 (Wei et al., 1992), *P. s.* pv. *syringae* 61 (He et al., 1993), *Pseudomonas* (*Burkholderia*) *solanacearum* GMI1000 (Arlat et al., 1994), *E. stewartii* (Bauer et al., 1994), *E. chrysanthemi* EC16 (Bauer et al., 1995), *P. s.* pv. *glycinea* race 4 (Preston et al., 1995), *P. s.* pv. *tomato* DC3000 (Preston et al., 1995), and *E. carotovora* subsp. *carotovora* Ecc71 (Cui et al., 1996; Mukherjee et al., 1997), have been identified, cloned and the corresponding elicitor proteins have been expressed, purified and characterized. The degree of nucleotide sequence homology between these harpin encoding genes, their locations in the respective *hrp* clusters, the degree of amino acid sequence homology between the corresponding elicitor proteins, as well as the role of these harpins in HR elicitation have been found to be highly variable. Nevertheless, all of the characterized harpin elicitors share a number of general characteristics. They are rich in glycine, lack cysteine, are heat-stable, and appear highly sensitive to proteolysis. Furthermore, they all lack an N-terminal signal peptide sequence, but are secreted via the *hrp* type III secretion system (Bonas, 1994; He, 1996).

Strobel et al. (1996) demonstrated the HrpZ protein of *P. s.* pv. *syringae* 61 to be an effective proteanaceous inducer of systemic acquired resistance (SAR) in cucumber and tobacco plants. The similarity in induction patterns of pathogenesis-related (PR) proteins, obtained in response to HrpZ and the bacterium that produces it, suggested that HrpZ is either a major inducer of SAR in *P. s.* pv. *syringae* 61, or is representative of the SAR inducers produced by this bacterium. The potential use of the harpin elicitor protein in the management of plant diseases was also highlighted (Strobel et al., 1996). We wish to apply harpin in a similar fashion in the assessment of the resistance mechanisms induced in commercially important South African stone fruit cultivars in response to infection with *P. s.* pv. *syringae* stain NV, one of the most virulent strains of the bacterial canker pathogen isolated from a diseased stone fruit tree in South Africa. A prerequisite for such experiments is the production of adequate quantities of purified, biologically active elicitor protein.

In this study we have amplified the harpin encoding gene of *P. s.* pv. *syringae* NV from its genome and determined the exact nature and extent of the homology between the harpin elicitors produced by *P. s.* pv. *syringae* NV and other *P. syringae* strains. We have also cloned the harpin encoding gene of *P. s.* pv. *syringae* NV into the pMAL-c2 expression system. The recombinant elicitor protein purified from this system was shown to be biologically active and share the common characteristics of harpin elicitor proteins.

Materials and Methods

Bacterial strains, plasmids and culture media

Pseudomonas syringae pathovar *syringae* strain NV (Roos and Hattingh, 1987) was obtained from the Infruitec culture collection. The bacterium was routinely grown on King's B agar plates (King et al., 1954) at 28 °C.

Plasmid pSYH10, containing the open reading frame (ORF) of the *P. s.* pv. *syringae* 61 *hrpZ* gene (He et al., 1993), was a gift from Alan Collmer (Cornell University, Ithaca, NY, USA). Plasmids used as vectors were pGEM-T (Promega Corporation, Madison WI, USA) and pMAL-c2 (New England Biolabs, Beverly, MA, USA). *Escherichia coli* strains DH5α, JM109 and TB1 were used as hosts in transformations. Starter cultures of *E. coli* DH5α and TB1 were obtained from Promega and New England Biolabs respectively. Freezer stocks of competent cells were prepared according to the protocol of Sambrook et al. (1989) and stored at −80 °C. High efficiency competent *E. coli* JM109 cells were obtained from Promega. Transformed DH5α and JM109 cells were routinely grown at 37 °C in Luria-Bertani medium, supplemented with 50 µg/mL ampicillin, for DNA manipulations. Transformed TB1 cells were grown at 37 °C in Rich broth (1.0 % Bacto-tryptone, 0.5 % Bacto-yeast extract, 0.5 % NaCl, 0.2 % glucose), supplemented with 100 µg/mL ampicillin, for DNA isolation and protein expression.

DNA isolations and amplification strategies

Genomic DNA was isolated from 24-hour old strain NV plate cultures, using the method of Goodwin and Lee (1993). The protocol was modified by replacing the microwave incubation with a 30 min incubation in a waterbath at 80 °C. Plasmid DNA was isolated from *E. coli* DH5α(pSYH10) according to the manufacturer's instructions, using the Wizard Plus Miniprep DNA Purification Kit (Promega).

The first set of polymerase chain reactions (PCRs) were performed with primers MAPf1 (5′-ATAGGAATTCATGCAGAGTCTCAGTCTTAACAGCAGC) and MAPr1 (5′-TAATCTAGATCAGGCTGCAGCCTGATTGC), synthesized by MWG-Biotech (Ebersburg, Germany). These primers were respectively based on the first (5′ terminal) 27 and last (3′ terminal) 20 nucleotides of the *P. s.* pv. *syringae* 61 *hrpZ* ORF (He et al., 1993). Random oligonucleotides (ATAG for MAPf1 and TAA for MAPr1), as well as restriction sites (for *Eco*RI and *Xba*I respectively) were included in the primer sequences to facilitate cloning of the PCR product. Strain NV genomic DNA (200 ng/reaction) or control plasmid pSYH10 DNA (100 ng/reaction) were used as templates in the amplification reactions. Reactions were performed in a total volume of 50 µL, with the following final concentrations of components: 0.5 pmol · µL^{-1} of each primer, 2.5 U Bio-X-Act DNA polymerase per reaction, 1× Optiperform reaction buffer, 1.5 mmol · L^{-1} $MgCl_2$ and 0.2 mmol · L^{-1} of each dNTP (all from Bioline, London, UK). Reaction mixtures were overlaid with mineral oil and amplifications performed in a Minicycler (MJ Research, Watertown, MA, USA) with an in-sample probe. Cycling parameters were: 94 °C (3 min); 35 cycles of 94 °C (1 min), 60 °C (2 min), 72 °C (2 min); and 72 °C for 15 min. Reaction products were electrophoresed in a 1 % agarose gel in 1× TAE buffer.

The second set of PCRs were performed using the same cycling parameters as the first, with two exceptions. The Bio-X-Act DNA polymerase and Optiperform reaction buffer were replaced with Biotaq DNA polymerase and NH_4^+ reaction buffer (Bioline), and primers MAPf2 (5′-CAAGCAAGATCCGGAACTCG) and MAPr2 (5′-GCAGCGGTGAAGATGTCGAC), synthesized by the University of Cape Town Synthetic DNA Laboratory (Cape Town, South Africa), were used. These primers were based on nucleotides 193–

212 of the *hrpA* and 97–116 of the *hrpB* ORFs of *P. s.* pv. *syringae* 61 (Preston et al., 1995) respectively. Plasmid pSYH10 DNA was not used as control template, as the regions flanking the *hrpZ* ORF in pSYH10 do not extend far enough to contain the sequences of primers MAPf2 and MAPr2. PCR products were electrophoresed in a 1% agarose gel as described above.

Cloning strategies

The 1.0-kb PCR product obtained with primers MAPf1 and MAPr1 was purified from a 1% low-melting agarose gel according the manufacturer's instructions, using the Wizard PCR Purification kit (Promega). The purified DNA fragment was cloned into the 3′ T-insertion site of the pGEM-T vector (3,005 bp), transformed into *E. coli* JM109 cells, and successful transformants were identified (pGEM-T Vector System II, Promega). Plasmid DNA was isolated from successful transformants as described above. The presence and orientation of the cloned PCR product in recombinant plasmids were determined by digestion for 1 h at 37 °C with *Eco*RI and *Pst*I in SuRE/cut Buffer H (Boehringer, Mannheim, Germany), respectively. Products were electrophoresed as described above.

Directional subcloning of the *P. s.* pv. *syringae* NV *hrpZ* gene into the protein expression vector, pMAL-c2 (New England Biolabs) was performed using *Eco*RI and *Sal*I. Plasmid DNA from *E. coli* JM109(pGNV8) and *E. coli* JM109(pGNV12) was digested at 37 °C for 1.5 h with *Sal*I and 1 h with *Eco*RI in SuRE/cut buffer H (Boehringer). pMAL-c2 DNA (New England Biolabs) was treated in the same way. Reaction products were electrophoresed in a 1% agarose gel. The linearized vector (6,618 bp) and the 1.1-kb *Eco*RI-*Sal*I fragments of pGNV8 and pGNV12 were purified from the gel as described before. Each *Eco*RI-*Sal*I fragment was ligated to the vector (to yield plasmids pMNV1 and pMNV2) according to the manufacturer's instructions, using the Fastlink Ligation and Screening Kit (Epicentre Technologies, Madison, WI, USA). The ligation reaction was allowed to proceed for 15 min. Competent *E. coli* TB1 cells were transformed with the ligation mixture and successful transformants identified, using the same protocol as described above.

Sequencing of PCR products and recombinant plasmids and analysis of sequence data

The 1.0-kb strain NV PCR product in recombinant pGNV plasmids was sequenced, using the ThermoSequenase cycle sequencing kit (Amersham, Little Chalfont, UK), a Model 4000L sequencer (LI-COR Inc., Lincoln, NE) and universal IRD-41 infrared fluorescent dye labelled primers Uf (5′-CGCCAGGGTTTTCCCAGTCACGAC) and Ur (5′-AGCGGATAACAATTTCACACAGGA), synthesized by MWG-Biotech. Sequencing reactions were carried out according to the manufacturer's instructions, using 50–100 fmol plasmid DNA template per reaction. The cycling profile consisted of an initial denaturation of 2 min at 95 °C, followed by 38 cycles of 95 °C (45 sec), 65 °C (60 sec), 72 °C (75 s). Subcloned *Eco*RI-*Sal*I fragments of pGNV8 and pGNV12 in pMNV1 and pMNV2 were sequenced in the same way, with the exception that the reverse primer Ur was replaced with reverse primer MASr1 (5′-GGTCGTCAGACTGTCGATGAAGCC) from MWG-Biotech.

The 1.2-kb PCR product obtained with primers MAPf2 and MAPr2 was purified from a 1% agarose gel as described before. Direct sequencing of the purified PCR product was performed by the University of Stellenbosch core DNA Sequencing Facility on an ABI PRISM model 377 sequencer (Perkin-Elmer, Branchburg, NJ, USA), using an ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) and the same primers as for the PCR. Sequencing reactions were carried out with 40 ng of DNA per reaction, according to the manufacturer's instructions, with the exception that annealing was done at 60 °C. Each sequencing reaction was repeated four times.

Sequence data was analyzed using the programme DAPSA version 4.02 (E. H. Harley, University of Cape Town, South Africa, 1996). The putative *P. s.* pv. *syringae* NV *hrpZ* nucleotide sequence was aligned with the corresponding and flanking genes (*hrpAZB*) of *P. s.* pv. *syringae* 61 (EMBL/GenBank Data Library accession numbers: *hrpA* – L41863, *hrpZ* – L14775 and *hrpB* – L41864), *P. s.* pv. *glycinea* race 4 (EMBL/GenBank Data Library accession number: *hrpAZB* – L41862) and *P. s.* pv. *tomato* DC3000 (EMBL/GenBank Data Library accession number: *hrpAZBCDE* – L41861). The predicted amino acid sequence of the HrpZ protein of *P. s.* pv. *syringae* NV was determined and aligned with that of the HrpZ proteins of the abovementioned bacteria. Homologies between the aligned nucleotide and amino acid sequences of the different bacteria were also calculated, using the same programme.

For phylogenetic analysis, sites involving gaps were excluded from the aligned *hrpZ* nucleotide sequences. Phylogenetic trees were inferred using the neighbour-joining (NJ) method (Saitou and Nei, 1987). Bootstrap analyses were performed to estimate the significance level of the NJ tree internal branches.

Expression, purification and partial characterization of recombinant P. s. pv. syringae NV HrpZ proteins

Recombinant *P. s.* pv. *syringae* NV HrpZ proteins were expressed in *E. coli* TB1(pMNV1) and *E. coli* TB1(pMNV2) as soluble, cytoplasmic fusion proteins with the *E. coli* maltose binding protein (MBP), following the manufacturer's instructions (pMAL expression system, New England Biolabs). To protect the recombinant proteins against proteolysis, all prescribed buffers were supplemented with 1 mmol · L^{-1} phenyl-methyl-sulphonyl fluoride (PMSF). Affinity chromatography using an amylose resin (New England Biolabs) was performed in a 2.5 × 10 cm perspex column as prescribed. The protein content of the column eluate was monitored spectrophotometrically at 280 nm. Fusion protein containing fractions were pooled, and incubated with factor Xa (New England Biolabs) at room temperature for 72 h.

To purify the recombinant harpin from the cleavage reaction, the relatively cumbersome methods suggested by the manufacturer (DEAE- or Q-Sepharose ion exchange chromatography, or removal of maltose by hydroxyapatite chromatography and domain separation by re-binding MBP to amylose) were replaced with a simple $(NH_4)_2SO_4$ precipitation and dialysis protocol. Saturated $(NH_4)_2SO_4$ was added to cleavage reaction mixes, to a final concentration of 25 %. Mixtures were incubated at 4 °C for 30 min and centrifuged at 4 °C for 20 min at 27,200 g_n. Supernatants were removed and pellets resuspended in original reaction volumes of column buffer (10 mmol · L^{-1} Tris-HCl pH 7.4, 0.2 mmol · L^{-1} NaCl, 1 mmol · L^{-1} EDTA, 1 mmol · L^{-1} PMSF). The precipitations were repeated. The final, recombinant harpin containing pellets were resuspended in half of the original reaction volumes of 10 mmol · L^{-1} MES pH 5.5, supplemented with 0.1 mmol · L^{-1} PMSF. Residual $(NH_4)_2SO_4$ was removed by overnight dialysis at 4 °C against 1,000 volumes of the same buffer. A similar protocol was employed by Preston et al. (1995) in the purification of native and recombinant *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 HrpZ proteins.

An aliquot of each fusion protein and recombinant harpin was boiled for 10 min at 100 °C in a waterbath, followed by centrifugation at 13,000 g_n for 5 min. The heat stability of the proteins were subsequently assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and HR analysis. Aliquots of the purified, recombinant harpins were dialyzed overnight at 4 °C against 1,000 volumes of distilled water, supplemented with

0.1 mmol · L^{-1} PMSF, and subjected to analysis by electrospray-mass spectrometry (ES-MS) on a Quattro triple quadropole mass spectrometer (Micromass, Manchester, UK) at the University of Stellenbosch core ES-MS facility, for accurate molecular mass determination.

All the steps of the protein expression and purification protocol were monitored by SDS-PAGE in 8 % gels, according to the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250. The protein content of all fractions were determined using the method of Bradford (1976), as adapted for use in a microtiter plate reader (Appel, 1996). A 1 mg · mL^{-1} bovine serum albumin (Bayer Miles Laboratories, Cape Town, South Africa) solution was used to prepare a reference dilution series (0–1 mg · mL^{-1} protein) for these determinations.

Plant material and HR tests

Tobacco plants (*Nicotiana tabacum* L. cv. ‹White Burley›) were grown in the laboratory at 23–25 °C, with a photoperiod of 24 h. Tobacco leaves were infiltrated on their abaxial surfaces with needleless syringes. Proteins were prepared for infiltration at different concentrations in 10–60 mmol · L^{-1} MES pH 5.5, supplemented with 0.1–0.6 mmol · L^{-1} PMSF. The relevant buffers were consistently infiltrated as controls. HR phenotypes were evaluated daily over a period of one week after infiltration and photographed.

Results

Amplification, cloning and sequencing of a putative P. s. pv. syringae NV hrpZ ORF

Using primers MAPf1 and MAPr1, a single DNA fragment of approximately 1.0 kb was amplified from the genomic DNA of *P. s.* pv. *syringae* NV. The PCR product was cloned into pGEM-T and the recombinant plasmids transformed into *E. coli* JM109. Eleven clones yielded a single *Eco*RI digestion product of approximately 4.0 kb, which indicated that they contained the PCR product. Seven of these clones yielded two bands (of approximately 3.0 kb and 1.0 kb respectively) after digestion with *Pst*I and the remaining four a single band of approximately 4.0 kb. These restriction patterns corresponded to the two possible orientations of the PCR product in the vector.

All eleven clones were sequenced. Alignment of the sequence data to the *hrpZ* ORFs of *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 (not shown), revealed that all eleven clones contained a 1,032-bp putative *hrpZ* ORF, of which nucleotides 1 to 27 and 1,013 to 1,032 respectively constituted the sections of primers MAPf1 and MAPr1 that were based on *P. s.* pv. *syringae* 61 sequence. The remaining nucleotides (numbers 28 to 1,012) in this ORF were identical in seven (63 %) of the clones; this was accepted to be the consensus sequence for this region of the *hrpZ* ORF in *P. s.* pv. *syringae* NV.

To determine whether the first 27 and last 20 bases of the *P. s.* pv. *syringae* NV *hrpZ* ORF were identical to that of *P. s.* pv. *syringae* 61 or not, a second set of PCRs was performed with primers MAPf2 and MAPr2. A single DNA fragment of approximately 1.2 kb was amplified from the genomic DNA of *P. s.* pv. *syringae* NV using these primers. The sequence of a stretch of 1,158 bp in this PCR product was obtained by direct sequencing. Results obtained from repeat reactions were identical. Alignment of this sequence to the *hrpAZB* gene segment of the abovementioned bacteria (not shown) confirmed that the sequence of the last 17 bp the *hrpAZ* spacer region, the entire 1,032-bp *hrpZ* ORF, the entire 27-bp *hrpZB* spacer region, as well as the first 82 bp of the *hrpB* ORF of *P. s.* pv. *syringae* NV had been determined. As was expected, bases 28 to 1,012 of the *hrpZ* ORF were identical to the consensus sequence determined before. The 5′ terminal 27 bases of the *hrpZ* ORFs in the 1.0- and 1.2-kb PCR products were also identical, which meant that the *hrpZ* genes of *P. s.* pv. *syringae* NV and *P. s.* pv. *syringae* 61 were identical in this region. In the last 20 bases of the ORF, two nucleotide differences were consistently detected between the two PCR products. The 1.2-kb product had a C at position 1,023 and a G at position 1,026, compared to a T and an A respectively in these positions in the *P. s.* pv. *syringae* 61 based sequences in the 1.0-kb product. This result confirmed that the *hrpZ* ORF of the two strains were not identical in the terminal 3′ region. These changes were incorporated to finalize the consensus sequence of the *P. s.* pv. *syringae* NV *hrpZ* gene, which was deposited in the EMBL/GenBank Data Library under accession number AF031667.

Sequence homology between the hrpZ gene and HrpZ protein of P. s. pv. syringae NV and that of other P. syringae pathovars and strains and molecular phylogeny

Alignment of the *P. s.* pv. *syringae* NV *hrpZ* nucleotide sequence ($hrpZ_{PssNV}$) with that of its presumed closest known relative, the *hrpZ* gene of *P. s.* pv. *syringae* 61 ($hrpZ_{Pss61}$), yielded a homology of 96.2 % (expressed in terms of identical nucleotides) between aligned base pairs. In total, 44 base pair differences were found between the *hrpZ* genes of the two strains. These included two three-nucleotide insertions (resulting in $hrpZ_{PssNV}$ being 1,032 bp in size, compared to the 1,026 bp of $hrpZ_{Pss61}$) and 38 single nucleotide substitutions. As expected, $hrpZ_{PssNV}$ and $hrpZ_{Pss61}$ shared similar degrees of nucleotide identity with the *hrpZ* genes of *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 (Table 1) and less than 30 % identity with any of the *Erwinia hrpN* genes or the *P. solanacearum popA* gene (results not shown).

Comparison of the sequences obtained for the regions flanking $hrpZ_{PssNV}$ with the corresponding regions of *P. s.* pv. *syringae* 61 revealed only 2 nucleotide differences. These substitutions fell within the *hrpAZ* spacer region and the *hrpZB* spacer region respectively and did not affect the ribosome binding sites identified by Preston et al. (1995) for either *hrpZ* or *hrpB*. The sequence obtained for the 3′ terminal flanking region of $hrpZ_{PssNV}$ traversed the *hrpZB* spacer region and identically matched the first 82 nucleotides of the *P. s.* pv. *syringae* 61 *hrpB* ORF.

The programme DAPSA version 4.02 was used to obtain the predicted sequence of the 343-amino acid *P. s.* pv. *syringae* NV HrpZ protein ($HrpZ_{PssNV}$) (Fig. 1). From its amino acid composition, the theoretical molecular mass of this protein was calculated to be 34,718.5 u. This correlated with the molecular mass of 34.7 Ku reported for the *P. s.* pv. *syringae* NV HrpZ protein ($HrpZ_{Pss61}$) (He et al., 1993). Alignment of the predicted $HrpZ_{PssNV}$ amino acid sequence with the sequence

Table 1: Homology between the nucleotide sequences of the *hrpZ* genes (A) and the predicted amino acid sequences of the HrpZ proteins (B) of *P. s.* pv. *syringae* NV, *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000. Figures for *P. s.* pv. *syringae* NV were obtained by alignment of its *hrpZ* nucleotide and predicted HrpZ amino acid sequences with that of the other pathovars and strains, using the programme DAPSA version 4.02. Results for the other bacteria were recorded previously by Preston et al. (1995) and were confirmed by the relevant sequence alignments with DAPSA in this study.

	P. s. pv. *syringae* NV	*P. s.* pv. *syringae* 61	*P. s.* pv. *glycinea* race 4	*P. s.* pv. *tomato* DC3000
A. *hrpZ* nucleotide sequence homology.				
Number of base pairs	1032	1026	1038	1113
% Identity between aligned nucleotides				
P. s. pv. *syringae* NV		96.2	78.7	66.4
P. s. pv. *syringae* 61			78.7	67.5
P. s. pv. *glycinea* race 4				66.6
P. s. pv. *tomato* DC3000				
B. HrpZ amino acid sequence homology.				
Number of amino acids	343	341	345	370
% Identity between aligned amino acids				
P. s. pv. *syringae* NV		96.4	78.4	62.5
P. s. pv. *syringae* 61			78.8	63.5
P. s. pv. *glycinea* race 4				64.8
P. s. pv. *tomato* DC3000				

of $HrpZ_{Pss61}$ revealed an identity of 96.4 % between aligned amino acids. The two three-nucleotide insertions in the $hrpZ_{PssNV}$ nucleotide sequence resulted in the insertion of an alanine residue between residues 29 and 30 and a glycine residue between residues 224 and 225, compared to the *P. s.* pv. *syringae* 61 amino acid sequence. The other nucleotide differences between the *hrpZ* gene sequences of the two strains resulted in twelve single amino acid substitutions between their predicted HrpZ proteins. Of these, five were conservative substitutions. The predicted amino acid sequence also confirmed that $HrpZ_{PssNV}$ lacks a putative N-terminal signal peptide sequence, is rich in glycine (47 of 343 amino acids = 13.7%) and lacks cysteine and tyrosine. These sequence characteristics agree with those reported for other *P. syringae* harpins (He et al., 1993; Preston et al., 1995; Alfano et al., 1996). Again, the degree of identity found with the HrpZ proteins of *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 was very similar for *P. s.* pv. *syringae* NV and *P. s.* pv. *syringae* 61 (Table 1). No more than 20% amino acid identity was shown by the NV HrpZ protein with the *P. solanacearum* or any of the *Erwinia* harpins (results not shown).

Phylogenetic relationships between the four *P. syringae* *hrpZ* sequences, as inferred by the NJ method, are shown in Fig. 2. The significance of internal branches of the tree was assessed by applying the NJ algorithm to 1,000 bootstrap replicates. The sequences of *P. s.* pv. *syringae* NV and *P. s.* pv. *syringae* 61 grouped together, and were clustered with the *P. s.* pv. *glycinea* race 4 sequence outside the *P. s.* pv. *tomato* DC3000 lineage. The nucleotide sequences of the *Erwinia* (*hrpN*) and *P. solanacearum* (*popA*) harpin encoding genes were not useful as outgroups for phylogenetic analysis, as a result of their low degree of sequence homology with the *P. syringae hrpZ* genes (He, 1996; Mukherjee et al., 1997).

Subcloning of the P. s. pv. syringae NV hrpZ gene into pMAL-c2

Directional subcloning of the *P. s.* pv. *syringae* NV *hrpZ* gene from the pGEM-T vector into the pMAL-c2 vector was performed using the *Eco*RI site incorporated in primer MAPf1 and the *Sal*I site in pGEM-T, and the *Eco*RI and *Sal*I sites of pMAL-c2. *Eco*RI was chosen for the 5′ restriction reaction, as this would minimize the number of residual amino acids linked to the N-terminal of the recombinant *P. s.* pv. *syringae* NV HrpZ protein after cleavage of the fusion protein with factor Xa. The pGEM-T *Sal*I site was preferred for the 3′ restriction reaction as it yielded more consistent results than the *Xba*I site incorporated in the 5′ end of primer MAPr1 (results not shown).

Of all the clones sequenced, only plasmids pGNV8, pGNV12, pGNV13 and pGNV14 contained the PCR product in the orientation meeting the restriction site requirements for subcloning of the *hrpZ* ORF into pMAL-c2. Of these, pGNV13 and pGNV14 lacked the correct *hrpZ* stop codon and were thus not suitable for protein expression. Both the remaining plasmids, pGNV8 and pGNV12, showed differences with the consensus *P. s.* pv. *syringae hrpZ* sequence at three positions. These were a G for an A (at position 260 for pGNV8 and position 275 for pGNV12), a C for a T at position 1,023 and a G for an A in position 1,026. As explained earlier, the latter two substitutions were the result of the *P. s.* pv. *syringae* 61 based primer MAPr1.

pGNV8 and pGNV12 were used for subcloning into pMAL-c2. Sequencing of plasmid DNA isolated from successful *E. coli* TB1 transformants confirmed that two recombinant plasmids, pMNV1 and pMNV2, were generated in the subcloning process. Both of these plasmids contained the 1,032 strain NV *hrpZ* ORF in pMAL-c2 in the same reading frame as the maltose binding protein (MBP) encoding gene (*malE*). As expected, the subcloned NV *hrpZ* ORFs in pMNV1 and pMNV2 were identical to each other and to the consensus NV *hrpZ* sequence, except for the abovementioned substitutions.

Translation of the *hrpZ* ORFs in pMNV1 and pMNV2, using the programme DAPSA version 4.02, confirmed that each of these plasmids encoded a 343-amino acid HrpZ protein. Compared to the amino acid sequence derived from the consensus nucleotide sequence of the *P. s.* pv. *syringae* NV *hrpZ* ORF, the nucleotide substitutions in pMNV1 and pMNV2 would both result in only one conservative substitution of arginine (A*G*G) for lysine (A*A*G), in positions 87 and 92 in the recombinant harpins expressed by *E. coli* TB1(pMNV1) and (pMNV2) respectively (Fig. 1). The substitutions introduced via primer MAPr1 in pGNV8 and pGNV12 were silent, and the amino acid sequences of the recombinant proteins were identical to that of the consensus sequence in the 3′ terminal region.

```
                                                                1    2
Pss NV    (ISEF)MQSLSL NSSSLQTPAM ALVLVRPETE TTGASSSSKA LQEVVVKLAE ELMRNGQLDD   56
Pss 61    ----······ ·····  ·····A· ····-T···· ····· ·····  ·····              55
Psg       ----·····  ···T··S·S· ····I····· ···P·T··R· ····IAQ··Q ··TH·····E   56
Pst       ----··A·N- SI·····S·S LFPVSLNSDV SANT·T···E ·KA·IDQ·VQ A·TQS····E   55

Pss NV    SSPLGKLLAK SMAADGKAGG GIEDVIAALD KLIHEKLGDN FGASAD---- ----------   102
Pss 61    ·····  ·· ·· ····· ····· ····· ····· ·····---- ----------            101
Psg       ·····G· A··S····· ·L··IK···· T····· ·····---- ----------            102
Pst       T·····M··· A·····SAN S·D·IT·S·· ····· ·····GIGAG GGGGGIGGAG         115

Pss NV    ---------- NASGTGQQDL MTQVLNGLVK SMLDDLLT-K QDGGTSFSED DMPMLNKIAQ   151
Pss 61    ---------- S····· ·····A· ····· ·····-- ····· ·····                 150
Psg       ---------- N··D···H·· ·····A· ···N·····- ··D··R···· ·····K···E      151
Pst       SGSGVGGGLS SDA·A··S·· ·S·····G· AV·····PS GE···T··S·· ···T·E·V··   175

Pss NV    FMDDNPAQFP KPDSGSWVNE LKEDNFLDGD ETAAFRSALD IIGQQLGNQQ SGAGGLAG--  209
Pss 61    ····· ····· ····· ····· ····· ·····  ·D··S·----                     208
Psg       ····· ····· ····· ···Q····· ·····S·· NA·····DS                       211
Pst       ·····K···· TR·G···M·· ····G··AQ ···Q····· V·····Q·· GD·S·VT---       232

Pss NV    TGGGLGTPSS FSSNSSGVKG DPLIDANTGP GDSGTTSGEA GQLIGELIDR GLQSVLAGGG   269
Pss 61    ·····  ·· ··N····-M· ····· ····N·R··· ····· ·····                   267
Psg       S·····S·V· NTE··P·SL· ····· ASNSNSN·DV ····· ·····                   271
Pst       S·····S·V· D·----SL· N·A····· AAN·NA·VDV ·····Q···· ·····SS···      287

Pss NV    LGTPV--NTP QTGTA----A NGGQSAQDLD QLLGGLLLKG LEATLKDAGQT ATDVQSSAA   323
Pss 61    ····-····· ····S----- ····· ····· ····· ····· ··G·····              321
Psg       ····--S·A N·ALV----P G·E·PN···G ·····Q·· ·····Q···· G·G····T·      325
Pst       ····DNS·Q P··GTPVANP T·NV·N···G ···S··RQR· ·····Q···N· GA·L·····   347

Pss NV    QIATLLVSTL LQGTRNQ--- AAA                                          343
Pss 61    ····· ·····---- ···                                                341
Psg       ·V·L···NM· ··S·K·---- ···                                          345
Pst       ·V·AQ·INA· ····N··TNQ ·V·                                          370
```

Fig. 1: Predicted amino acid sequence of the *P. s.* pv. *syringae* NV HrpZ protein (Pss NV), aligned to the sequences of the *P. s.* pv. *syringae* 61 (Pss61), *P. s.* pv. *glycinea* race 4 (Psg) and *P. s.* pv. *tomato* DC3000 (Pst) HrpZ proteins. Conservative amino acid substitutions between the proteins of *P. s.* pv. *syringae* NV and *P. s.* pv. *syringae* 61 are indicated in **boldface** in the latter sequence. The N-terminal tetrapeptide ISEF, indicated in brackets in the PssNV sequence, is not encoded by the bacterium's *hrpZ* gene. It is a residual peptide which forms part of the purified recombinant harpins after cleavage of the MBP-harpin fusion proteins expressed in *E. coli* TB1(pMNV1) and *E. coli* TB1 (pMNV2) with factor Xa. The positions of the lysine to arginine mutations encoded in recombinant plasmids pMNV1 and pMNV2 are marked with «1» and «2» above the PssNV sequence. The boxes indicate the three groups of amino acid repeats implicated in the activity of the *P. s.* pv. *syringae* 61 HrpZ protein (He et al., 1993; Alfano et al., 1996).

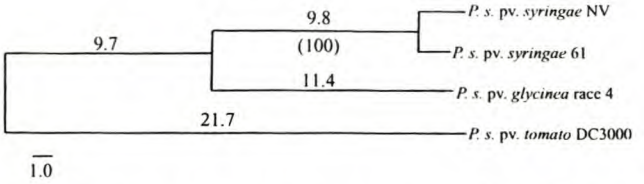


Fig. 2: Neighbour-joining phylogenetic tree derived from *Pseudomonas syringae hrpZ* nucleotide sequences. Horizontal distances are proportional to phylogenetic distances expressed in substitutions per 100 sites. Vertical separations are for clarity only. The root-containing branch is arbitrarily divided into two parts. Bootstrap values are indicated in brackets were applicable.

Expression, purification and partial characterization of recombinant P. s. pv. syringae NV HrpZ proteins

The protein profiles of *E. coli* (pMNV1) before and after addition of isopropyl-β-D-thiogalactoside (IPTG) are shown in Fig. 3. Induction with IPTG led to the expression of a large amount of protein of approximately 73 Ku, as calculated from the electrophoretic mobility of molecular mass marker proteins. This corresponded well with the theoretical molecular mass of the MBP-harpin fusion protein of 77.4 Ku (42.7 Ku + 34.7 Ku). This protein was effectively released from the cells by sonication and collected in the «crude cellular extract», from which it was purified by affinity chromatography on an amylose column (Fig. 4). In addition to the 73 Ku fusion protein, a number of smaller proteins (46–60 Ku) were detected in the fractions eluted with maltose from the amylose column (Fig. 3). These were believed to correspond to incomplete translation products of *malE-hrpZ* transcripts.

Cleavage with factor Xa yielded two major protein bands, of approximately 43 and 35 Ku respectively (Fig. 3). These values correlated with the theoretical molecular masses of the MBP and the recombinant harpin (rHrpZ$_{PssNV}$). The two

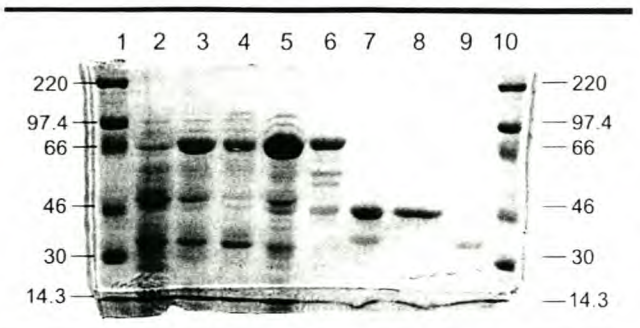


Fig. 3: Protein profiles on 8 % SDS-PAGE of *E. coli* TB1 (pMNV1) fractions collected during the expression and purification of recombinant *P. s.* pv. *syringae* NV HrpZ protein. Lanes 1 and 10, «Rainbow» molecular mass marker proteins (Amersham); lane 2, uninduced cells; lane 3, cells after induction with IPTG; lane 4, insoluble proteins after sonication; lane 5, fusion protein containing «crude cell extract»; lane 6, fractions eluted from the amylose column with maltose (compare with Fig. 4); lane 7, reaction products of cleavage with factor Xa; lane 8, MBP containing supernatant of $(NH_4)_2SO_4$ precipitation; lane 9, purified recombinant harpin. Molecular masses of «Rainbow Marker» protein standards are given in kDa. Identical protein profiles were obtained for corresponding fractions using *E. coli* TB1 (pMNV2).

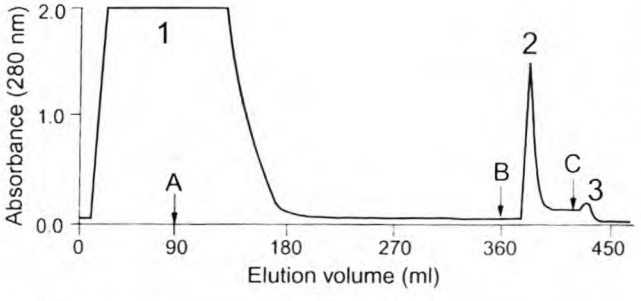


Fig. 4: Typical chromatogram obtained for the resolution of *E. coli* TB1(pMNV1) «crude cellular extract» on an amylose column. The column was equilibrated in column buffer (10 mmol · L^{-1} Tris-HCl pH 7.1, 0.2 mol · L^{-1} NaCl, 1 mmol · L^{-1} EDTA, 1 mmol · L^{-1} PMSF). 45 mL «crude cellular extract» was diluted 1 : 1 in column buffer to a protein concentration of 2.7 mg · mL^{-1}, and loaded on the column at a flow speed of 1 mL · min^{-1}. The column was washed with 270 mL (18 column volumes) column buffer (introduced to the column at A). Fusion protein was subsequently eluted with 60 mL column buffer, supplemented with 10 mmol/L maltose (introduced to the column at B). 15 fractions of 2 mL each, followed by 5 fractions of 3 mL each were collected and pooled for subsequent cleavage of the fusion proteins. The column was regenerated by washing with 30 mL distilled water (introduced to the column at C), followed by 90 mL 0.1 % SDS, 90 mL distilled water and 60 mL column buffer. The protein content of the column eluate was monitored spectrophotometrically at 280 nm. Numbered peaks correspond to: soluble cytoplasmic proteins (excluding MBP fusions) contained in the *E. coli* TB1(pMNV1) «crude cellular extract» (1); MBP fusion proteins (2; compare to lane 6 in Fig. 3) and maltose (3). Similar results were obtained for the chromatography of the *E. coli* TB1 (pMNV2) «crude cellular extract».

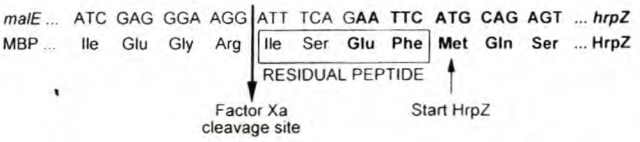


Fig. 5: Cleavage site for factor Xa in plasmids pMNV1 and pMNV2. Nucleotides and amino acids contributed by the vector pMAL-c2 in pMNV1 and pMNV2 are indicated in normal typescript and those corresponding to the 5′ region of the cloned *Eco*RI-*Sal*I *P. s.* pv. *syringae* NV *hrpZ* containing insert in **boldface**.

proteins were separated successfully from each other by $(NH_4)_2SO_4$ precipitation, leaving the MBP in the supernatant and the $rHrpZ_{PssNV}$ in the pellet. The identity of the 43 Ku band as MBP was supported by the presence of a band of identical mobility on SDS-PAGE in a purified preparation of MBP from New England Biolabs (result not shown).

SDS-PAGE of the supernatants and pellets obtained after heat-treatment of the fusion proteins and purified recombinant harpins revealed that the purified recombinant harpins displayed the expected heat-stability. The purified fusion proteins were, however, not heat-stable (results not shown).

Due to the construction of the pMAL-c2 multiple cloning site, cleavage of the MBP-HrpZ fusion protein with factor Xa would theoretically result in a residual tetrapeptide, Ile-Ser-Glu-Phe, at the N-terminal of the purified $rHrpZ_{PssNV}$ (Fig. 5). Taking this into account, the molecular masses of the recombinant harpins purified from *E. coli* TB1 (pMNV1) and (pMNV2) were calculated to be 35,223 u (34,746.5 u + 476.5 u for the residual peptide). These masses were confirmed by ES-MS (results not shown), indicating that the recombinant harpins that were purified corresponded to the protein products of the *P. s.* pv. *syringae* NV hrpZ gene contained in pMNV1 and pMNV2.

Biological activity of MBP-HrpZ fusion proteins and the purified, recombinant P. s. pv. syringae NV HrpZ proteins

The biological activity of the MBP-HrpZ fusion protein and the purified, recombinant *P. s.* pv. *syringae* NV HrpZ protein was assessed by their ability to elicit the HR in tobacco plants (Klement, 1963, 1982; Klement et al., 1964). Both the recombinant proteins purified from *E. coli* TB1 (pMNV1) and (pMNV2) displayed a positive HR at concentrations higher than 20 µmol · L^{-1}. The HR was characterized by watersoaking of the infiltrated region within 12 – 24 h after injection. Confluent necrosis of approximately 50 % of the infiltrated area subsequently appeared within 48 h (Fig. 6 A). This correlated with the results obtained by Preston et al. (1995) for the biological activity of recombinant and native HrpZ proteins of *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000, and resembled the type II HR described by Alfano et al. (1996). Heat treatment of recombinant harpins did not affect their HR eliciting ability (results not shown).

At concentrations of the recombinant harpins lower than 20 µmol · L^{-1}, shown in Fig. 6 B, watersoaking was limited to a small area surrounding the point of infiltration and appeared within 24 h. Infiltrated regions became chlorotic

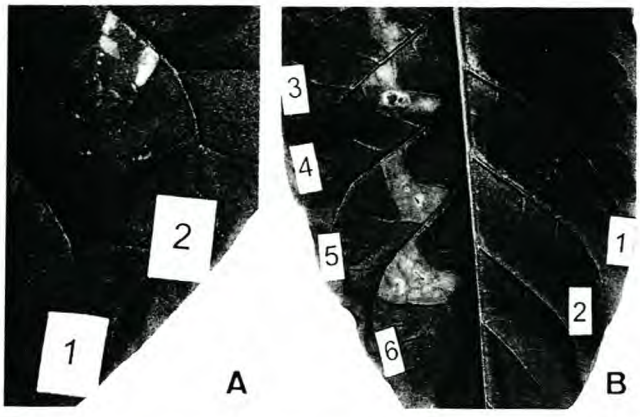


Fig. 6: HR phenotypes of recombinant harpins and MBP-HrpZ fusion proteins purified from *E. coli* TB1 (pMNV1) in tobacco. Buffers that were used for protein preparation and infiltrated as controls are: A1, buffer 1 (60 mmol/L MES + 0.6 mmol/L PMSF); B1, buffer 2 (10 mmol/L MES pH 5.5 + 0.1 mmol/L PMSF); B2, buffer 3: 30 mmol/L MES pH 5.5 + 0.3 mmol/L PMSF. Protein preparations are: A2, purified recombinant harpin (36 µmol/L, in buffer 1); B3, MBP-HrpZ fusion protein (6 µmol/L) in buffer 2; B4, MBP-HrpZ fusion protein (24 µmol/L) in buffer 3; B5, purified recombinant harpin (2.5 µmol/L) in buffer 2; B6, purified recombinant harpin (16 µmol/L) in buffer 3. Results were photographed 5 days after infiltration. Similar results were obtained for proteins from *E. coli* TB1 (pMNV2).

within 48 h and the limited watersoaked areas only appeared necrotic after 72 h. Chlorosis remained limited to the infiltrated area. This response resembled the HR symptoms observed with dilute suspensions ($<10^6$ cells · mL^{-1}) of phytopathogenic bacteria infiltrated into non-host plants (Klement and Goodman, 1967; Turner and Novacky, 1974; Lummerzheim et al., 1993), and was markedly different to the symptoms obtained with control buffers. Similar HR symptoms were obtained with MBP-HrpZ fusion proteins isolated from *E. coli* TB1 (pMNV1) and (pMNV2), infiltrated at concentrations of 2.5–24 µmol · L^{-1} (Fig. 6 B).

Discussion

In this study, a segment of the *hrpAZB* operon of *Pseudomonas syringae* pathovar *syringae* NV was amplified, cloned and sequenced. The complete harpin elicitor encoding gene ($hrpZ_{PssNV}$) was subsequently identified and compared to the corresponding gene of related *P. syringae* pathovars and strains. The NV *hrpZ* gene was then subcloned into the pMAL-c2 system, from which biologically active recombinant HrpZ protein was purified.

To our knowledge, this study is the first to compare the *hrpZ* nucleotide and HrpZ amino acid sequences of different strains of the same pathovar of a phytopathogenic bacterium. A phylogenetic analysis of the three known *P. syringae hrpZ* sequences, and the sequence obtained in this study, confirmed the phylogenetic relationship between *P. syringae* pathovars and strains inferred previously from total DNA-DNA hybridization data (Pecknold and Grogan, 1973). The degree of sequence identity between the two *P. s.* pv. *syringae hrpZ* genes fell within the range of 95–100 %, reported by Pecknold and Grogan (1973) for total DNA homology between *P. s.* pv. *syringae* strains. However, the sequence identity of the pv. *glycinea* (79 %) and pv. *tomato* (66–68 %) *hrpZ* genes with the pv. *syringae* sequences exceeded the homology limits (60–66 % and 58 % respectively) reported by Pecknold and Grogan for total DNA homology between these pathovars and a pv. *syringae* reference strain (Pecknold and Grogan, 1973). The best comparative sequence data for other *hrp* loci from *P. syringae* pathovars are available for *hrpA, hrpB* (Preston et al., 1995) and *hrpL* (Mansfield, et al., 1994; Xiao et al., 1994; Liang and Jones, 1995; Cournoyer et al., 1996). Of these, *hrpB* and *hrpL* follow the same trend as *hrpZ*, being more homologous between pathovars than expected from values reported by Pecknold and Grogan (1973). These findings support the concept that *hrp* genes occur within «pathogenicity islands» and are hence more highly conserved (Alfano and Collmer, 1996).

Like all the other characterized *hrp* encoded and secreted elicitor proteins, the newly identified *P. s.* pv. *syringae* NV HrpZ protein also proved to be rich in glycine, devoid of cysteine and lacking an N-terminal signal peptide. Extensive analysis of the structure of the *P. s.* pv. *syringae* 61 HrpZ protein by He et al. (1993) and Alfano et al. (1996) has led, amongst others, to the identification of three sets of identical repeated sequences. One set, LAKSM, was identified in the N-terminal portion of the protein, whilst the other two, GGGLGTP and QTGT, reside closer to the C-terminal. Comparison of the predicted HrpZ sequences of *P. s.* pv. *syringae* NV, *P. s.* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 (Fig. 1) shows both of the GGGLGTP repeats to be extremely well-conserved in all four pathogens, with only one conservative substitution (serine for threonine) in the first of these repeats in the *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 proteins (Preston et al., 1995). Mutations in the other two sets of repeats are, however, much more abundant among the four pathogens.

In conclusion, we have recovered from the South African stone fruit pathogen biologically active recombinant harpin protein. This has provided us with a useful tool in our investigations of the resistance mechanisms induced in stone fruits.

Acknowledgements

The authors wish to thank Fritz Tiedt (Department of Biochemistry, University of Stellenbosch) for the production of photographs, and Thinus van der Merwe (University of Stellenbosch core ES-MS Facility) and Carel van Heerden (University of Stellenbosch core DNA Sequencing Facility) for their expert technical contributions. Prof. Eric Harley (Department of Chemical Pathology, University of Cape Town) is thanked for his assistance with the phylogenetic analysis. Financial support from the South African Foundation of Research and Development (FRD), the University of Stellenbosch and ARC-Infruitec, is gratefully acknowledged.

References

ALFANO, J. R., D. W. BAUER, T. M. MILOS, and A. COLLMER: Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ har-

pin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. Mol. Microbiol. *19,* 715–728 (1996).

Alfano, J. R. and A. Collmer: Bacterial pathogens of plants: Life up against the wall. Plant Cell *8,* 1684–1698 (1996).

Appel, M.: Investigations into the production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae* isolated from a nectarine tree. M. Sc. thesis, University of Stellenbosch (1996).

Arlat, M., F. van Gijsegem, J. C. Huet, J. C. Pernollet, and C. A. Boucher: PopA1, a protein that induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum.* EMBO J. *13,* 543–553 (1994).

Bauer, D. W., A. J. Bogdanove, S. V. Beer, and A. Collmer: *Erwinia chrysamthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. *7,* 573–581 (1994).

Bauer, D. W., Z. M. Wei, S. V. Beer, and A. Collmer: *Erwinia chrysanthemi* harpin$_{Ech}$: an elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. Mol. Plant-Microbe Interact. *8,* 484–491 (1995).

Bonas, U.: *Hrp* genes of phytopathogenic bacteria. In: Dangl, J. L. (ed.): Curr. Top. Microbiol. Immunol. *192:* Bacterial pathogenesis of plant and animals – molecular and cellular mechanisms, pp. 79–98. Springer-Verlag, Berlin–Heidelberg–New York (1994).

Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. *72,* 248–254 (1976).

Cournoyer, B., D. Arnold, R. Jackson, and A. Vivian: Phylogenetic evidence for a diversification of *Pseudomonas syringae* pathovar *pisi* race 4 isolates into two distinct lineages. Phytopathology *86,* 1051–1056 (1996).

Cui, Y., L. Madi, A. Mukherjee, C. Korsi Dumenyo, and A. K. Chatterjee: The RsmA$^-$ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN*$_{Ecc}$ and elicit a hypersensitive reaction-like response in tobacco leaves. Mol. Plant-Microbe Interact. *9,* 565–573 (1996).

Goodwin, D. C. and S. B. Lee: Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechniques *15,* 438–444 (1993).

Hattingh, M. J., I. M. M. Roos, and E. L. Mansvelt: Infection and systemic invasion of deciduous fruit trees by *Pseudomonas syringae* in South Africa. Plant Disease *73,* 784–789 (1989).

He, S. Y.: Elicitation of plant hypersensitive response by bacteria. Plant Physiol. *112,* 865–869 (1996).

He, S. Y., H. C. Huang, and A. Collmer: *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. Cell *73,* 1255–1266 (1993).

King, E. O., M. K. Ward, and D. E. Raney: Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. *44,* 301–307 (1954).

Klement, Z.: Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature *199,* 299–300 (1963).

– Hypersensitivity. In: Mount, M. S. and G. H. Lacy (eds.): Phytopathogenic prokaryotes, vol. *2,* pp. 149–177. Academic Press, New York (1982).

Klement, Z., G. L. Farkas, and L. Lovrekovich: Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology *54,* 474–477 (1964).

Klement, Z. and R. N. Goodman: The hypersensitive reaction to infection by bacterial plant pathogens. Annu. Rev. Phytopathol. *5,* 17–44 (1967).

Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature *227,* 680–685 (1970).

Liang, L. Z. and A. L. Jones: Organization of the *hrp* gene cluster and nucleotide sequence of the *hrpL* gene from *Pseudomonas syringae* pv. *morspronorum.* Phytopathology *85,* 118–123 (1995).

Lindgren, P. B., R. C. Peet, and N. J. Panopoulos: Gene cluster of *Pseudomonas syringae* pv. «phaseolicola» controls pathogenicity on bean and hypersensitivity on non-host plants. J. Bacteriol. *168,* 512–522 (1986).

Lummerzheim, M., D. de Oliveira, C. Castresana, F. C. Miguens, E. Louzada, D. Roby, M. van Montagu, and B. Timmermann: Identification of compatible and incompatible interactions between *Arabidopsis thaliana* and *Xanthomonas campestris* pv. *campestris* and characterization of the hypersensitive response. Mol. Plant-Microbe Interact. *5,* 532–544 (1993).

Mansfield, J., C. Jenner, R. Hockenhull, M. A. Bennet, and R. Stewart: Characterization of *avrPphE,* a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY,* a new *hrp* gene identified in the haloblight bacterium. Mol. Plant-Microbe Interact. *7,* 726–739 (1994).

Mukherjee, A., Y. Cui, Y. Liu, and A. K. Chatterjee: Molecular characterization and expression of the *Erwinia carotovora hrpN*$_{Ecc}$ gene, which encodes an elicitor of the hypersensitive reaction. Mol. Plant-Microbe Interact. *10,* 462–471 (1997).

Pecknold, P. C. and R. C. Grogan: Deoxyribonucleic acid homology groups among phytopathogenic *Pseudomonas* species. Intl. J. Syst. Bacteriol. *23,* 111–121 (1973).

Preston, G., H. C. Huang, S. Y. He, and A. Collmer: The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae, glycinea,* and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. Mol. Plant-Microbe Interact. *8,* 717–732 (1995).

Roos, I. M. M. and M. J. Hattingh: Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. Phytopathology *77,* 900–908 (1987).

Saitou, N. and M. Nei: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4,* 406–425 (1987).

Sambrook, J., E. F. Fritsch, and T. Maniatis: Molecular cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor NY (1989).

Strobel, N. E., C. Ji, S. Gopalan, J. A. Kuc, and S. Y. He: Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZ$_{Pss}$ protein. Plant J. *9,* 431–439 (1996).

Turner, J. G. and A. Novacky: The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology *64,* 885–890 (1974).

Xiao, Y., S. Heu, J. Yi, Y. Lu, and S. W. Hutcheson: Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. *176,* 1025–1036 (1994).

Wei, Z. M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer: Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora.* Science *257,* 85–88 (1992).

CHAPTER 4

DIFFERENTIAL DISPLAY OF EUKARYOTIC mRNA: THE TECHNOLOGY CHOSEN FOR THE IDENTIFICATION OF PLANT GENES INVOLVED IN INTERACTIONS BETWEEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* AND STONE FRUIT TREES

4.1 INTRODUCTION

The first part of this study was dedicated to cloning of the harpin encoding gene of the bacterial canker pathogen, *Pseudomonas syringae* pv. *syringae* NV. In the second part, the emphasis was shifted to the responses elicited by this pathogen and its harpin elicitor protein in a stone fruit host. For such investigations, a technique for the analysis of differential gene expression in plants had to be employed. The differential display (DD) technique of Liang and Pardee (1992) was identified as the most suitable for these investigations, for reasons that are outlined in section 4.2. The paper included in section 4.3 comprises an extensive review of the principles of this technology, criticisms against it and modifications aimed at extending its usefulness, and serves as a background for the experimental work presented in Chapter 5.

4.2 WHY DIFFERENTIAL DISPLAY?

Higher organisms contain thousands of genes of which only a small fraction is expressed in any individual cell/tissue at a given time. The choice of which ones are expressed is determined by genetic, environmental and/or experimentally induced variation and underlies development, differentiation, maintenance, responses to endogenous and exogenous stimuli, aging and programmed cell death (Liang and Pardee 1992, Wan et al. 1996, Hansen and Harper 1997). The approach followed in an investigation of any of these processes is determined by whether the focus will be on expression patterns of selected genes known to participate in the process, or whether differentially expressed genes involved in the process are to be identified without any prior information of such genes.

The second part of the current study, presented in Chapter 5, was aimed at the identification of genes that are induced in a stone fruit host of *P. s.* pv. *syringae* in response to challenge with the bacterium and/or its harpin elicitor. In a previous study conducted in our laboratory, colorimetric assays for two families of pathogenesis-related proteins (chitinases and β-1,3-glucanases; see section 2.3.3.4) were employed successfully to show temporal differences in the induction of these proteins in potato cultivars exhibiting different degrees of resistance to the dry rot fungus, *Fusarium solani* (Appel et al. 1994, Ries et al. 1994, Appel et al. 1995). Although these assays were applicable to the current study, differential display (DD; Liang and Pardee 1992) was identified as the technology of choice for the task at hand, for the following reasons:

a. None of the studies on the induction of plant defense genes by *P. syringae* pathovars, and/or recombinant harpin proteins published up to 1997 had been performed with stone fruit trees. A technique capable of making a broad-scale survey of gene expression patterns, without any prior information of the genes that are differentially expressed between different experimental conditions, thus seemed to be the most appropriate for the current study. (Our colorimetric assays would allow an analysis of only a fraction of the genes potentially induced in the *P. s.* pv. *syringae*-stone fruit interaction.)

b. The responses of two cultivars of *Prunus salicina* to four different treatments were to be compared in the current study (see section 5.3). It would have been extremely cumbersome, or even impossible (Wan et al. 1996), to perform such a complex analysis using other established techniques for the assessment of differential gene expression, such as two-dimensional polyacrylamide gel electrophoresis (2-D PAGE; see Bauw and Van Montagu 1997), or differential or subtractive hybridization methods (see Sargent 1987)*.

c. Although our research team did not have any expertise in DD, all of the technologies

---

* A technique that is in principle related to DD, **RNA fingerprinting by arbitrarily-primed PCR** (RAP-PCR) was also introduced in 1992. Although the inventors of this technique proposed it to be more useful than DD (Welsh et al. 1992, McClelland and Welsh 1995), the literature showed that DD had become the method of choice for differential mRNA expression analysis (see section 4.3.3).

DNA array technology (Lockhardt et al. 1996, Marshall and Hodgson 1998, Ramsay 1998) was introduced in the middle nineties as a radically new alternative for differential gene expression analysis. Being capable of the simultaneous identification of large numbers of differentially expressed genes through the immobliziation of known sequences on silicone chips, it is more powerful than any of its predecessors. Highly specialized computer technology is, however, required to interpret the results of these hybridization experiments. By 1998, the use of this technology was in its infancy in South Africa, and is only now (2001) being established for routine use.

on which it is based (PCR, PAGE and recombinant DNA technology) were either established in our laboratory or were accessible to us.

The events leading to the author's visit to the University of Tennessee during 1997-1998 to gain expertise in DD for the purpose of establishing this technology in our laboratory, were outlined in Chapter 1. The review paper on DD technology published during this visit is included in the next section.

4.3 DIFFERENTIAL DISPLAY OF EUKARYOTIC mRNA: MEETING THE DEMANDS OF THE NEW MILLENNIUM?

4.3.1 Contributions of co-authors

The author of this dissertation conducted all the research for the review paper included in this section, compiled the manuscript and performed the DD analyses aimed at identifying genes that are differentially expressed in supernodulation mutants of soybean.

The contribution of each of the co-authors were as follows:

a. *Prof. Dirk U. Bellstedt* is an associate professor of Biochemistry at the University of Stellenbosch, and the promoter of this dissertation. For the purpose of establishing DD technology in his laboratory for this and future studies, he negotiated the author's research visit to the Prof. Gresshoff's laboratory and provided financial support for this visit.

b. *Prof. Peter M. Gresshoff** held the Racheff Chair for Plant Molecular Genetics in the Institute of Agriculture and Center for Legume Research at the University of Tennessee (Knoxville, Tennessee, USA) from 1989-1999. He offered the author the opportunity to gain expertise in DD in his laboratory, against the background of his research team's extensive experience in other arbitrarily-primed PCR technologies. He initiated the writing of the review paper, constructed Figs. 1 and 2 and co-authored the final text of the conclusion.

Minor revisions of the original text suggested by the co-authors were incorporated in the final manuscript.

---

* Currently Head of the School of Life Sciences and Director of the Center for Molecular Plant Sciences, University of Queensland, Brisbane, Australia.

4.3.2 Errata

The published review paper contains three errors:

a. "... the 0.2 **mg** total RNA typically used ..." should read 0.2 μg (p. 564).

b. **û** in front of "... <lock-docking> ..." should be removed (Table 1 on p. 566).

c. The following publication cited in the text (p. 567) was omitted from the list of references in the published paper:

MEN, A.E. and P.M. GRESSHOFF: Efficient cloning of DAF polymorphic markers from silver stained polyacrylamide gels. BioTechniques 24, 593-595 (1998).

J. Plant Physiol. Vol. 154. pp. 561–570 (1999)
http://www.urbanfischer.de/journals/jpp

JOURNAL OF
Plant Physiology

Review

Differential Display of Eukaryotic mRNA: Meeting the Demands of the New Millennium?

M. Appel[1,2], D. U. Bellstedt[2], and P. M. Gresshoff[1]*

[1] Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, University of Tennessee, Knoxville, P.O. Box 1071, Knoxville, TN 37901-1071, USA

[2] Department of Biochemistry, University of Stellenbosch, Private Bag X1, Stellenbosch 7602, South Africa

Received June 14, 1998 · Accepted October 29, 1998

Summary

Differential display of eukaryotic mRNA (DD) was introduced in 1992 as a powerful new method for the identification and cloning of differentially expressed eukaryotic genes. Compared to existing methods, DD was technically simple and fast, and offered superior sensitivity and information generation capabilities. It quickly found wide-ranging application in many areas of biology. Ever since, many modifications have been suggested to deal with the criticisms inherent to the design of the technique. The majority of these proposed improved primer designs, which would facilitate the detection of rare mRNAs and eliminate false positives. Others sought to simplify the process through which DD results are verified. Even though molecular genetics and biology are in the process of being revolutionized by DNA array technology, DD remains a merited companion for the molecular biologist of the new millennium.

Key words: Gene expression, third world, technology transfer, plant genomics, arbitrary PCR, mRNA.

Introduction

Researchers in many areas of biology, such as cancer research, developmental biology, pathology and plant physiology, have been pursuing the identification and isolation of eukaryotic genes that are differentially expressed in cells/tissues as a result of genetic, environmental and/or experimentally induced variation. Up to the early nineties, the methodologies available to this end, such as differential and subtractive hybridization (Sargent, 1987), were cumbersome and yielded limited information. In 1992, Liang and Pardee introduced a powerful new technique, called differential display of eukaryotic mRNA (Liang and Pardee, 1992) (DD; also referred to as «differential display reverse transcription PCR» or DDRT-PCR; Bauer et al., 1993). Although a multistep process, DD was based on simple, widely-accessible and established techniques, such as arbitrarily-primed PCR, polyacrylamide gel electrophoresis (PAGE), recombinant DNA technology and Northern hybridization (see Fig. 1). Furthermore, it offered unprecedented sensitivity, reproducibility and speed, as well as the possibility to compare multiple conditions simultaneously (Liang and Pardee, 1992). A different, but related strategy, named RNA fingerprinting by arbitrarily primed PCR or RAP (Welsh et al., 1992) was introduced shortly after Liang and Pardee's strategy. However, DD quickly became the preferred method for the identification and cloning of differentially expressed genes; a fact that was reflected by a 1996 Medline database search (GenHunter Corp. 1997). Yet, criticisms inherent to the design of DD have repeatedly been raised, and many modifications aimed at extending the usefulness of the technique have been suggested over the past seven years (Wan et al., 1996). Some of these modifications were discussed in an early review by Liang and Pardee (Liang and Pardee, 1995), but many others have appeared ever since.

We are on the threshold of a new millennium, and molecular genetics and biology is being revolutionized by fast emerging DNA array technology (reviewed in a recent issue

* Correspondence (e-mail: pgresshoff@soybean.ag.utk.edu)

0176-1617/99/154/561 $ 12.00/0

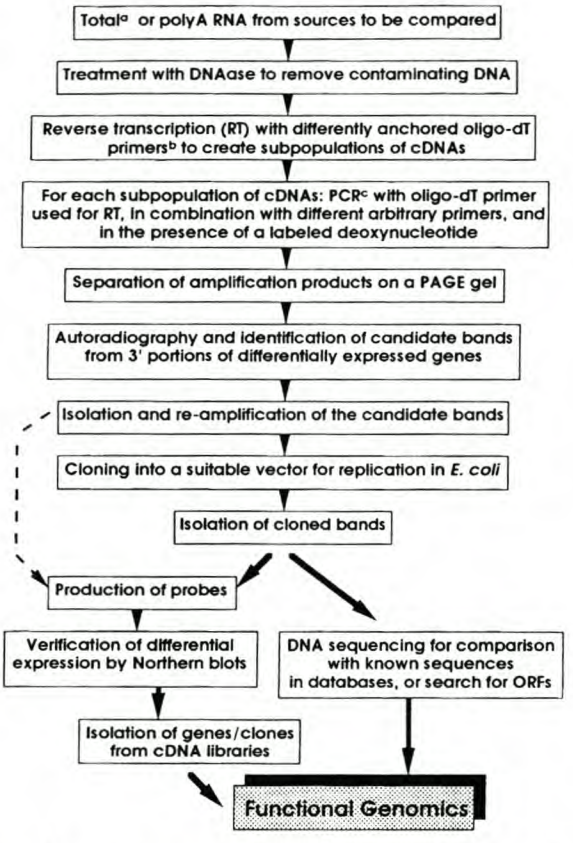


Fig. 1: The overall strategy of mRNA differential display. a. Total RNA has become the preferred substrate for DD PCR (Liang et al., 1993). b. The following oligo-dT primer combinations are commonly used: (i) 12 two-base anchored primers (T_nMN, where M = dA/dC/dG and N = dA/dC/dG/dT) (Liang and Pardee, 1992), (ii) 4 degenerate two-base anchored primers (T_nVN, where V = an equimolar mix of dA, dC and dG; Liang et al., 1993), or (iii) 3 one-base anchored primers (T_nM) (Liang et al., 1994). n = 9 to 12, and 5′ restriction sites have been incorporated to enhance specificity and facilitate the cloning of amplification products (Liang et al., 1994). c. The RAP strategy (Welsh et al., 1992) differs from DD in two respects: only one arbitrary primer (18- to 20-mer) is used for both cDNA synthesis and amplification, and reverse transcription is followed by a PCR using a few initial low-stringency cycles. During these cycles, second cDNA strands are synthesized by extension of the same arbitrary primer at adequately matched sites on the first strands. The resulting products, tagged with the arbitrary primer at both ends, are amplified specifically during subsequent high-stringency cycles.

of Nature Biotechnology (Ramsay, 1998; Marshall and Hodgson, 1998). This prompted us to evaluate the current status of DD and its merit as a partner in gene analysis and discovery in the new millennium.

The principles of differential display

The principles of DD have been described extensively (Liang and Pardee, 1995) and are outlined in Fig. 1. In a multi-step process such as this, it is evident that the eventual result (i.e. the verification that a cloned band truly represents a differentially expressed mRNA species) depends on the successful execution of each consecutive step, as this determines the quality of the starting material for the next step. Success with DD thus resides in the ability to obtain intact, DNA-free RNA, and to convert this to amplification products representative of the gene expression pattern in each of the sources to be compared. In our opinion, a sound knowledge and understanding of the biochemical and physical principles governing each individual technique in this process goes a long way towards the successful application of DD.

It is important to understand that the generation of amplification products during DD is based on the principles of arbitrarily-primed PCR (Welsh and McClelland, 1990) rather than that of «classical» (i.e. specific) PCR (Mullis and Faloona, 1987). Although DD differs in its design from arbitrary PCR techniques typically used for DNA and RNA fingerprinting (Vogt et al., 1997), DD amplicons are also defined by the stochastic behaviour of short arbitrary primers, under the specific parameters (cDNA, Mg^{2+} and deoxynucleotide (dNTP) concentrations, as well as annealing temperature) employed in DD (Liang and Pardee, 1992; Caetano-Anollés et al., 1992).

The design of arbitrary primers for DD has been the subject of much controversy and discussion. Originally, it was argued that arbitrary primers should be short, in order to randomly anneal frequently enough to the 5′ ends of cDNA species to generate amplification products which are representative of the majority of mRNAs in the cell/tissue, and can be resolved on a «sequencing» gel (i.e. have different sizes, preferably all smaller than 500 bp). Theoretically, this was best obtainable using 6- and 7-mers. On the other hand, these primers should be long enough to enable specific amplification by PCR when using *Taq* polymerase at its optimal temperature (72 °C). In the presence of largely decreased dNTP concentrations (typically around $2\,\mu mol \cdot L^{-1}$) used in DD, 10-mer arbitrary primers were found to meet this criterion (Liang and Pardee, 1992).

In practice, 10-mer arbitrary primers display many more amplification products than predicted from their theoretical annealing probabilities (e.g. one recognition site per 4^{10} or 1.05×10^3 kb for any arbitrary 10-mer sequence; Liang and Pardee, 1992). This discrepancy is attributed to 5′ mismatches in the annealing of arbitrary primers during the low-stringency PCR (Bauer et al., 1993). Annealing specificity and the initiation of amplification by *Taq* polymerase has been shown to be dependent on the 3 to 6 most 3′ nucleotides of arbitrary primers during a DD PCR. Sequencing of cloned amplification products has confirmed that one to four mismatches in the remaining 5′ bases often occur without abolishing amplification, leading to observed banding patterns characteristic of shorter arbitrary primers. Similar banding patterns have, however, not been observed consistently with 10-mers containing identical 3′ terminals. This suggests that the 5′ portion of the primer plays an important role in stabilizing (or destabilizing) the primer-cDNA complex and explains why longer primers variably display more than the predicted number of bands (Bauer et al., 1993; Caetano-Anollés et al., 1992; Galindo et al., 1997).

Longer oligomers (10–15 bp; including or excluding restriction endonuclease sites for cloning purposes) have gained increased popularity as arbitrary primers for DD. These oligonucleotides achieve the amplification specificity of longer

primers, yet possess the displaying capabilities characteristic of shorter primers due to 5′ mismatching. To increase their efficiency and specificity, following criteria have been recommended for arbitrary primers: a 50 % GC content, dG or dC as the preferred most 3′ base, and the absence of self-complementary that can lead to secondary structures (Bauer et al., 1993; Galindo et al., 1997; Liang et al., 1994; Mou et al., 1994) (in contrast, mini-hairpin primers have been shown highly successful in other arbitrary PCR applications (Caetano-Anollés and Gresshoff, 1994).

Criticisms and modifications

The successful application of differential display has been documented extensively (GenHunter Corp. 1997). Yet, many researchers have experienced frustration and failure with this technique (Wan et al., 1996). The four most frequently raised criticisms, and the most prominent methodological modifications aimed at addressing each of these complaints, are outlined below.

(i) Limited usefulness of information derived from DD

The complaint has been raised that amplification products identified as candidate differentially expressed mRNA species do not usually comprise the complete coding regions of genes for subsequent cloning and manipulation (Galindo et al., 1997). Although this is true, and a limitation inherent to the design of the technique, the availability of rapidly expanding sequence databases, combined with advances in bio-informatics, render the information derived from differential display increasingly useful (Wan et al., 1996). The 5′-RACE (rapid amplification of cDNA ends) technique (Frohman et al., 1988) also offers the possibility to obtain full-length genes from differentially displayed amplification products; this approach was employed successfully in one of the first reports using DD for the identification of differentially expressed genes in plant tissues (Goormachtig et al., 1995). To generate useful information from DD of sea urchin mRNA (which contains long, untranslated 3′ regions), Haag and Raman (1994) successfully replaced the oligo-dT primer with a second arbitrary primer during the DD PCR. This approach presents the possibility to combine the principles of DD and DNA amplification fingerprinting (DAF), which successfully generates multi-amplicon profiles from complex substrates using short arbitrary primers (5- to 10-mers) under high annealing temperatures (up to 60 °C; Caetano-Anollés et al., 1991, 1992) and promises to extend the usefulness of DD.

(ii) Cumbersome verification of putative positives

The sensitivity and technical simplicity of the initial stages of the DD process (i.e. up to the production of an autoradiogram of separated amplification products) are generally acknowledged. For improved results, minor modifications, such as the use of flat-bottomed combs (rather than the traditional «sharkstooth» ones; Galindo et al., 1997) and non-denaturing gels (as opposed to sequencing gels which typically contain 7–8 mol · L^{-1} urea; Bauer et al., 1993), or even horizontal PAGE (Lohmann et al., 1995), have been suggested; and caution has been expressed that the sources of reaction tubes (Chen et al., 1994) and *Taq* polymerase (Haag and Raman, 1994) can influence the performance of the DD PCR. In debates weighing safety considerations against sensitivity, ^{33}P emerged as the label of choice for DD, although ^{35}S and ^{32}P are still being used routinely (Liang and Pardee, 1995). Non-radioactive detection methods, such as silver staining (Lohmann et al., 1995), or chemiluminescence (An et al., 1996) have also been proposed. To increase the throughput of DD, a variety of protocols for the analysis of DD results by automated sequencing have been developed (primer design and PCR reaction conditions were specifically adapted for use with fluorescent labels in these protocols; Bauer et al., 1993; Ito et al., 1994; Smith et al., 1997; Motlik et al., 1998). Alternatively, the generation of amplification products can be accelerated drastically through the use of an air-thermocycler for DD PCR (McKendree et al., 1995).

However, a major criticism remains: the verification of putative positives obtained by DD is time-consuming and requires large quantities of RNA (Galindo et al., 1997). Whereas the initial stages can be completed in a few days, the cloning of candidate bands to generate DNA for sequencing and/or probes for the final (and essential) verification by Northern hybridization is indeed cumbersome. Yet, this part of the process (also) employs accessible and established molecular biology techniques (and still beats the efforts required by alternative, antiquated methods; Wan et al., 1996).

Methodological modifications addressing this issue can be divided into two categories. The first contains protocols for the generation of sequencing data and probes for Northern hybridization directly from re-amplified candidate bands (thus circumventing the cloning process; Reeves et al., 1995; Wang and Feuerstein, 1995; Yeatman and Mao, 1995; Brenz Verca et al., 1998). The second category focuses on new and improved Northern hybridization protocols for the verification of putative positives. Most notable of these is the reverse Northern hybridization technique (Mou et al., 1994). In the original protocol, labelled cDNA probes were generated from the total RNA isolated from each of the different conditions to be compared in an experiment. The probes were then hybridized, separately but simultaneously, to re-amplification products of all the candidate bands isolated in the experiment, immobilized on a Nylon membrane. Variations on this theme have subsequently been introduced (see section on false positives). Since reverse Northern strategies are fast, sensitive and require small amounts RNA, they have become the preferred method for the verification of DD results (Galindo et al., 1997).

(iii) Questionable ability to detect rare mRNAs

Approximately 99 % of the 20,000 to 30,000 mRNA species present in a cell are rare (i.e. present at 1/20,000 to 1/70,000; Wan et al., 1996). Liang and Pardee (1992) argued that rare mRNA species were detectable with their original protocol, since they could reproducibly detect the thymidine kinase gene (present at about 30 copies per cell) in «cycling» mouse A31 cells (using a perfectly matching arbitrary primer). However, the use of different quantities of each RNA sample (for RT and/or DD PCR) was recommended,

since non-reproducible bands in duplicate reactions were presumed to originate from rare mRNA species that are not present in sufficient quantities (in the 0.2 mg total RNA typically used) to be amplified to detectable levels (Mou et al., 1994; Liang et al., 1993).

Bertioli et al. (1995) specifically addressed the issue of rare mRNA detection in a 1995 study, and presented compelling experimental evidence that DD showed a strong bias towards high copy number mRNAs. This was mainly attributed to the competition for substrates by the many PCR products in a DD reaction. Using a computer-simulated DD model, it was shown that many more bands were generated in a DD PCR than were visualized on a gel. It was argued that the PCR products displayed on a gel are those that were amplified to detectable levels by the time that the dNTPs (which are the limiting substrate in the reaction) were depleted. It follows logically that templates present at higher levels at the start of the reaction (i.e. abundant transcripts) would reach these levels more quickly and would be preferentially displayed (Bertioli et al., 1995).

The inefficiency of 10-mers (used in the original protocol) as PCR primers was identified as the second factor limiting the sensitivity of DD PCR. It was concluded that DD was unable of displaying the vast majority of rare mRNAs, regardless of the number of primer combinations employed (abundant transcripts, however, would be displayed efficiently by very few primer combinations). Increased DD sensitivity was speculated to reside in reducing the competition for PCR limiting substrates, while using longer, more selective and efficient primers at higher annealing temperatures (Bertioli et al., 1995).

These principles were applied in Ikonomov and Jacob's «differential display with selected primers» (SPR) strategy, in which different combinations of arbitrary, gene specific and oligo-dT primers were used (Ikonomov and Jacob, 1996). Analysis of putative positives showed that the majority of amplification products corresponding to highly abundant transcripts were amplified by a single primer or with a primer having a 50–65 % GC content at the 3′ end. In contrast, only 5 % of the amplification products which showed amplification dependency on two primers corresponded to highly abundant mRNAs. The study concluded that the bias of DD could be shifted towards moderate- and low-abundance mRNAs (concomitantly reducing the percentage of false positives) by experimentally selecting combinations of primers (50 % GC rich 15- to 21-mers) to avoid the amplification of highly abundant ribosomal and mitochondrial transcripts, using a higher annealing temperature (50 °C) in all PCR cycles, and by only considering bands that are dependent on both primers for amplification as putative positives (Ikonomov and Jacob, 1996). In contrast, Guimarães et al. (1995) reported the cloning of low abundance genes, some of which were represented by the amplification products of only one primer (oligo-dT or arbitrary) on the DD gel. Other researchers have also demonstrated DD capable of displaying both abundant and rare mRNAs, using protocols with little variation to the original one (Benito et al., 1996; Wan et al., 1996).

The limited sensitivity of «standard» Northern hybridization protocols has been highlighted as an additional factor contributing to the failure of detecting rare mRNA species, especially in cases where probes were derived from differentially displayed bands smaller than 200 bp (Yeatman and Mao, 1995; Liang et al., 1993; Ikonomov and Jacob, 1996). Southern blot and quantitative RT-PCR protocols and the reverse Northern hybridization technique have been introduced to address this problem (Ikonomov and Jacob, 1996).

A controversial issue related to the detection of rare mRNA species (and hence of arbitrary primer design) is the question of how many primer combinations are required to display all differentially expressed genes in a cell/tissue. Depending on the preferred set of anchored oligo-dT primers (see Fig. 1), arbitrary primer length, and different interpretations of 5′ mismatching during DD, answers ranging from 240^{2} to 312^{3} and even $1{,}024^{14}$ have been put forward. This debate may benefit the commercial suppliers of DD kits and reagents, but in our opinion, achieves little more than discouraging newcomers to the technique. It seems relevant to replace question: «how many primer combinations are needed to display all mRNAs?» with «how many primer combinations are required to sufficiently display mRNAs for the purpose of my particular study?».

The most practical approach would be a pragmatic one: to test as many primers and primer combinations as it takes to achieve the objective of the study. In cases where target genes are represented by rare mRNA species, this process will obviously be more exhaustive than in the case of abundant target mRNAs. (For example, Wilkinson et al. (1995) used only 3 primer combinations to identify 5 mRNAs showing ripening-enhanced expression in strawberry (3 of which were positively identified on the basis of the homology of their corresponding cDNA clones with known proteins), whereas Ikeda et al. (1997) exhausted 240 combinations in their identification of an aquaporin-like gene required for the *Brassica* self-incompatibility response.)

For expression analysis studies focused on specific and restricted sets or families of genes (i.e., which do not require the detection of all differentially expressed genes within a given system), a modified DD technique called «restriction fragment length polymorphism-coupled domain-directed differential display» (RC4D) was developed by Fischer et al. (1995). RC4D exploits the possibility to replace 5′ primers of arbitrary sequence with domain specific primers in such studies. This use of limited and well-defined primer sets under improved PCR conditions proved to eliminate many of the above mentioned frustrations experienced with DD (Fischer et al., 1995). Similar strategies were applied by Joshi et al. (1996) in the cloning of 3′ regions of putative wheat heat shock protein encoding genes, as well as in the adaptation of DD for the detection of amplification products by bromide staining on agarose gels (Hsu et al., 1993; Sokolov and Prokop, 1994).

(iv) High percentages of false positives

Throughout its existence, most resounding criticism against DD has been that the percentage of false positives are too high (Liang and Pardee, 1995; Galindo et al., 1997). The complexity of the entire process, from RNA isolation to Northern hybridization has to be appreciated in any assess-

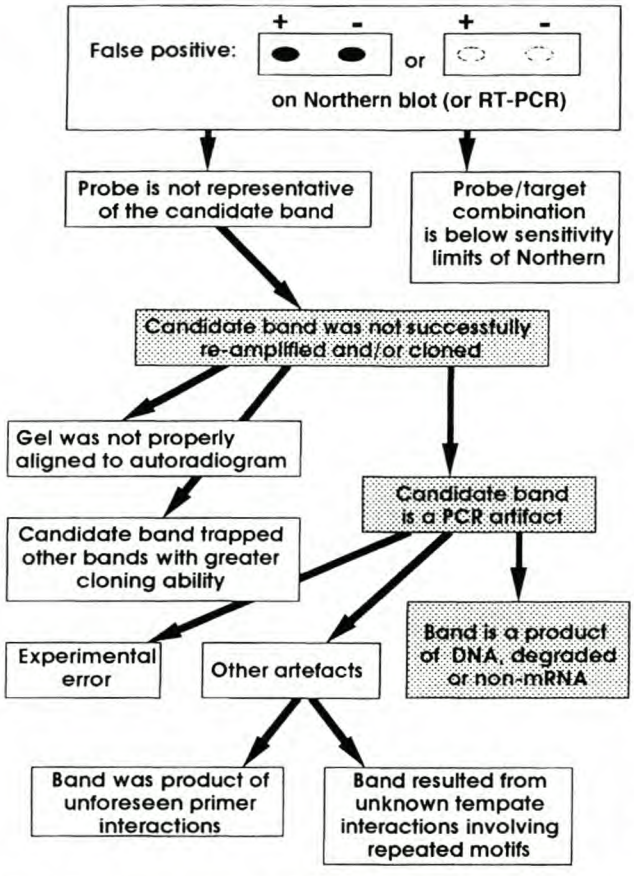


Fig. 2: Retrospective analysis of the origins of false positives during differential display.

ment of the origin of false positives. A false positive result (i.e., that a candidate band on a DD gel ultimately yields a hybridization signal in both or neither of the compared sources in a Northern blot) may have its origin in any one (or a combination) of the steps outlined in Fig. 1. From our «retrospective» analysis of the possible origins of false positives (Fig. 2), three «hot-spots» (other than the sensitivity limits of existing Northern blot protocols) can be identified: the quality of the starting material (total or poly(A)$^+$ enriched RNA), the generation DD PCR artifacts, and the re-amplification and cloning of candidate bands representing putative positives.

Contamination of RNA with residual DNA from the isolation procedure is widely acknowledged as a source of false positives (Liang and Pardee, 1995). DNA contamination can be limited to a large extent by using an RNA isolation protocol employing CsCl gradient ultracentrifugation rather than the more popular (and quicker) protocols based on differential precipitation of RNA with LiCl (Ausubel et al., 1995), or the use of magnetic beads for mRNA isolation (McKendree et al., 1995). Regardless of the method of choice, treatment with (RNAse free) DNAase I prior to reverse transcription and DD PCR should be standard practice (Liang et al., 1993). An alternative emerging approach to deal with DNA contamination, is to side-step the problem by labelling the oligo-dT primer (instead of incorporating a labelled deoxynucleotide into the PCR mix; Tokuyama and Takeda, 1995). This results in the display on the autoradiogram of only those amplification products which are dependent on reverse transcription (i.e. are amplified from cDNAs generated from the RNA sample), and undoubtedly simplifies the identification of candidate bands for cloning. Yet, invisible, «undesired» amplification products are still generated during the PCR and care must thus be taken with this approach for two reasons. Firstly, high levels of contamination may outcompete cDNAs corresponding to rare mRNA species as preferred amplicons. Furthermore, the presence of «invisible» amplification products (particularly in areas surrounding candidate bands) may complicate the cloning and re-amplification of such bands to an extent that might increase the percentage of false positives (Liang and Pardee, 1995).

The generation of DD PCR artifacts that eventually lead to false positives has been attributed to two main causes (other than DNA contamination): experimental variability and the inherent design of the DD PCR (using low stringency conditions in the presence of short primers; Zhao et al., 1995). Limiting experimental variability should not be a major source of concern for skilled and knowledgeable researchers/ technicians and will not be discussed. On the other hand, design of the DD PCR has been a primary target for modification of the original DD protocol. Not surprisingly, «improved» protocols demonstrated highly reduced rates of false positives through the use of longer arbitrary primers (with specific and/or alternative features) in combination with higher annealing temperatures (Table 1). In essence, these protocols represent a marriage between the conceptually related, but methodologically different principles of DD and RAP. As such, lower ratios of amplification products per primer combination (Welsh et al., 1992) (and, consequently, the potential need to test more primer combinations to achieve success) in exchange for higher true positive hit rates appear to be a reasonable compromise. Successful applications of DD using protocols based on both the original and modified PCR design has been documented. Whichever protocol is preferred, PCR artifacts can largely be excluded from a pool of putative positives by only considering bands that appear reproducibility in duplicate PCR reactions and are dependent on reverse transcription (Galindo et al., 1997; Liang et al., 1993).

The re-amplification and cloning of candidate bands from complex amplification profiles is an asperity common to many DNA and RNA profiling techniques. This stems mainly from the fact that re-amplification products from isolated candidate bands are often not heterogeneous (Bauer et al., 1993). As a result, re-amplification products are not suitable for the direct use as probes in Northern hybridization (to verify positives) or in the screening of cDNA libraries, and give rise to clones with inserts of heterogeneous sequence after cloning (Callard et al., 1994). To eliminate false positives arising from the use of unrepresentative probes, several methods for the selection of clones containing the correct cDNA probe have been devised (Ito et al., 1994; Callard et al., 1994; Li et al., 1994; Zhao et al., 1996; Gery and Lavi, 1997). An alternative approach applied the principles of reverse Northern hybridization (Mou et al., 1994) to concomitantly screen clones and verify that their inserts represent true differentially expressed mRNAs (Galindo et al., 1997; Liu and Raghot-

Table 1: Representative modifications to the DD PCR design aimed at improving the rate of true positives

Modified primers for DD PCR	Modified PCR components and conditions	Other modifications	Ref.
18- to 20-mer arbitrary primers and elongated oligo-dT primers, E1-T_{12}MN[a] (E1 = GGAATTCGGT[b]) or LH-T_{11}C (LH = TGCCGAAGC)	1 low-stringency annealing cycle (40°C for 4 min) +35 high-stringency cycles (60°C for 2 min)	–	A
22-mer arbitrary (containing a *Hin*dIII site in the 5′ portion) and 22-mer oligo-dT primers (X-T_{10}MN, where X = GCGCAAGCTT[c])	4 low-stringency annealing cycles (41°C for 1 min) +18 high-stringency cycles (60°C for 45 s)	–	B
25-mer arbitrary primers and «lock-docking»	Higher [dNTP] (50 µmol/L^{-1} each)	Reverse transcription with a single oligo-dT primer	C
29-mer oligo-dT primers (CATTATGCTGATGATATCT$_9$MM)	3 low-stringency annealing cycles (40°C for 5 min) +22 high-stringency annealing cycles (60°C for 1 min)		
	Mixture of 2 thermostable DNA polymerases to achieve long and accurate amplification		
	«Hot-start» PCR using antibodies against *Taq* polymerase		
–	25 cycles of two step annealing (42°C for 30 s, followed by 57°C for 1 min)	Reverse transcription with RTases from 2 different sources (cDNAs generated thus are used as duplicates in DD PCR)	D

[a] M = dA/dC/dG; N = dA/dC/dG/dT.
[b] GAATTC = *Eco*RI restriction site.
[c] AAGCTT = *Hin*dIII restriction site.
References: A = Zhao et al., 1995; B = Liskens et al., 1995; C = Diachenko et al., 1996; D = Sung and Denman, 1997.

hama, 1996; Vögeli-Lange et al., 1996; Zhang et al., 1996). In cases where quantities of isolated RNA are limited, the usefulness of this approach has been extended through the use of amplified RNA (Van Gelder et al., 1990) for the production of cDNA probes corresponding to each of the RNA sources (Galindo et al., 1997).

An aspect related to high false positive rates is that of redundancy (i.e. cloning the same cDNA more than once; Wan et al., 1996). Mismatching is the major factor contributing to redundancy. Other factors include «weak primers» (for unknown reasons, some arbitrary primers do not contribute to amplification in combination with an oligo-dT primer), the oligo-dT primers themselves (subpopulations of mRNAs are not always separated completely by differently anchored oligo-dT primers), as well as the molar ratio between the oligo-dT and arbitrary primer used (Galindo et al., 1997). Redundancy was shown to be minimized through the use of one-base anchored oligo-dT primers (Liang et al., 1994). Additionally, the use of arbitrary primers which are as diverse as possible in their last 4 bases, control PCRs in the absence of arbitrary primers to eliminate «weak primers», a different set of arbitrary 10-mers for each degenerate two-base anchored oligo-dT primer, and an equal molar ratio of arbitrary and oligo-dT primers, were recommended to eliminate redundancy (Galindo et al., 1997).

From our own experience

We are currently using DD in the search for genes involved in the autoregulation of nodulation in legumes (Gresshoff, 1993). Our research is focused on an interesting single recessive mutation that leads to supernodulation (Carroll et al., 1985). Mutants carrying this gene (*nts-1*) develop abundant nodules (up to several thousand per plant) as a result of diminished regulation of a transition control point during meristem formation (Kolchinsky et al., 1997; Mathews et al., 1989). Grafting experiments revealed that the *nts-1* gene functions in the shoot (Delves et al., 1986, 1992), which led to the discovery that nodule numbers in legumes are regulated systemically (Bauer, 1981; Kosslak and Bohlool, 1984; Olsson et al., 1989). As yet, the signal molecules have not been identified, although the involvement of auxin has been proposed (Gresshoff, 1993). The *nts-1* gene was mapped onto linkage group H of soybean, perhaps as close as 0.3 cM (representing between 50 to 150 kb, depending on which genetic distance conversion factor is being used) of marker pUTG-132a (Kolchinsky et al., 1997; Landau-Ellis et al., 1991; A. Men, pers. comm.), which provides an excellent starting point for a chromosome walk (Young and Phillips, 1994; Gresshoff et al., 1998). However, the soybean genome is rather large (about 1,100 Mb), and plagued with repeated DNA elements (Kolchinsky and Gresshoff, 1995), constituting as much as 65 % of the whole genome.

In the absence of molecular information concerning the *nts-1* gene, its product, or the affected biochemical pathway, we felt that DD could provide an alternative tool to define gene products affected directly or indirectly by the supernodulation mutation. It is unlikely that this approach will differentiate mutant from wild-type RNA, unless the mutation affects transcriptional levels or RNA stability. Nevertheless,

alterations of gene expression functionally downstream from the *nts-1* gene are expected.

For this purpose, total RNA was extracted from young shoots of *Bradyrhizobium japonicum* inoculated (or uninoculated) wild-type (cv. «Bragg») and mutant (*nts382*) plants, using the guanidium thiocyanate/CsCl ultracentrifugation method (Ausubel et al., 1995). Using a well-established, commercially available DD system (RNAImage kit, GenHunter Corp., Nashville, TN), 200–250 amplification products (typically ranging between <100 and 1,000 bp) were routinely displayed for each primer combination on a standard sequencing gel. Eighty to 95 % of these bands were reproducibly obtained between duplicate samples (using the same or duplicate reverse transcription reaction mixes). According to expectations, a very low percentage (0.17 %) of the ca. 1,800 amplification products (obtained with 8 primer combinations) constituted putative differentially expressed genes.

Our application of DD has benefited greatly from extensive expertise in the generation and analysis of arbitrarily-primed amplification products from complex templates, gained particularly through the development and application of DAF (Caetano-Anollés et al., 1991) and silver staining (Bassam et al., 1991) technologies in our laboratory. To facilitate cloning of candidate bands, we routinely silver stain our DD gels after autoradiography (Fig. 3), using a modified version of the method developed by Bassam et al. (1991). Although silver staining does not achieve the sensitivity of autoradiography, this procedure has three advantages. Firstly, it allows very accurate alignment of autoradiograms to gels for the excision of candidate bands (these bands are sometimes visibly stained, which simplifies the process even more). Secondly, it allows the single-round re-amplification of candidate bands from rehydrated gels, which has recently proved very efficient in the cloning of DAF-markers (Men and Gresshoff, 1998). Additionally, it allows the use of standard, non-labeled molecular size markers (instead of having to specially prepare or purchase expensive labeled ones), as the positions of the markers can simply be marked on the autoradiogram after silver staining. Some of these advantages have also been recognized in other studies (Lohmann et al., 1995). The use of silver-stained, polyester-backed mini-PAGE gels (Bassam et al., 1991) to verify the sizes of re-amplification products from excised bands, as well as from positive transformants after cloning, has also proved more useful and sensitive than the traditional use of agarose gels and ethidium bromide staining.

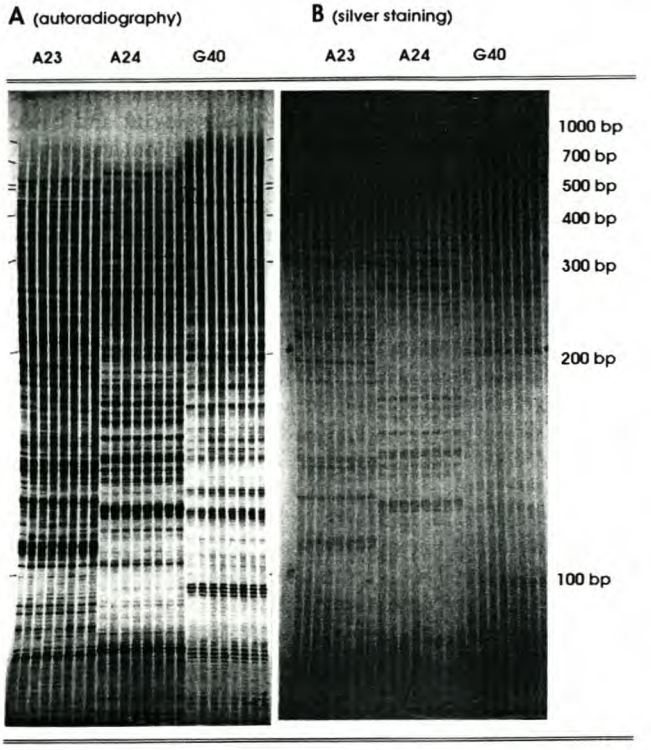


Fig. 3: Autoradiogram (A) of a typical non-denaturing DD gel, subjected to silver staining (B). Reverse transcription and DD PCRs, using total RNA from uninoculated and inoculated soybean (wild type cv. 'Bragg', supernodulating mutant nts382) were performed in the presence of ^{35}S-dATP, using the RNAImage kit (GenHunter Corp., Nashville TN). Oligo-dT primer H-T_{11}A was combined with arbitrary primers H-AP23 and H-AP24, and primer H-T_{11}G with H-AP40. The gel was left on the glass plate, fixed in 5 % methanol/5 % acetic acid, dried and exposed to x-ray film (BioMax MR, Kodak) for 24 h. Silver staining was performed after autoradiography, essentially as described by Bassam et al. (1991). Staining and washing times were extended to adapt to the larger gel surface.

In conclusion: when the 'chips are up', what is the use for DD?

The detection of mRNA differences in complex mixtures is a challenge for both the chemist and molecular biologist. Genes which are of pivotal importance through their control of key processes are often expressed at low levels; in some cases representing just a few mRNA molecules per cell. In contrast, a small number (approximately 300 out of 20,000 to 30,000 distinct mRNA species) of highly expressed genes make up 50 % of the total cellular mRNA mass (Wan et al., 1996). How does one find the needle in the haystack? Classical approaches, employing differential screening of cDNA libraries, were laborious and yielded limited information. Differential display (and RAP) ushered in a new generation of gene discovery technologies, applying technically elegant and established DNA profiling techniques on RNA. Although offering superior sensitivity and information generation capabilities, problems pertaining to labor intensity, false positives and the detection of rare mRNA species persisted.

Automation has always been an attractive proposition to extend the usefulness of an analytical technique. It often requires manual and time-consuming separation technologies to be circumvented or replaced. This avenue has indeed been explored with the adaption of DD to allow analysis with automated sequencers, but it has not streamlined the process by which DD results are verified.

Yet another generation of gene discovery technologies has emerged in the form of oligonucleotide arrays. Based either on selected oligo-motifs, or random oligonucleotide tethers, DNA chips overcome the restrictions inherent to separation technologies, as «order» is established through the positioning of known sequences in addressable positions. This technology

has achieved the detection of RNAs present at a frequency of 1:300,000 (Lockhardt et al., 1996) and undoubtedly is more powerful than any of its predecessors.

Does this leave a place for DD in the new millennium? We believe it does, because DD is technically elegant and based on technologies which are distributed throughout the world. Gene chips, on the other hand, are only accessible through very expensive data documentation and reading systems. Few research laboratories will end up with chip readers. Although central chip reading services may arise, chip manufacture is envisaged to focus on «elite» species, and «elite» organs, leaving large areas of the biological landscape unresolved. The quest for understanding the complexities of life that compels us to discover new genes, calls for a global involvement, which acknowledges the diversity of living systems. It would be sad if all our biological knowledge is based on man, *Arabidopsis* and yeast. The ongoing efforts to address the difficulties inherent to the design of DD, which have been reviewed here, speak of a commitment by the international research community to exploit the full potential offered by this technology. Moreover, DD is a multi-step process which relies on a huge pool of basic and applied chemistry and molecular biology for its successful application. A such, it will remain a valued educational tool for the molecular biologist of the new millennium.

Acknowledgements

M.A. was supported by a fellowship from the South African Foundation for Research and Development. Soybean research was supported by the Tennessee Soybean Promotional Board and the Racheff Endowment. Dr. Keishi Senoo (Mie University, Japan) is thanked for helpful discussions and duplicate RNA.

References

An, G., G. Luo, R. W. Veltri, and S. M. O'Hara: Sensitive, nonradioactive differential display method using chemiluminescent detection. BioTechniques *20,* 342–346 (1996).

Ausubel, F. M., R. Brent, R. E. Kinston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.): Current Protocols in Molecular Biology. John Wiley & Sons, New York (1995).

Bassam, B. J., G. Caetano-Anollés, and P. M. Gresshoff: Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. *196,* 80–83 (1991).

Bauer, D., H. Müller, J. Reich, H. Riedel, V. Ahrenkiel, P. Warthoe, and M. Strauss: Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucl. Acids Res. *21,* 4272–4280 (1993).

Bauer, D. W.: Regulation of nodulation in legumes. Annu. Rev. Plant Physiol. *32,* 407–447 (1981).

Benito, E. P., T. Prins, and A. L. van Kan: Application of differential display RT-PCT to the analysis of gene expression in a plant-fungus interaction. Plant Molec. Biol. *32,* 947–957 (1996).

Bertioli, D. J., U. H. A. Schlichter, M. J. Adams, P. R. Burrows, H.-H. Steinbiss, and J. F. Antoniw: An analysis of differential display shows a strong bias towards high copy number mRNAs. Nucl. Acids Res. *23,* 4520–4523 (1995).

Brenz Verca, M. S., S. Brenz Verca, S. Rusconi, and J.-L. Dreyer: Modification of primer design facilitates the use of differential display. BioTechniques *24,* 374–380 (1998).

Caetano-Anollés, G. and P. M. Gresshoff: DNA amplification fingerprinting using arbitrary mini-hairpin oligonucleotide primers. Bio/Technology *12,* 619–623 (1994).

Caetano-Anollés, G., B. J. Bassam, and P. M. Gresshoff: DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology *9,* 553–557 (1991).

– – – Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. Mol. Gen. Genet. *235,* 157–165 (1992).

Callard, D., B. Lescure, and L. Mazzolini: A method for the elimination of false positives generated by the mRNA differential display technique. BioTechniques *16,* 1096–1103 (1994).

Carroll, B. J., D. L. McNeil, and P. M. Gresshoff: Isolation and properties of soybean mutants which nodulate in the presence of high nitrate concentrations. Proc. Natl. Acad. Sci. USA *82,* 4162–4166 (1985).

Chen, Z., K. Swisshelm, and R. Sager: A cautionary note on reaction tubes for differential display and cDNA amplification in thermal cycling. BioTechniques *16,* 1003–1006 (1994).

Delves, A. C., A. Higgins, and P. M. Gresshoff: The shoot apex is not the source of the systemic signal controlling nodule numbers in soybean. Plant, Cell and Environment *15,* 249–254 (1992).

Delves, A. C., A. Mathews, D. A. Day, A. S. Carter, B. J. Carroll, and P. M. Gresshoff: Regulation of the soybean-*Rhizobium* symbiosis by shoot and root factors. Plant Physiol. *82,* 588–590 (1986).

Diachenko, L. B., J. Ledesma, A. A. Chenchik, and P. D. Siebert: Combining the technique of RNA fingerprinting and differential display to obtain differentially expressed mRNA. Biochem. Biophys. Res. Comm. *219,* 824–828 (1996).

Fischer, A., H. Saedler, and G. Theissen: Restriction fragment length polymorphism-coupled domain-directed differential display: a highly efficient technique for expression analysis in multigene families. Proc. Natl. Acad. Sci. USA *92,* 5331–5335 (1995).

Frohman, M. A., M. K. Dush, and G. R. Martin: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA *85,* 8998–9002 (1988).

Galindo, J. E., G. M.-C. Poirier, H. Guo, A. Huvar, P. C. Wagaman, J. Zhu, J. Tench, J. S. Wan, and M. G. Erlander: Differential display of RNA. In: Caetano-Anollés, G. and P. M. Gresshoff (eds.): DNA markers: protocols, applications and overviews, pp. 225–236. Wiley-Liss, New York (1997).

GenHunter Corp: Your complete source for differential display: 1998/1999 catalogue. GenHunter Corp, Nashville (1997).

– Comparison of differential display with other methodologies for cloning differentially expressed genes. Differential Display News *2*(2), 3 (1997).

Gerahty, N., G. Caetano-Anollés, P. Joshi, and P. M. Gresshoff: Anatomical analysis of nodule development in soybean reveals a new autoregulatory control point. Plant Science *85,* 1–7 (1992).

Gery, S. and S. Lavi: Purification and cloning of differential display products. BioTechniques *23,* 198–202 (1997).

Goormachtig, S., M. Valerio-Lepiniec, K. Szczyglowski, M. van Montagu, M. Holsters, and F. J. De Bruijn: Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. Mol. Plant-Microbe Interact. *8,* 816–824 (1995).

Gresshoff, P. M., J. Stiller, A. Men, S. Radutoiu, S. Pillai, D. Landau-Ellis, R.-J. Chian, F. Ghassemi, M. Bhattacharyya, A. Hussain, D. Lohar, and Q. Jiang (1998): Functional genomics of legumes: map-based cloning and gene trapping advances

in soybean and *Lotus japonicus*. In: MARTINEZ, E. and G. HERNÁNDEZ (eds.): Highlights on Nitrogen Fixation Research. Plenum Press, New York (in press) (1999).

GRESSHOFF, P. M.: Molecular genetic analysis of nodulation genes in soybean. Plant Breeding Reviews *11*, 275–318 (1993).

GUIMARÃES, M. J., F. LEE, A. ZLOTNIK, and T. MCCLANAHAN: Differential display by PCR: novel findings and applications. Nucl. Acids Res. *23*, 1832–1833 (1995).

HAAG, E. and V. RAMAN: Effects of primer choice and source of *Taq* DNA polymerase on the banding patterns of differential display RT-PCR. BioTechniques *17*, 226–228 (1994).

HSU, D. K. W., P. J. DONOHUE, G. F. ALBERTS, and J. A. WINKLES: Fibroblast growth factor-1 induces phosphofructokinase, fatty acid synthase and Ca^{2+}-ATPase mRNA expression in NIH 3T3 cells. Biochem. Biophys. Res. Comm. *197*, 1483–1491 (1993).

IKEDA, S., J. B. NASRALLAH, R. DIXIT, S. PREISS, and M. E. NASRALLAH: An aquaporin-like gene required for the *Brassica* self-incompatibility response. Science *276*, 1564–1566 (1997).

IKONOMOV, O. C. and M. H. JACOB: Differential display protocol with selected primers that preferentially isolates mRNAs of moderate- to low-abundance in a microscopic system. BioTechniques *20*, 1030–1042 (1996).

ITO, T., K. KITO, N. ADATI, Y. MITSUI, H. HAGIWARA, and Y. SAKAKI: Fluorescent differential display: arbitarily primed RT-PCR fingerprinting on an automated DNA sequencer. FEBS Letters *351*, 231–236 (1994).

JOSHI, C. P., S. KUMAR, and H. T. NGUYEN: Application of modified differential display technique for cloning and sequencing of the 3′ region from three putative members of wheat HSP70 gene family. Plant Molec. Biol. *30*, 641–646 (1996).

KOLCHINSKY, A. and P. M. GRESSHOFF: A major satellite DNA of soybean is a 92-base pairs tandem repeat. Theor. Appl. Genet. *90*, 621–626 (1995).

KOLCHINSKY, A., D. LANDAU-ELLIS, and P. M. GRESSHOFF: Map order and linkage distances of molecular markers close to the supernodulation (*nts-1*) locus of soybean. Molec. Gen. Genet. *254*, 29–36 (1997).

KOSSLAK, R. M. and B. B. BOHLOOL: Suppression of nodule development on one side of a split-root system of soybean caused by prior inoculation of the other side. Plant Physiol. *75*, 125–130 (1984).

LANDAU-ELLIS, D., S. A. ANGERMÜLLER, R. SHOEMAKER, and P. M. GRESSHOFF: The genetic locus controlling supernodulation cosegregates tightly with a cloned molecular marker. Mol. Gen. Genet. *228*, 221–226 (1991).

LI, F., E. S. BARNATHAN, and K. KARIKÓ: Rapid method for screening and cloning cDNAs generated in differential mRNA display: application of northern blot for affinity capturing of cDNA. Nucl. Acids Res. *22*, 1764–1765 (1994).

LIANG, P. and A. B. PARDEE: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967–971 (1992).

– – Recent advances in differential display. Curr. Opin. Immunol. *7*, 274–280 (1995).

LIANG, P., L. AVERBOUKH, and A. B. PARDEE: Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucl. Acids Res. *21*, 3269–3275 (1993).

LIANG, P., W. ZHU, X. ZHANG, Z. GUO, R. P. O'CONNELL, L. AVERBOUKH, F. WANG, and A. B. PARDEE: Differential display using one-base anchored oligo-dT primers. Nucl. Acids Res. *22*, 5763–5764 (1994).

LISKENS, M. H. K., J. FENG, W. H. ANDREWS, B. E. ENLOW, S. M. SAATI, L. A. TONKIN, W. D. FUNK, and B. VILLEPONTEAU: Cataloging altered gene expression in young and senescent cells using enhanced differential display. Nucl. Acids Res. *23*, 3244–3251 (1995).

LIU, C. and K. G. RAGHOTHAMA: Practical method for cloning cDNAs generated in an mRNA differential display. BioTechniques *20*, 576–580 (1996).

LOCKHARDT, D. J., H. DONG, M. C. BRYNE, M. T. FOLLETTIE, M. V. GALLO, M. S. CHEE, M. MITTMANN, C. WANG, M. KOBAYASHI, H. HORTON, and E. L. BROWN: Expression monitoring by hybridization to high-density oligonucleotide arrays. Nature Biotechnology *14*, 1675–1680 (1996).

LOHMANN, J., H. SCHICKLE, and T. C. G. BOSCH: REN display, a rapid and efficient method for nonradioactive differential display and mRNA isolation. BioTechniques *18*, 200–202 (1995).

MARSHALL, A. and J. HODGSON: DNA chips: an array of possibilities. Nature Biotechnology *16*, 27–31 (1998).

MATHEWS, A., B. J. CARROLL, and P. M. GRESSHOFF: Development of *Bradyrhizobium* infections in supernodulating and non-nodulating mutants of soybean (*Glycine max* (L.) Merrill). Protoplasma *150*, 40–47 (1989).

MCKENDREE, W. L. Jr., C. J. NARIN III, and M. G. BAUSHER: Differential display from plant leaves using oligo(dT) magnetic bead mRNA isolation and hot air PCR. BioTechniques *19*, 715–719 (1995).

MOU, I., H. MILLER, J. LI, F. WANG, and L. CALIFOUR: Improvements to the differential display method for gene analysis. Biochem. Biophys. Res. Comm. *199*, 564–569 (1994).

MULLIS, K. B. and F. A. FALOONA: Specific synthesis of DNA *in vitro* via a polymerase catalyzed reaction. Methods Enzmol. *255*, 335–350 (1987).

OLSSON, J. E., P. NAKAO, B. B. BOHLOOL, and P. M. GRESSHOFF: Lack of systemic suppression of nodulation in split root systems of supernodulating soybean (*Glycine max.* (L.) Merr.) mutants. Plant Physiol. *90*, 1347–1352 (1989).

RAMSAY, G.: DNA chips: state-of-the-art. Nature Biotechnology *16*, 40–44 (1998).

REEVES, S. A., M.-P. RUBVIO, and D. N. LOUIS: General method for PCR amplification and direct sequencing of mRNA differential display products. BioTechniques *18*, 18–20 (1995).

SARGENT, T.: Isolation of differentially expressed genes. Methods Enzymol. *152*, 423–432 (1987).

SMITH, N. R., M. ALDERSLEY, A. LI, A. S. HIGH, T. P. MOYNIHAN, A. F. MARKHAM, and P. A. ROBINSON: Automated differential display using a fluorescently labeled universal primer. BioTechniques *23*, 274–279 (1997).

SOKOLOV, B. P. and D. J. PROCKOP: A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes. Nucl. Acids Res. *22*, 4009–4015 (1994).

TOKUYAMA, Y. and J. TAKEDA: Use of ^{33}P-labeled primer increases the sensitivity and specificity of mRNA differential display. BioTechniques *18*, 424–425 (1995).

VAN GELDER, R. N., M. E. VON ZASTROW, A. YOOL, W. C. DEMENT, J. D. BARCHAS, and J. H. EBERWINE: Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc. Natl. Acad. Sci. USA *87*, 1663–1667 (1990).

VÖGELI-LANGE, R., N. BRÜCKERT, T. BOLLER, and A. WIEMKEN: Rapid selection and classification of positive clones generated by mRNA differential display. Nucl. Acids Res. *24*, 1385–1386 (1996).

VOGT, T., F. MATHIEU-DAUDE, F. KULLMANN, J. WELSH, and M. MCCLELLAND: Fingerprinting of DNA and RNA using arbitrarily primed PCR, pp. 55–74 in *DNA* markers: protocols, applications and overviews. CAETANO-ANOLLES, G., and GRESSHOFF, P. M. (eds.). Wiley-Liss, New York (1997).

WAN, J. S., S. J. SHARP, G. M.-C. POIRIER, P. C. WAGAMAN, J. CHAMBERS, J. PYATI, Y.-L. HOM, J. E. GALINDO, A. HUVAR, P. A. PETERSON, M. R. JACKSON, and M. C. ERLANDER: Cloning differentially expressed mRNAs. Nature Biotechnology *14*, 1685–1691 (1996).

Wang, X. and G. Z. Feuerstein: Direct sequencing of DNA isolated from mRNA differential display. BioTechniques *18,* 448–452 (1995).

Welsh, J. and M. McClelland: Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. *18,* 7213–7218 (1990).

Welsh, J., K. Chada, S. S. Dalal, R. Cheng, D. Ralph, and M. McClelland: Arbitrarily primed PCR fingerprinting of RNA. Nucl. Acids Res. *20,* 4965–4970 (1992).

Wilkinson, J. Q., M. B., Lanahan, T. W. Conner, and H. J. Klee: Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. Plant Mol. Biol. *27,* 1097–1108 (1995).

Yeatman, T. J. and W. Mao: Identification of a differentially-expressed message associated with colon cancer liver metastasis using an improved method of differential display. Nucl. Acids Res. *23,* 4007–4008 (1995).

Young, N. D. and R. I. Phillips: Cloning plant genes known only by phenotype. Plant Cell *6,* 1193–1195 (1994)..

Zhang, H., R. Zang, and P. Liang: Differential screening of gene expression difference enriched by differential display. Nucl. Acids Res. *24,* 2454–2455 (1996).

Zhao, S., S. I., Ooi, F.-C. Yang, and A. B. Pardee: Three methods for identification of true positive cloned cDNA fragments in differential display. BioTechniques *20,* 400–404 (1996).

Zhao, S., S. I. Ooi, and A. B. Pardee: New primer strategy improves precision of differential display. BioTechniques *18,* 842–850 (1995).

CHAPTER 5

IDENTIFICATION OF PLANT GENES INVOLVED IN THE INTERACTION BETWEEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* NV AND PLUM CULTIVARS USING DIFFERENTIAL DISPLAY

5.1 INTRODUCTION

This chapter describes the identification, by means of differential display (DD) technology, of plant genes that are involved in the interaction between the bacterial canker pathogen *Pseudomonas syringae* pv. *syringae* and plum cultivars. This work fulfills the fourth and final objective of the study presented in this dissertation (as outlined in Chapter 1) and is presented in the form of a manuscript in section 5.3. To assist the reader, background information on the following aspects is included in section 5.2:

5.2.1 The DD system and strategies used in the study.

5.2.2 Why and how a reverse transcription polymerase chain reaction (RT PCR) strategy was developed for verification of DD results.

5.2.3 Why and how an internal RT PCR standard was developed.

5.2 BACKGROUND INFORMATION

5.2.1 DD system and strategies

5.2.1.1 DD system components

The analysis of differentially expressed mRNAs by differential display is a multi-step process, comprised of commonly used molecular biology techniques (as outlined in Chapter 4). Equipment and reagents needed to perform these techniques are widely available, and laboratories tend to establish preferences in brand names and suppliers. Many steps of a DD experiment, such as the analysis of DD PCR products by polyacrylamide gel electrophoresis (PAGE), as well as the re-amplification, cloning and further manipulation and analysis of candidate bands may be performed according to standard protocols, and published DD studies demonstrate the variety of suitable systems that are

available.

The successful establishment of DD in any laboratory rests on the ability to get started. This depends on whether (i) intact, DNA-free mRNA (or total RNA) can be isolated from the experimental source(s), (ii) full-length cDNAs that adequately represent mRNA populations can be generated, and (iii) these cDNAs can be converted to arbitrarily-primed amplification products capable of displaying differences in the expressed mRNA populations. It is therefore not surprising that commercially developed DD systems ("kits"), which particularly focus on aspects (ii) and (iii), were introduced shortly after the technique was described in 1992. The most popular of these are the products of GenHunter Corporation (Nashville, TN, USA), which are developed and marketed by the inventors of the technique, Peng Liang and Arthur B. Pardee.

When our research team set out to gain expertise in DD technology and establish it in our laboratory, one of the primary choices was whether to use the relatively expensive GenHunter RNAimage® system, or to develop a RT and DD PCR system from our available molecular biology basis. This question was addressed by the author during her visit to the Plant Molecular Genetics laboratory of Prof. Peter Gresshoff (University of Tennessee, Knoxville, TN, USA).

The Gresshoff laboratory had extensive expertise in the development and application of other arbitrarily-primed PCR techniques, particularly DNA amplification fingerprinting (DAF), and constituted an ideal environment in which to build up our own DD system. The RNAimage® system* was, however, initially tested and found to be extremely efficient for the generation of cDNA and DD PCR products. Results obtained with this system contrasted sharply with attempts to construct and optimize DD PCRs using cDNA populations generated with other commercially available RT systems. The cost of the RNAimage® system seemed warranted by the consistent quality of results generated with

---

* Two DD reagents (DNA polymerase and labelled dATP) are not included in RNAimage® kits. The system is optimized for use with either Taq (Qiagen) or AmpliTaq (Perkin-Elmer) DNA polymerase, and either α-[^{33}P]- or α-[^{35}S]-labelled dATP.

Both of the polymerases and labels were evaluated in the Gresshoff laboratory and found to produce similar results. In the author's home laboratory Qiagen polymerase was preferred as it was much less expensive. The cost of using either label was approximately equal, but ^{35}S-dATP was preferred for two reasons: (i) working with ^{33}P-labels require special precautions and such facilities were not readily available, and (ii) possible exposure to radioactive fumes produced during the thermocycling of ^{35}S-containing reactions could be limited by placing the thermocycler in an available, dedicated fume hood.

it, and it was decided to use this system for the generation of DD profiles in future: in the Gresshoff laboratory, as well as in the author's home laboratory.

The GenHunter range of DD products includes kits for the isolation of total RNA, the treatment of RNA with DNase I, the cloning of candidate DD bands, and verification that cloned bands were derived from differentially expressed mRNAs. During the establishment of DD in our laboratory, the following choices regarding these aspects were made:

a. The GenHunter RNApure™ total RNA isolation kit was never tested by the author. A variety of methods, none entailing the use of commercial kits, were already established in the Gresshoff laboratory for the isolation of total RNA (which is preferred for DD; Liang et al. 1993, Adamovicz and Gause 1995). Two of these, a guanidinium thiocyanate/CsCl ultracentrifugation* method and a phenol/SDS extraction, LiCl precipitation "miniprep" method, were evaluated by the author. Although the former was more laborious, it yielded large quantities of intact RNA from two different plants (soybean and the model legume *Lotus japonicus*). Back home, excellent results were obtained with this method during trial isolations from plum tree shoots.

   One commercial kit (the Qiagen RNeasy Plant Mini kit) was tested for the isolation of RNA from plum tree leaves. It yielded intact RNA, but of such a low concentration that it was not practical to use with seasonal plants. The ultracentrifugation method was thus established as the preferred method for the isolation of total RNA from plum tree material for DD analyses.

b. Formaldehyde-agarose electrophoresis of total RNA isolated with the ultracentrifugation method did not show the presence of contaminating DNA†. Nevertheless, all RNA isolates were treated with DNase I prior to cDNA synthesis (as suggested by Liang et al. 1993). The GenHunter MessageClean® kit was highly recommended by fellow researchers at home and abroad (many of whom have experienced difficulties with DNAse I treatment of RNA). Since the author consistently obtained high yields of DNAse-free RNA‡ with this kit, it was used in all treatments of RNA for DD and RT PCR.

---

* Very popular for the isolation of RNA from plants (Adamovicz and Gause 1995).

† Contaminating DNA is visible on an agarose gel as a band of higher molecular mass than the largest ribosomal RNA band (for plants, the 25S rRNA band; Anderson and Beardall 1991).

‡ Determined by RNAse digestion of treated RNA, followed by agarose electrophoresis of digestion products (results not shown).

c. For the re-amplification and cloning of candidate DD bands, a method developed in the Gresshoff laboratory (Men and Gresshoff 1998) was preferred above the RNAimage® protocol and GenHunter's PCR-TRAP® cloning system. The former method was much simpler and allowed the use of any T-A cloning system, one of which was already established in the author's home laboratory (see Chapter 3).

d. The strategy which was developed to verify whether cloned bands truly represented differentially expressed mRNAs is described in detail in the next section.

5.2.1.2 Negative controls for DNA contamination

"False positives" (i.e. cloned DNA fragments that were not derived from differentially expressed mRNAs) are frequently encountered in DD analyses. DNA contamination of the RNA used in DD is a major source of false positives (see section 4.3.3). It is therefore expedient to ascertain whether DD bands selected for cloning were generated in a reverse transcriptase (RTase)-dependent manner. Ideally, a "minus RT" negative control should be performed for each candidate band*.

Due to the high cost of ^{35}S-dATP, "minus RT" controls were not performed for candidate bands selected for cloning in the current study. Instead, two precautions were taken to minimize the likelihood of selecting DNA-derived bands for further analysis:

a. RNA samples were always treated with DNase I prior to RT (see previous section).

b. Only bands that were reproducibly obtained in duplicate DD PCR profiles were considered for selection as candidate bands†.

5.2.1.3 Candidate band sequencing strategy

In most studies, only those DD bands confirmed to represent differentially expressed

---

* Briefly, such a negative control is performed by (i) repeating the RT reaction used to generate the cDNA population from which the candidate DD band was generated, **in the absence of RTase enzyme**, and (ii) repeating the DD reaction in which the candidate band was generated (in duplicate), using this "minus RTase" reaction as template for arbitrarily-primed cDNA amplification.

† When the RNAimage® system was evaluated by the author in the Gresshoff laboratory, a large number of negative controls were performed to investigate the nature of DD PCR products generated in the absence of any one of the components of the RT or DD reactions. Amplification products were generated in almost all of these controls, and the collective results from these experiments indicated that products not derived from mRNA (or cDNA) were likely to be represented by non-reproducible bands in the profiles of duplicate DD PCR products (results not shown). A similar conclusion was reached by Liang et al. (1993) and Galindo et al. (1997).

genes (or clones from cDNA libraries corresponding to these bands) are sequenced (upon which sequence databases are perused in attempts to assign identity and/or function to the genes represented by these bands). In this study, **all** cloned bands were sequenced for the following reasons:

a. A pair of primers specific for each band was required for the verification of its expression pattern by RT PCR (see next section). The arbitrary and anchored primers used in the DD PCR and for the re-amplification of candidate bands could have been used for this purpose, but were not preferred for two reasons: (i) arbitrary primers often bind with a number of mismatches during the DD PCR (see section 4.3.3), and (ii) anchored oligo-dT primers are unsuitable as standard PCR primers due to their low annealing temperatures. Sequence data would thus allow the design of "nested" primers (corresponding to sequences internal to that of the flanking DD PCR primers) with higher specificities for the mRNAs represented by each of the cloned DD bands.

b. Automated sequencing technology was readily accessible and relatively inexpensive. Sequencing reactions were performed in the laboratory and sent to the University of Stellenbosch Core DNA Cycle Sequencing Facility for clean-up and analysis.

5.2.1.4 BLAST strategy for sequence similarity searches

The BLAST* 2.0 search engine (provided on-line by the National Center for Biotechnology Information on the NCBI website, http://www.ncbi.nlm.nih.gov/blast) is not the only search engine available for sequence similarity searches. However, it is the most popular and widely-used one, and also the search engine used in the current study to ascertain whether cloned candidate bands showed any similarity to genes of known biological function.

Basic† BLAST searches, were performed throughout the current study. The Basic BLAST

---

* BLAST = Basic Local Alignment Search Tool. The BLAST algorithm represents a balance between speed and increased sensitivity for distant sequence relationships. Global alignments are underscored and regions of local alignment emphasized, in order to detect relationships among sequences which share only isolated regions of similarity with a view to comparing structure and function (Altschul et al. 1990, 1997).

BLAST version 2.0 was created to offer several advanced features (which were not available in previous versions). Most important of these is the Gapped BLAST algorithm, which allows gaps (insertions and/or deletions) to be introduced into the alignments that are returned. By doing this, similar regions are not broken into several fragments, and search results reflect biological relationships more closely (see BLAST Overview and BLAST Tutorial on the NCBI website).

† The BLAST 2.0 server also offers Advanced BLAST searches, in which advanced search parameters may be customized for specialized applications.

search page contains a number of default search parameters, which were used throughout the current study. A detailed description of each of these parameters may be found in the BLAST documentation on the NCBI website, and will not be repeated here. To assist the reader in the interpretation of the BLAST search results given in section 5.3, three aspects of the BLAST search strategy are, however, discussed briefly below.

(i) Choice of BLAST programme:

The BLAST 2.0 server offers five different programmes, each representing a different search strategy (see Table 5-1). The BLASTN and TBLASTX programs were used in the current study.

(ii) Choice of sequence database:

The NCBI website offers access to several protein and nucleotide databases against which query sequences may be compared. Some of these, such as the **nr** ("non-redundant") databases, are inclusive (contains all non-redundant sequences submitted to various Data Libraries in the USA, Europe and Asia), whilst others are more specialized. Specialized databases include collections of sequences that (i) are organism-specific (e.g. human, mouse, yeast or *Escherichia coli*), (ii) belong to a certain sequence type (e.g. expressed sequence tags (ESTs) or Alu repeats), or (iii) have a particular characteristic or application (e.g. sequences of immunological interest or vector

Table 5-1. BLAST search programmes.

Programme	Description
BLASTN	Compares a nucleotide query sequence against a nucleotide sequence database
BLASTP	Compares an amino acid query sequence against a protein sequence database
BLASTX[a]	Compares a nucleotide query sequence translated in all reading frames against a protein sequence database
TBLASTX[a]	Compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database[b]
TBLASTN	Compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames.

[a] Useful to find potential translation products of an unknown nucleotide sequence.

[b] May not be used with some non-redundant databases, as it is computationally too intensive.

sequences). Sequence databases (which may be searched with the BLAST 2.0 or other search engines) are also available on a variety of other websites, many of which are linked to genome sequencing projects*.

The purpose of a particular search largely dictates the choice of database (and, to a certain extent the choice of BLAST programme†). In the current study, a comprehensive BLASTN search of the NCBI nr database was performed as a first approach to ascertain whether any sequences with similarity to the sequences of cloned DD bands were represented in the Data Libraries. In order to find more statistically significant matches (see next (iii) below) with sequences of known function, the DD band sequences were also searched with (i) the TBLASTX programme against all Viridiplantae (higher plant) DNA sequences in the GenBank Data Library (provided on The *Arabidopsis* Information Resource (TAIR) website, http://www.arabidopsis.org/blast), and (ii) the BLASTN programme against the BCM Human Transcript Database (provided on the Baylor College of Medicine Human Genome Sequencing Center website, http://www.hgsc.bcm.tmc.edu/SearchLauncher).

(iii) Expect (E)-value:

One of the most important parameters in a BLAST search is the Expect (E)-value, which indicates the statistical significance an alignment ("match" or "hit") between a query sequence and a sequence in a database. The default Basic BLAST 2.0 E-threshold setting is 10. This means that for a particular query, all possible alignments for which 10 or less hits of similar bit score‡ are expected to occur **by chance** in a database of similar size, will be returned in the search (as such the E-threshold determines the amount of "background noise" allowed in a particular search).

The E-value of a particular match is dependent on (i) the bit score and (ii) the size of the database. The lower the E-value, the more likely it is that the alignment did not occur

---

* Each website that offers searchable sequence databases normally contains a list of the available databases and their content/application.

† Certain BLAST programmes may only be used in combination with databases of a particular type, e.g. BLASTP and BLASTX may only be used with **protein** databases (see Table 5-1).

‡ The length of the alignment between a query sequence and a sequence in a database is reflected in the bit score (S) returned with search results. The higher the bit score, the better the alignment. Factors such as the scoring system and the introduction of gaps (to enable the best alignment) are included in the calculation of the bit score. The bit score may thus be used to compare alignments returned from different searches (see BLAST documentation on NCBI website for more details).

randomly, but reflects true sequence similarity. As a rule, E-values higher than 0.1 are not regarded to reflect statistically significant sequence similarity. Although this guideline was followed in the current study, it was applied with caution for the following reasons:

a. Query sequences were relatively short (≤ 350 bp). Random alignments are more likely to occur with short sequences than with longer sequences, resulting in higher E-values.

b. The combination of a short query sequences and large database promotes increased E-values*.

c. The E-value reflects the statistical significance of a match, but may not be directly correlated with the **biological** significance thereof. The biological significance of each match must be evaluated individually and can only really be confirmed when the match can be correlated with additional information (i.e. full-length cDNA sequence, mRNA expression patterns or protein levels).

5.2.2 Strategy for the verification of DD results by RT PCR

5.2.2.1 Northern hybridization vs. *RT PCR*

The success of a DD experiment depends on the ability to verify that cloned bands represent differentially expressed mRNAs, using the original RNA sample set. Different aspects of the verification process, including the methods used, modifications of standard protocols aimed at making the verification process less cumbersome, and the variable and complex origins of "false positives" are discussed in detail in Chapter 4 and will not be repeated here.

For this discussion it is important to note that Northern hybridization is most the commonly used technique for the verification of DD results (Liang and Pardee 1995). In short, labelled hybridization probes are generated from cloned bands and hybridized to an aliquot of each of the original RNA samples. A signal with all or none of the samples denotes a false positive†, whereas the absence or reduced intensity of a signal in some of

---

* For this reason, a larger number of statistically significant matches were obtained when the cloned DD band sequences were searched against the GenBank Viridiplantae DNA database, as opposed to the NCBI nr database (see section 5.3).

† A signal with none of the samples may also be the result of a probe-target combination that is below the sensitivity threshold of the hybridization protocol. A Northern blot using total RNA may, for example, be unable to yield visible signals with short probes derived from low-abundance transcripts (Liang et al. 1992, 1993).

the samples confirms that the cloned band was derived from a differentially expressed mRNA. When large numbers of candidate bands have to be evaluated, this process is extremely cumbersome and requires large quantities of RNA for multiple blots. If the number of RNA conditions is small compared to the number of cloned bands, the verification process may be facilitated by using the reverse Northern hybridization technique (Mou et al. 1994, Galindo et al. 1997). In this method, labelled cDNA probes are generated from the original RNA samples. These probes are hybridized simultaneously to all of the cloned bands or plasmid DNA of colonies picked from each cloning experiment (spotted on a membrane), performing one blot per RNA condition*.

In the current study, neither of these methods seemed appropriate for the verification of DD results, for the following reasons:

a. The number of cloned bands equalled the number of RNA conditions (see section 5.3.2), which rendered reverse Northern blots impractical. Standard Northern blots would require more RNA than what was available, even if membranes were stripped and reprobed a number of times†.

b. All of the candidate bands cloned in this study were short (≤ 351 bp) and AT-rich (see section 5.3.2). Probes generated from such bands are very likely to fail in the detection of their corresponding mRNAs in Northern hybridizations, especially if these mRNAs are of low abundance and total RNA is blotted (Liang et al. 1992, 1993).

These factors demanded a different approach to ensure the successful verification of the DD results generated in this study. An RT PCR strategy was developed for this purpose, as it would:

a. require much less RNA (≤ 5 μg total RNA per sample), and could therefore be performed in duplicate with the available amounts of RNA.

b. be much more sensitive and thus have a better chance of verifying the expression of low abundance mRNAs, if any were represented in the pool of cloned bands (Gause and Adamovicz 1995).

---

* One advantage of standard Northern blots (compared to reverse Northerns or RT PCRs) is that information about the sizes of mRNA transcripts represented by cloned bands can be obtained.

† A standard Northern blot was performed with an α-[^{32}P]-labelled probe prepared from one of the cloned bands (band DDe; probe = 185 bp). The hybridization signal was very weak and only detectable on Kodak BioMax MR film after exposure of one week. The hybridization result (non shown) corresponded to the result obtained for this band using the RT PCR protocol described in section 5.3.2.

5.2.2.2 Experimental considerations

The RT PCR strategy that was developed is described in detail in section 5.3.2. Briefly, cDNA was generated from each of the eight RNA samples with an oligo-dT primer. These cDNAs were used as templates in eight series of standard PCRs, each of which was performed with a pair of primers specifically designed to amplify one of the cloned DD bands. Amplification products were visualized on ethidium bromide-stained agarose gels. A number of experimental aspects had to be considered in the design of the RT PCR strategy and are discussed below.

(i) One-step or two-step procedure:

RT PCR may be performed as a one-step or a two-step procedure. In the former, cDNA synthesis and amplification are performed with one pair of gene-specific primers in the same tube. Two-step procedures offer a choice of using one or two tubes. In a two-tube, two-step protocol, cDNA synthesis is performed in the first tube and an aliquot of the product transferred to a separate tube as template for the amplification reaction. A one-tube, two-step protocol may be preferred when the amount of template is limited. cDNA synthesis is then performed in the first step, after which PCR reagents are added and the entire RT reaction used as template for the amplification reaction (Roche Diagnostics 1999).

The main advantages of one-step procedures are that pipetting steps are minimized and the chances of contamination reduced. Additionally, cDNA synthesis is performed at a higher temperature, which increases the specificity of the reaction by eliminating secondary mRNA structure. These procedures are, on the other hand, less efficient than two-step protocols, as reaction conditions have to be adapted for the needs of both the reverse transcriptase and DNA polymerase enzymes. Two-step procedures are more laborious, but more flexible and efficient, as reaction conditions for the RT and PCR steps may be optimized separately (Roche Diagnostics 1999).

In this study, a two-tube, two-step procedure was preferred, as it permitted the cDNA generated from each RNA condition to be used in a series of PCRs, each employing a different pair of "DD band-specific" primers.

(ii) Choice of reverse transcriptase enzyme:

A number of reverse transcriptases (RTases) with different characteristics are commercially available (reviewed in Dieffenbach et al. 1995a). Superscript™ II RTase (an RNase H$^-$ Moloney murine leukemia virus RTase, marketed by Life Technologies) was highly recommended by fellow researchers for its ability to yield higher quantities of longer cDNAs than most other RTases. In a comparative experiment, aimed at the amplification of the elongation factor *eIF-4A* gene from tobacco (see section 5.2.3.2), cDNA generated with this enzyme yielded orders of magnitude more target PCR product than cDNA synthesized with another commercial RTase (Omniscript, Qiagen). Superscript™ II was subsequently used for all non-DD RT PCR applications in our laboratory.

(iii) Choice of primer for cDNA synthesis:

First-strand cDNA synthesis in an RT reaction may be primed in one of three ways (reviewed in Dieffenbach et al. 1995a). When random hexamers are used, all of the RNAs in a sample serve as templates for cDNA synthesis. This method is not recommended when total RNA is used as starting material, as 95-99% of the total RNA in a cell consists of ribosomal and transfer RNAs (Adamovicz and Gause 1995). If mRNAs are to be transcribed specifically, an oligo-dT primer or gene-specific primer may be used. The latter (in which the reverse PCR primer is used for the priming of RT) is more specific, but the former was preferred in this study for the same reason that motivated the choice of a two-tube, two-step RT PCR procedure.

(iv) Choice of DNA polymerase:

Thermostable DNA polymerases from a variety of suppliers have been used in our laboratory for different PCR applications. More expensive enzymes (with special properties and/or reaction buffers) are used for specialist applications (such as DD PCR), whereas an inexpensive system (SuperTherm DNA polymerase, 10 x reaction buffer JMR-455 and $MgCl_2$; all from JMR Holdings, Sidcup, Kent, UK) is used in all standard PCRs. This system yielded consistent results during the development of the RT PCR protocol, and was subsequently used in the routine verification of DD results.

(v) Quantitative or semi-quantitative RT PCR:

Accurate quantification of differences in the expression levels of genes represented by

cloned DD bands is possible with RT PCR. A truly quantitative RT PCR requires careful, empirical optimization for each individual target sequence, as a variety of factors contribute to the fact that a non-linear relationship between the amount of starting material and reaction product exists in practice under standard PCR conditions. To ensure that the amount of RNA corresponding to a target gene is quantitatively reflected by the amount of RT PCR product, input RNA normally has to be diluted and the number of PCR cycles reduced (typically to less than 30). This often does not produce sufficient product for detection by ethidium bromide staining, and requires the use of labelled nucleotides, labelled primers or Southern hybridization to a labelled probe to detect and quantitate the reaction product (Foley et al. 1993, Gause and Adamovicz 1995).

In this study, a "standard", semi-quantitative, 35-cycle RT PCR strategy was developed for the verification of cloned bands, for the following reasons:

a. The individual, empirical optimization of a quantitative RT PCR strategy for every cloned band appeared to be cumbersome and impractical to perform in the routine verification of DD results*.

b. Standard RT PCR strategies, in which targets were amplified for 30 or more cycles and amplification products visualized by ethidium bromide staining, have been used successfully in other published studies to reflect differences in the expression levels of defense-related genes in *Arabidopsis* (Penninckx et al. 1996, Gómez-Gómez et al. 1999).

(vi) Internal standard for the control of source variability:

A prerequisite in any quantitative or semi-quantitative RT PCR (or Northern hybridization) protocol, is the capacity to distinguish between differences in signal intensity related to actual differences in gene expression, and differences resulting from variation in the concentration and integrity of a target mRNA between individual samples (Gause and Adamovicz 1995).

---

* Recent advances in PCR technology has led to the development of "real-time PCR", which allows the fluorescence monitoring of amplification product formation throughout the course of a PCR (see Higuchi et al. 1992, 1993, Ririe et al. 1997 and Wittwer et al. 1997). This technique lends itself to the fast and efficient development and optimization of quantitative RT PCR protocols. However, real-time PCR technology requires specialized (and expensive!) equipment, which was not accessible to us at the time when these experiments were performed.

The quality and quantity of RNA samples is normally evaluated in one of two ways. The first simply entails agarose gel electrophoresis and ethidium bromide staining of an equal amount (5-10 μg) of each sample. If rRNA bands have the same intensity and appear intact, the overall quality and concentration of the sample is regarded to be similar. This method (which is routinely employed in Northern hybridization analyses) requires large quantities of RNA, and cannot display variations in the content and condition of individual mRNAs within different total RNA samples (Gause and Adamovicz 1995).

The second, and more efficient way to deal with source variability in RT PCR protocols, is to include an endogenous internal standard*. This involves the amplification of a gene that is known to be expressed constitutively in the tissue/cell population from which RNA samples were derived. The quantity of target RT PCR product obtained for each sample is first normalized against the amount of control product for that sample before differences between samples are evaluated (Gause and Adamovicz 1995)†.

The development of such an internal RT PCR standard for the routine verification of DD results is described in section 5.2.3.

5.2.2.3 Design of "DD band specific" primers and choice of RT PCR conditions

The nucleotide sequence of each cloned DD band (obtained by automated cycle sequencing as described in section 5.3.2) was used as the basis to design a pair of primers that would specifically amplify (the part of) the cDNA represented by that band. Primer design was performed with the programme Primer Designer (version 1.01; Scientific & Educational Software, 1990), according to the criteria outlined in Chapter 3 (section 3.2.2). Primer design was influenced by the following factors:

a. Cloned bands were short. In order to obtain the longest possible amplification product for each band, primers were based on suitable regions that were closest to the 5'- and

---

* In quantitative protocols, **exogenous** standards (which closely mimic the target sequence and are amplified with the same primer pair as the target) are often added. The same amplification efficiency is assumed for both the control and target. A known quantity of the standard is used to titrate a constant quantity of the target in a competitive RT PCR, and the concentration at which the target reaction product equals that of the standard reaction product, is taken as the starting concentration of the target mRNA in the sample. Such exogenous standards, can however, not compensate for variability in the integrity of different mRNAs between multiple RNA samples in an assay (Gause and Adamovicz 1995).

† If DD results are verified by Northern hybridization, the control gene is used for the production of a hybridization probe. Results are normalized by expressing the intensity of hybridization signals for individual samples relative to the signal obtained with the control probe in each sample.

3'-ends of each sequence.

b. Cloned bands were AT-rich (57-70%, exclusive of sequences corresponding to the DD PCR primers). As a result, the minimum permissible G+C-content was dropped from the default value of 50% to 30%.

c. The optimal melting temperature (T_m) range of the actin primers used to generate an internal RT PCR standard (see section 5.2.3.3) was 50-55°C. All primer pairs thus had to be effective in this T_m range.

d. A 3'-terminal G or C was not possible in all cases. For two primers, a G or C could only be achieved at the third position from the 3'-end.

Optimal reaction conditions for each of these "DD band specific" primer pairs had to determined empirically. Theoretically, the preferred operational T_m was 50°C for six of the primer pairs and 55°C for the other two. It would, however, be convenient if the same reaction conditions could be used for the simultaneous verification of all the cloned DD bands.

As a first approach, each primer pair was tested at an annealing temperature of 50°C in the presence of 1.5 mM $MgCl_2$. Original DD profiles (from which candidate bands were identified for cloning) were used to select a cDNA that was expected to yield an amplification product as the template for each primer pair. A single amplification product of the expected size was obtained for each of the primer pairs (Fig. 5-1). Since the intensities of these RT PCR products were proportional to their molecular sizes, further optimization of the reaction conditions for any of the primer pairs was not considered to be necessary.

5.2.3 Development of an internal RT PCR standard

5.2.3.1 Genes used as internal controls in other studies

At the time when the RT PCR strategy was developed, DD was used exclusively in our laboratory for the identification of genes involved in interactions between *Prunus salicina* and different necrotizing pathogens. Theoretically, any gene that is **not** differentially expressed in plum tissue under the experimental conditions employed in these studies would have constituted a suitable internal control gene for our verification strategy. To identify such a gene, two different approaches were followed:

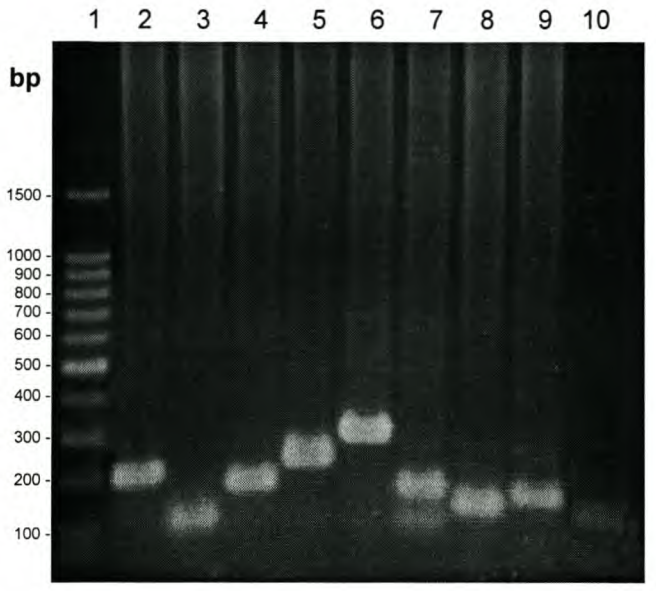


Fig. 5-1. Agarose gel electrophoresis of RT PCR products obtained with "DD band specific" primers, using selected cDNAs as templates.

cDNA synthesis was performed in 20 µL reactions, essentially as described in the Superscript™ II RTase package insert (Life Technologies, Paisley, Glasgow, UK). The only exception was that 50 units of RTase (instead of 200) was added with the rest of the reaction components to each reaction before the 42°C incubation step. An oligo-$(dT)_{12\text{-}18}$ primer (Life Techologies) was used to prime cDNA synthesis. 5 µg of a selected total RNA sample (from the set originally used for DD) was used as template in each of the eight RT reactions.

PCRs were performed in 20 µL reactions. Each reaction contained 1 U SuperTherm DNA polymerase, 1 x reaction buffer and 1.5 mM $MgCl_2$ (all from JMR holdings, Sidcup, Kent, UK), 0.2 mM of each dNTP (ABgene, Epsom, Surrey, UK) and 0.5 µM each of the relevant forward and reverse "DD band specific" primers (synthesized by the DNA synthesis laboratory, University of Cape Town, South Africa). 2 µL of a selected RT reaction or double distilled water (for negative controls) were used as template. Reactions were performed in a PCR Express thermocycler (Hybaid, Ashford, Middlesex, UK) for 35 cycles of 45 sec at 94°C, 45 sec at 50°C and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C.

5 µL of each PCR product were electrophoresed in 1 x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in a 2% agarose gel. For optimal visualization of small bands, the gel was stained after electrophoresis in a 1 µg/mL ethidium bromide solution for 45 min.

The sizes of molecular marker fragments are given on the left. The expected size of the RT PCR product for each cloned DD band is included in brackets below.

Lanes 1: Promega 100 bp DNA ladder; ***2:*** Primers for band **DD3 (225 bp)** with *P. s.* pv. *syringae* NV treated 'Songold' cDNA as template; ***3:*** Primers for band **DD4 (111 bp)** with untreated control 'Laetitia' cDNA as template; ***4:*** Primers for band **DD5 (184 bp)** with wounding control 'Laetitia' cDNA as template; ***5:*** Primers for band **DD6 (236 bp)** with untreated control 'Laetitia' cDNA as template; ***6:*** Primers for band **DD7 (298 bp)** with wounding control 'Laetitia' cDNA as template; ***7:*** Primers for band **DDa (160 bp)** with harpin treated 'Laetitia' cDNA as template; ***8:*** Primers for band **DDc (127 bp)** with harpin treated 'Laetitia' cDNA as template; ***9:*** Primers for band **DDe (151 bp)** with harpin treated 'Laetitia' cDNA as template; ***10:*** Negative control for primers DDa.

Similar results were obtained when 2 µg total RNA was used for cDNA synthesis (not shown). The less intense band obtained with the DDa-specific primer pair correspond to primer-dimers, as shown in the negative control in lane 10.

a. Firstly, the GenBank nucleotide databases were searched for all *Prunus* nucleotide sequences to ascertain whether any constitutively expressed *Prunus* genes have been characterized. A total of 239 nucleotide sequences were retrieved in this search. Since it was impractical to trace the expression patterns of such a large number of genes, the focus was narrowed to the six *P. salicina* sequences retrieved in the search (Table 5-2).

   The chloroplast *trnL* intron and ITS sequences of nuclear rRNA are commonly used in phylogenetic analyses (White et al. 1990, Taberlet et al. 1991). The *P. salicina trnL* and ITS sequences deposited in GenBank were all generated in unpublished studies of this kind* and were thus not suitable for the development of an internal RT PCR control.

   Ribonucleases (S-RNases) are associated with gametophytic self-incompatibility in two plant families, the Solanaceae and the Rocaceae. Although no information was available on the expression patterns of the two *P. salicina* S-RNase genes deposited in

Table 5-2. *Prunus salicina* nucleotide sequences retrieved from the GenBank nucleotide database[a].

Accession no.	Gene(s)/sequences included	Type of sequence	Cultivar/ strain	Ref.
AB026981	Sa-RNase	Partial mRNA for coding region	Sordum	ds
AB026982	Sb-RNase	Partial mRNA for coding region	Sordum	ds
AF115481	Chloroplast transfer RNA-Leu (*trnL*)	Complete chloroplast gene sequence (including intron)	ns	ds
AF179486[b]	Internal transcribed spacer (ITS) 1 and 5.8 S ribosomal RNA	Complete nuclear DNA sequence Partial nuclear DNA sequence	Wen 3020 (CS)	ds
AF179487	5.8 S ribosomal RNA and ITS 2	Partial nuclear DNA sequence Complete nuclear DNA sequence	Wen 3020 (CS)	ds
AF185618	ITS 1 and 5.8 S ribosomal RNA and ITS 2	Complete nuclear DNA sequence Complete nuclear DNA sequence Complete nuclear DNA sequence	ns	ds

[a] Website: http://www.ncbi.nlm.nih.gov/entrez. Date of data retrieval: 16 August 2000.

[b] The same sequence data is retrievable under accession number AH009373.

Abbreviations: **ds** = direct submission, **ns** = not specified.

* According to information supplied with sequence submissions.

GenBank, previous studies have proposed that these genes may be involved in plant defense (Lee et al. 1992, Lusso and Kuc 1995, Galiana et al. 1997). The *P. salicina* S-RNase genes thus also did not offer a suitable basis for the development of an internal RT PCR control in the current study.

b. The second approach was to scrutinize the literature and databases for genes known to be constitutively expressed in a wide range of plant tissues. In studies involving mammalian cells and/or tissues "housekeeping" genes, such as β-actin (Svetic et al. 1991, Kinoshita et al. 1992), β_2-microglobulin (Murphy et al. 1990), hypoxanthine-guanine phosphoribosyl transferase (HPRT; Pieretti et al. 1991) and glyceraldehyde phosphate dehydrogenase (GAPDH; Gendelman et al. 1990) are routinely used as internal controls.

   A survey of the literature describing the use of DD in plant gene expression analysis failed to reveal any such commonly used internal "plant" standards. In a few studies, genes known to be expressed constitutively in the particular tissue from which RNA was isolated, were used as controls (e.g. Szczyglowski et al. 1997). The majority, however, offered ethidium bromide stained rRNA bands on agarose gels as controls, or used hybridization probes derived from rRNAs* to demonstrate the absence of source variability.

   In some published plant DD studies, Northern hybridizations performed with total RNA were not sensitive enough to verify the expression of some of the cloned bands. Detection was, however, achieved when blots were repeated with poly(A)$^+$ RNA extracted from the original samples used for DD. To demonstrate equal loading of lanes in such blots (which do not contain rRNA), Seehaus and Tenhaken (1998) used a probe derived from an ascorbate peroxidase gene, known to be expressed constitutively in soybean under the experimental conditions used. In another study, a probe derived from the eukaryotic elongation factor 4A encoding gene (*eIF-4A*) was used for this purpose (Szczyglowski et al. 1997). This gene was previously demonstrated to be highly and constitutively expressed in plants (Owttrim et al. 1991, 1994) and was also used as an internal control in the analysis of S-like RNAse gene expression in *Arabidopsis* by Northern hybridization (Taylor et al. 1993).

---

* Although rRNAs are constitutively expressed in eukaryotic cells, their suitability as internal controls in mRNA analyses is questionable, as the kinetics of rRNA transcription and degradation is very different to that of mRNA (Jones et al. 1994).

5.2.3.2 *Attempts to design* eIF-4A *specific control primers**

The documented application of the *eIF-4A* gene as an internal standard in plant gene expression analysis rendered it a good candidate for a RT PCR control in our DD verification strategy. To test its applicability in our system, a pair of primers had to be designed that would allow the amplification of an *eIF-4A* fragment from cDNAs generated by oligo-dT primed reverse transcription of the RNA samples originally used for DD.

To this end, *eIF-4A* mRNA sequences of *Nicotiana plumbaginifolia* (curled-leaf tobacco), *Zea mays* (maize), *Arabidopsis thaliana* and *Triticum aestivum* (wheat) were obtained from the GenBank data library†. These sequences were aligned and used as a basis to design a primer pair (MAC-f and MAC-r), according to the procedure described in Chapter 3 (section 3.2.2). The regions of highest similarity between the aligned sequences that were closest to the 5'- and 3'-termini of the open reading frame (ORF) were identified as the preferred primer sites, as this would yield the longest possible *eIF-4A* amplification product.

Primers MAC-f and MAC-r‡ were first used in PCRs with genomic DNA of *N. tabacum* cv. 'White Burley' (common tobacco) and several stone fruit cultivars (*Prunus salicina* cvs. 'Songold', 'Laetitia' and 'Lady Red', and *P. persica* var. *nectarina* cv. 'Flavorine') as templates. The results from a series of PCRs, performed at two annealing temperatures and three $MgCl_2$ concentrations (Fig. 5-2) suggested the following:

a. Primers MAC-f and MAC-r were specific for tobacco *eIF-4A*. This was confirmed by partial sequencing of the dominant (1.5 kb) tobacco amplification product. 280 of the 5'-terminal 370 nucleotides sequenced with MAC-f and all 467 nucleotides of the 3'-terminal fragment obtained with MAC-r aligned with 97% identity to the corresponding *N. plumbaginifolia eIF-4A* mRNA sequence. The remaining 90 nucleotides in the 5'-terminal fragment were identified as one (of at least two) introns in the genomic *N. tabacum eIF-4A* fragment bordered by the sequences of MAC-f and MAC-r.

---

* This series of experiments did not yield the desired result and is presented in a summarized form to demonstrate the process of designing control RT PCR primers.

† GenBank accession numbers of sequences are: ***N. plumbaginifolia eIF-4A2*:** X61205, ***Z. mays eIF-4A*:** U17979, ***A. thaliana eIF-4A1*:** X65052, ***A. thaliana eIF-4A2*:** X65053 and ***T. aestivum eIF-4A*:** Z21510.

‡ Primer sequences are: **MAC-f:** 5'-CAGGAGGAGTTCTTCACATC-3', corresponding to nucleotides 82-101 of the *N. plumbaginifolia eIF-4A2* ORF and **MAC-r:** 5'-TGCAAGTAGTTCTCTGGCTG-3', corresponding to nucleotides 1075-1094 of the same sequence.

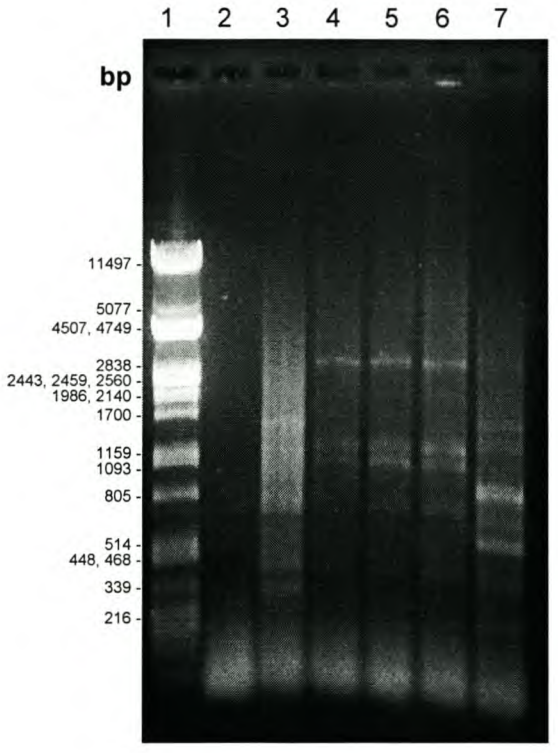


Fig. 5-2. Agarose gel electrophoresis of PCR products obtained with *eIF-4A* specific primers MAC-f and MAC-r, using genomic DNA from tobacco and stone fruit as templates.

PCRs were performed in 50 µL reactions. Each reaction contained 2.5 U SuperTherm DNA polymerase, 1 x reaction buffer and 2.5 mM $MgCl_2$ (all from JMR Holdings, Sidcup, Kent, UK), 0.2 mM of each dNTP (ABgene, Epsom, Surrey, UK) and 0.5 µM each of primers MAC-f and MAC-r (synthesized by the DNA synthesis laboratory, University of Cape Town, South Africa). Genomic plant DNA (40 ng, isolated according to a method adapted from Doyle and Doyle 1987) or double distilled water (for negative controls) was used as template. Reactions were performed in a PCR Express thermocycler (Hybaid, Ashford, Middlesex, UK) for 35 cycles of 45 sec at 94°C, 45 sec at 50°C and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. 15 µL of each PCR product were electrophoresed in 1 x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in a 1% agarose gel containing 0.25 µg/mL ethidium bromide.

The sizes of molecular marker fragments are given on the left.

Lanes 1: *Pst* I/ϕλ DNA ladder; ***2:*** Negative control; ***3:*** *Nicotiana tabacum* cv. 'White Burley'; ***4:*** *Prunus salicina* cv. 'Laetitia'; ***5:*** *P. salicina* cv. 'Lady Red'; ***6:*** *P. salicina* cv. 'Songold'; ***7:*** *P. persica* var. *nectarina* cv. 'Flavorine'.

A second series of PCRs were performed as described above, but with varying concentrations of $MgCl_2$ (1.5 and 3.5 mM, respectively). At 1.5 mM, no significant amplification products were obtained for any the plum cultivars, but at 3.5 mM two more bands (1.6 and 1.3 kb respectively) were observed (results not shown).

When the PCRs were repeated as described, but at an annealing temperature of 55°C over the range of $MgCl_2$ concentrations (1.5, 2.5 and 3.5 mM), the smaller (1.0 kb) tobacco band consistently failed to amplify. At this temperature, none of the $MgCl_2$ concentrations yielded any plum amplification products. At 1.5 and 2.5 mM $MgCl_2$, no nectarine products were obtained, but at 3.5 mM $MgCl_2$ the 0.9 kb and 0.55 kb bands seen above were weakly amplified (results not shown).

b. The stone fruit *eIF-4A* sequence was probably quite divergent from the tobacco, maize, *Arabidopsis* and wheat sequences in the regions corresponding to primers MAC-f and MAC-r. Reaction conditions that seemed optimal for the amplification of a single *eIF-4A* fragment in tobacco did not yield any stone fruit amplification products. Although some products were obtained under conditions that permit amplification from less specific priming events (i.e. a higher $MgCl_2$ concentration and lower annealing temperature), these primers would probably not be suitable for the amplification of an *eIF-4A* fragment that could serve as an internal RT PCR control.

Attempts were nevertheless made to generate *eIF-4A* fragments by RT PCR from *N. tabacum* and *P. salicina* cv. 'Songold' total RNA. A series of experiments were performed in which (i) the amount of total RNA used for cDNA synthesis, (ii) the RT primer (oligo-dT or MAC-r), (iii) the amount of cDNA used as template for the PCR, (iv) the PCR annealing temperature, and (v) the $MgCl_2$ concentration in the PCR were varied. The salient results of these experiments were the following:

a. A 1.0 kb fragment was consistently amplified from tobacco cDNA. An optimal yield of this product was obtained when (i) at least 1 μg total RNA was used for cDNA synthesis, (ii) RT was primed with the oligo-dT primer, and (iii) the PCR was performed at an annealing temperature of 50°C in the presence of 1.5 mM $MgCl_2$. This product (predicted on the basis of the *N. plumbaginifolia eIF-4A* sequence to be 1013 nucleotides in length) was purified and subjected to automated cycle sequencing with primers MAC-f and MAC-r*. The 946 nucleotides of sequence obtained in this way aligned with 98% identity to nucleotides 106-1051 of the *N. plumbaginifolia eIF-4A* mRNA sequence.

b. Plum cDNA synthesized with the oligo-dT primer consistently failed to yield any amplification products in subsequent PCRs. When MAC-r was used for RT, and the PCR performed at $MgCl_2$ concentrations higher than 1.5 mM, two amplification products (1.2 and 0.95 kb respectively) were faintly recognizable against a very smeary background on an agarose gel. Whichever way reaction conditions were manipulated,

---

* Briefly, the PCR was scaled up fivefold and the total reaction product electrophoresed in 1 x TAE in an 1% agarose gel containing 0.125 μg/mL ethidium bromide. The *eIF-4A* band was excised from the gel and purified using the Wizard PCR prep DNA purification kit (Promega, Madison, WI). DNA eluted from the Wizard columns were concentrated by evaporation. A aliquot was electrophoresed in an agarose gel to estimate the concentration (relative to DNA fragments of a known concentration). Cycle sequencing was performed as described in section 5.3.2.

MAC-f and MAC-r appeared to be unsuitable for the generation of an *eIF-4A* internal RT PCR standard.

The final approach in the development of a possible *eIF-4A* control rested on attempts to isolate and purify some of the amplification products that were obtained with the MAC primers from plum genomic DNA (Fig. 5-2). If these fragments could be sequenced, and proved to be *eIF-4A* related, a different pair of primers that would specifically amplify a part of the plum *eIF-4A* sequence could be designed. Even when reaction volumes were scaled up fivefold, and reaction products pooled and concentrated before agarose electrophoresis, the intensities of these bands relative to background smears were never high enough to achieve this.

In summary, these experiments produced an *eIF-4A* specific primer pair that was potentially useful to generate an internal RT PCR control when tobacco material was used for differential gene analysis. Further attempts to apply this control in the verification of DD results obtained with plum material were, however, abandoned.

5.2.3.3 Actin as an internal "plant" RT PCR standard

The author's efforts to develop a plant *eIF-4A* internal RT PCR standard co-incided with a similar venture undertaken at Stellenbosch University's Institute of Plant Biotechnology (IPB) under the guidance of Prof. Frikkie Botha. This group investigated the possibility to use a plant **actin** gene as internal standard for gene expression analysis in grapevine (in a similar fashion to the general use of the β-actin gene in mammalian tissues; Gause and Adamovicz 1995). Like their mammalian counterparts, plant actin encoding genes may be regarded as "housekeeping" genes as (i) cytoplasmic actin is an essential component of the cell cytoskeleton of eukaryotic cells, including that of plant cells (Pollard and Cooper 1986), and (ii) a number of cellular processes, such as cytoplasmic streaming, cell shape determination, organelle movement, cell division and extension growth, are believed to involve cytoskeletal actin proteins in higher plants (Staiger and Schliwa 1987).

A pair of actin specific primers was generously made available by Anita Burger of IPB for evaluation with plum material. These primers, actin-F (5'-TCACACTTTCTACAATGAGCT) and actin-R (5'-GATATCCACATCACACTTCAT) were based on homologous 5'- and 3'-regions in the nucleotide sequences of plant actin mRNAs obtained from the GenBank

database. This primer pair was tested under the reaction conditions described in Fig. 5-1, with cDNAs generated from the plum RNAs used for DD as templates. A 600 bp band of high and equal intensity was obtained from all eight samples (Fig 5-3).

The eight RT PCR reaction products were subsequently pooled for the purification and automated sequencing* of the 600 bp band. Sequence data obtained with the 5-' and 3-' primers were integrated with the programme DAPSA (version 4.61; E.H. Harley, University of Cape Town, South Africa, 1999). The entire sequence of 607 nucleotides† (inclusive of the two 21-mer actin-F and actin-R primer sequences) was obtained in this way.

A basic BLAST search (Altschul et al. 1990, 1997) of the GenBank nucleotide database recorded 183 "hits" and retrieved 100 sequences with significant sequence similarity to

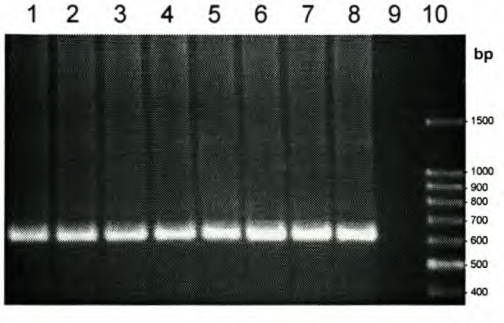


Fig. 5-3. Agarose gel electrophoresis of PCR products obtained with actin specific primers, using cDNA generated from the *Prunus salicina* RNA samples used for DD analysis.

cDNA synthesis was performed in 20 µL reactions as described in Fig. 5-1, using 2 µg of *P. salicina* total RNA as template in each of the eight RT reactions. For a negative RT control, RNA was replaced with an equal volume of double distilled water.

PCRs were performed in 20 µL reactions as described in Fig 5-1, except that the final concentration of each actin primer was reduced with one-tenth to 0.05 µM. (This was done since amplification with the standard 0.5 µM yielded such intense bands that their apparent size could not be assessed properly.) For the negative PCR control, 2 µL of the negative RT control was used as template.

5 µL of each PCR product were electrophoresed and visualized as described in Fig. 5-1. Similar results were obtained from triplicate PCRs, each performed with cDNAs generated in a different series of RT reactions.

Lanes 1: Untreated control 'Laetitia' cDNA; ***2:*** Wounding control 'Laetitia' cDNA; ***3:*** Harpin treated 'Laetitia' cDNA; ***4:*** *P. s.* pv. *syringae* NV treated 'Laetitia' cDNA; ***5:*** Untreated control 'Songold' cDNA; ***6:*** Wounding control 'Songold' cDNA; ***7:*** Harpin treated 'Songold' cDNA; ***8:****P. s.* pv. *syringae* treated 'Songold' cDNA; ***9:*** Negative control; ***10:*** Promega 100 bp DNA ladder.

The sizes of molecular marker fragments are given on the right.

---

* Performed essentially as described for the tobacco *eIF-4A* RT PCR product, except that "quarter" sequencing reactions (in which all reagent volumes were halved) were used.

† Deposited into the GenBank nucleotide database (accession number AF321852).

this partial *Prunus salicina* actin sequence. The majority (76%)* of these matches were with actin nucleotide sequences (complete or partial mRNA, cDNA or genomic DNA) from other plants. A cartoon of the ten complete plant actin sequences in the database that showed the highest degree of similarity to the partial plum sequence, is given in Fig. 5-4. The partial sequence of the only *Prunus* actin sequence in the database is also included in the figure.

Having successfully amplified an actin fragment from oligo-dT primed *P. salicina* cDNA, two questions remained to be addressed. The first was whether the actin gene represented a legitimate internal control under the conditions used in the DD experiments. If so, the final decision to be made was whether the control fragment was to be co-amplified with individual DD bands, or whether the control amplification was to be performed in a separate tube (Gause and Adamovicz 1995).

To answer the first of these questions experimentally, a signal of equal intensity for all eight RNA samples used in the DD experiment had to be shown in a Northern hybridization or RT PCR, using an actin probe or the abovementioned actin primers. Such a result was indeed obtained (Fig. 5-3), but without an internal standard to demonstrate the absence of source variability. The decision to use actin as an internal control in the verification of our DD results was therefore additionally based on the following indirect evidence†:

a. The RT PCR products obtained with the actin primer pair from the eight different plum RNAs were pooled before sequencing of the 0.6 kb band. The sequence data had a

---

* The remaining 14% of the hits were with actin sequences of *Trichosurus vulpecula* (the silvergray brushtail possom; two hits), *Xenopus laevis* (the African clawed frog), *Danio rerio* (the zebrafish; two hits), *Gallus gallus* (the chicken; two hits), *Carassius auratus* (the goldfish), *Coturnix coturnix* (the Japanese quail), *Biompharalia glabrata* (the bloodfluke planorb; two hits), *Cyprinus carpio* (the common carp), *Pseudorasbora parva* (a fish species) and *Rattus rattus* (the black rat). 75-80% identity was obtained with the region in these non-plant actin sequences that corresponded to the partial *P. salicina* actin sequence.

† The following facts have bearing on the arguments that follow:

(i) Actin is expressed by gene families in all higher plants studied to date (Meagher 1991).

(ii) Unlike mammalian actin genes, the gene structure of plant actin encoding genes are uniquely conserved, and consists of four exons of conserved length and three introns of variable length (Shah et al. 1983).

(iii) Despite the high conservation of gene organization, a relatively high degree of amino acid divergence exists within the protein sequences of actins expressed in the same plant, as well as within the actins expressed in different plant families (Shah et al. 1983, McDowell et al. 1996).

(iv) Different subclasses of actin genes are expressed at different developmental stages and in different tissues (Meagher 1991, McDowell et al. 1996).

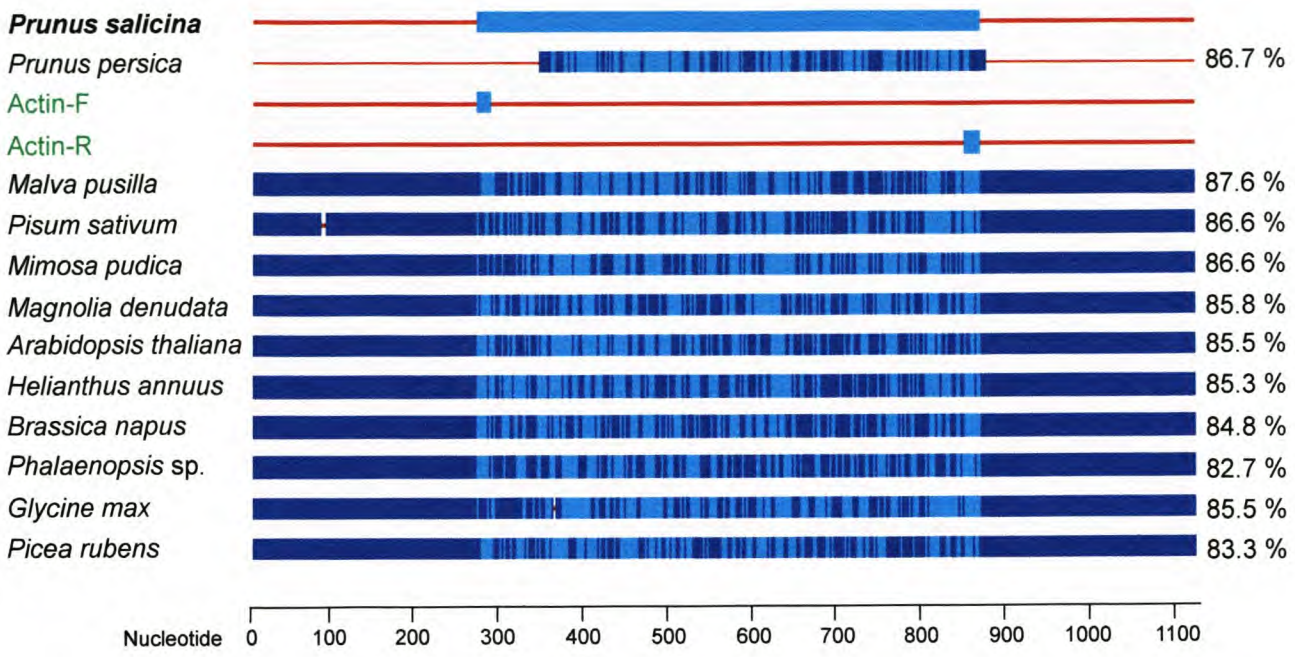


Fig. 5-4. Cartoon of plant actin mRNA sequences, aligned to the partial *Prunus salicina* actin mRNA sequence obtained by RT PCR with primers actin-F and actin-R.

mRNA sequences of the 10 complete plant actin mRNAs that showed the highest degree of similarity with the partial *P. salicina* actin mRNA sequence in the BLAST search, as well as the partial *P. persica* sequence, were obtained from the GenBank database (accession numbers are given below; the *G. max* mRNA sequence was deducted from the genomic DNA sequence). The cartoon of aligned sequences was generated with the programme DAPSA 4.61. Light blue areas in sequences aligned to the partial *P. salicina* sequence denote identical nucleotides in the corresponding region, whereas dark blue areas represent nucleotide differences. Since the amplified *P. salicina* fragment is incomplete, the 5'- and 3'-ends of the complete actin genes are also indicated in dark blue.

All of the complete actin mRNAs are 1134 nucleotides long (corresponding to a protein of 378 amino acids), with the exception of the *P. sativum* and *G. max* sequences, which has a 3-nucleotide deletion each. The partial *P. salicina* sequence (607 nucleotides) aligned with nucleotides 267-873 of the other plant sequences. The percentage of identical nucleotides between the partial plum sequence and each of the other sequences in this region is given on the right. In the comparison of the partial plum and peach sequences, only the 528 nucleotides common to both were used to calculate the percentage of identity. The relative positions of the actin primers are also indicated. Actin-F corresponds to positions 267-287 and actin-R to positions 853-873 of the aligned sequences. Actin-F is not 100 % identical to the relevant region in any of genes obtained from GenBank, but actin-R corresponds with 100 % identity to nucleotides 853-873 of the *M. pusilla*, *B. napus* and *G. max* mRNAs.

Accession numbers of complete actin mRNA sequences included in the alignment are: ***Malva pusilla* Act1:** AF112538, ***Pisum sativum* actin:** X68649, ***Mimosa pudica* actin isoform B:** AB032361, ***Magnolia denudata* actin:** AF281323, ***Arabidopsis thaliana* actin-2:** U37281, ***Helianthus annuus* actin:** AF282624, ***Brassica napus* actin:** AF111812, ***Phalaenopsis* sp. 'True Lady' ACT1:** AF246714, ***Glycine max* SAc3 genomic DNA:** J01297, ***Picea rubens* actin:** AF172094, ***Prunus persica* partial mRNA for actin:** AB046952.

very low signal-to-noise-ratio, which indicated that only one sequence was present in this pooled sample. This suggested that only one plum actin gene was expressed in plant material from which RNA was isolated for DD analysis.

b. Despite the documented sequence divergence between the coding regions of plant actin genes, the partial *P. salicina* actin sequence showed significant similarity with more than one actin isoform of several plants in the BLAST search: *Pisum sativum*: 2 isoforms; *Mimosa pudica*: 3 isoforms; *Arabidopsis thaliana*: 7 isoforms; *Glycine max*: 8 isoforms; *Phalaenopsis* sp.: 2 isoforms; *Oryza sativa*: 3 isoforms; *Nicotiana tabacum*: 6 isoforms; *Daucus carota*: 2 isoforms; *Anemia phyllitidis*: 2 isoforms; *Solanum tuberosum*: 8 isoforms and *Zea mays*: 3 isoforms. Together with the sequencing data, this suggested that the actin primer pair was not isoform-, but rather actin-specific, and that only one actin isoform (gene) was probably expressed in plum shoot tips under the experimental conditions used for DD sample generation.

c. Results similar to those shown in Fig. 5-3 were consistently obtained in several series of amplification experiments in which cDNA synthesis was performed with (i) different aliquots of each RNA (which were DNase I-treated and stored separately), and (ii) different dilutions of each RNA (made to the desired concentration from aliquotted stocks). Likewise, PCRs were performed with (i) a cDNA set generated in different RT reactions and (ii) different dilutions of the actin primers.

   In experiments where the amount of total RNA used for cDNA synthesis was decreased (from 5 μg to 2 μg to 1 μg), or the final concentration of the actin primers was decreased (from 0.5 μM each to 0.05 μM each to 0.017 μM each), the intensity of the actin band decreased proportionally and evenly for all eight conditions. Only when the final concentration of each primer was decreased 100-fold (i.e. to 0.005 μM each) in the PCR, did the actin band fail to amplify from any of the eight cDNAs. This suggested that the actin gene was expressed at high levels in actively growing plum shoots, and at comparative levels between the cultivars, independent of treatment.

d. Amplification of the *Arabidopsis thaliana* actin-1 gene (*AAc1*; Nairn et al. 1988) has been used in similar RT PCR strategies as an internal control for source variability (Penninckx et al. 1996, Gómez-Gómez et al. 1999).

Having demonstrated that primers actin-F and actin-R were capable of amplifying an actin fragment which seemed to be expressed at high and comparative levels in all eight RNA conditions, the final decision was whether to co-amplify the control and target sequences in a single tube during a PCR, or whether to use separate tubes. Co-amplification is often not desired, as the levels of control gene expression may be very high relatively to that of some target genes (Gause and Adamovicz 1995). In this study target fragments were also very short (25-50% of the length of the control fragment), therefore the intensity of the control band had to be limited to achieve comparable staining of control and target amplification products in ethidium bromide stained agarose gels.

Co-amplification of selected DD bands and the actin band was initially done in the same tube and seemed to yield acceptable results when the final concentration of each actin primer was reduced to one-tenth of the concentration of each "DD band specific" primer. These initial experiments were, however, performed with selected cDNAs, and when the experiment was extended to include the entire sample set for each particular band, the co-amplification approach proved to be unsuitable in cases where cloned DD bands truly corresponded to differentially expressed mRNAs. The following results were typically obtained for "false positive" and "true positive" DD bands, respectively:

a. When a cloned band did **not** appear to correspond to a differentially expressed mRNA, the "DD band specific" primer pair amplified a target of more or less the same intensity from all eight cDNAs. Competition for reagents (particularly dNTPs and $MgCl_2$) during co-amplification of the target and control (actin) fragments seemed to be fairly consistent, as the intensity of both bands were always comparable between the eight conditions.

b. When a cloned band, however, appeared to represent a **differentially** expressed mRNA, the cDNA for that gene would be absent in some of the samples. In such samples, more dNTPs would be available for the amplification of the control band. The subsequent increase in the intensity of the control band in samples deficient in the target band rendered the control useless for the normalization and interpretation of the final result.

The co-amplification or "multiplex" approach could have been optimized further, by increasing the concentration of dNTPs and $MgCl_2$ in the final reaction (Edwards and Gibbs

1995), or by adding the control primers at a later stage during the PCR (Kinoshita et al. 1992). Separate amplification of the control and target sequences was, however, preferred, as it allowed greater flexibility (Murphy et al. 1990). Variability introduced during the detection of amplification products (such as lane-to-lane variation during electrophoresis) was normalized by electrophoresis of the target and control reaction products in the same lane (see section 5.3.2).

5.3 IDENTIFICATION OF PLANT GENES INVOLVED IN INTERACTIONS BETWEEN THE BACTERIAL CANKER PATHOGEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* NV AND PLUM TREES USING DIFFERENTIAL DISPLAY

5.3.1 Contributions of co-authors

The manuscript that follows contains the original and independent work of the author of this dissertation. The manuscript was compiled entirely by the author and all experimental work presented in it was devised and conducted by her, with the exception of the clean-up and analysis of cycle sequencing reactions, which was performed as a routine and paid service by the University of Stellenbosch Core DNA Cycle Sequencing Facility.

Each of the co-authors contributed to the manuscript in the following way:

a. *Ms Sonja Vorwerk* visited the laboratory of Prof. Dirk Bellstedt from 21 January to 15 May 2000. She had just completed her "Diplomarbeit" under the supervision of Prof. Elmar Weiler at the Department of Plant Physiology (Rühr-Universität, Bochum, Germany) and wished to gain experience in differential display technology before she continued with her Ph. D. studies. The second series of DD experiments described in the manuscript was used for this purpose. Under supervision of the author of this dissertation, Ms Vorwerk thus assisted in the generation of the second series of RT and DD PCR products, the separation of DD PCR products by PAGE, and the excision, re-amplification, cloning and sequencing of candidate bands DD3, DD4, DD5, DD6 and DD7.

b. *Prof. E. Lucienne Mansvelt** is a senior researcher at the Plant Biotechnology and Pathology Division of the ARC-Fruit, Wine and Vine Research Institute. She has collaborated with the author and her supervisor on molecular investigations of the

* Recently appointed as special associate professor of Plant Pathology at Stellenbosch University.

bacterial canker pathogen since 1994 and is the co-promoter of this dissertation. As such, she has provided logistical support and partial funding for this project. For this study, she supplied the stock cultures of *P. s.* pv. *syringae* NV, as well as tobacco plants, plum trees and greenhouse facilities. The bacterium was cultivated and all plant infiltrations were performed under her supervision. She also provided the facilities for and assistance with the photography of RT PCR results.

c. *Prof. Dirk U. Bellstedt* is an associate professor of Biochemistry at the University of Stellenbosch and the promoter of this dissertation. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He promoted all collaborations, provided funding for the project and facilitated all logistical aspects within the Department of Biochemistry.

IDENTIFICATION OF PLANT GENES INVOLVED IN INTERACTIONS BETWEEN THE BACTERIAL CANKER PATHOGEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* NV AND PLUM TREES USING DIFFERENTIAL DISPLAY

Maryke Appel[1], Sonja Vorwerk[1]*, E. Lucienne Mansvelt[2] and Dirk U. Bellstedt[1]

[1]*Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, SOUTH AFRICA*

[2]*ARC-Fruit, Wine and Vine Research Institute, Private Bag X5013, Stellenbosch 7599, SOUTH AFRICA*

* Present address: *Department of Plant Breeding and Yield Physiology, Max Planck Institute for Plant Breeding, Carl-von-Linnè Weg 10, Cologne 50829, GERMANY*

ABSTRACT

To identify genes involved in the interaction between the bacterial canker pathogen *Pseudomonas syringae* pv. *syringae* and stone fruit trees, actively growing shoot tips of two *Prunus salicina* cultivars, cv. 'Laetitia' (moderately resistant) and cv. 'Songold' (highly susceptible) were infiltrated with either (i) live *P. s.* pv. *syringae* NV bacteria, (ii) recombinant harpin$_{PssNV}$ protein, or (iii) MES buffer (wounding control). RNA was isolated from these, as well as from untreated shoot tips, 24 hours later for comparative mRNA analysis using the differential display (DD) technique.

Fifteen combinations of arbitrary and anchored primers were used to generate DD profiles. Eleven candidate bands were selected, of which eight were successfully re-amplified, cloned and sequenced. A semi-quantitative reverse transcription polymerase chain reaction (RT PCR) strategy was employed to verify the expression patterns of these differentially amplified cDNA fragments. Two of the candidate bands (DDc and DD4) appeared to represent mRNAs that are differentially expressed between cultivars and/or treatments at the 24 hour time point, whereas no differences in the expression levels of the other six candidate bands (DDa, DDe, DD3, DD5, DD6 and DD7) could be detected.

BLAST searches of sequence databases yielded significant matches with plant defense-related mRNAs for DDe, DD4 and DD7. Matches for DD4, which appeared to represent an mRNA that is more strongly down-regulated as a result of these three treatments in cv. 'Laetitia' than in cv. 'Songold', was obtained with sequences of the *Arabidopsis* disease resistance gene *RPM1* and a

mammalian inhibitor of apoptosis protein (IAP). This suggests that hypersensitive cell death may play an important role in the defense of *Prunus* spp. against the bacterial canker pathogen.

Key words:

Apoptosis, bacterial canker of stone fruit, differential display, hypersensitive response, phenylpropanoid pathway, plant defense responses, *Pseudomonas syringae* pv. *syringae*.

Abbreviations:

4CL = 4-coumarate:CoA ligase, **ACC** = 1-aminocyclopropane-1-carboxylate, ***Avr*** = avirulence (gene), **BSA** = bovine serum albumin, **cfu** = colony forming units, **cv.** = cultivar, **DD** = differential display, **DEPC** = diethylpyrocarbonate, **DTT** = dithiotreitol, **E-value** = expect value, **HR** = hypersensitive response, **IAP** = inhibitor of apoptosis protein, **MES** = 2-N-morpholino-ethanesulphonic acid, **MOPS** = 3-(N-morpholino) propanesulphonic acid, **NCBI** = National Center for Biotechnology Information, **PAL** = phenylalanine ammonia lyase, **PAGE** = polyacrylamide gel electrophoresis, **PCD** = programmed cell death, **PCR** = polymerase chain reaction, **PMSF** = phenylmethylsulphonylfluoride, **PR** = pathogenesis-related, **pv.** = pathovar, **PVP** = poly(1-vinyl-2-pyrrolidone), ***R*** = resistance (gene), **RIP** = ribosome-inactivating protein, **RT** = reverse transcription, **RT PCR** = reverse transcription polymerase chain reaction, **RuBisCO** = ribulose-1,5-bisphosphate carboxylase/oxygenase, **SAR** = systemic acquired resistance, **TAIR** = The *Arabidopsis* Information Resource, $\mathbf{T_m}$ = melting temperature, **TMV** = tobacco mosaic virus, **UTR** = untranslated region.

INTRODUCTION

Bacterial canker of stone fruit is an economically important crop disease in temperate and Mediterranean fruit-producing countries of the world. In South Africa this disease appears to be more destructive than elsewhere. Reasons for this are obscure, but a number of predisposing factors such as climatic and soil conditions and poor horticultural practices have been implicated. Chemical control of the disease has failed completely in this country, and the breeding of resistant host trees has been identified as a priority in the effective, long-term management of the disease (Hattingh et al. 1989).

Pseudomonas syringae pathovar *syringae* is the major causative agent of bacterial canker in South Africa. It is a weak, but opportunistic, systemic plant pathogen with a complex life cycle characterized by an epiphytic phase on non-hosts or symptomless host plants (Hattingh et al. 1989). A large number of *P. s.* pv. *syringae* strains have been isolated from fruit trees in this country and have been characterized with regard to their cultural, biochemical, nutritional and physiological features (Roos and Hattingh 1987). South African commercial cultivars of *Prunus persica* (peach), *P. persica* var. *nectarina* (nectarine), *P. salicina* (Japanese plum), *P. domestica* (French plum), *P. armeniaca* (apricot), *P. avium* (sweet cherry) and *P. amygdalus* (almond) have been shown to be susceptible to the pathogen, with the exception of *P. salicina* cv. 'Laetitia' which has demonstrated an appreciable degree of resistance against local *P. s.* pv. *syringae* strains (Hattingh et al. 1989). The molecular basis of interactions between *P. s.* pv. *syringae* and any of these stone fruit cultivars has, however, not been investigated to date.

The ability of *P. syringae* and other Gram-negative phytopathogenic bacteria from the genera *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia* to cause disease in hosts and a hypersensitive response (HR) in non-hosts or resistant hosts has a common molecular basis and is controlled by *hrp* genes (Lindgren et al. 1986, Lindgren 1997). *Hrp* genes are clustered in pathogenicity islands and encode the components of a conserved type III protein secretion system, regulatory proteins and secreted proteins (Collmer 1998, Alfano et al. 2000). It is proposed that the Hrp type III secretion system is capable of delivering many, diverse products of bacterial avirulence (*avr*) genes, which are the primary effectors of pathogenesis, into plant cells (Alfano and Collmer 1996, Collmer 1998).

Plants possess a number of constitutive "barriers" against pathogen attack, but respond mainly through the local and systemic activation of an array of inducible defense mechanisms. These include the production of active oxygen species, cell wall reinforcement (through the deposition of callose and lignin and the cross-linking of cell wall proteins), the production of low molecular weight antimicrobial compounds (phytoalexins), the expression of pathogenesis-related (PR) proteins, and the HR (which is regarded as a form of programmed cell death) (Hammond-Kosack and Jones 1996, Heath 2000). All plants are genetically programmed for disease resistance, and the outcome of an interaction between a particular plant and a potential pathogen depends on whether the plant is able to recognize the pathogen as "non-self" and mobilize its defenses to counteract the pathogen's advances. The outcome of a plant-pathogen interaction (i.e. resistance or disease development) does not only depend on the spatial co-ordination and magnitude of defense-related gene expression, but also on the speed at which the expression of these genes can be induced (Dean

and Kuc 1985, Baron and Zambryski 1995). Different defense-related genes display different temporal expression patterns which may be correlated with their various functions in the overall defense strategy (Lamb et al. 1989, Esnault et al. 1993, Kombrink et al. 1993).

Recognition of attackers depends on the plant's perception of pathogen-derived exogenous elicitors (Ebel and Cosio 1994). "Pathogen-specific" (or "race-specific") elicitors are proposed to be the products of "maladapted" *avr* genes acquired through horizontal transfer between co-evolving bacterial populations. Incompatibility results when such an *avr* gene product is recognized by a receptor encoded by one of a variety of disease resistance (*R*) genes carried by plants (De Wit 1997, Vivian and Gibbon 1997). "Non-specific" or general elicitors elicit plant defenses independent of *avr-R* gene interaction. Only two such elicitors from *bacterial* pathogens have been identified thus far: flagellin (a component of the eubacterial flagellum; Felix et al. 1999) and harpins. Harpins are heat-stable, glycine-rich, *hrp* encoded proteins that lack cysteine and are secreted via the Hrp type III secretion system. They contribute to the pathogenicity and HR-eliciting ability of Hrp^+ bacteria in variable ways, and elicit the HR and systemic acquired resistance (SAR) in plants when injected in their purified form. Despite the fact that harpins have been associated with the induction of several plant-defense responses, their biological functions in these processes are still unknown (Alfano and Collmer 1996, 1997; Collmer 1998).

We have recently cloned the harpin encoding gene (*hrpZ*) of one of the most virulent South African strains of the bacterial canker pathogen, *P. s.* pv. *syringae* NV, and have expressed it in *E. coli* for the production of biologically-active, recombinant $harpin_{PssNV}$ that may be used as a tool in molecular investigations of *P. s.* pv. *syringae*-stone fruit interactions (Appel et al. 1999b). In this study we have compared the induction of gene expression by *P. s.* pv. *syringae* NV and $harpin_{PssNV}$ in interactions with the moderately resistant *P. salicina* cv. 'Laetitia' and the highly susceptible *P. salicina* cv. 'Songold'. The differential display (DD) technique was chosen for this comparative mRNA analysis, as it is based on technologies accessible to us and is capable of comparing multiple conditions simultaneously (Liang and Pardee 1992, 1995). This has allowed us to identify a number of differentially amplified cDNA fragments that may represent plant genes involved in the interaction between *P. s.* pv. *syringae* and plum trees.

MATERIALS AND METHODS

Maintenance of plants

Tobacco plants (*Nicotiana tabacum* cv. 'White Burley') were grown and maintained in a greenhouse at an average temperature of 23°C and relative humidity of 70-80%. Plants of uniform height (15-20 cm) were used for HR tests. *Prunus salicina* cultivar 'Laetitia' and cv. 'Songold' trees were obtained from a commercial nursery. They were maintained in sandy soil and fed with Hoagland's solution once per week. Two year old trees were used for DD experiments.

Cultivation of *P. syringae* pv. *syringae* NV, harpin preparation and plant infiltration

Pseudomonas syringae pathovar *syringae* strain NV (Roos and Hattingh 1987) was routinely maintained on King's medium B (King et al. 1954) at 27°C. For HR testing in tobacco (to confirm the pathogenicity of the culture) and infiltration of plum trees (for RNA isolation), small quantities of plated culture were suspended in double distilled water until the density of the suspension visually approached that of a reference suspension containing 10^8 to 10^9 colony forming units (cfu)/mL. The actual concentrations of inocula were determined after infiltration by dilution plating on King's medium B, as described previously (Appel 1996).

Recombinant $harpin_{PssNV}$ was purified from *Escherichia coli* TB1 (pMNV1) as described previously (Appel et al. 1999b) and stored at -70°C. Before infiltration of tobacco (to confirm the HR eliciting ability of the preparation) and plum trees (for RNA isolation), the concentration of recombinant protein (in 10 mM 2-N-morpholino-ethanesulphonic acid [MES] buffer, pH 5.5 + 0.1 mM phenylmethylsulphonylfluoride [PMSF]) was determined to be 0.34 mg/mL (= 9.7 µM) by the Bradford method, using bovine serum albumin (BSA) as standard protein (Bradford 1976).

Actively growing plum shoots were infiltrated with either the abovementioned recombinant $harpin_{PssNV}$ preparation or *P. s.* pv. *syringae* NV (2 x 10^8 cfu/mL). A hypodermic syringe fitted with a 25-gauge needle was used to inject 250 µL test solution into the shoot, just below the growth tip. Droplets forming on the shoot at the infiltration site were allowed to be absorbed into the tissue. A third infiltration was done with MES buffer as a wounding control (10 mM MES, pH 5.5, supplemented with 0.1 mM PMSF; He et al. 1993, Appel 1996). Three shoots on the same tree were used for the three different treatments, while a fourth shoot was tagged as an "untreated" control. Treatments were performed in duplicate on two different trees of each cultivar.

RNA isolation, evaluation and treatment

Twenty-four hours after treatment, shoot tips were excised 0.5 cm below the infiltration site, collected in pre-weighed foil bags and snap-frozen in liquid nitrogen. "Untreated" shoots were excised at similar positions, in order to yield samples of comparative mass. Frozen tissue was kept at -70°C until use.

Total RNA was isolated according to the guanidinium/CsCl method described in Ausubel et al. (1995), with a number of modifications. Briefly, samples were taken from -70°C and kept in liquid nitrogen. Each sample was weighed quickly and transferred from the foil bag to a mortar (precooled overnight at -70°C) filled with liquid nitrogen. The sample was ground to a fine powder (using a precooled pestle) and mixed with a spatula tip of poly(1-vinyl-2-pyrrolidone) (PVP, mol. wt. 25 000 to 30 000; Merck, Darmstadt, Germany). The powder was mixed with 15 mL guanidinium solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% m/v sarkosyl [sodium lauroyl sarcosinate], 0.1 M 2-mercapto-ethanol) and allowed to thaw at room temperature. The resulting suspensions were transferred to 50 mL centrifuge tubes and centrifuged for 30 min at 17 400 x *g* at room temperature. Each clear supernatant was transferred to a polyallomer, bell-top Quick-Seal ultracentrifuge tube (25 x 64 mm; Beckmann Instruments, Palo Alto, CA) containing 9 mL CsCl (5.7 M in 0.1 M EDTA). Tubes were balanced with guanidinium solution, sealed and centrifuged in a swinging bucket rotor (SW 25.1; Beckmann Instruments) at 25 000 rpm (90 200 x *g*) for 25 hours. After ultracentrifugation, the content of each tube was carefully aspirated through a hole cut into the top. The RNA pellet in the bottom of each tube was recovered by suspension in 400 µL TES solution (10 mM Tris HCl pH 7.4, 5 mM EDTA, 1% m/v SDS). This was followed by extraction with phenol:choloroform:isoamylalcohol (25:24:1, pH of phenolic phase = 6.6; Sigma, St. Louis, MO) and precipitation of RNA (overnight at -20°C) with 0.1 volume sodium acetate (3 M, pH 3.2) and 2.5 volumes ethanol (100%). RNA pellets were recovered by centrifugation (13 000 x *g* for 15 min at 4°C) washed in 70% ethanol and dissolved in double distilled water treated with diethylpyrocarbonate (DEPC; Sigma).

For all subsequent analyses, RNA was treated with DNase I to remove any contaminating DNA. Treatments were performed with the MessageClean kit (GenHunter Corporation, Nashville, TN), essentially as prescribed by the manufacturer. To increase yields of treated RNA, 200 µL DEPC-treated water was added to each 56.7 µL sample before extraction with 250 µL phenol:chloroform:isoamylaclohol (the same solution used during RNA isolation, instead of the

recommended phenol:chloroform 3:1). DNA-free RNA was precipitated and resuspended as described above.

RNA samples were diluted in DEPC-treated water and absorbances read at 230, 260 and 280 nm to determine their concentration and purity. To check the integrity of the rRNA bands, an aliquot of each sample was mixed with 0.25 volumes 5 x RNA loading buffer (Qiagen GmbH 1999), incubated at 65°C for 15 min and cooled on ice for 10 min. After the addition of 1 µL ethidium bromide (5 mg/mL) to each sample, electrophoresis was performed in a 1.5% agarose gel (containing 1.8% v/v formaldehyde) in a 3-(N-morpholino) propanesulphonic acid (MOPS) buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), at 8 V/cm.

RNAs were stored as aqueous suspensions at -20°C for frequent use, or mixed with 0.1 volume sodium acetate (3 M, pH 3.2) and 2.5 volumes ethanol (100%) to be stored for long periods at -70°C.

Differential display reactions and analysis of reaction products

Reverse transcription (RT) and differential display (DD) polymerase chain reactions (PCRs) were performed with the RNAimage system (GenHunter), according to the manufacturer's instructions, using Taq DNA polymerase (5 U/µL; Qiagen, Hilden, Germany) and α-[^{35}S]-dATP (1000 Ci/mmol; New England Nuclear, Zaventem, Belgium).

For each batch of RT reactions, a fresh dilution (0.1 µg/µL) of each total RNA sample was used. Three RT reactions, each containing a different one-base anchored primer (H-T_{11}M, where M = A, C or G), were performed for each of the eight RNA samples. RT products were used immediately for DD PCRs, or stored at -20 °C for later use.

Two series of DD PCRs were performed. In the first series, anchored primers used for RT were not included in the DD PCR and priming was done in both directions with a single arbitrary primer (analogous to the strategy described in Haag and Raman 1994). In the second series, the anchored primer used in the RT reaction was combined with an arbitrary primer according to the manufacturer's protocol. Primer combinations used in the two series of reactions are given in Table 1. All DD PCRs were performed in duplicate in thin-walled, flat-capped 0.2 mL PCR tubes (Quality Scientific Plastics/Porex, Petaluma, CA) in a PCR Express thermocycler (Hybaid, Ashford, Middlesex, UK). To minimize contamination from ^{35}S-labelled gases which may be released during

the PCRs (Clinton and Scougall 1995), reactions were overlaid with mineral oil and thermocycling was carried out in a vented fume hood. PCR products were stored at -20 °C for analysis.

DD PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) in 6% "sequencing" gels, as described in Ausubel et al. 1995. During the preparation of gels, one glass plate of each set was treated with "bind-silane" (1% v/v γ-methacryloxypropyltrimethoxy silane [Sigma] in 80% v/v ethanol, 1.67% v/v acetic acid) to bind the polyacrylamide gel after electrophoresis, whereas the opposite plate was treated with Spray-and-Cook® (Colman Foods, N'dabeni, South Africa) to release the gel. Spacers and combs were 0.4 mm thick, and flat-bottomed, well-forming combs were used as recommended by Galindo et al. (1997). Samples were prepared for loading according to the RNAimage kit instructions. Duplicate DD PCR products were always loaded in adjacent lanes to facilitate the identification of candidate bands. To assess the size of DD fragments after autoradiography (see below), two 5 μL aliquots of 100 bp DNA ladder (Promega, Madison, WI; diluted 1:20 in double distilled water), were prepared in the same way as the DD PCR products, and loaded in the outside lanes of each gel. Electrophoresis was stopped when the slower (xylene cyanol) dye front was about 5 cm from the bottom of the gel, which allowed fragments of ±100 bp to reach the bottom of the gel.

After electrophoresis, glass plates were allowed to cool down (to <35°C) and separated. The gel was soaked in 5% v/v methanol, 5% v/v acetic acid for 20 min at room temperature (with occasional agitation) to remove urea, and dried on the silane-treated glass plate in an incubator (≥90 min at 60°C). The gel was then covered with a sheet of Kodak BioMax MR film (Amersham, Little Chalfont, Buckinghamshire, UK), sealed in a light-tight container and autoradiography was allowed to proceed at room temperature for 30-65 hours (depending on the age of the ^{35}S-dATP used in the PCR). Autoradiographs were developed according to the manufacturer's instructions, using Chronex Premix Developer and Fixer solutions (Protea Medical Services, Midrand, South Africa).

Excision, re-amplification and cloning of candidate bands

Autoradiographs were inspected and the positions of candidate bands were marked. DD PCR products containing those candidate bands selected for further manipulations were re-electrophoresed in duplicate on a single gel. To facilitate the identification of the candidate bands after electrophoresis, selected RNA conditions in which these bands were absent were also loaded on the gel. Electrophoresis and autoradiography were performed as described previously. After the autoradiograph was developed and candidate bands were marked, the gel was subjected to silver

staining as described in Appel et al. (1999a). Briefly, the gel was rehydrated in distilled water (preheated to ±40°C) for 30 min. This was followed by staining in a silver solution (0.2% m/v silver nitrate, 0.056% v/v formaldehyde) for 1 hour, a brief rinse in distilled water, and developing in a cold (8-10°C) carbonate solution (3% m/v sodium carbonate, 0.056% v/v formaldehyde, 32 µM sodium thiosulphate). Developing was stopped by rinsing the gel in a cold acetic acid solution (7.5% v/v) for 30 min, after which the gel was rinsed in 3 changes of distilled water (10 min per change).

The gel was allowed to drip dry, after which it was positioned on top of its corresponding autoradiograph on a light box, taking care to align lanes perfectly. Each candidate band was excised with a 2-10 µL pipette tip, transferred to a PCR tube containing 20 µL TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and incubated at 95°C for 20 min to elute the DNA. Eluates were used immediately for re-amplification, or stored at -20°C.

For the re-amplification of excised bands, 2 µL of each DNA eluate was used as the template in a 20 µL PCR. PCR mixes and cycling parameters were identical to those used for the DD PCR, with the exception that the ^{35}S-dATP was omitted and the relevant anchored primer was included in all reactions (also for the re-amplification of bands generated in DD PCRs which did not include an anchored primer). A duplicate re-amplification reaction was performed for each band, in which the final concentration of each dNTP was increased from 2 µM to 20 µM. Re-amplification products were analyzed on silver-stained, polyester-backed 10% PAGE mini-gels as described by Bassam et al. (1991). Samples were prepared for loading and DNA molecular size fragments included in each gel as described for the analysis of DD PCR products by PAGE.

Of each successful re-amplification reaction (performed with 20 µM of each dNTP), 1 µL was ligated directly into the pGEM-T Easy vector (Promega). Recombinant plasmids were transformed into High Efficiency *E. coli* JM109 competent cells (Promega) according to the manufacturer's instructions.

Analysis of clones and sequencing of cloned DD bands

Clones from each transformation were selected randomly and plasmid DNA was isolated according to the manufacturer's instructions using the Wizard *Plus* Miniprep DNA purification system (Promega). The presence and sizes of inserts were determined by restriction digestion with *Eco*RI in SuRE cut buffer H (Roche, Mannheim, Germany), for 1 hour at 37°C. Digestion products were

mixed with 0.2 volumes of DNA loading dye (0.1% m/v bromophenol blue, 100 mM Tris HCl pH 8.0, 50 mM EDTA, 50% glycerol) and electrophoresed in 1 x TAE (40 mM Tris HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in 1.5% agarose gels containing 0.125-0.5 μg/mL ethidium bromide. The sizes of *Eco*RI fragments were estimated relative to the electrophoretic mobility of 100 bp DNA ladder (Promega) and *Hind*III digested λϕ-DNA (Roche) fragments.

Miniprep DNA of clones that showed an insert of the expected length for each particular cloned DD band was subjected to automated cycle sequencing with the ABI PRISM BigDye Terminator Ready Reaction DNA Sequencing Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer, Warrington, UK). Sequencing was performed in 20 μL reactions, each containing 2 μL M13(-21) primer (0.8 pmol/μL), 4 μL BigDye Terminator RR Mix, 10 μL 5 x dilution buffer (0.4 M Tris HCl pH 9.0, 10 mM $MgCl_2$) and 2 μL plasmid DNA (175 ng). Reactions were performed in a PCR Express thermocycler for 35 cycles of 96°C (10 sec), 50°C (15 sec), 60°C (4 min). Reaction products were sent to the University of Stellenbosch Core DNA Cycle Sequencing Facility for clean-up, and analysis on an ABI PRISM model 377 sequencer (Perkin-Elmer).

Analysis of sequence data and the design of "DD band specific" primers

Sequence data were analyzed and aligned with the programme DAPSA version 4.61 (E.H. Harley, University of Cape Town, 1999). For each of the cloned candidate bands, clones were analyzed by *Eco*RI digestion and sequenced until at least three clones with uniform insert length (corresponding to the apparent length of the particular DD fragment) and >98% nucleotide identity were found. The data from these clones were used to compile the consensus sequence for each of the cloned DD bands.

These consensus sequences were used to design a set of nested primers (internal to the sequences of the primers used for the DD PCR and re-amplification) that would allow the specific amplification of each band by RT PCR (see below). The Primer Designer software package (version 1.01; Scientific & Educational Software, 1990) was used for this purpose. Built-in criteria were adhered to as far as possible to find the primer pair that would yield the longest possible amplification product for each band (Fig. 6). Due to the high A+T-content of most of the bands, the default minimum G+C-content value had to be reduced from 50% to 30%. The default minimum melting temperature (T_m) was also reduced from 55°C to 50°C.

Verification of DD results by RT PCR

The expression patterns of differentially amplified cDNA fragments were verified in duplicate, using a semi-quantitative RT PCR protocol. The first set of PCRs was performed with cDNA templates generated by RT from 5 µg of each of the eight total RNA samples used for the DD analysis. For the duplicate set of PCRs, cDNA templates generated from only 2 µg total RNA per RT reaction was used.

cDNA synthesis was performed with Superscript II reverse transcriptase (Life Technologies, Paisley, Glasgow, UK), essentially according to the manufacturer's instructions. Briefly, each aliquot of RNA was mixed with 1 µL oligo-$(dT)_{12-18}$ primer (0.5 mg/mL; Life Technologies) and DEPC-treated water to a volume of 12.75 µL in a 0.2 mL thin-walled PCR tube. A "minus template" negative control reaction, in which RNA was replaced with an equal volume DEPC-treated water, was also prepared.

Reactions were heated at 70°C for 15 min in a PCR Express thermocycler and chilled in ice water, after which 7.25 µL of a master mix containing 4 µL 5 x First Strand buffer, 2 µL 0.1 M dithiothreitol (DTT), 0.25 µL Superscript II reverse transcriptase (all from Life Technologies) and 1 µL dNTP mix (10 mM of each dNTP; Roche) were added to each tube. Reaction tubes were returned to the thermocycler for a 50 min incubation at 42°C, followed by a 15 min incubation at 70°C to inactivate the enzyme. RT reactions were kept on ice while PCRs were set up, or stored at -20°C for later use.

PCRs were performed in 20 µL reaction volumes, containing 1 U SuperTherm DNA polymerase, 1 x reaction buffer and 1.5 mM $MgCl_2$ (all from JMR Holdings, Sidcup, Kent, UK), 0.2 mM of each dNTP (Roche), 0.5 µM each of a forward and reverse "DD band specific" primer (synthesized by the DNA synthesis laboratory, University of Cape Town, South Africa), and 2 µL of a selected RT reaction (or the "minus template" negative control) as template. Reactions were performed in a PCR Express thermocycler for 35 cycles of 45 sec at 94°C, 45 sec at 50°C and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. For internal control reactions, the "DD band specific" primers were replaced with 0.05 µM each of the primers actin-F (5'-TCACACTTTCTACA ATGAGCT) and actin-R (5'-GATATCCACATCACACTTCAT). All PCRs were performed in thin-walled, flat- capped 0.2 mL PCR tubes. To minimize variation between reactions due to pipetting, master mixes were used as far as possible.

RT PCR products were visualized by electrophoresis in 2% agarose gels in 1 x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0). A separate gel was run for the products for each "DD band specific" primer pair. For each of the eight RNA conditions, 5 μL of RT PCR product obtained with the "DD band specific" primer pair was mixed with 5 μL of RT PCR product obtained with the internal control actin primer pair and 2 μL DNA loading dye (0.1% m/v bromophenol blue, 100 mM Tris HCl pH 8.0, 50 mM EDTA, 50% glycerol) and co-electrophoresed in one lane. For optimal visualization of small bands, gels were stained after electrophoresis in an ethidium bromide solution (1 μg/mL) for 45 min. The molecular sizes of RT PCR products were estimated according to the electrophoretic mobility of 100 bp DNA ladder fragments (Promega).

BLAST searches

To ascertain whether the cloned candidate bands showed similarity to any genes of known biological function, BLAST algorithms (Altschul et al. 1990, 1997) were employed to compare each of the candidate band consensus sequences (after deletion of the sequences of the DD PCR primer sequences) with nucleotide sequences in a number of databases. The BLASTN and TBLASTX programmes provided by the National Center for Biotechnology Information (NCBI) BLAST 2.0 server were used in combination with the following databases: **nr** (all non-redundant GenBank+EMBL+DDBJ+PDB nucleotide sequences, excluding EST, STS, GSS or HTGS sequences) on the NCBI website (http://www.ncbi.nlm.nih.gov/blast); **All Higher Plant Sequences** (all Viridiplantae DNA sequences from GenBank) on The *Arabidopsis* Information Resource (TAIR) website (http://www.arabidopsis.org/blast), and the **BCM Human Transcript Database** on the Human Genome Sequencing Center, Baylor College of Medicine website (http://www.hgsc.bcm.tmc.edu/SearchLauncher). Gapped alignments and filtering for low complexity regions and repeats were allowed. Default search settings were used throughout. The default Expect (E) value threshold was 10 for all searches (i.e. only those matches for which 10 or less hits with scores equal to or better than the defined alignment score were expected to occur by chance in a database of similar size, were retrieved). Matches with E-values of 0.1 and lower were regarded to reflect true (statistically significant) sequence similarity (as recommended in the BLAST tutorial on the NCBI website). Nevertheless, all matches with $E \leq 10$ were evaluated.

RESULTS

RNA isolation

Intact RNA was consistently isolated with the guanidinium/CsCl ultracentrifugation method (Fig. 1). Based on A_{260} = 1.0 for 40 µg/mL RNA (Jones et al. 1994), yields of total RNA were calculated to range between 0.02 and 0.1% (m/m) of the mass of the plant material.

A_{260}/A_{280} ratios were constantly lower than expected (1.5 for all samples, instead of 1.7 to 2.0 according to Ausubel et al. 1995). The ratios for DNAse I treated RNAs were higher, typically ranging from 1.6 to 1.7. Increases were attributed to the removal of protein contaminants during the phenol extraction step of the treatment protocol. Additional phenol extractions did, however, not result in any significant increases in A_{260}/A_{280} ratios. Lower than typical A_{260}/A_{280} ratios are not uncommon for guanidinium-based RNA extraction protocols (Yamaguchi et al. 1992) and did not appear to affect the efficiency of subsequent RT and DD reactions. A_{260}/A_{230} ratios were consistently >2.0, indicating that the isolated RNAs were free from carbohydrate contamination (Jones et al. 1994).

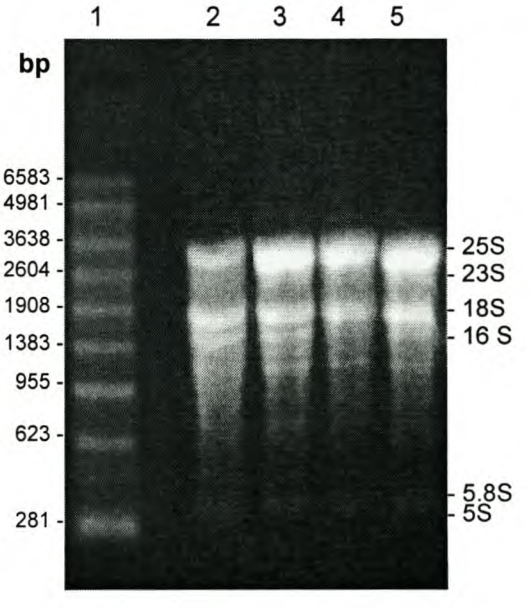


Fig. 1. Formaldehyde-agarose electrophoresis of total RNA isolated from *Prunus salicina* cultivars.

Lanes 1: RNA markers (Promega); ***2:*** 'Laetitia' untreated control; ***3:*** 'Laetitia' wounding control; ***4:*** 'Songold' harpin treatment; ***5:*** 'Songold' *P. s.* pv. *syringae* NV treatment.

RNA was isolated as described in Materials and Methods. Of each of the selected samples, 10 µg was electrophoresed as described. Similar results were obtained for the other four samples used in this study.

The positions of 25S, 18S, 5.8S and 5S cytoplasmic ribosomal RNA bands, as well as the 23S and 16S chloroplast rRNA bands (Anderson and Beardall 1991) are indicated on the right.

The sizes of RNA marker fragments (total mass per lane = 3 µg) are given on the left.

Differential display profiles and the selection of candidate bands

Fifteen primer combinations (i.e. combinations of an arbitrary DD PCR primer and an anchored RT primer for cDNA synthesis) were used to generate differential display profiles in this study (see Table 1). Three subpopulations of cDNAs were generated for each of the eight RNA conditions (four treatments of two cultivars), using the three one-base anchored primers H-T_{11}M (where M = A, C or G) supplied in the RNAimage system (Liang et al. 1994). To avoid redundancy in the cloning of candidate bands (Galindo et al. 1997), all three of these cDNA subpopulations were not always used as DD PCR templates in combination with a particular arbitrary primer. Arbitrary primers were chosen randomly from four available RNAimage primer sets.

A typical set of DD profiles is shown in Fig. 2. All fifteen primer combinations yielded complex profiles consisting of 130-210 bands per lane. The size-distribution of amplified fragments (typically <100 to ±800 bp, but up to and exceeding 1500 bp in a few cases) indicated that isolated RNAs were of a high quality (GenHunter Corporation 1997). An average of 90 to 98% reproducibility between bands in duplicate samples was obtained for all primer combinations, except for 55C (arbitrary primer H-AP**55**, combined with cDNA synthesized with anchored primer H-T_{11}**C**). For reasons that were unclear, <70% reproducibility between duplicate DD PCRs was obtained with this primer combination for all eight RNA conditions. This rendered the 55C profile set unsuitable for candidate band selection. The results obtained with each of the fifteen primer combinations are summarized in Table 1.

Very strict criteria were applied in the definition and identification of "candidate bands" for further analysis. In a complex sample set such as the one used in this study, it seemed more appropriate to first identify the "variable positions" in each set of DD profiles. Variation in the intensity of a band across all sixteen lanes generated with a particular primer combination could be attributed to a number of possible reasons. Variation that seemed to be experimentally-induced (e.g. the result of non-reproducible amplification (see Appel et al. 1999a), differences in PCR template concentration or uneven loading of PCR products on the gel) was excluded from further consideration, leaving a total of 439 variable positions in the 14 useful sets of DD profiles.

In experiments such as these where plants are inoculated with a bacterium, contamination of the isolated plant RNA with bacterial RNA may be expected. Bacterial RNA is, however, not polyadenylated, and should therefore not be amplified reproducibly using the GenHunter DD system. Only 7 of the 439 variable positions (1.6%) showed the induction of a band specifically in

Table 1. Summary of differential display results.

Arbitrary primer used in PCR		Anchored primer used for RT ($H\text{-}T_{11}M$)[b,c]	Average no. of bands per lane[d]	No. of variable positions	Number of candidate bands					Bands verified by RT PCR
H-AP #[a,c]	5'→3' sequence				In total[e]	Selected for further analysis	Re-amplified successfully	Cloned	Consensus sequence determined	
SERIES 1:										
29	AAGCTT AGCAGCA	G	169	20	12	0	-	-	-	-
31	AAGCTT GGTGAAC	A	209	43	22	0	-	-	-	-
32	AAGCTT CCTGCAA	C	187	23	10	0	-	-	-	-
50	AAGCTT TGAGACT	C	132	45	13	2	2 (+1)[f]	2 (+1)[f]	2 (+1)[f]	DDa, DDc (+ DDe)[f]
51	AAGCTT CGAAATG	C	132	20	11	0	-	-	-	-
53	AAGCTT CCTCTAT	A	149	32	25	0	-	-	-	-
55	AAGCTT ACGTTAG	C	133	ND[g]						
66	AAGCTT GCCTTTA	G	182	24	14	2	-	-	-	-
68	AAGCTT CTTTGGT	G	163	25	11	0	0	0	0	-
71	AAGCTT GTAGTAA	A	153	30	13	0	-	-	-	-
SERIES 2:										
7	AAGCTT AACGAGC	C	170	30	17	3	2	2	2	DD3, DD4
27	AAGCTT CTGCTGG	A	172	20	7	0	-	-	-	-
65	AAGCTT CAAGACC	A	191	54	20	1	1	1	1	DD7
65	"	C	179	35	17	2	1	1	1	DD6
65	"	G	131	38	9	1	1	1	1	DD5
		TOTAL	**(164)**	**439**	**201**	**11**	**7 (+1)[f]**	**7 (+1)[f]**	**7 (+1)[f]**	

[a] As supplied in the RNAimage system. H designates the 5'-terminal *Hind* III restriction site (AAGCTT) included in each of the arbitrary primer sequences.

[b] Only the anchoring base (M) is given. In all of the anchored primers this base is preceded by 5'-AAGCTT TTTTTTTTT, where AAGCTT is a *Hind* III restriction site (and H = AAGC).

[c] In series 1, only the arbitrary primer was added to DD PCR mixes. In series 2, both the arbitrary primer and the anchored primer used for cDNA synthesis were included in PCR mixes.

[d] Calculated from 4 randomly chosen lanes out of the 16 lanes (8 samples in duplicate) generated with each primer combination.

[e] Of the total amount of candidate bands (201), only 34 (17%) were strong bands that displayed prominent differences between RNA conditions.

[f] Band DDe was reproducibly re-amplified from the excised gel slice containing band DDc. Since more than one amplification product may occupy the same position on a DD gel (Bauer et al. 1993, Liang and Pardee 1995), the expression pattern of DDe was provisionally considered to be the same as that of DDc, and DDe was included in subsequent analyses.

[g] Not determined. The number of bands per lane and size-distribution of bands obtained with this primer combination were similar to results obtained with the other 14 combinations. Reproducibility between duplicate DD PCRs for all eight RNA conditions were, however, uncharacteristically poor (<70%) and rendered this set of DD profiles unsuitable for candidate band selection.

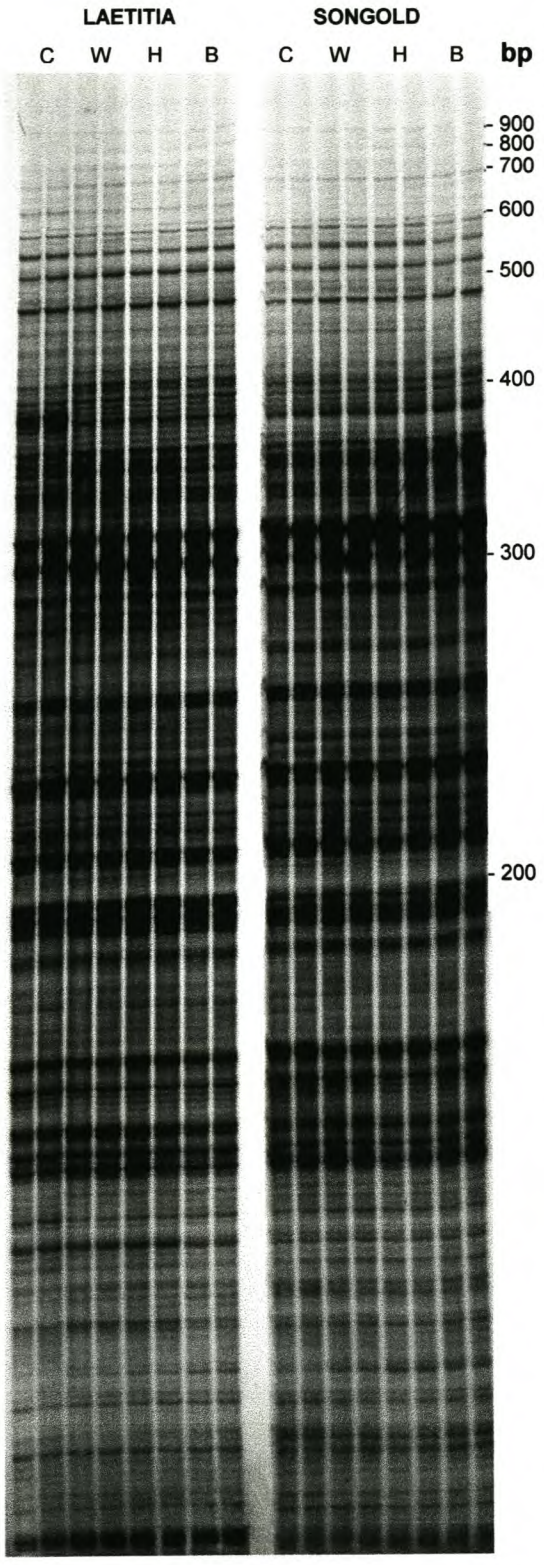


Fig. 2. Autoradiograph of a typical set of DD profiles generated from *Prunus salicina* cultivars 'Laetitia' and 'Songold' total RNA, representing four different treatments.

Treatments: ***C*** = untreated control; ***W*** = wounding control; ***H*** = treatment with recombinant harpin protein; ***B*** = treatment with live *P. s.* pv. *syringae* NV bacteria.

Profiles were generated with primer combination **66G** and electrophoresed as described in Materials and Methods. Duplicate DD PCR products for each RNA condition are loaded in adjacent lanes. The sizes of Promega 100 bp ladder fragments (given on the right) were transferred to the autoradiograph after silver staining of the gel.

bacterium-treated conditions. These positions were possible candidates for bacterial contamination and were excluded from the selection of candidate bands (as recommended in Seehaus and Tenhaken 1998).

The remaining variable positions could be divided into two categories. The first included those positions (231 of 432 = 53.5%) in which all the bands corresponding to one cultivar were of similar intensity, but different to the intensity of all the bands obtained for the other cultivar. This form of "differential expression" appeared to be the result of genotypic differences between the two cultivars and therefore not relevant to the objectives of this investigation. The remaining pool of variable positions (201 of 432 = 46.5%) consisted only of bands which were reproducibly displayed with varied intensity between different treatments of the same cultivar, the same treatment of different cultivars, or combinations of both. Bands occupying such variable positions (in one or more RNA condition) were considered to be "candidate bands".

Due to the fact that the vast majority (167 of 201 = 83%) of candidate bands were weak/faint and did not display prominent differences between RNA conditions, not all candidate bands were considered for further analysis. In the first DD series, it was decided to verify the differential expression patterns of selected candidates by repeating the DD PCRs (using templates from duplicate RT reactions). For such comparative analyses to be meaningful, all sixteen lanes had to be repeated for each primer combination that was used for the generation of a selected candidate. Duplicate DD profiles were thus generated for the four primer combinations (50C, 53A, 66G and 68G) that yielded the most prominent bands representing what was regarded to be the most relevant differential expression patterns (e.g. absent in controls, but present in harpin and/or bacterium treatments). Although this approach seemed to be useful for the early elimination of some false positives, it proved to be costly and time-consuming due to the complexity of the sample set. In the second DD series, the cloning of a larger number of candidate bands was regarded to be a more economical approach to compensate for a possible high rate of false positives.

In total, 4 candidate bands from the first DD series (for which the differential expression patterns were verified in duplicate profiles), and 7 candidate bands from the second DD series (for which this was not done), were selected for re-amplification. The various patterns of differential gene expression represented by these bands are shown in Fig. 3.

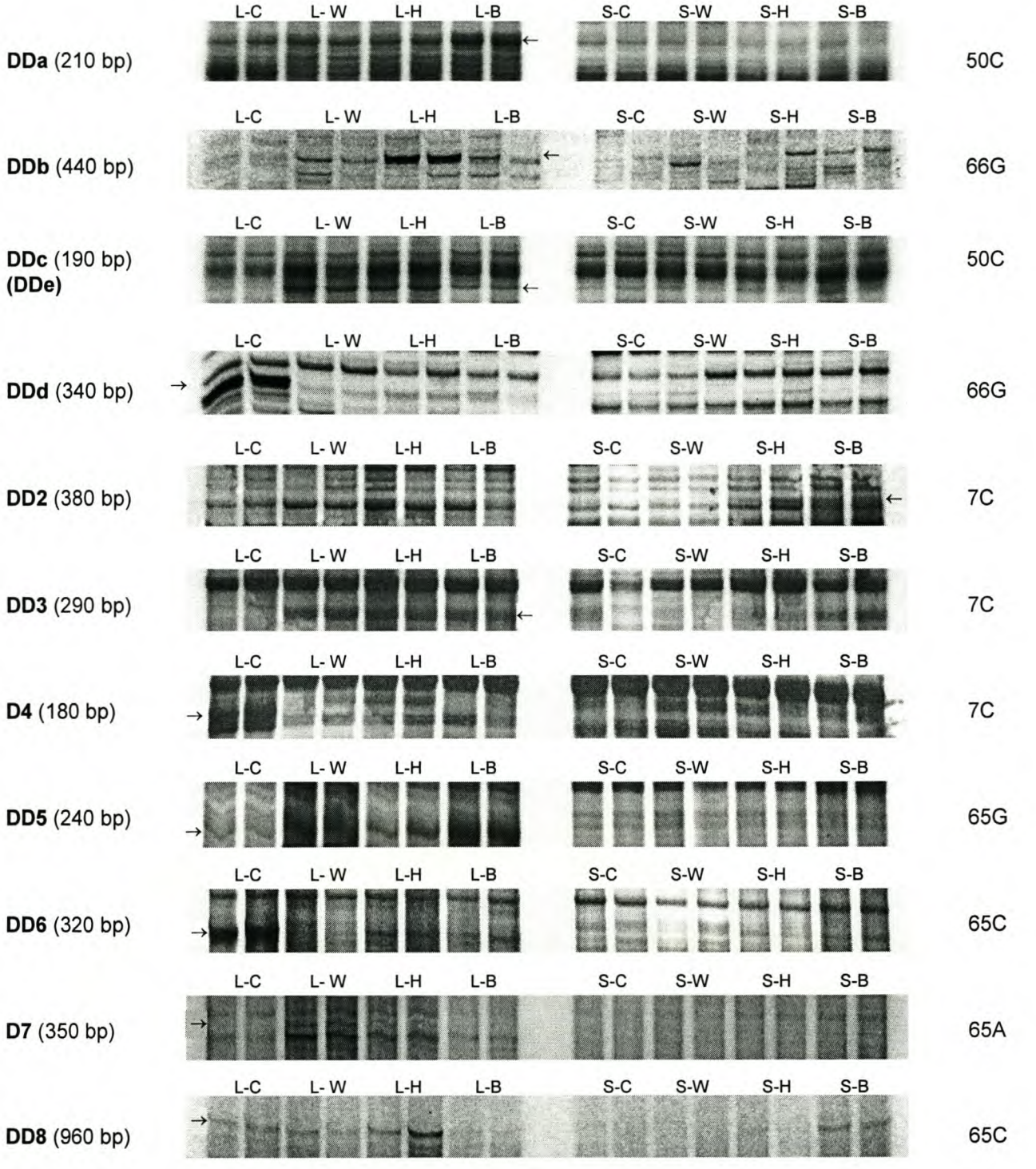


Fig. 3. Sections of DD gels showing the expression patterns of candidate bands selected for further analysis.

RNA conditions: **L-C** = 'Laetitia' untreated control; **L-W** = 'Laetitia' wounding control; **L-H** = 'Laetitia' treated with recombinant harpin; **L-B** = 'Laetitia' treated with *P. s.* pv. *syringae* NV bacteria; **S-C** = 'Songold' untreated control; **S-W** = 'Songold' wounding control; **S-H** = 'Songold' treatment with recombinant harpin; **S-B** = 'Songold' treatment with *P. s.* pv. *syringae* NV bacteria.

Duplicate DD PCR products for each RNA condition were loaded in adjacent lanes. The designation of each candidate band is given on the left, with its approximate molecular size in parenthesis. The primer combination used to generate each set of the profiles from which the candidate band was selected, is given on the right.

Candidate bands DDa, DDc, DD3 and DD8 (shown in Fig. 3) exhibited complex expression patterns (i.e. did not appear to be strongly induced in only one or two RNA conditions, but to be induced at different levels in multiple conditions). In each of these cases the candidate band was excised for re-amplification from **two** of the conditions in which it was identified. This was done to confirm that the candidate band represented the same amplification product throughout the sample set in each particular case.

Re-amplification and cloning of candidate bands

We have developed a strategy for the re-amplification and cloning of excised candidate bands which differ from approaches used in most published studies in three respects: (i) DNA is eluted from excised DD gel strips by a simple high-temperature incubation step (i.e. no electro-elution or chemical purification), (ii) re-amplification products are evaluated on silver-stained PAGE mini-gels, and (iii) re-amplification products are cloned directly into a T-A cloning vector, without isolation and/or precipitation of the band corresponding to the excised DD candidate band (Men and Gresshoff 1998, Appel et al. 1999a). The assessment of re-amplification products on silver-stained PAGE gels offers three advantages compared to the traditional use of agarose electrophoresis and ethidium bromide staining: (i) the superior sensitivity of silver staining allows the detection of re-amplification products after a single round of re-amplification, (ii) the high resolution of PAGE makes it easy to verify whether the major re-amplification product corresponds in size to the excised candidate band in each case, and (iii) "background" re-amplification products are visible on silver stained PAGE gels (but not on ethidium bromide stained agarose gels) and may be correlated with clones showing undesired insert sizes.

Another aspect of our strategy is that re-amplification reactions are performed in duplicate for all excised candidate bands. One reaction is done in the presence of 2 μM of each dNTP (which was shown to be optimal for the generation of radio-actively labelled, arbitrarily-primed DD PCR products) and the other in the presence of 20 μM of each dNTP (which is recommended for optimal specificity and yield in specifically-primed re-amplification reactions performed the absence of a radio-active label; Liang and Pardee 1992; GenHunter Corporation 1997). This approach does not only show whether an excised candidate band can be re-amplified reproducibly, but also confirms the "background" re-amplification patterns in each case. Low dNTP reaction products contain lower concentrations of "background" amplification products, which is advantageous for direct cloning.

High dNTP reaction products are, however, often preferred for cloning as they contain a higher concentration of the desired re-amplified candidate band (Fig. 4).

Candidate bands from the first DD series were initially re-amplified in the absence of the anchored primer used for RT (as this primer was also excluded from the DD PCRs). Surprisingly, none of the four bands could be re-amplified in this way, even when the amount of template (DNA eluted from excised gel strips) was increased five-fold (results not shown). When the anchored primer was included in the re-amplification mix, candidate bands DDa and DDc were re-amplified reproducibly from both gel strips excised for each of these bands (Fig. 5). This result suggested that the anchored primer carried over with cDNA from the RT reaction into the DD PCR must have participated in the generation of at least a fraction of the DD PCR products. The omission of the anchored primer in the DD PCRs thus did not seem to offer the same advantages as replacing it with a second arbitrary primer (as described by Haag and Raman 1994), but rather to complicate the DD process downstream of the PCR. As a result, the second series of DD PCRs were performed according to the original RNAimage protocol.

Re-amplification products of candidate bands DDb and DDd (generated in the presence of both the arbitrary and anchored primers) yielded different profiles for the low and high dNTP reactions. The low dNTP DDb reaction and the high dNTP DDd reaction contained bands corresponding to the sizes of the respective candidate bands. These bands were excised from the mini-gel, eluted and used as templates for duplicate re-amplification reactions as before. This yielded reproducible profiles for both dNTP concentrations, but the target bands (corresponding in size to DDb and DDd

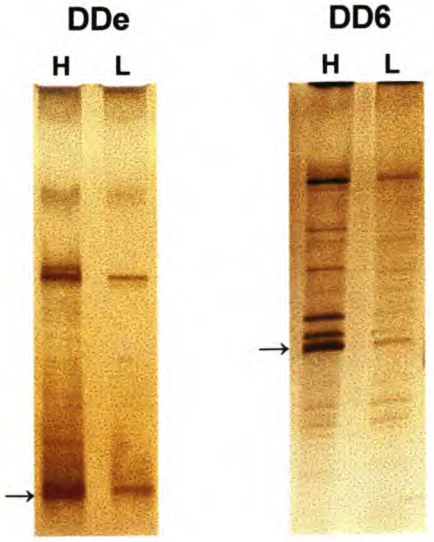


Fig. 4. Re-amplification products of two DD candidate bands, visualized by silver staining on PAGE mini-gels.

Excised candidate bands **DDe** and **DD6** were re-amplified as described in Materials and Methods. Duplicate re-amplification reactions were performed in the presence of high dNTPs (**H** = 20 μM of each dNTP) or low dNTPs (**L** = 2 μM of each dNTP).

The re-amplified candidate band is indicated with an arrow in each case. All other bands are "background" amplification products. The use of lower dNTP concentrations resulted in lower concentrations of both target (candidate) and "background" bands. In both cases, the product of the high dNTP reaction was preferred for cloning, as it contained a higher concentration of the re-amplified candidate band.

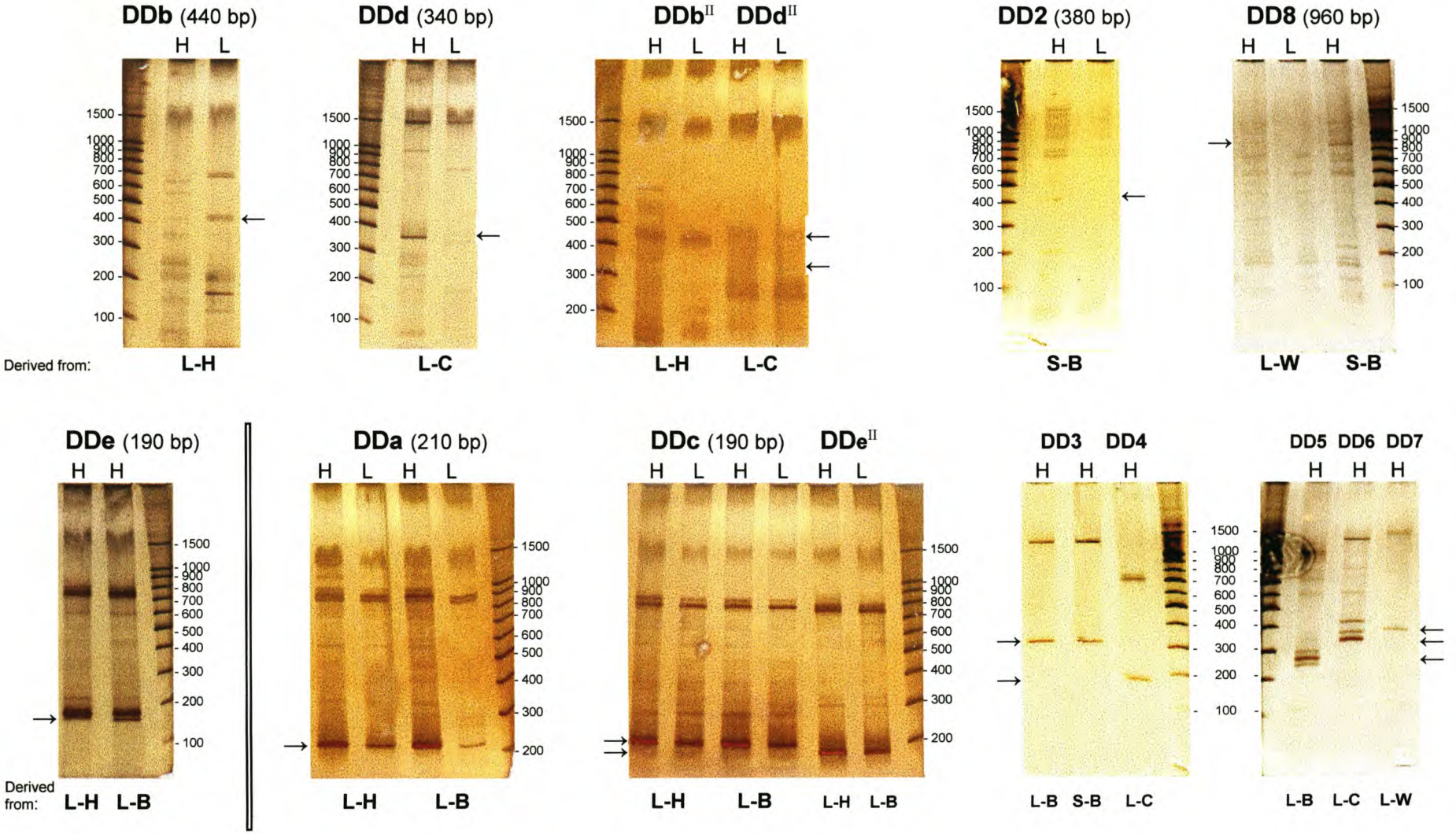


Fig. 5. Products from the re-amplification of excised DD candidate bands visualized on silver stained PAGE mini-gels. ***Top row:*** Unsuccessful (non-reproducible) re-amplifications. ***Bottom row:*** Generation of band DDe; candidate bands which were re-amplified successfully and cloned.

Re-amplifications were performed as described in Materials and Methods and Results, with high dNTPs (**H** = 20 μM each) or low dNTPs (**L** = 2 μM each). The positions of candidate bands are indicated with arrows. The RNA condition from which each candidate band was derived is indicated at the bottom of each gel (abbreviations are the same as in Fig. 3). The superscript ^{II} denotes the products of a second round of re-amplification, generated from a band excised from the first round (see relevant gels to the left) as template. The sizes of Promega 100 bp ladder fragments are given in bp. The approximate size of candidate band **DD3** = 290 bp, **DD4** = 180 bp, **DD5** = 240 bp, **DD6** = 320 bp and **DD7** = 350 bp.

respectively) were either absent or diffuse and of a low concentration and were not deemed suitable for cloning purposes (Fig. 5). Candidate bands DD2 and DD8 from the second DD series also failed to re-amplify reproducibly from gel strips originally excised from the DD gel and were subsequently excluded from further analysis. The other five candidate bands from the second DD series (DD3, DD4, DD5, DD6 and DD7) were re-amplified successfully (Fig. 5).

An interesting result from the re-amplification of candidate band DDc was that a band just smaller than the major amplification product was consistently present in the re-amplification products generated from both gel strips excised for DDc (Fig. 5). It has been demonstrated previously that more than one amplification product may occupy the same position on a DD gel (Bauer et al. 1993, Liang and Pardee 1995), and it was possible that the smaller band was only distinguishable on the 10% mini-PAGE gel due to its higher resolving power for smaller molecules (compared to that of the 6% DD gel). The expression pattern of the smaller band was provisionally considered to be the same as that of DDc. The band was excised from the silver-stained PAGE mini-gel, re-amplified independently and designated "DDe" for further analysis.

In summary, of the eleven candidate bands that were originally excised, seven (DDa, DDc, DD3, DD4, DD5, DD6 and DD7) were re-amplified successfully. Candidates DDa, DDc and DD3 were excised and re-amplified in duplicate and DDe was generated during the re-amplification process. Eleven sets of transformants were thus generated after direct ligation of the respective (high dNTP) re-amplification reaction products into the T-A cloning vector, pGEM-T Easy, and transformation of *E. coli* JM109 with the ligation reaction products. The results of transformation controls included in these experiments correlated well with the manufacturer's specifications (not shown). The number of clones obtained from each transformation was within the specified range, but the ratio of blue to white colonies obtained in all eleven transformations were higher than specified (blue:white >2:3). This was expected, since (i) re-amplification reaction products used for ligation were not homogeneous, (ii) optimal vector:insert ratios were not calculated, and (iii) many of the candidate bands (inserts) were short and probably did not interrupt the sequence of the *lacZ* gene (Promega Corporation 1996). As a result, light blue colonies were also screened for the presence of inserts corresponding to cloned candidate DD bands.

Analysis of clones and sequences of cloned candidate bands

The multiple cloning region of pGEM-T Easy is flanked by recognition sites for *Eco*RI. Provided that insert DNA does not contain *Eco*RI recognition sites, digestion of recombinant pGEM-T

plasmid DNA with this enzyme yields two fragments, one of 3.0 kb corresponding to the vector, and another of 18 bp + size of insert. Selected colonies from each set of transformants were therefore screened by inspection of *Eco*RI digestion products on ethidium bromide stained agarose gels (not shown).

Despite the fact that candidate bands were not purified from re-amplification reaction products containing significant concentrations of "background" bands prior to cloning (as shown in Fig. 5), a small percentage of the clones obtained from each transformation had to be analyzed to obtain the consensus sequence of each cloned candidate band (Table 2). Similar results were obtained before (M. Appel, unpublished results), and demonstrated the effectiveness of our cloning strategy.

The consensus sequences of the cloned candidate bands are given in Fig. 6. In each of the three "complicated" cases (DDa, DDc and DD3), where gel strips were excised and bands were successfully re-amplified from more than one RNA condition, the same sequence was derived from these "duplicate" determinations. This confirmed that the band which appeared at different intensities in multiple conditions (as shown in Fig. 3) represented the same mRNA in each case.

Table 2. Analysis of clones containing candidate DD bands.

Candidate band[a]	Number of clones				Band size (bp) (incl. DD primers)
	Analyzed	showing correct insert size (*Eco*RI digest)	Sequenced	Corresponding to consensus sequence	
DDa (1)	5	4	3	3	210
DDa (2)	5	5	4	3	210
DDc (1)	3	2	2	2	186
DDc (2)	3	3	3	2	186
DDe	17	4	3 (+1)[b]	2 (+1)[b]	185
DD3 (1)	3	3	3	3	290
DD3 (2)	3	3	3	2	290
DD4	3	3	3	3	177
DD5	6	5	4	3	237
DD6	4	4	3	3	316
DD7	8	3	3	3	351

[a] Bands DDa, DDc and DD3 were excised for further analysis from **two** of the RNA conditions from which it was generated by DD PCR (see discussion on p. 144). DDa (1) was derived from L-NV and DD2 (2) from L-H DDc (1) was derived from L-H and DDc (2) from L-NV, and DD3 (1) and DD3 (2) from S-NV and L-NV, respectively. Compare to Figs. 3 and 5 for abbreviations of conditions.

[b] One of the clones that did not show the correct insert size was also sequenced. The recombinant plasmid contained two inserts flanked by the sequences of arbitrary primer H-AP50 and anchored primer H-T_{11}C (which were used to generate band DDe, see Table 1). These inserts were ligated "tail-to-tail" into the multiple cloning region, in such a way that they shared some of the nucleotides of the anchored primer. The sequence of the longer one of the two inserts was identical to the sequence of the inserts in two of the other three DDe clones that were sequenced.

Band DDa, generated with 50C:

```
AAGCTTTGAG ACTGACTTGG GTGTATTTGG GATTTGGGTT GTGAGAGTTA AACTCTGTAA TTCCTAGGAT   70
AGGTGAGAGG GTTAATCTCC TTTGGTTGTA ATCTCTTTTG GGATACTAGT GGAATTTCTC GCTGTCTTCG  140
AACTGGACGT AGGCTACACA CGGGCCAAAC CAGTATAAAT CCTTGTGTTG TTTGGAAAAA AAAAAAGCTT  210
```

Band DDc, generated with 50C:

```
AAGCTTTGAG ACTGGGCAA TAATTGAGTG AGGATAGAGA AATTAATAGT TCATTTTTTT GGAGGATCCA   70
TATGATTCTG TAATTTCATA TGTTTTGATG TGAATCTTTT GCTTTGGGAT TTCAATTTGG CAGACTTTGT  140
GAGTGAAATA TATATTGGTT TATTCATTTG GAAAAAAAAA AAGCTT                            186
```

Band DDe, generated with 50C:

```
AAGCTTTGAG ACTGGTCAGA GTAAATTATA TGTGATCTCA ATGTACATAA ACACACTAAG GGATAGTACT   70
AGTAGACTCA GTATGAAGTT GTTCTCTTTC TTTTTCTTAA CATAAAGTGG AAAGAAACCA TGCTGCTTGA  140
CCAACTTAAT GTGATCTCCT GAATCTCTTG AAAAAAAAAA AGCTT                             185
```

Band DD3, generated with 7C:

```
AAGCTTAACG AGGCCAGTAC CACTTACCCC AATTCATTCA TCAGGATCAT CGGATTCGAC AACATCCGCC   70
AAACACAGTG CGTTAGTTTC ATCGCTTACA AGCCCCCTAG TTTCTAAGTT TTGAGGCTAC CCTAGCTATC  140
CGGCTTTACT TCAATTTCAC TTTCAATTCG TTTTGTTGCT TCCCTTATTT TCCTTTTTAC TCCGTAATTT  210
CTCTTCTTGT AAACCTCCGT TTATTCTGTG AATTGATTTA GATGTGAACA GAGATGTGTT GATCGAAAAA  280
AAAAAAGCTT                                                                   290
```

Band DD4, generated with 7C:

```
AAGCTTAACG AGGGGATCAA GTAGTGGCCG TTCATAGATT TAGAATGTAC TCTATTTTTT GCACATGTAT   70
GTTTATATGT TTCATTCGGC CAAGACCGGC CGTTCTTTTT GTAACTGCGA CCCTTATCTT TTAATCTAAA  140
TGAAGTATGA CTTTTTTCCC TGAAAAAAAA AAAGCTT                                      177
```

Band DD5, generated with 65G:

```
AAGCTTCAAG ACCCAAGCTT CAAGACCAAT GTATGTGTCC TATGTTTAGT GTCATTATAT ATAGTACAGT   70
ATAAGACATT AAGCATGATG TTGGTAGAAA ATATGATGTC GACACGTGGC AAAAAATGGC ATAAAGCCTG  140
TGAACCTATG ATCAAATTGG TGCCTGGGAT GTCAATACGT ACTTGTACCC TCAGTTTTCA TGATATATAT  210
ATTTTTTTCC CCAAAAAAAA AAAGCTT                                                 237
```

Band DD6, generated with 65C:

```
AAGCTTCAAG ACCCATCATT GCAGAGTGAA AGAGACATTT ACTTCATAGA AGAAGTCAGT ATTTACTGCC   70
AGAAGTGTTA AATTTTCCAT GTATGTTTAT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTAAAATAAT  140
ATAAGGTATA GTTGAGGAGC AAACAATAAG TTGCTATGCA CTTGTAATAT CTGATATGAA GCCAAAACTT  210
TTGCTACCCA CCTTAGGGGC CCAGGCTGCA TTATTATTCT AAATCATAAT GATCATGGTG GTCTAATCTG  280
ATAGTGAATT ATATTTCAAT GAAAAAAAAA AAGCTT                                       316
```

Band DD7, generated with 65A:

```
AAGCTTCAAG ACCGATAAGT AGCCTCAATT TGTGTCTTGC TAGTTGTTGC CAATCTGATT ATATATTTCT   70
CGTCATGGCA TGTAGGGCTG CTTCTATAAC TTGGTGGGGT GGAGGGATTG CAGCATGCCA TTGGGAGCCA  140
CAGGTTGGCG CATCGCAGTT GTTCTGAAAG GTTTAAAATG TCTTGACTTA TGCATATGTT GAATAATTTC  210
CACTCTGCTG GTTTATTTTA TTGGTTATTC TATTACTCTG AACTACTTGC CTCCATTGTT GTAATATCTG  280
GAATAAATAA ACAGGTTGGC TGTATAATTG TTATTGTATG CAGCAAGGCC TTTTGTAAAA AAAAAAGCT  350
T
```

Fig. 6. Consensus nucleotide sequences of cloned candidate DD bands.

The consensus sequence of each cloned candidate band, inclusive of the DD primer sequences, was determined as described in Materials and Methods. The sequence of the arbitrary primer used to generate a particular band is highlighted in red and the complement of the anchored primer in blue. Putative polyadenylation signals corresponding to the eukaryotic consensus sequence AAUAAA, or the signal (C/G)AUAA$_{1-3}$ often found in plants, are underlined. The regions on which primers for the verification of the expression patterns by RT PCR were based, are highlighted in yellow (forward primer) and green (reverse primer), respectively.

As expected from the results of re-amplification experiments, all of the cloned candidate bands were flanked by the sequences of the arbitrary and anchored DD primers used to generate them. The exact size of each cloned band (given in Table 2) was derived from the sequence data and correlated with the size of the candidate band estimated from the DD gel (given in Fig. 3). The sequence of band DDe, which was derived from the excised DDc gel strip during re-amplification, proved to be completely different to and one nucleotide shorter than that of DDc.

Because the cloned candidate bands theoretically represent the 3'-ends of polyadenylated mRNAs, sequences were inspected for the presence of putative polyadenylation signals. The signal AAUAAA, which is found 10-35 bp upstream of the site of poly(A) addition and is highly conserved and essential for polyadenylation in the majority of eukaryotic (especially mammalian) genes, was identified in only one of the candidate band sequences (DD7, 45 bp upstream from the poly(A) tail). In other studies, a perfect copy of this signal was found in less than half of plants examined, and at variable distances from the poly(A) addition site, which suggested that polyadenylation in plants may depend on a different mechanism than that in animals (Wahle and Keller 1992). One or more copies of the signal (C/G)AUAA$_{1-3}$, which is often found in plants (Anderson and Beardall 1991), was identified at variable distances from the poly(A) tail in bands DDe and DD5 (Fig. 6).

Verification of the differential expression of cloned candidate bands by RT PCR

An essential part of any DD experiment is to verify the expression patterns of candidate bands using original RNA samples, to determine whether these bands truly represent mRNAs which are differentially expressed in the experimental setup. Standard or reverse Northern blots are generally used for this purpose (Liang and Pardee 1995). A semi-quantitative RT PCR verification strategy was, however, preferred in this study for the following reasons: (i) reverse Northern blots would be impractical, as the number of cloned bands equalled the number of RNA conditions, (ii) standard Northern blots would require more RNA than what was available, even if membranes were stripped and reprobed a number of times, and (iii) all of the candidate bands cloned in this study were short (≤351 bp) and AT-rich (57 to 70% A+T content). Probes generated from such bands are very likely to fail in the detection of their corresponding mRNAs in Northern hybridizations, especially if these mRNAs are of low abundance and total RNA is blotted (Liang et al. 1992, 1993).

Primer pairs that would specifically amplify each of the cloned bands (using cDNA generated with a poly(dT) primer from the total RNA isolates analyzed by DD as templates) were subsequently

designed (see Table 3 for sequences). To achieve optimal specificity for each of the cloned bands, these primers were based on regions of candidate band sequences internal to the sequences of the flanking DD primers (see Fig. 6). Each of the primer pairs yielded a single amplification product from a cDNA expected to contain the candidate band in each case (selected from the original DD profiles). All of these amplification products exhibited the expected size of the corresponding cloned band (given in Table 3; result not shown). A primer pair that amplifies a 0.6 kb plant actin fragment was used to generate an internal RT PCR control (in a similar fashion as the *Arabidopsis* actin-specific primers used by Penninckx et al. 1996, Gómez-Gómez et al. 1999).

RT PCR verification results for each of the cloned candidate bands are given in Fig. 7. For half of the eight candidate bands (DDa, DDe, DD3 and DD7) an amplification product of similar intensity was obtained for each of the eight RNA conditions. This implied that these bands either (i) did not represent differentially expressed mRNAs (i.e. were "false positives"), or (ii) that the 35-cycle RT PCR protocol was not capable of differentiating the expression levels of mRNAs represented by these bands due to the fact that amplification plateaus for these templates had been reached (see Gause and Adamovicz 1995).

The DD5 and DD6 specific primer pairs yielded a single amplification product of similar intensity for all RNA isolates from cultivar 'Laetitia', and two bands (both of equal intensity in all four RNA

Table 3. "DD band specific" primers used in the RT PCR verification of cloned candidate bands.

Band to be amplified	Forward primer (5'→3')	Reverse primer (5'→3')	Expected size of amplification product (bp)
DDa	GGGTGTATTTGGGATTTGGG	TTATACTGGTTTGGCCCGTG	160
DDc	GGCAATAATTGAGTGAGGAT	TCACAAAGTCTGCCAAATTG	127
DDe	GGTCAGAGTAAATTATATGT	ATTCAGGAGATCACATTAAG	151
DD3	ACTTACCCCAATTCATTCAT	TCAATTCACAGAATAAACGG	225
DD4	AAGTAGTGGCCGTTCATAGA	AGATAAGGGTCGCAGTTACA	111
DD5	CTTCAAGACCAATGTATGTG	ATGAAAACTGAGGGTACAAG	184
DD6	AGAAGTCAGTATTTACTGCC	CACTATCAGATTAGACCACC	236
DD7	TGTGTCTTGCTAGTTGTTGC	CCTTGCTGCATACAATAACA	298
Actin control	TCACACTTTCTACAATGAGCT	GATATCCACATCACACTTCAT	607

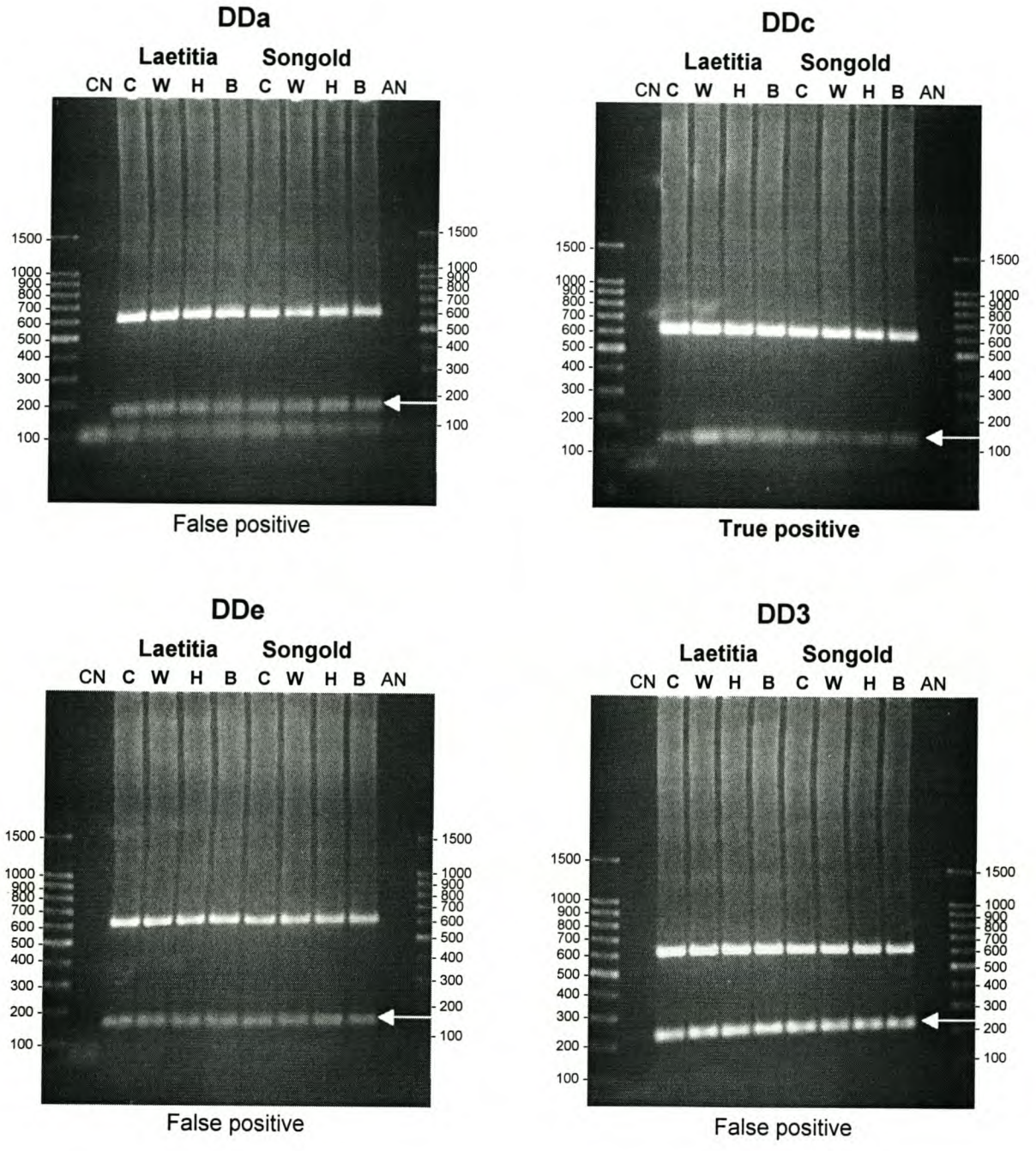


Fig. 7. Agarose gel electrophoresis of RT PCR products generated with DD candidate band specific primers *(continued on next page)*.

RNA conditions: **C** = untreated control; **W** = wounding control; **H** = treatment with recombinant harpin; **B** = treatment with *P. s.* pv. *syringae* NV bacteria.

Amplification products were generated and analyzed as described in Materials and Methods. Amplification products corresponding to each of the cloned candidate bands are indicated with arrows. The larger (0.6 kb) set of bands on each gel comprises the actin fragments amplified from each RNA condition as an internal control. Lanes **CN** and **AN** on each gel represent "minus template" negative RT PCR controls. These controls were generated by using one-tenth of an RT reaction containing DEPC-treated water instead of RNA as the PCR template, with either the relevant "DD band specific" primer pair (**CN**) or the actin specific primer pair (**AN**). The sizes of 100 bp DNA ladder fragments are given in bp.

The results shown here were generated with 2 μg of each total RNA sample in the RT step. In duplicate experiments using 5 μg of total RNA per RT reaction, higher band intensities but similar expression patterns were obtained with all of the primer pairs (not shown).

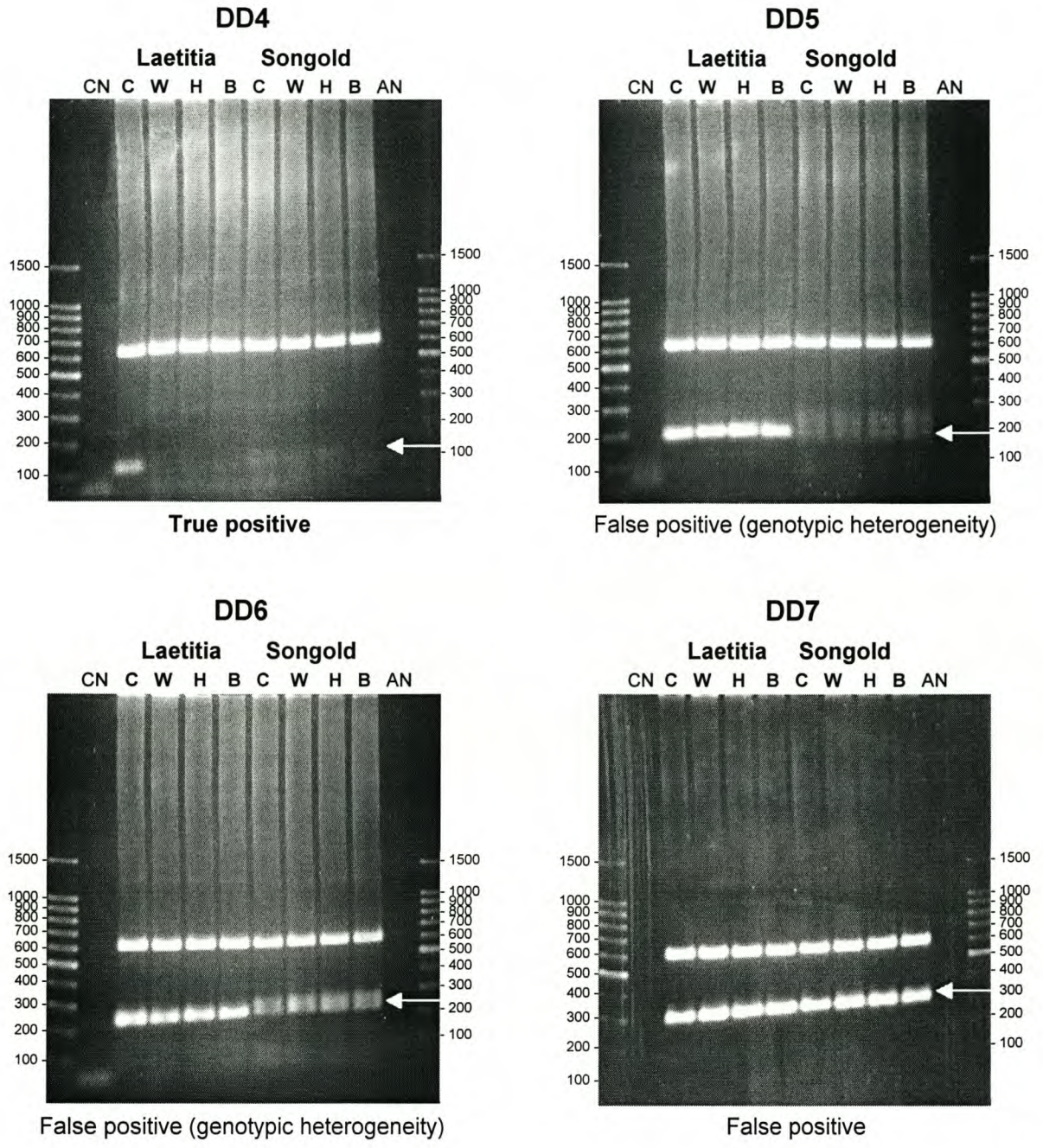


Fig. 7. Agarose gel electrophoresis of RT PCR products generated with DD candidate band specific primers *(continued from previous page)*.

isolates and less intense than the 'Laetitia' bands) for cultivar 'Songold'. These results suggested that genotypic heterogeneity exists between the two cultivars in the mRNAs represented by these bands. However, differences in the expression levels of these mRNAs could not be detected between different cultivars and/or treatments.

The results obtained for bands DDc and DD4 indicated that these bands were derived from mRNAs that are differentially expressed between the two cultivars as a result of the different experimental treatments. For both of these bands, the relative amounts of RT PCR product obtained for each of the eight RNA samples correlated with the original DD pattern (compare Figs. 3 and 7).

BLAST searches

DD primers have been demonstrated to bind with several mismatches during the DD PCR (Liang and Pardee 1992, Haag and Raman 1994). For this reason, the arbitrary DD primer sequences were deleted from the consensus sequences of cloned candidate bands before these sequences were subjected to BLAST similarity searches against sequence databases. Anchored primer sequences were also deleted, as search engines were set to filter out repeated and/or low complexity regions (such as poly(A) tracts). Despite the fact that the differential expression of bands DDa, DDe, DD3, DD5, DD6 and DD7 could not be verified by RT PCR, these bands were included in sequence similarity searches (see Discussion).

A comprehensive BLASTN search, in which the shortened nucleotide sequence of each candidate band (i.e. after deletion of the DD primer sequences) was compared to all non-redundant nucleotide sequence databases, was performed as a first approach (Table 4). Hits reflecting statistically significant sequence similarities (i.e. with E-values ≤ 0.1) were only obtained for bands DDc, DD3 and DD7. From these results, a possible identity could only be assigned to band DD3. The 5'-terminal half of this band consistently showed a high degree of similarity (84 to 88% identity for different plants) with a 3'-region of the *rbcS* gene, which encodes the small subunit of the highly abundant photosynthesis enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; Anderson and Beardall 1991). The two statistically significant hits for DDc (both involving nucleotides 137 to 157 of the shortened DDc sequence), as well as the only statistically significant hit for DD7 (involving nucleotides 286-307 of the shortened DD7 sequence) were with *Drosophila melanogaster* genomic scaffolds (sets of contigs that are ordered, oriented and positioned with respect to each other; Myers et al. 2000), but the regions of similarity were not associated with any sequence of known function. A different region of DD7 (nucleotides 105-123 of the shortened sequence) consistently matched a coding region of human coagulation factor VIII sequences (7 hits out of 87, all with E = 2.3).

Table 4. Results of BLASTN searches: candidate DD band nucleotide sequences compared with non-redundant nucleotide sequence databases.[a]

Candidate band	No. of hits	E-value range[b]		No. hits with E ≤ 0.1	Comments: hits with significant E values or possible significance in the context of plant-pathogen interactions
		Min.	Max.		
DDa	11	1.3	4.9	0	Six of the 11 hits (E = 1.3 or 4.9) were with uncharacterized plant sequences: 2 with *Arabidopsis thaliana* BAC clones, 3 with chromosome 4 of *A. thaliana*, and 1 with the complete *Zea mays* chloroplast genome.
DDc	38	0.068	4.2	2	Both significant hits involved the last 19 nucleotides[c] of the DDc sequence and gave identical bit scores (42.1)[d] In both cases 100% identity was obtained with *Drosophila melanogaster* sequences: nucleotides 102 194 to 102 214 of the complete genomic scaffold 142000013386046 sequence [AE003583] and nucleotides 6775 to 6795 of the chromosome 2L DNA sequence P1 DS01020 (D139) [AC004276]. In neither case was the region of similarity associated with any sequence of known function.
DDe	43	1.1	4.2	0	Twelve of the 43 hits (E = 1.1 or 4.2, all involving nucleotides 71 to 88 or 89 of the DDe sequence) were with exon 6 of plant catalase sequences (*A. thaliana* [AF021937, U43147, X64271]; *Raphanus sativus* [AF248491, AF031318]; *Helianthus annuus* [AF243517], *Brassica juncea* [AF104451, AF104452]; *Nicotiana tabacum* [U03473, U07627 = both "salicylic acid binding catalases"], *N. plumbaginifolia* [Z36976] and *N. sylvestris* [U07626]). Four of the remaining hits (E = 4.2, all involving nucleotides 82 to 99) were with a coding region of eukaryotic initiation factor 5 (*eIF-5*) of *Homo sapiens* [NM_001969, U49436] and *Rattus norvegicus* [L11651, NM_020075].
DD3	105	1 e-10	1.9	64	Seventy-nine hits (53 with E < 0.1 and 26 with E > 0.1, bit scores ranging from 72 to 38) with genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Almost all of these hits involved regions within 5'-terminal 100 nucleotides of the 261 bp DD3 sequence.
DD4	32	0.25	3.9	0	Only one hit with a plant sequence (an uncharacterized *A. thaliana* genomic BAC sequence) was obtained. Two of the remaining 31 hits were with *D. melanogaster* genomic scaffold sequences and the other 29 with uncharacterized human sequences. None of these were associated with sequences of known function.
DD5	16	1.5	5.8	0	Hits were obtained with uncharacterized/non-coding sequences from *A. thaliana* (1), human (8), *Saccharomyces cerevisiae* (2), *Caenorhabditis elegans* (2),*Cochliobolus heterostropus* (1) and *Mus musculus* (1). The only hit corresponding to a known coding sequence was with a *Dictyostelium discoideum* vacuolar proton ATPase subunit [U38803] (E = 5.8).
DD6	37	0.13	8.2	0	The only four hits with plant sequences were with uncharacterized *A. thaliana* sequences. Of the remaining hits, only three were of interest (E = 8.2 for all three). They corresponded to the 3'-untranslated regions (UTRs) of an *Artemia franciscana* partial ubiquitin mRNA [L19056], a hamster insulin tumor cell helix-loop-helix transcription factor mRNA [S80870] and a *Mesocricetus aureus* (golden hamster) cytochrome P450 IIC mRNA [X63022].
DD7	87	0.038	9.3	1	Twenty-six of the 87 hits, including the only one with an E-value < 0.1, were with *D. melanogaster* genomic scaffold sequences. All of these involved nucleotides 208 to 226 of the DD7 sequence, except the significant hit, which involved nucleotides 286 to 307 of the 322 bp DD7 sequence. None of these hits were with sequences to which a known function has been ascribed. Of the other hits, seven (E = 2.3, involving nucleotides 105-123) were with human coagulation factor VIII coding sequences [K01740, M14113, M88646, M90707, NM_019863, NM_000132 and X01179].

[a] Data was retrieved on 22 October 2000, when NCBI database nr = 712 155 sequences or 2 383 860 932 letters (nucleotides). Candidate bands shown by RT PCR to correspond to differentially expressed mRNAs are indicated in **boldface**. Accession numbers of sequences of interest with which similarity was scored, are given in [square brackets].

[b] Hits were not evenly distributed over the reported E-range. Most of the hits were clustered around one or two E-values in the range, one of which normally was the highest one.

[c] DD primer sequences were deleted from cloned DD sequences prior to BLAST searches. Numbers of nucleotides referred to in the discussion of BLAST results do therefore not correspond directly to the numbering in Fig. 6. Instead, nucleotide 1 of a sequence referred to here corresponds to nucleotide 14 of the same sequence in Fig. 6, and the "last" nucleotide of this "shortened sequence" to the 16th nucleotide from last in Fig. 6.

[d] Bit scores are derived from raw alignment scores and have been normalized with respect to the scoring system. They may thus be used to compare alignment scores from different searches. The higher the score, the better the alignment.

Two consistent (yet statistically non-significant) matches with coding sequences of known function were obtained for two different sections of DDe: nucleotides 71 to 88 or 89 showed 100% identity with a region in the sixth exon of several plant catalase genes (12 out of the 43 hits), whereas nucleotides 82 to 99 matched a stretch of nucleotides in a 3'-region of the eukaryotic (translation) initiation factor 5 (*eIF-5*) transcript with 100% identity (4 hits).

In an attempt to obtain a larger number of statistically significant matches, a similarity search was conducted against a nucleotide database containing only plant sequences. In this case, all GenBank Viridiplantae (higher plant) DNA sequences were searched with the TBLASTX algorithm. This algorithm (which compares all six translation frames of a nucleotide sequence against the six-frame translations of each sequence in a nucleotide database) was deemed more applicable for similarity searches with DD products, as they theoretically represent expressed sequences (W. Hide, personal communication). The results of this search session are summarized in Table 5. Although the smaller size of the plant sequence database yielded a marked increase in the number of statistically significant hits, the usefulness of these search results was restricted by the fact that the majority of sequences in the database have not yet been characterized in terms of coding regions/motifs of known function. Nevertheless, matches with coding regions of known plant defense-related genes were obtained for DDe, DD4, and DD7. The similarity of DD3 with *rbcS* was also confirmed in the TBLASTX search.

Another attempt was made to correlate the cloned sequences with genes of known function. In this case, a BLASTN search against the well-characterized Baylor College of Medicine Human Genome Sequencing Center Human Transcript Database was performed. This nucleotide database is much smaller than either of the abovementioned ones (19 242 sequences or 35 825 453 nucleotides on 22 October 2000), yet statistically significant matches were only obtained for DDe, DD4 and DD7. A single significant hit (out of 39, E = 0.064) for DDe yielded the same match with human *eIF-5* [U49436] as in the original BLASTN search. Likewise, the four statistically significant hits for DD7 (out of 19, all with E = 0.035) corresponded to four of the previous matches with human coagulation factor VIII sequences [K01740, M14113, M90707 and X01179]. Two statistically significant matches (out of 15, both with E = 0.060) were obtained for DD4. Both involved nucleotides 125 to 142 of the DD4 sequence, and showed 100% identity with a stretch of nucleotides the 5'-coding region of two human members of the inhibitors of apoptosis protein (IAP) family [L49431 and U45879, respectively] (see Fig. 8).

Table 5. Results of TBLASTX searches: candidate DD band nucleotide sequences compared against all higher plant nucleotide sequences.[a]

DD band	No. of hits[b]	E-value range[c]		No. hits with E < 0.1	Sequences of known function with which statistically significant similarity was obtained			Accession no.	E of hit (bit score)[d]
		Min.	Max.		Organism	Gene	Part of gene		
DDa	100+	0.028	0.94	25	*Lupinus albus* *Betula pendula*	L-asparaginase Phenylalanine ammonia lyase	Exon 4 (of 4) Promoter region	L19141 AJ278116	0.039 (35.0) 0.100 (33.6)
DDc	100+	3 e-5	0.077	32	*Glycine max* *Canavaila lineata*	4-coumarate:CoA ligase Aspartate aminotransferase 2	Intron 1 (of 5) 3'-untranslated region (UTR)	AF002257 AJ001360	0.022 (35.9) 0.077 (34.0)
DDe	100+	0.016	1.3	2	*Nicotiana glutinosa*	Virus resistance (*N*) gene	Exon 2 (of 5)	U15605	0.016 (36.3)
DD3	100+	1 e-16	2 e -14	100+	*Glycine max*	Rubisco small subunit (*rbcS*)	Exon 3 (of 3)	Various[e]	See E- range (85-78)
DD4	100+	7 e-4	0.28	49	*Dioscorea tokoro* *Arabidopsis thaliana* *Capsicum annuum* *Lupinus albus* *Oryza sativa* *Brassica rapa* *Brassica napus* Cucurbita pepo	Phosphoglucose isomerase Shaggy-like kinase gamma Thionin 1-aminocyclopropane-1-carboxylate synthase 4 (ACS4) β-amylase S-locus glycoprotein Disease resistance gene (*RPM1*) Aspartic endopeptidase	5'-flanking (promoter) region 5'-flanking region Intron 3'-UTR Intron 4 (of 6) 5'-flanking region 3'-UTR **(shown in Fig. 8)** Coding region	AB16716 Y12710 X95363 AF119410 L10346 D88192 AF105140 AB002695	0.002 (39.1) 0.008 (37.3) 0.011 (37.3) 0.020 (36.3) 0.041 (35.0) 0.041 (35.0) 0.078 (34.0) 0.098 (34.0)
DD5	100+	7 e-4	0.28	78	*Nicotiana tabacum* *Zea mays* " " " " *Chlamydomonas reinhartii*	- Zein " (*fluory2*) " " (*ZSF4C2*) " Flagellar outer arm dynein ATPase heavy chain, α gene	Cryptic constitutive promoter Exon 2 (of 2) " " " " Intron 12 (of 21)	AF133844 E01145 L34340 V01478 X55723 V01480 L26049	0.016 (37.7) 0.022 (37.3) 0.022 (37.3) 0.022 (37.3) 0.078 (35.4) 0.078 (35.4) 0.078 (35.4)
DD6	100+	0.005	8.1	3	*Triticum aestivum*	Viviparous transcription factor (*vp1B*)	End of intron 5 plus start of exon 6 (of 6)	AJ400713	0.005 (40.5)
DD7	100+	0.003	1.1	13	*Iris hollandica*	Ribosome inactivating type 1 protein (RIP)	Coding region of mature peptide	U78041	0.024 (38.6)

[a] Searches were performed on 22 October 2000, when the GenBank Viridiplantae nucleotide database = 823 875 sequences or 632 276 766 letters (nucleotides).

[b] The search engine was set to report the 100 most significant hits with E < 10. Where 100+ is listed in this column, the number of hits with an E < 10 (default value) hits exceeded 100.

[c] E-value range for the 100 reported hits. Hits were not evenly distributed over the entire E-range, but were typically clustered around a limited number of E-values in the range.

[d] Bit scores are derived from raw alignment scores and have been normalized with respect to the scoring system. They may thus be used to compare alignment scores from different searches. The higher the score, the better the alignment.

[e] The 100 most significant hits were all with *rbcS*-associated clones of *Glycine max*. Accession numbers are therefore not reported.

A. TBLASTX match with the 3'-UTR of the *Brassica napus* disease resistance gene *RPM1* (2781 nucleotides = 927 amino acids)

```
DD4 DNA:        1 ... 29                                                                94 ... 177
                   CGT TCA TAG ATT TAG AAT GTA CTC TAT TTT TTG CAC ATG TAT GTT TAT ATG TTT CAT TCG GCC AAG
DD4 RF +1:         R   S   *   I   *   N   V   L   Y   F   L   H   M   Y   V   Y   M   F   H   S   A   K
                   +   +       +       |   |   |   +   |   +           |       |       +       |       |
RPM1 RF +1:        K   N   M   L   I   N   V   L   F   F   V   I   S   Y   F   Y   N   F   *   S   I   K
RPM1 mRNA:      ... 3937                                                                        4002 ...
```

ALIGNMENT PARAMETERS: Bit score = **34.0** E = **0.078** % similarity = **14/22 (62%)** % identity = **9/22 (40%)**

B. BLASTN matches with coding region of two *Homo sapiens* members of the inhibitor of apoptosis protein (IAP) family

i. *Homo sapiens* TNFR2-TRAF singalling complex protein c-IAP1 (1857 nucleotides = 619 amino acids) [L49431]

```
DD4 DNA:        1 ... 125                                                  142 ... 177
                   A A A T G A A G T A T G A C T T T T
                   | | | | | | | | | | | | | | | | |
c-IAP1 ORF:        A A A T G A A G T A T G A C T T T T
                1 ... 113                                                  130 ... 1857
```

ALIGNMENT PARAMETERS: Bit score = **36.2** E = **0.060** % similarity = **18/18 (100%)** % identity = **18/18 (100%)**

ii. *Homo sapiens* inhibitor of apoptosis protein 2 (1857 nucleotides = 619 amino acids) [U45879]

```
DD4 DNA:        1 ... 125                                                  142 ... 177
                   A A A T G A A G T A T G A C T T T T
                   | | | | | | | | | | | | | | | | |
H-IAP2 ORF:        A A A T G A A G T A T G A C T T T T
                1 ... 113                                                  130 ... 1857
```

ALIGNMENT PARAMETERS: Bit score = **36.2** E = **0.060** % similarity = **18/18 (100%)** % identity = **18/18 (100%)**

Fig. 8. Sequence alignments for three statistically significant matches retrieved for band DD4 with in BLAST sequence similarity searches.

Nucleotide sequences are numbered in the 5'→3' direction. Blue numbers indicate the region of DD4 involved in each alignment. Although DD primer sequences were omitted for search purposes, nucleotide numbers correspond those of the complete DD4 sequence given in Fig. 6. Stretches of similar nucleotides retrieved from sequences in the databases are shown in red. Amino acids derived from the query and target sequences in the TBLASTX search are indicated with their one-letter abbreviations. Horizontal lines between aligned sequences indicate identical nucleotides/amino acids, whereas a + in the TBLASTX alignment indicates amino acids with similar properties. An * in the TBLASTX alignment denotes a stop codon. **Abbreviations:** 3'-UTR = 3'-untranslated region, RF = translation reading frame.

DISCUSSION

Several cDNA fragments were differentially amplified from actively growing shoot tips of two plum cultivars that were inoculated with either the bacterial canker pathogen, *Pseudomonas syringae* pv. *syringae*, or a purified preparation of biologically active recombinant $harpin_{PssNV}$ protein, or as controls, wounded or not treated. Differential display was performed 24 hours after treatment to ascertain whether early induced gene expression differences could be detected between the moderately resistant cultivar 'Laetitia' (representing an incompatible interaction with the pathogen) and the highly susceptible, compatible cv. 'Songold' (see Birch et al. 1999).

Plant treatments and DD profiles

The differential display technique lends itself to the analysis of differential gene expression patterns in complex sample sets such as the one generated in this study (Liang and Pardee 1992, 1995). Highly reproducible arbitrarily-primed cDNA profiles were generated from the set of eight mRNA conditions with this technique. Treatment-related variation displayed in these profiles was, however, somewhat obscured by the large (and unexpected) degree of genotypic variation between the two plum cultivars.

Published reports on the induction of defense-related mechanisms in response to high inocula (> 2 x 10^8 colony forming units (cfu)/mL) of pathogenic bacteria and/or harpin treatment (some of which included a 24 hour post-inoculation time-point; e.g. Bestwick et al. 1997, Wang and Liu 1999, Xie and Chen 2000), led us to expect some pertinent differences in the DD profiles generated from harpin- and/or bacterium-treated (compared to other) shoots in the current study. In most of the variable positions no distinction could, however, be made between the effects of wounding, harpin treatment or bacterial inoculation. This may have been attributable to one, or a combination of three factors.

Firstly, the limited number of DD primer combinations used in the study may not have been capable of displaying all of the harpin- and/or *P. s.* pv. *syringae* NV-induced mRNAs. More of these mRNAs may therefore have been detected if more primer combinations had been tested.

Secondly, although the pathogenicity of the *P. s.* pv. *syringae* NV culture used for the inoculation of plum trees was confirmed by of means an HR test in tobacco, the conditions under which trees were maintained may have favoured epiphytic survival of the bacterium, rather than pathogenicity. (*P.*

syringae is regarded as a weak pathogen, that only causes disease when its host is stressed, Roos et al. 1993.)

Finally, the concentration of harpin used for the treatment plum shoots may have been too low. This is, however, unlikely, as the concentration of $harpin_{PssNV}$ used in this study was much higher (5 to 340 times) than the concentration of harpins derived from *P. s.* pv. *syringae* 61 or *Erwinia amylovora* Ea321 reported previously to induce defense-related gene expression in *Arabidopsis* and tobacco cell cultures and/or intact plants (Gopolan et al. 1996, Ádám et al. 1997, Desikan et al. 1998, 1999, Xie and Chen 2000).

Unfortunately, no assay (such as the HR test) was available to assess whether the treatments applied in this study did in fact lead to the induction of defense responses in experimental trees. Such an assay will be invaluable in future molecular studies of this pathosystem.

Verification of DD results

Eight differentially amplified plum cDNA fragments were successfully re-amplified, cloned and sequenced. They ranged from 177 to 351 bp in size (including DD primer sequences; average size 244 bp, or 215 bp without primer sequences), which compared well with pathogen-induced plant cDNAs isolated in other studies by DD (Truesdell and Dickman 1997, Seehaus and Tenhaken 1998) and suppressive subtractive hybridization (Birch et al. 1999).

An RT PCR based strategy was employed in this study to verify the expression patterns of **all** eight of the cloned cDNAs, as sufficient quantities of RNA were not available to achieve this with a Northern hybridization approach (see Truesdell 1997, Seehaus and Tenhaken 1998). The differential amplification patterns of bands DDc and DD4 were successfully verified with the semi-quantitative RT PCR protocol used. However, in the other six cases, the results obtained with the 35-cycle protocol were not conclusive. No differences in the expression levels of DDa, DDe, DD3, DD5, DD6 and DD7 could be detected, and the protocol was not able to distinguish whether these bands were indeed "false positives", or whether differences in the expression levels of the mRNAs represented by these bands were masked by differences in their amplification efficiencies and the plateau-effect characteristic of RT PCR protocols with a high number (>30) amplification cycles (Gause and Adamovicz 1995). More conclusive results may have been obtained had the RT PCR protocol been optimized empirically for each band with respect to template concentration and the

number of cycles that would have ensured a linear relationship between template and amplification product concentration (Foley et al. 1993).

The abovementioned results suggest that the superior sensitivity and speed of RT PCR (compared to Northern hybridization) can only be fully exploited in the verification of DD results if the technique is applied in a fully quantitative manner. The optimization of conditions for each individual template is a cumbersome process, which has discouraged the routine use of RT PCR in DD studies up to now. The advent of "real-time" PCR technology has, however, simplified the development and application of quantitative (RT) PCR techniques.

BLAST similarity search results

The sequences of **all eight** cDNAs cloned in this study were subjected to BLAST sequence similarity searches for the following reasons: (i) our verification strategy did not prove conclusively that any of these cDNAs were "false positives", and (ii) inducible plant defense responses, which are known to display different temporal patterns, were only monitored at a single time-point in the current study. (A cloned defense-related cDNA that was not shown to be differentially expressed between cultivars and/or treatments at 24 hours post treatment, may exhibit differential expression at an earlier/later stage in this particular pathosystem.) The data from BLAST and RT PCR results were therefore interpreted collectively for each of the cloned bands.

DD3

Of all the cloned DD bands, the greatest sequence similarity with a gene of known function (reflected in the lowest E-values and highest bit scores) was obtained for DD3. This band matched the *rbcS* gene of several plant species in both the BLASTN and TBLASTX searches. RT PCR showed DD3 not to be differentially expressed, a result which appeared to correlate with the fact that the *rbcS*-encoded small subunit of the photosynthesis enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is highly and constitutively expressed during light periods (Anderson and Beardall 1991). It is interesting to note that a rapid, systemic decrease in the levels of *rbcS* expression was observed by Kombrink and Hahlbrock (1990) in fungus-infected or elicitor-treated potato leaves. The timing of this repression (which lasted past 24 hours post-inoculation) correlated with the transcriptional activation of defense-related genes such as the phenylpropanoid biosynthetic enzymes phenylalanine-ammonia lyase (PAL) and 4-coumarate:CoA ligase (4CL). It was subsequently argued that the induction of a massive, multi-component defense

response required the repression of other cellular functions to ensure metabolic balance, and that the small subunit of RuBisCO was apparently dispensable in pathogen defense (Kombrink and Hahlbrock 1990). It is possible that rbcS may play a similar role in the pathosystem investigated here, but that sub-optimal RT PCR conditions resulted in the failure to observe these expression patterns in the current study.

DDe and DD7

Differential expression of two of the three other bands (DDe and DD7), for which matches with known plant defense-related genes were obtained, could also not be verified.

The consistent match between the DDe sequence and the sixth exon of several plant catalase genes obtained in the first BLASTN search was initially viewed with interest, as catalases have been implicated in the establishment of a positive feedback loop for the production of the reactive oxygen intermediate H_2O_2 during plant-defense responses (Ryals et al. 1995, Schneider et al. 1996, Lamb and Dixon 1997). These hits were, however, not statistically significant in the BLASTN search, and were not retrieved among the 100 best hits for DDe with plant DNA sequences in the TBLASTX search.

A different part of DDe (nucleotides 112 to 150 of the shortened sequence) was matched with statistical significance to one sequence of known function in the TBLASTX search, namely the *Nicotiana glutinosa* disease resistance gene *N*. This tobacco *R* gene has previously been shown to confer pathogen-specific resistance in tobacco to most strains of tobacco mosaic virus (TMV), with the TMV replicase gene being implicated as the corresponding *avr* gene (Whitham et al. 1994). The "non-differential" expression pattern observed for DDe by RT PCR correlates with the notion that resistance genes are expressed at low levels in uninfected plants and are not greatly induced upon pathogen infection (Michelmore 1995, Hammond-Kosack and Jones 1997). If DDe therefore represents a *Prunus salicina N* gene homologue that is present in both cvs. 'Laetitia' and 'Songold', the difference in susceptibility between these cultivars to the bacterial canker pathogen can only be explained if this *R* gene does not play a role in the interaction between *Prunus* species and the bacterial canker pathogen. This implies that (i) a corresponding *avr* gene is not carried by *P. s.* pv. *syringae*, (ii) the moderate resistance shown by cv. 'Laetitia' resistance to the pathogen is not *avr-R* gene mediated, and/or (iii) the putative *N* homologue may play a role in *Prunus* resistance against other pathogens.

A statistically significant match with a gene generally associated with plant defense against **viral** pathogens was also retrieved for DD7: the highly expressed ribosome-inactivating protein (RIP) encoding gene of *Iris hollandica*. A RIP was previously demonstrated to be the most abundant protein in *Iris* bulbs (Van Damme et al. 1997), and the expression of RIPs are not expected to form part of the inducible defense response against *P. s.* pv. *syringae* infection in *Prunus* species. This suggests that the RT PCR protocol appropriately demonstrated DD7 to be a "false positive" in the context of this experiment.

DDc and DD4

The differential amplification of DDc and DD4 were verified by RT PCR. Unfortunately, no statistically significant matches with the coding regions of genes with known function were retrieved for DDc in any of the BLAST searches. Intensive searches of well-characterized, organism-specific databases (e.g. those that exclusively contain sequences derived from model organisms such as *Escherichia coli*, *Caenorhabditis elegans* or *Drosophila melanogaster*) were not undertaken in this study, but may yield useful results in this regard.

DD4 was matched with statistical significance to non-coding regions of several genes that may be involved in plant resistance (see Table 4). Matches with introns and/or non-coding, 5'-flanking regions of genes (including promoter areas; also compare with results for DDa and DD5) may, however, not be extrapolated to the possible involvement of such genes in the experimental system, as cloned DD bands are supposed to represent mRNA transcripts. In contrast, 3'-untranslated regions (3'-UTRs) of expressed genes are commonly cloned during DD analyses when anchored oligo-dT primers are used for cDNA synthesis (Haag and Raman 1994, Galindo et al. 1997).

Two different regions of DD4 were matched with the 3'-UTRs of genes with known function. Nucleotides 29-85 (of the shortened sequence) matched a region in the 3'-UTR of the *Lupinus albus ACS4* gene, which encodes 1-aminocyclopropane-1-carboxylate synthase (ACC synthase), the final and rate-limiting enzyme in the biosynthetic pathway of the endogenous signalling molecule ethylene (Taiz and Zeiger 1991). Although this match seemed promising with regard to the objectives of this study, the expression pattern of DD4 (down-regulation in treated samples relative to untreated controls, see Fig. 7) did not correlate with previous reports on the **induction** of ACC synthase expression in response to wounding and pathogen infection (Taiz and Zeiger 1991). Another factor that suggested the statistically significant similarity with *ACS4* to be **biologically**

non-significant, was the fact that the match was derived from an alignment between the -1 reading frame translation product of DD4 and the +3 reading frame translation product of ACS4.

The most exciting results of this study were the statistically significant matches of DD4 with the 3'-UTR of the *Brassica napus* disease resistance gene *RPM1* and the 5'-coding region of two human IAPs (depicted in Fig. 8). *RPM1* is a homologue of the *Arabidopsis R* gene which confers resistance to *P. syringae* strains carrying the corresponding, non-homologous *avrRpm1* or *avrB* genes (Grant et al. 1995). Both of the IAPs retrieved in the DD4 similarity search (members of a protein family originally identified in baculoviruses; Clem and Miller 1994) have been proposed to suppress apoptosis in healthy mammalian cells (Rothe et al. 1995, Liston et al. 1996).

Despite the fact that the interaction between the systemic pathogen *P. s.* pv. *syringae* NV and the resistant cv. 'Laetitia' does not lead to a visible HR (even when bacterial inocula exceeded 10^8 cfu/mL; Klement 1982), both of the abovementioned matches for DD4 suggest a role for hypersensitive cell death (which is now commonly accepted to represent a form of programmed cell death or PCD; Heath 1998) in this pathosystem. In the case of *RPM1*, rapid degradation of an epitope-tagged *RPM1* gene (over-expressed in transgenic *Arabidopsis* plants) coincided with the onset of the HR caused by incompatible *P. syringae* bacteria (as late as 22 hours post-infection in some interactions). This degradation was not observed in response to wounding or inoculation with non-HR inducing compatible bacteria. It was suggested that the down-regulation of *RPM1* (by which transduction of the bacterially-delivered *Avr* signal will be interrupted) may constitute one way in which the plant is able to control the extent of cell death and the overall resistance response at the site of infection (Boyes et al. 1998).

The expression patterns observed for DD4 by RT PCR may be correlated with such a role in PCD for the mRNA represented by DD4. The highest levels of DD4 were observed in the **untreated** controls of both cultivars (consistent with the constitutive expression of *R* genes in uninfected plants; Michelmore 1995, Hammond-Kosack and Jones 1997). Slightly reduced levels in the treated 'Songold' samples and very low levels in the three treated 'Laetitia' samples indicate a more effective "desuppression" of PCD in cv. 'Laetitia', and may reflect the resistance shown by this cultivar, but not by cv. 'Songold'. The same argument holds for a possible relation between DD4 and IAPs. The differential expression of DD4 between 'Laetitia' and 'Songold' samples was seen more clearly when the amount of total RNA used for cDNA synthesis was raised from 2 μg (shown in Fig. 7) to 5 μg (result not shown). The decrease in DD4 expression in the 'Laetitia' wounding

control was, however, puzzling with regard to a relation with either RPM1 or IAPs, as wounding is presumed not to induce hypersensitive cell death (Baron and Zambryski 1995).

The work presented here has produced some promising preliminary results with respect to the molecular dissection of the *Prunus* defense response to *P. s.* pv. *syringae*. More work is, however, needed to confirm that the sequence similarities between genes of known function and the relatively short cloned DD fragments retrieved in BLAST searches are of biological significance in this pathosystem. Future endeavours towards this goal will include: (i) the development of a quantitative RT PCR verification strategy and re-assessment of the expression patterns of cDNAs cloned in this study, (ii) the application of the 5'-RACE technique (Frohman et al. 1988) to obtain full-length sequences of the mRNAs represented by cloned bands of interest, (iii) repetition of BLAST searches with these full-length sequences, (iv) assessment (by RT PCR) of the temporal expression patterns of cloned bands in an experimental setup similar to the one used in this study and (v) the development of a strategy to correlate the temporal expression patterns of these mRNAs with protein levels in the pathosystem at the corresponding time intervals. The knowledge gained from these continued studies will be applied in the selection of commercial stone fruit cultivars which are resistant to the bacterial canker pathogen.

ACKNOWLEDGEMENTS

The authors thank Prof. Peter Gresshoff (formerly from Plant Molecular Genetics, University of Tennessee, Knoxville) for sponsoring a research visit for M.A. to gain expertise in the use of DD technology. We also thank Carel van Heerden (Core DNA Cycle Sequencing Facility, University of Stellenbosch) for analysis of sequencing reactions, Dr. Winston Hide (South African National Bioinformatics Institute) for his expert advice on BLAST searches, Drs. Johan Burger (Genetics, University of Stellenbosch) and Heidi de Wet (Biochemistry, University of Stellenbosch) for helpful discussions, Coral de Villiers (Biochemistry, University of Stellenbosch) for technical assistance, and Fritz Tiedt (Biochemistry, University of Stellenbosch) for assistance with the production of photographs. Actin primers were a generous gift from Anita Burger (Institute for Plant Biotechnology, University of Stellenbosch). Financial support for this work, M.A.'s postgraduate studies and research visit to the University of Tennessee from the South African National Research Foundation (NRF), the University of Stellenbosch and the ARC-Fruit, Wine and Vine Research Institute is gratefully acknowledged.

REFERENCES

Ádám, A.L., Pike, S., Hoyos, M.E., Stone, J.M., Walker, J.C. and Novacky, A. (1997). Rapid and transient activation of a myelin basic protein kinase in tobacco leaves treated with harpin from *Erwinia amylovora*. *Plant Physiol.* **115:** 853-861.

Alfano, J.R., Charkowski, A.O., Deng, W.-L., Badel, J.L., Petnicki-Ocwieja, T., Van Dijk, K. and Collmer, A. (2000). The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* **97:** 4856-4861.

Alfano, J.R. and Collmer, A. (1996). Bacterial pathogens in plants: life up against the wall. *Plant Cell* **8:** 1683-1898.

Alfano, J.R. and Collmer, A. (1997). The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* **179:** 5655-5662.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215:** 403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids. Res.* **25:** 3389-3402.

Anderson, J.W. and Beardall, J. (1991). Molecular activities of plant cells: an introduction to plant biochemistry. Blackwell Scientific Publications, Oxford.

Appel, M. (1996). Investigations into the production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae* isolated from a nectarine tree. M. Sc. thesis. University of Stellenbosch, Stellenbosch.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1999a). Differential display of eukaryotic mRNA: meeting the demands of the new millennium? *J. Plant Physiol.* **154:** 561-570.

Appel,. M., Hampf, M., Mansvelt, L., Hapgood, J. and Bellstedt, D. (1999b). Amplification, sequence and expression of the harpin encoding gene of the bacterial canker pathogen, *Pseudomonas syringae* pv. *syringae* strain NV. *J. Plant Physiol.* **154:** 489-497.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) (1995). Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Baron, C. and Zambryski, P.C. (1995). The plant response in pathogenesis, symbiosis and wounding: variations of a common theme? *Annu. Rev. Genet.* **29:** 107-129.

Bassam, B.J., Caetano-Anollés, G. and Gresshoff, P.M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **196:** 80-83.

Bauer, D., Müller, H., Reich, J., Riedel, H., Ahrenkiel, V., Warthoe, P. and Strauss, M. (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucl. Acids Res.* **21:** 4272-4280.

Bestwick, C.S., Brown, I.R., Bennet, M.H.R. and Mansfield, J.W. (1997). Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell* **9:** 209-221.

Birch, P.R.J., Avrova, A.O., Duncan, J.M., Lyon, G.D. and Toth, R.L. (1999). Isolation of potato genes that are induced during an early stage of the hypersensitive response to *Phytophtora infestans*. *Mol. Plant-Microbe Interact.* **12:** 356-361.

Boyes, D., Nam, J. and Dangl, J.L. (1998). The *Arabidopsis thaliana RPM1* disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. *Proc. Natl. Acad. Sci. USA* **95:** 15849-15854.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Clem, R.J. and Miller, L.K. (1994). Induction and inhibition of apoptosis by insect viruses. In: Tomei, D.L. and Cope, F.O. (eds.) Apoptosis II: the molecular basis of apoptosis in disease, pp. 89-110. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Clinton, M. and Scougall, R.K. (1995). Detection and capture of ^{35}S-labeled gas released from reaction tubes during differential display PCR. *BioTechniques* **19:** 798-799.

Collmer, A. (1998). Determinants of pathogenicity and avirulence in plant pathogenic bacteria. *Curr. Opin. Plant Biol.* **1:** 329-335.

Dean, R.A. and Kuc, J. (1985). Induced systemic protection in plants. *Trends in Biotechnol.* **3:** 125-129.

Desikan, R., Clarke, A., Atherfold, P., Hancock, J.T. and Neill, S.J. (1999). Harpin induces mitogen-activated protein kinase activity during defense responses in *Arabidopsis thaliana* suspension cultures. *Planta* **210:** 97-103.

Desikan, R., Reynolds, A., Hancock, J.T. and Neill, S.J. (1998). Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. *Biochem. J.* **330:** 115-120.

De Wit, P.J.G.M. (1997). Pathogen avirulence and plant resistance: a key role for recognition. *Trends in Plant Sci.* **2:** 452-457.

Ebel, J. and Cosio, E.G. (1994). Elicitors of plant defense responses. *Int. Rev. Cytol.* **148:** 1-36.

Esnault, R., El Turk, J., Asemota, O., Sallaud, C., Breda, C., Buffard, D. and Kondorosi, A. (1993). Structure and expression of several peroxidase cDNAs from *Medicago sativa*. In: Welinder, K.G., Rasmussen, S.K., Penel, C. and Greppin, H. (eds.). Plant peroxidases: biochemistry and physiology (Proceedings of the III International Symposium), pp. 362-368. University of Geneva, Geneva.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18:** 265-276.

Foley, K.P., Leonard, M.W. and Engel, J.D. (1993). Quantitation of RNA using the polymerase chain reaction. *Trends in Genet.* **9:** 380-385.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85:** 8998-9002.

Galindo, J.E., Poirier, G.M.-C., Guo, H., Huvar, A., Wagaman, P.C., Zhu, J., Tench, J., Wan, J.S. and Erlander, M.G. (1997). Differential display of mRNA. In: Caetano-Anollés, G. and Gresshoff, P.M. (eds.). DNA markers: protocols, applications and overviews, pp. 225-236. Wiley-Liss, New York.

Gause, W.C. and Adamovicz, J. (1995). Use of PCR to quantitate relative differences in gene expression. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 293-311. Cold Spring Harbor Laboratory Press, New York.

GenHunter Corporation (1997). RNAimage instruction manual. GenHunter Corporation, Nashville, TN.

Gómez-Gómez, L., Felix, G. and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18:** 277-284.

Gopolan, S., Wei, W. and He, S.Y. (1996b). *hrp* gene-dependent induction of *hin1*: a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant J.* **10:** 591-600.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J. et al. (1995). Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance. *Science* **269:** 843-846.

Haag, E. and Raman, V. (1994). Effects of primer choice and source of Taq DNA polymerase on the banding patterns of differential display RT-PCR. *BioTechniques* **17:** 226-228.

Hattingh, M.J., Roos, I.M.M. and Mansvelt, E.L. (1989). Infection and systemic invasion of deciduous fruit trees by *Pseudomonas syringae* in South Africa. *Plant Disease* **73:** 784-789.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996). Resistance gene-dependent plant defense responses. *Plant Cell* **8:** 1773-1791.

Hammond-Kosack, K.E. and Jones, J.D.G. (1997). Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48:** 757-607.

He, S.Y., Huang, H.-C. and Collmer, A. (1993). *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. *Cell* **73:** 1255-1266.

Heath, M.C. (1998). Apoptosis, programmed cell death and the hypersensitive response. *Eur. J. Plant Pathol.* **104:** 117-124.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. *Curr. Opin. Plant Biol.* **83:** 315-319.

Jones, P., Qiu, J. and Rickwood, D. (1994). RNA isolation and analysis. BIOS Scientific Publishers, Oxford.

King, E.O., Ward, M.K. and Raney, D.E. (1954). Two simple media for the demonstration of pyocyanini and fluorescein. *J. Lab. Med.* **22:** 301-307.

Klement, Z. (1982). Hypersensitivity. In: Mount, M.S. and Lacy, G.H. (eds.) Phytopathogenic Prokaryotes, vol. 2, pp. 149-177. Academic Press. New York.

Kombrink, E., Beerhues, L., Garcia-Garcia, F., Hahlbrock, K., Müller, M., Schröder, M., Witte, B. and Schmelzer, E. (1993). Expression patterns of defense-related genes in infected and uninfected plants. In: Fritig, B. and Legrand, M. (eds.) Mechanisms of plant defense responses, pp. 236-249. Kluwer Academic Publishers, Dordrecht.

Kombrink, E. and Hahlbrock, K. (1990). Rapid, systemic repression of the synthesis of ribulose 1,5-bisphosphate carboxylase small-subunit mRNA in fungus-infected or elicitor-treated potato leaves. *Planta* **181:** 216-219.

Lamb, C.J. and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48:** 251-275.

Lamb, C.J., Lawton, M.J., Dron, M. and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56:** 215-224.

Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. and Pardee, A.B. (1992). Differential display and cloning of messenger RNAs from human breast cancer *versus* mammalian epithelial cells. *Cancer Res.* **52:** 6966-6968.

Liang, P., Averboukh, L. and Pardee, A.B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucl. Acids Res.* **21:** 3269-3275.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257:** 967-971.

Liang, P. and Pardee, A.B. (1995). Recent advances in differential display. *Curr. Opin. Immunol.* **7:** 274-280.

Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connell, R., Averboukh, L., Wang, F. and Pardee, A.B. (1994). Differential display using one-base anchored oligo-dT primers. *Nucl. Acids Res.* **22:** 5763-5764.

Lindgren, P.B. (1997). The role of *hrp* genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* **35:** 129-152.

Lindgren, P.B., Peet, R.C. and Panopoulos, N.J. (1986). Gene cluster of *Pseudomonas syringae* pv. *"phaseolicola"* controls pathogenicity on bean and hypersensitivity on non-host plants. *J. Bacteriol.* **168:** 512-522.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. MacKenzie, A. and Korneluk, R.G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379:** 349-353.

Michelmore, R.W. (1995). Isolation of disease resistance genes from crop plants. *Curr. Opin. Biotechnol.* **6:** 145-152.

Men, A.E. and P.M. Gresshoff (1998). Efficient cloning of DAF polymorphic markers from silver stained polyacrylamide gels. *BioTechniques* **24:** 593-595.

Myers, E.W., Sutton, G.G., Delcher, A.L., Dew, I.M., Fasulo, D.P., Flanigan, M.J., Kravitz, S.A., Mobarry, C.M., Reinert, K.H.J., Remington, K.A., Anson, E.L., Bolanos, R.A., Chou, H.-H., Jordan, C.M., Halpern, A.L., Lonardi, S., Beasly, E.M., Brandon, R.C., Chen, L., Dunn, P.J., Lai, Z., Liang, Y., Nusskern, D.R., Zhan, M., Zhang, Q., Zheng, X., Rubin, G.M., Adams, M.D. and Venter, J.C. (2000). A whole-genome assembly of *Drosophila*. *Science* **287:** 2196-2203.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J.-P., Manners, J.M. and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* **8:** 2309-2323.

Promega Corporation (1996). Promega technical manual no. 042: pGEM-T vector system. Promega Corporation, Madison, WI, USA.

Qiagen GmbH (1999). RNeasy Mini Handbook, Qiagen GmbH, Hilden, Germany.

Roos, I.M.M. and Hattingh, M.J. (1987). Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. *Phytopathology* **77:** 900-908.

Roos, I.M.M., Mansvelt, E.L. and Hattingh, M.J. (1993). Pathological anatomy of infection and systemic invasion on deciduous fruit trees by bacterial pathogens. In: Biggs, A.R. (ed.) Handbook of cytology, histology and histochemistry of fruit tree diseases, pp. 281-298. CRC Press, Boca Raton.

Rothe, M., Pan, M.-G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V. (1995). The TNFR2-TRAF singaling complex contains two novel proteins related to Baculoviral inhibitor of apoptosis proteins. *Cell* **83:** 1243-1252.

Ryals, J., Lawton, K.A., Delaney, T., Friedrich, L., Kessmann, H., Neuenschwander, U., Uknes, S., Vernooij, B. and Weyman, K. (1995). Signal transduction in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA* **92:** 4202-4205.

Seehaus, K. and Tenhaken, R. (1998). Cloning of genes by mRNA differential display induced during the hypersensitive reaction of soybean after inoculation with *Pseudomonas syringae* pv. *glycinea*. *Plant Mol. Biol.* **38:** 1225-1234.

Schneider, M., Schweizer, P., Meuwly, P. and Métraux, J.P. (1996). Systemic acquired resistance in plants. *Intl. Rev. Cytol.* **168:** 303-340.

Taiz, L. and Zeiger, E. (1991). Plant physiology. The Benjamin/Cummings Publishing Company, Redwood city.

Truesdell, G.M. and Dickmann, M.B. (1997). Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. *Plant Mol. Biol.* **33:** 737-743.

Van Damme, E.J., Barre, A., Barbieri, L., Valbonesi, P., Rouge, P., Van Leuven, F., Stirpe, F. and Peumans, W.J. (1997). Type I ribosome-inactivating proteins are the most abundant proteins in iris (*Iris hollandica* var. Professor Blaauw) bulbs: characterization and molecular cloning. *Biochem. J.* **324:** 963-970.

Vivian, A. and Gibbon, M.J. (1997). Avirulence genes in plant-pathogenic bacteria: signals or weapons? *Microbiology* **143:** 693-704.

Wahle, E. and Keller, W. (1992). The biochemistry of 3'-end cleavage and polyadenylation of messenger RNA precursors. *Annu. Rev. Biochem.* **61:** 419-440.

Wang, W.-C. and Liu, Z.-H. (1999). $Harpin_{Pss}$-induced peroxidase and lignin accumulation in tobacco during the hypersensitive response. *Aust. J. Plant Physiol.* **26:** 265-272.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C. and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the interleukin-1 receptor. *Cell* **78:** 1101-1115.

Xie, Z. and Chen, Z. (2000). Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. *Mol. Plant-Microbe Interact.* **13:** 183-190.

Yamaguchi, M., Dieffenbach, C.W., Connolly, R., Cruess, D.F., Baur, W. and Sharefkin, J.B. (1992). Effect of different laboratory techniques for guanidium-phenol-chloroform RNA extraction on A_{260}/A_{280} and on accuracy of mRNA quantitation by reverse transcriptase-PCR. *PCR Methods Appl.* **1:** 286-290.

CHAPTER 6

CONCLUSIONS

The research presented in this dissertation had two broad objectives: (i) to clone, sequence and express the *Pseudomonas syringae* pv. *syringae* NV harpin encoding (*hrpZ*$_{PssNV}$) gene, and (ii) to establish a molecular system in which the recombinant HrpZ$_{PssNV}$ protein could be applied to identify genes involved in the interaction between the bacterial stone fruit pathogen and a stone fruit host (*Prunus salicina*). The first objective was fully met. To fulfill of the second objective, a complex technique had to be established and applied to a poorly characterized pathosystem. Success was hampered by several pitfalls, in spite of which promising preliminary results were obtained.

To conclude this dissertation, the achievements of this study, as well as the pitfalls and proposals for future success in investigations of this nature are outlined below.

6.1 ACHIEVEMENTS

The salient achievements of the current study were the following:

1. The *P. s.* pv. *syringae* NV *hrpZ*$_{PssNV}$ nucleotide and deduced HrpZ$_{PssNV}$ amino acid sequences were described and compared to the *hrpZ* and HrpZ sequences of other phytopathogenic *P. syringae* strains.
2. A recombinant system, which may be used for the production of biologically active *P. s.* pv. *syringae* harpin protein, was established. The well-documented ability of this protein (and other harpins) to induce localized and systemic defense responses in a variety of plant species renders it a valuable and accessible tool for local (South African) investigations into a variety of pathosystems involving necrogenic phyto-pathogens.
3. Progress towards the molecular dissection of the poorly characterized *P. s.* pv. *syringae-Prunus* pathosystem has been made.

6.2 PITFALLS AND PROPOSALS FOR FUTURE SUCCESS

Progress in the second phase of the study was hampered by several factors which were discussed in Chapter 5. The brief discussions that follow are aimed at (i) extending the usefulness of the preliminary results obtained in the current study, and (ii) benefitting continued molecular studies of this nature:

(i) Assessment of plant responses to experimental treatment:

A disadvantage of the *P. s.* pv. *syringae-Prunus salicina* pathosystem investigated in this study was that no assay was available to assess whether experimental treatments (wounding, infiltration with $harpin_{PssNV}$ protein or inoculation with *P. s.* pv. *syringae* NV) indeed induced defense-related responses in either the resistant or the susceptible plum cultivar. This was compounded by two factors. Firstly, it was not possible to ascertain whether inoculation with the *P. s.* pv. *syringae* NV culture (which was confirmed to be pathogenic on a non-host, tobacco) would establish a pathogenic or epiphytic bacterial population in experimental plants. Secondly, although a relatively high concentration of $harpin_{PssNV}$ (compared to that of harpins infiltrated into other plants in previously published studies) was used in the current study, it was not possible to determine whether it would be adequate to elicit a response in plum trees.

It was hoped that this study would yield a DD fragment that could be used as a "marker" for the induction of plant defense responses (at least at 24 hours post-inoculation) in future studies. This was unfortunately not achieved.

Future studies of this nature may therefore benefit from the use of "model" plants such as tobacco or *Arabidopsis* for three reasons: (i) these plants were employed in many published studies in combination with harpin-producing pathogens and purified preparations of harpin proteins, (ii) a visible hypersensitive response (HR) develops in response to harpin inoculations in many cultivars/ecotypes of these plants, and (iii) much progress has been made with the genetic characterization of *A. thaliana* and, to a lesser extent, some *Nicotiana* species, and a great deal of sequence information is available for these plants in internationally accessible databases.

(ii) Verification of DD results:

Reasons for the employment of a semi-quantitative reverse transcriptase polymerase chain reaction (RT PCR) strategy for the verification of DD results were explained in detail in previous chapters and will not be repeated here. Although this strategy was considered to be the most appropriate at the time, the expression patterns of many of the cloned DD bands could not be verified conclusively. Some of these cDNAs showed preliminary sequence similarity with known plant defense-related genes, and may yet be demonstrated to be differentially expressed in this experimental system once an optimized, quantitative verification protocol is applied.

Most Northern hybridization-based verification protocols do not include an amplification step* and are therefore easier than RT PCR-based protocols to adapt for quantitative applications. Advances in Northern hybridization technology offer some possibilities for the development of a highly sensitive Northern hybridization verification protocol for future studies of this nature†. This may, for example, be achieved through the use of high specific activity radiolabelled riboprobes and high sensitivity membranes. However, the speed and sensitivity of RT PCR still render this technique the most attractive for the routine verification of DD results. Access to "real-time" PCR technology offers the possibility optimizing protocols for individual templates easily and quickly to yield fully quantitative results (without the use of radio-active labels), and promises to extend the usefulness of the DD system established in this study significantly.

(iii) BLAST sequence similarity searches:

Sequence data derived from cloned DD fragments are generally of limited use in sequence similarity searches, as they are normally short and do not comprise the coding regions of the mRNAs from which they were derived. Additionally, sequence similarity searches with short fragments frequently yield matches that are (i) of statistical, but not biological significance, (ii) not statistically significant (but may be of biological significance), or (iii) of neither statistical nor biological significance.

The BLAST results obtained in this study, although promising, must therefore be regarded

---

* An exception is the reverse Northern hybridization technique combined with the use of amplified RNA (see section 4.3.3).

† Highly sensitive protocols are not only required for the detection of low-abundance transcripts, but are imperative when RNA availability is limited (e.g. in a study such as this where seasonal plants were used).

as preliminary until they can be confirmed with full-length cDNA sequences*.

(iv) Confirming biological significance within a particular system:

Once full-length cDNAs have been cloned and statistically significant sequence similarities have been confirmed, the biological significance of corresponding mRNAs may be assessed within the experimental system. This may be achieved in one, or a combination of the following ways:

a. An RT PCR (or Northern hybridization) strategy may be developed to assess the temporal expression patterns of individual mRNAs in the experimental system. In studies of this nature, such data will predict whether a particular mRNA is expressed in early or late defense responses. Should a sequence similarity match with a known defense-related gene be available, the response may be compared with published data on other or related pathosystems.

b. Protocols may be developed to measure the levels of protein derived from a particular mRNA at different time-points in the experimental system, and correlate these with the temporal expression patterns of the mRNA. The stability of transcripts and their relative importance in a particular defense response may be assessed in this way.

c. mRNAs may be expressed in transgenic plants to assess their biological functions in a particular pathosystem *in vivo*.

6.3 CONCLUDING REMARKS

This dissertation is the first report documenting resistance mechanisms of stone fruit trees to bacterial canker on a molecular level. It builds on the internationally recognized contributions to the field of bacterial fruit tree diseases made by Prof. Martin J. Hattingh (formerly Head of the Department of Plant Pathology and Dean of Agriculture of Stellenbosch University), Dr. Isabel M.M. Roos (formerly Head of the Plant Biotechnology and Pathology Section of Infruitec) and Dr. E. Lucienne Mansvelt (co-promoter of this dissertation).

---

* Full-length cDNAs corresponding to cloned DD fragments may be obtained by using the 5'-RACE technique, or through the screening of a *Prunus salicina* cDNA library (should such a library become available).

In spite of certain technical shortcomings, this study has led to the identification of several *Prunus* genes previously implicated by other research groups in plant defense and disease resistance. This confirms that the approach followed here is appropriate and holds particular promise for future studies of this nature. In the long term, the collective knowledge gained from these projects will be applied in the design of programmes for the establishment of commercial cultivars which are resistant to the major South African stone fruit pathogens.

LITERATURE CITED

Ádám, A.L., Pike, S., Hoyos, M.E., Stone, J.M., Walker, J.C. and Novacky, A. (1997). Rapid and transient activation of a myelin basic protein kinase in tobacco leaves treated with harpin from *Erwinia amylovora*. *Plant Physiol.* **115:** 853-861.

Adamovicz, J. and Gause, W.C. (1995). RNA purification. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 113-129. Cold Spring Harbor Laboratory Press, New York.

Alfano, J.R., Bauer, D.W., Milos, T.M. and Collmer, A. (1996). Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally nonpolar deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. *Mol. Microbiol.* **19:** 715-728.

Alfano, J.R., Charkowski, A.O., Deng, W.-L., Badel, J.L., Petnicki-Ocwieja, T., Van Dijk, K. and Collmer, A. (2000). The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* **97:** 4856-4861.

Alfano, J.R. and Collmer, A. (1996). Bacterial pathogens in plants: life up against the wall. *Plant Cell* **8:** 1683-1898.

Alfano, J.R. and Collmer, A. (1997). The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* **179:** 5655-5662.

Alfano, J.R., Kim, H.-S., Delaney, T.P. and Collmer, A. (1997). Evidence that the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in a *hrp*-dependent manner with tobacco cells. *Mol. Plant-Microbe Interact.* **10:** 580-588.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215:** 403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids. Res.* **25:** 3389-3402.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92:** 773-784.

Anderson, J.W. and Beardall, J. (1991). Molecular activities of plant cells: an introduction to plant biochemistry. Blackwell Scientific Publications, Oxford.

Appel, M. (1996). Investigations into the production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae* isolated from a nectarine tree. M. Sc. thesis. University of Stellenbosch, Stellenbosch.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1998a). Differential display of eukaryotic mRNA: current state of the art. Lecture presented at the joint 15th congress of the South African Society for Biochemistry and Molecular Biology and 2nd congress of the Federation of African Societies for Biochemistry and Molecular Biology. Potchefstroom, South Africa, 29 September - 3 October 1998.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1998b). False positives in differential display: analysis and antidotes originating from DNA amplification fingerprinting (DAF) technology. Poster presented at the joint 15th congress of the South African Society for Biochemistry and Molecular Biology and 2nd congress of the Federation of African Societies for Biochemistry and Molecular Biology. Potchefstroom, South Africa, 29 September - 3 October 1998.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1999). Differential display of eukaryotic mRNA: meeting the demands of the new millennium? *Plant Physiol.* **154:** 561-570.

Appel, M., Mansvelt, E.L. and Bellstedt, D.U. (2000). Identification of genes involved in the interaction between *Pseudomonas syringae* pv. *syringae* and plum trees using differential display. Poster presented at the BioY2K Combined Millennium Meeting. Grahamstown, South Africa, 23-28 January 2000.

Appel, M., Ries, G.F. de W. and Bellstedt, D.U. (1994). The quantitative assessment of chitinase activity in potato tubers using Remazol Brilliant Violet labelled carboxymethyl-chitin. Poster presented at the 12th congress of the South African Biochemical Society. Stellenbosch, South Africa, January 1994.

Appel, M., Ries, G.F. de W., Hofmeyr, J.-H.S. and Bellstedt, D.U. (1995). A method for the quantitative assessment of wound-induced chitinase activity in potato tubers. *J. Phytopathol.* **143:** 525-529.

Arlat, M., Van Gijsegem, F., Huet, J.C., Pernollet, J.C. and Boucher, C.A. (1994). PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* **13:** 543-553.

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S.P. (1997). Signalling in plant-microbe interactions. *Science* **276:** 726-733.

Baker, C.J. and Orlandi, E.W. (1995). Active oxygen species in plant pathogenesis. *Annu. Rev. Phytopathol.* **33:** 299-321.

Baker, C.J., Orlandi, E.W. and Mock, N.M. (1993). Harpin, an elicitor of the hypersensitive response in tobacco cause by *Erwinia amylovora*, elicits active oxygen production in suspension cells. *Plant Physiol.* **102:** 1341-1344.

Barney, M. (1995). *Erwinia amylovora hrpN* mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. *Eur. J. Plant Pathol.* **101:** 333-340.

Baron, C. and Zambryski, P.C. (1995). The plant response in pathogenesis, symbiosis and wounding: variations of a common theme? *Annu. Rev. Genet.* **29:** 107-129.

Barz, W. and Welle, R. (1992). Biosynthesis and metabolism of isoflavones and pterocarpan phytoalexins in chickpea, soybean and phytopathogenic fungi. *Recent Adv. Phytochem.* **26:** 139-164.

Bauw, G. and Van Montagu, M. (1997). Two-dimensional polyacrylamide gel electrophoresis- based analysis for the identification of proteins and corresponding genes. In: Hansen, E. and Harper, G. (eds.). Differentially expressed genes in plants: a bench manual, pp. 105-118. Taylor and Francis, London.

Baydoun, E. A.-H. and Fry, S.C. (1985). The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. *Planta* **165:** 269-276.

Beattie, G.A. and Lindow, S.E. (1994). Epiphytic fitness of phytopathogenic bacteria: physiological adaptions for growth and survival. In: Dangl. L. (ed.) Current Topics in Microbiology and Immunology, vol. 192: Bacterial pathogenesis of plants and animals - molecular and cellular mechanisms, pp. 1-27. Springer-Verlag, Berlin.

Beer, S.V., Bauer, D.W., Jiang, X.H., Laby, R.J., Sneath, B.J., Wei, Z.-M., Wilcox, D.A. and Zumoff, C.H. (1991). The hrp gene cluster of Erwinia amylovora. In: Hennecke, H. and Verma, D.P.S. (eds.) Advances in molecular genetics of plant-microbe interactions, pp. 53-60. Kluwer Academic Publishers, Dordrecht.

Bell, A.A. (1981). Biochemical mechanisms of disease resistance. *Annu. Rev. Plant Physiol.* **32:** 21-81.

Benhamou, N. (1996). Elicitor-induced plant defence pathways. *Trends in Plant Sci.* **1:** 233-240.

Bennet, R.N. and Wallsgrove, R.M. (1994). Secondary metabolites in plant defense mechanisms. *New Phytol.* **127:** 617-633. (Transley review no. 72).

Bent, A.F. (1996). Plant disease resistance genes: function meets structure. *Plant Cell* **8:** 1757-1771.

Blumwald, E., Aharon, G.S. and Lam, B.C.-H. (1998). Early signal transduction pathways in plant-pathogen interactions. *Trends in Plant Sci.* **3:** 342-346.

Bogdanove, A.J., Bauer, D.W. and Beer, S.V. (1998a). *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. *J. Bacteriol.* **180:** 2244-2247.

Bogdanove, A.J., Beer, S.V., Bonas, U., Boucher, C.A., Collmer, A., Coplin, D.L., Cornelis, G.R., Huang, H.-C., Hutcheson, S.W., Panopoulos, N.J., and Van Gijsegem, F. (1996). Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol. Microbiol.* **20:** 681-683.

Bogdanove, A.J., Kim, J.F., Wei, Z., Kolchinsky, P., Charkowski, A.O., Conlin, A.K., Collmer, A. and Beer, S.V. (1998b). Homology and functional similarity of a *hrp*-linked pathogenicity operon, *dspEF*, of *Erwinia amylovora* and the *avrE* locus of *Pseudomonas syringae* pathovar *tomato*. *Proc. Natl. Acad. Sci. USA* **95:** 1325-1330.

Bol, J.F. and Linthorst, H.J.M. (1990). Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* **28:** 113-138.

Boller, T. (1995). Chemoperception of microbial signals in plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46:** 189-214.

Bolwell, G.P. (1999). Role of active oxygen species and NO in plant defence responses. *Curr. Opin. Plant Biol.* **2:** 287-294.

Bonas, U. (1994). *hrp* genes of phytopathogenic bacteria. In: Dangl. L. (ed.) Current Topics in Microbiology and Immunology, vol. 192: Bacterial pathogenesis of plants and animals - molecular and cellular mechanisms, pp. 79-98. Springer-Verlag, Berlin.

Bonas, U. and Van den Ackerveken, G. (1997). Recognition of bacterial avirulence proteins occurs inside the plant cell: a general phenomenon in resistance to bacterial diseases? *Plant J.* **12:** 1-7.

Bowles, D.J. (1990). Defense-related proteins in higher plants. *Annu. Rev. Biochem.* **59:** 873-907.

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *J. Exp. Bot.* **48:** 181-199.

Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., Van Daelen, R., Van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P. (1997). The barley *Mlo* gene: a novel control element for plant pathogen resistance. *Cell* **88:** 695-705.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88:** 57-63.

Cassab, G.I. and Varner, J.E. (1988). Cell wall proteins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **39:** 321-353.

Chandra, S., Stennis, M. and Low, P.S. (1997). Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. *J. Biol. Chem.* **272:** 28274-28280.

Charkowski, A.O., Alfano, J.R., Preston, G., Yuan, J., He, S.Y. and Collmer, A. (1998). *Pseudomonas syringae* pv. *tomato* secretes a protein via the Hrp (type III) pathway that has domains similar to harpins and pectate lyases and the capacity to elicit the plant hypersensitive response and to bind to pectate. *J. Bacteriol.* **180:** 5211-5217.

Chester, K.S. (1933). The problem of acquired physiological immunity in plants. *Quart. Rev. Biol.* **8:** 129-154, 274-324.

Chinnaiyan, A.M. and Dixit, V.M. (1996). The cell-death machine. *Curr. Biol.* **6:** 555-562.

Clark, J.M (1988). Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl. Acids Res.* **16:** 9677-9686.

Collinge, D.B. and Slusarenko, A.J. (1987). Plant gene expression in response to pathogens. *Plant Mol. Biol.* **9:** 389-410.

Collmer, A. (1998). Determinants of pathogenicity and avirulence in plant pathogenic bacteria. *Curr. Opin. Plant Biol.* **1:** 329-335.

Collmer, A. and Bauer, D.W. (1994). *Erwinia chrysanthemi* and *Pseudomonas syringae*: plant pathogens trafficking in extracellular virulence proteins. In: Dangl. L. (ed.) Current Topics in Microbiology and Immunology, vol. 192: Bacterial pathogenesis of plants and animals - molecular and cellular mechanisms, pp. 43-78. Springer-Verlag, Berlin.

Condit, C.M. and Meagher, R.B. (1987). Expression of a gene encoding a glycine-rich protein in petunia. *Mol. Cell. Biol.* **7:** 4273-4279.

Corbin, D.R., Sauer, N. and Lamb, C.J. (1987). Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. *Mol. Cell. Biol.* **7:** 4337-4444.

Cornelis, G.R. (1994). *Yersinia* pathogenicity factors. In: Dangl. L. (ed.) Current Topics in Microbiology and Immunology, vol. 192: Bacterial pathogenesis of plants and animals - molecular and cellular mechanisms, pp. 243-263. Springer-Verlag, Berlin.

Cruickshank, I.A.M. (1963). Phytoalexins. *Annu. Rev. Phytopathol.* **1:** 351-374.

Dangl, J.L. (1994). The enigmatic avirulence genes of phytopathogenic bacteria. In: Dangl. L. (ed.) Current Topics in Microbiology and Immunology, vol. 192: Bacterial pathogenesis of plants and animals - molecular and cellular mechanisms, pp. 99-118. Springer-Verlag, Berlin.

Dangl, J.L. (1995). Pièce de Résistance: novel classes of plant disease resistance genes. *Cell* **80:** 363-366.

Dangl, J.L., Dietrich, R.A. and Richberg, M.H. (1996). Death don't have no mercy: cell death programs in plant-microbe interactions. *Plant Cell* **8:** 1793-1807.

Daniels, M.J., Dow, J.M. and Osbourn, A.E. (1988). Molecular genetics of pathogenicity in phytopathogenic bacteria. *Annu. Rev. Phytopathol.* **26:** 285-312.

Das, A. (1990). Overproduction of proteins in *Escherichia coli*: vectors, hosts and strategies. *Methods Enzymol.* **182:** 93-112.

Davis, K.R. and Hahlbrock, K. (1987). Induction of defense responses in cultured parsley cells by plant cell wall fragments. *Plant Physiol.* **85:** 1286-1290.

De Wit, P.J.G.M. (1995). Fungal avirulence genes and plant resistance genes: unravelling he molecular basis of gene-for-gene interactions. *Adv. Bot. Res.* **21:** 147-185.

De Wit, P.J.G.M. (1997). Pathogen avirulence and plant resistance: a key role for recognition. *Trends in Plant Sci.* **2:** 452-457.

Dean, R.A. and Kuc, J. (1985). Induced systemic protection in plants. *Trends in Biotechnol.* **3:** 125-129.

Del Pozo, O. and Lam, E. (1999). Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Curr. Biol.* **8:** 1129-1132.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. *Science* **266:** 1247-1249.

Desikan, R., Clarke, A., Atherfold, P., Hancock, J.T. and Neill, S.J. (1999). Harpin induces mitogen-activated protein kinase activity during defense responses in *Arabidopsis thaliana* suspension cultures. *Planta* **210:** 97-103.

Desikan, R., Hancock, J.T., Coffey, M.J. and Neill, S.J. (1996). Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. *FEBS Lett.* **382:** 213-217.

Desikan, R., Reynolds, A., Hancock, J.T. and Neill, S.J. (1998). Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. *Biochem. J.* **330:** 115-120.

Dieffenbach, C.W., Dveksler, G.S. and Rashtchian, A. (1995a). PCR starting from RNA: Introduction. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 287-292. Cold Spring Harbor Laboratory Press, New York.

Dieffenbach, C.W., Lowe, T.M.J. and Dveksler, G.S. (1995b). General concepts for PCR primer design. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 133-142. Cold Spring Harbor Laboratory Press, New York. Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C. and Dangl, J.L. (1997). A novel zinc finger protein is encoded by the *Arabidopsis LSD1* gene and functions as a negative regulator of plant cell death. *Cell* **88:** 685-694.

Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C. and Dangl, J.L. (1997). A novel zinc finger protein is encoded by the *Arabidopsis LSD1* gene and functions as a negative regulator of plant cell death. *Cell* **88:** 685-694.

Dixon, R.A. (1986). The phytoalexin response: elicitation, signalling and the control of host gene expression. *Biol. Rev.* **61:** 239-291.

Dixon, R.A. and Harrison, M.J. (1990). Activation, structure and organization of genes involved in microbial defense in plants. *Adv. Genet.* **28:** 165-233.

Dixon, R.A., Harrison, M.J., and Lamb, C.J. (1994). Early events in the activation of plant defense responses. *Annu. Rev. Phytopathol.* **32:** 479-501.

Dixon, R.A. and Lamb, C.J. (1990). Molecular communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41:** 339-367.

Doke, N. (1983). Involvement of superoxide anion generation in hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophtora infestans*. *Physiol. Plant Pathol.* **23:** 345-347.

Dong, H., Delaney, T.P., Bauer, D.W. and Beer, S.V. (1999). Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* **20:** 207-215.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19:** 11-15.

Dröge-Laser, W., Kaiser, A., Lindsay, W.P., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A. and Lamb, C. (1997). Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. *EMBO J.* **16:** 726-738.

D'Silva, I., Poirier, G.G. and Heath, M.C. (1998). Activation of cysteine proteases in cowpea plants during the hypersensitive response - a form of programmed cell death. *Exp. Cell. Res.* **245:** 389-399.

Ebel, J. and Cosio, E.G. (1994). Elicitors of plant defense responses. *Int. Rev. Cytol.* **148:** 1-36.

Edwards, M.C. and Gibbs, R.A. (1995). Multiplex PCR. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 157-171. Cold Spring Harbor Laboratory Press, New York.

Elhers, S. and Smith, K.A. (1991). Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *J. Exp. Med.* **173:** 25-36.

Ellis, J., Dodds, P. and Pryor, T. (2000). Structure, function and evolution of plant disease resistance genes. *Curr. Opin. Plant Biol.* **3:** 278-284.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K. and Somssich, I.E. (1999). Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J. 18: 4689-4699.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18:** 265-276.

Flor, H.H. (1955). Host-parasite interactions in flax rust - Its genetics and other implications. *Phytopathology* **45:** 680-685.

Flor, H.H. (1971). Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9:** 275-296.

Foley, K.P., Leonard, M.W. and Engel, J.D. (1993). Quantitation of RNA using the polymerase chain reaction. *Trends in Genet.* **9:** 380-385.

Fritig, B., Heitz, T. and Legrand, M. (1998). Antimicrobial proteins in induced plant defense. *Curr. Opin. Immunol.* **10:** 16-22.

Fukuda, H. (1997). Tracheary element differentiation. *Plant Cell* **9:** 1147-1156.

Gabriel, D.W. and Rolfe, B. (1990). Working models of specific recognition in plant-microbe interactions. *Annu. Rev. Phytopathol.* **28:** 365-390.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993). Requirements of salicylic acid for the induction of systemic acquired resistance. *Science* **261:** 754-756.

Galiana, E., Bonnet, P., Conrod, S., Keller, H., Panabières, F., Ponchet, M., Poupet, A. and Ricci, P. (1997). RNase activity prevents growth of a fungal pathogen in tobacco leaves and increases upon induction of systemic acquired resistance with elicitin. *Plant Physiol.* **115:** 1557-1567.

Galindo, J.E., Poirier, G.M.-C., Guo, H., Huvar, A., Wagaman, P.C., Zhu, J., Tench, J., Wan, J.S. and Erlander, M.G. (1997). Differential display of mRNA. In: Caetano-Anollés, G. and Gresshoff, P.M. (eds.). DNA markers: protocols, applications and overviews, pp. 225-236. Wiley-Liss, New York.

Gaudriault, S., Brisset, M.-N. and Barny, M.-A. (1998). HrpW of *Erwinia amylovora*, a new Hrp-secreted protein. FEBS Lett. 28: 224-228.

Gause, W.C. and Adamovicz, J. (1995). Use of PCR to quantitate relative differences in gene expression. In: Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 293-311. Cold Spring Harbor Laboratory Press, New York.

Gebhardt, C. (1997). Plant genes for pathogen resistance - variation on a theme. *Trends in Plant Sci.* **2:** 243-244.

Gendelman, H.E., Friedman, R.M., Joe, S., Baca, L.M., Turpin, J.A., Dveksler, G., Meltzer, M.S. and Dieffenbach, C. (1990). A selective defect of interferon α production in human immunodeficiency virus-infected monocytes. *J. Exp. Med.* **172:** 1433-1442.

Germino, J., Gray, J.G., Charbonneau, H., Vanaman, T. and Bastia, D. (1983). Use of gene fusions and protein-protein interaction in the isolation of a biologically active regulatory protein: the replication initiator protein of plasmid R6K. *Proc. Natl. Acad. Sci. USA* **80:** 6848-6852.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. (1997). Use of *Arabidopsis* for genetic dissection of plant defense responses. *Annu. Rev. Genet.* **31:** 547-569.

Gold, L. (1990). Expression of heterologous proteins in *Escherichia coli*. *Methods Enzymol.* **185:** 11-14.

Gómez-Gómez, L., Felix, G. and Boller, T. (1999). A single locus determines the sensitivity to bacterial flagellin in *Arabidopsis*. *Plant J.* **18:** 277-284.

Gopolan, S., Bauer, D.W., Alfano, J.R., Loniello, A.O., He, S.Y. and Collmer, A. (1996a). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8:** 1095-1105.

Gopolan, S., Wei, W. and He, S.Y. (1996b). *hrp* gene-dependent induction of *hin1*: a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant J.* **10:** 591-600.

Graham, T.L., Kim, J.E. and Graham, M.Y. (1990). Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Mol. Plant-Microbe Interact.* **3:** 157-166.

Gray, J., Close, P.S., Briggs, S.P. and Johal, G.S. (1997). A novel suppressor of cell death in plants encoded by the *lls1* gene of maize. *Cell* **98:** 25-31.

Greenberg, J.T. (1997). Programmed cell death in plant-pathogen interactions. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48:** 525-545.

Greenberg, J.T., Guo, A., Klessig, D.F. and Ausubel, F.M. (1994). Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. *Cell* **77:** 551-563.

Groisman, E.A. and Ochman, H. (1996). Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* **87:** 791-794.

Gross, D. (1991). Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* **29:** 247-278.

Guan, C., Li, P., Riggs, P. and Inouye, H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene* **67:** 21-30.

Hacker, J., Blum-Oehler, G., Muhldorfer, I. and Tschape, H. (1997). Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol. Microbiol.* **23:** 1089-1097.

Hahlbrock, K. (1981). Flavanoids. In: Conn, E. (ed.) The Biochemistry of plants - a comprehensive treatise, vol. 7: Secondary plant products, pp. 425-456. Academic Press, New York.

Hahlbrock, K. and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40:** 347-369.

Hale, A.J., Smith, C.A., Sutherland, L.C., Stoneman, V.E.A., Longthorne, V.L., Culhane, A.C. and Williams, G.T. (1996). Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.* **236:** 1-26.

Ham, J.H., Bauer, D.W., Fouts, D.E. and Collmer, A. (1998). A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proc. Natl. Acad. Sci. USA* **95:** 10206-10211.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996). Resistance gene-dependent plant defense responses. *Plant Cell* **8:** 1773-1791.

Hammond-Kosack, K.E. and Jones, J.D.G. (1997). Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48:** 757-607.

Hanekamp, T., Kobayashi, D., Hayes, S. and Stayton, M.M. (1997). Avirulence gene D of *Pseudomonas syringae* pv. *tomato* may have undergone horizontal gene transfer. *FEBS Lett.* **415:** 40-44.

Hansen, E. and Harper, G. (1997). Preface. In: Hansen, E. and Harper, G. (eds.) Differentially expressed genes in plants: a bench manual, p. ix. Taylor and Francis, London.

Hattingh, M.J., Roos, I.M.M. and Mansvelt, E.L. (1989). Infection and systemic invasion of deciduous fruit trees by *Pseudomonas syringae* in South Africa. *Plant Disease* **73:** 784-789.

Hausladen, A. and Stamler, J.S. (1998). Nitric oxide in plant immunity. *Proc. Natl. Acad. Sci. USA* **95:** 10345-10347.

He, S.Y. (1996). Elicitation of plant hypersensitive response by bacteria. *Plant Physiol.* **112:** 865-869.

He, S.Y., Huang, H.-C. and Collmer, A. (1993). *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. *Cell* **73:** 1255-1266.

Heath, M.C. (1998). Apoptosis, programmed cell death and the hypersensitive response. *Eur. J. Plant Pathol.* **104:** 117-124.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. *Curr. Opin. Plant Biol.* **83:** 315-319.

Heath, M.C., Howard, R.J., Valent, B. and Chumley, F.G. (1992). Ultrastructural interactions of one strain of *Magnaporthe grisea* with goosegrass and weeping lovegrass. *Can. J. Bot.* **70:** 779-787.

Heil, M. (1999). Systemic acquired resistance: available information and open ecological questions. *J. Ecology* **87:** 341-346.

Heu, S. and Hutcheson, S.W. (1993). Nucleotide sequence and properties of the hrmA locus associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. *Mol. Plant-Microbe Interact.* **6:** 553-564.

Heyns, A.J. (1960). Bacterial canker of apricot trees. *S. Afr. J. Agric. Sci.* **3:** 449-457.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology* **10:** 413-417.

Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993). Kinetic PCR: real time monitoring of DNA amplification reactions *Bio/Technology* **11:** 1026-1030.

Hirano, S.S. and Upper, C.D. (1990). Population biology and epidemiology of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* **28:** 155-177.

Holmes, F.O. (1929). Local lesions in tobacco mosaic. *Bot. Gaz.* **87:** 39-55.

Hooykans, P.J.J. and Beijersbergen, A.G.M. (1994). The virulence system of *Agrobacterium tumefaciens*. *Annu. Rev. Phytopathol.* **32:** 157-179.

Hoyos, M.E., Stanley, C.M., He, S.Y., Pike, S., Pu, X.-A. and Novacky, A. (1996). The interaction of $Harpin_{Pss}$ with plant cell walls. *Mol. Plant-Microbe Interact.* **9:** 608-616.

Huang, H.C., Hutcheson, S.W. and Collmer, A. (1991). Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and TnphoA tagging of genes encoding exported membrane-spanning proteins. *Mol. Plant-Microbe Interact.* **4:** 469-476.

Huang, H.C., Schuurink, R., Denny, T.P., Atkinson, M.M., Baker, C.J., Yucel, I., Hutcheson, S.W. and Collmer, A. (1988). Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *J. Bacteriol.* **170:** 4748-4756.

Hunt, M.D., Delaney, T.P., Dietrich, R.A., Weymann, K.B., Dangl, J.L. and Ryals, J.A. (1997). Salicylate-independent lesion formation in *Arabidopsis lsd* mutants. *Mol. Plant-Microbe Interact.* **10:** 531-536.

Hunt, M.D., Neuenschwander, U.H., Delaney, T.P., Weymann, K.B., Friedrich, L.B., Lawton, K.A., Steiner, H.-Y. and Ryals, J.A. (1996). Recent advances in systemic acquired resistance research - a review. *Gene* **179:** 89-95.

Hunt, M.D. and Ryals, J.A. (1996). Systemic acquired resistance signal transduction. *Crit. Rev. Plant Sci.* **15:** 583-606.

Ji, C., Smith-Becker, J. and Keen, N.T. (1998). Genetics of plant-pathogen interactions. *Curr. Opin. Biotechnol.* **9:** 202-207.

Johal, G.S. and Briggs, S.P. (1992). Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* **258:** 985-987.

Jones, A. (2000). Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? *Trends in Plant Sci.* **5:** 225-230.

Jones, P., Qui, J. and Rickwood, D. (1994). RNA isolation and analysis. BIOS Scientific Publishers, Oxford.

Kawalleck, P., Schmelzer, E., Hahlbrock, K. and Somssich, I.E. (1995). Two pathogen-responsive genes in parsley encode a tyrosine-rich hydroxyproline-rich glycoprotein (HRGP) and an anionic peroxidase. *Mol. Gen. Genet.* **247:** 444-452.

Keen, N.T. (1982). Specific recognition in gene-for-gene host-parasite systems. *Adv. Plant Pathol.* **2:** 35-82.

Keen, N.T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D. and Staskawicz, B. (1990). Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. *Mol. Plant-Microbe Interact.* **3:** 112-121.

Keith, L.W., Boyd, C., Keen, N.T. and Partridge, J.E. (1997). Comparison of *avrD* alleles from *Pseudomonas syringae* pv. *glycinea*. *Mol. Plant-Microbe Interact.* **10:** 416-422.

Kessman, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S. and Ryals, J. (1994). Induction of systemic acquired disease resistance in plants by chemicals. *Annu. Rev. Phytopathol.* **32:** 439-459.

Kim, J.F. and Beer, S.V. (1998). HrpW of *Erwinia amylovora*, a new harpin that is a member of a proposed class of pectate lyases. *J. Bacteriol.* **180:** 5203-5210.

Kinoshita, T., Imamura, J., Nagai, H., and Shimotohno, K. (1992). Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal. Biochem.* **206:** 231-235.

Klement, Z. (1963). Method for the rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature* **199:** 299-300.

Klement, Z. (1982). Hypersensitivity. In: Mount, M.S. and Lacy, G.H. (eds.) Phytopathogenic Prokaryotes, vol. 2, pp. 149-177. Academic Press. New York.

Klement, Z., Farkas, G.L. and Lovrekovich, L. (1964). Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology* **54:** 474-477.

Klüsener, B. and Weiler, E.W. (1999). Pore-forming properties of elicitors of plant defense reactions and cellulolytic enzymes. *FEBS Lett.* **459:** 263-266.

Köhle, H., Jelick, W., Poten, F., Blaschek, W. and Kauss, H. (1985). Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. *Plant Physiol.* **77:** 544-551.

Kolattukudy, P.E., Mohan, R., Bajar, M.A. and Sherf, B.A. (1992). Plant peroxidase gene expression and function. *Biochem. Soc. Trans.* **20:** 333-337.

Kombrink, E., Beerhues, L., Garcia-Garcia, F., Hahlbrock, K., Müller, M., Schröder, M., Witte, B. and Schmelzer, E. (1993). Expression patterns of defense-related genes in infected and uninfected plants. In: Fritig, B. and Legrand, M. (eds.) Mechanisms of plant defense responses, pp. 236-249. Kluwer Academic Publishers, Dordrecht.

Kombrink, E. and Somssich, I.E. (1995). Defense responses of plants to pathogens. *Adv. Bot. Res.* **21:** 2-33.

Kuc, J. (1972). Phytoalexins. *Annu. Rev. Phytopathol.* **10:** 207-232.

Kuc, J. (1982). Induced immunity to plant disease. *BioScience* **32:** 854-860.

Kuc, J. (1995). Phytoalexins, stress metabolism, and disease resistance in plants. *Annu. Rev. Phytopathol.* **33:** 275-297.

Lacomme, C. and Santa Cruz, S. (1999). Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proc. Natl. Acad. Sci. USA* **96:** 7956-7961.

Lamb, C.J. (1994). Plant disease resistance genes in signal perception and transduction. *Cell* **76:** 419-422.

Lamb, C.J. and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48:** 251-275.

Lamb, C.J., Lawton, M.J., Dron, M. and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56:** 215-224.

Lawrence, J.G. and Roth, J.R. (1993). Selfish operons: horizontal transfer may drive the evolution of gene clusters. *Genetics* **143:** 1843-1860.

Lawton, M.A. and Lamb, C.J. (1987). Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. *Mol. Cell. Biol.* **7:** 335-341.

Leach, J.E. and White, F.F. (1996). Bacterial avirulence genes. *Annu. Rev. Phytopathol.* **43:** 153-179.

Lee, H.S., Singh, A. and Koa, T.H. (1992). RNase X2, a pistil-specific ribonuclease from *Petunia inflata*, shares sequence similarity with solanaceous S proteins. *Plant Mol. Biol.* **20:** 1131-1141.

Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. and Pardee, A.B. (1992). Differential display and cloning of messenger RNAs from human breast cancer *versus* mammalian epithelial cells. *Cancer Res.* **52:** 6966-6968.

Liang, P., Averboukh, L. and Pardee, A.B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucl. Acids Res.* **21:** 3269-3275.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257:** 967-971.

Liang, P. and Pardee, A.B. (1995). Recent advances in differential display. *Curr. Opin. Immunol.* **7:** 274-280.

Ligterink, W., Kroj, T., Zur Nieden, U., Hirt, H. and Scheel, D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. *Science* **276:** 2054-2057.

Lindgren, P.B. (1997). The role of *hrp* genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* **35:** 129-152.

Lindgren, P.B., Peet, R.C. and Panopoulos, N.J. (1986). Gene cluster of *Pseudomonas syringae* pv. *"phaseolicola"* controls pathogenicity on bean and hypersensitivity on non-host plants. *J. Bacteriol.* **168:** 512-522.

Linthorst, H.J.M. (1991). Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* **10:** 123-150.

Lockhardt, D.J., Dong, H., Bryne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. and Brown, E.L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* **14:** 1675-1680.

Lorang, J.M. and Keen, N.T. (1995). Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato*: a *hrp*-linked avirulence locus consisting of at least two transcriptional units. *Mol. Plant-Microbe Interact.* **8:** 49-57.

Lusso, M. and Kuc, J. (1995). Increased activities of ribonuclease and protease after challenge in tobacco plants with induced systemic resistance. *Physiol. Mol. Plant Pathol.* **47:** 419-428.

Maina, C.V., Riggs, P.D., Grandea, A.G. III, Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, L.A. and Guan, C. (1988). A vector to express and purify foreign proteins in *Escherichia coli* by fusion to, and separation from, maltose binding protein. *Gene* **74:** 365-373.

Maleck, K. and Dietrich, R.A. (1999). Defense on multiple fronts: how do plants cope with diverse enemies? *Trends in Plant Sci.* **4:** 215-219.

Maleck, K. and Lawton, K. (1998). Plant strategies for resistance to pathogens. *Curr. Opin. Biotechnol.* **9:** 208-213.

Mansfield, J., Jenner, C., Hockenhull, R., Bennet, M.A. and Stewart, R. (1994). Characterization of *avrPphE*, a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY*, a new hrp gene identified in the halo-blight bacterium. *Mol. Plant-Microbe Interact.* **7:** 726-739.

Marshall, A. and Hodgson, J. (1998). DNA chips: an array of possibilities. *Nature Biotechnology* **16:** 27-31.

Marston, A.O. (1986). The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240:**1-12.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262:** 1432-1436.

McClelland, M. and Welsh, J. (1995). RNA fingerprinting using arbitrarily primed PCR. In Dieffenbach, C.W. and Dveksler, G.S. (eds.). PCR primer: a laboratory manual, pp. 213-234. Cold Spring Harbor Laboratory Press, New York.

McDowell, J.M. and Dangl, J.L. (2000). Signal transduction in the plant immune response. *Trends in Biochem. Sci.* **25:** 79-82.

McDowell, J.M., Huang, S., McKinney, E.C., An, Y.-Q. and Meagher, R.B. (1996). Structure and evolution of the actin gene family in *Arabidopsis thaliana*. *Genetics* **142:** 587-602.

Meagher, R.B. (1991). Divergence and differential expression of actin gene families in higher plants. *Int. Rev. Cytol.* **125:** 139-163.

Melchers, L.S. and Stuiver, M.H. (2000). Novel genes for disease-resistance breeding. *Curr. Opin. Plant Biol.* **3:** 147-152.

Men, A.E. and Gresshoff, P.M. (1998). Efficient cloning of DAF polymorphic markers from silver stained polyacrylamide gels. *BioTechniques* **24:** 593-595.

Mezei, L.M. and Storts, D.R. (1994). Cloning of PCR products. In: Griffin, H.G. and Griffin, A.M. (eds.) PCR technology: current innovations. CRC Press, Boca Raton, FL, USA. pp. 21-27.

Mitsuhara, I., Malik, K.A., Miura, M. and Ohashi, Y. (1999). Animal cell-death suppressors Bcl-x_L and Ced-9 inhibit cell death in tobacco plants. *Curr. Biol.* **9:** 775-778.

Mittler, R., Herr, E.H., Orvar, B.L., Van Camp, W., Willkens, H. and Ellis, B.E. (1999). Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proc. Natl. Acad. Sci. USA* **96:** 14156-14170.

Mou, I., Miller, H., Li, J., Wang, F. and Califour, L. (1994). Improvements to the differential display method for gene analysis. *Biochem. Biophys. Res. Comm.* **199:** 564-569.

Muller, K.O. (1961). The phytoalexin concept and its methodological significance. *Recent Adv. Bot.* **1:** 396-400.

Murphy, L.D., Herzog, C.E., Rudick, J.B., Fojo, A.T. and Bates, S.E. (1990). Use of the polymerase chain reaction in the quantitation of *mdr*-1 gene expression. *Biochemistry* **29:** 10351-10356.

Nairn, C.J., Winesett, L. and Ferl, R.J. (1988). Nucleotide sequence of an actin gene from *Arabidopsis thaliana*. *Gene* **65:** 247-257.

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H. and Ryals, J. (1995). Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *Plant J.* **8:** 227-233.

New England BioLabs (1993). pMAL™-2 expression system technical manual. New England BioLabs, Beverley, MA.

Nothnagel, E.A., McNeil, M., Albersheim, P. and Dell, A. (1983). Host-pathogen interactions XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiol.* **71:** 916-926.

Osbourn, A.E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **8:** 1821-1831.

Owttrim, G.W., Hofmann, S. and Kuhlemeier, C. (1991). Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco. *Nucl. Acids. Res.* **19:** 5491-5496.

Owttrim, G.W., Mandel, T., Trachsel, H., Thomas, A.A.M. and Kuhlemeier, C. (1994). Characterization of the tobacco eIF-4A family. *Plant. Mol. Biol.* **26:** 1747-1757.

Panopoulos, N.J. and Peet, R.C. (1985). The molecular genetics of plant pathogenic bacteria and their plasmids. *Annu. Rev. Phytopathol.* **23:** 381-419.

Parker, E. and Coleman, M.J. (1997). Molecular intimacy between proteins specifying plant-pathogen recognition. *Trends in Biol. Sci.* **22:** 291-296.

Paul, N.D., Hatcher, P.E. and Taylor, J.E. (2000). Coping with multiple enemies: an integration of molecular and ecological perspectives. *Trends in Plant Sci.* **5:** 220-225.

Paxton, J.D., Groth, J. and Graham, T. (1994). Constraints on pathogens attacking plants. *Crit. Rev. Plant. Sci.* **13:** 77-95.

Peñaloza-Vázquez, A., Rangaswamy, V., Ullrich, M., Bailey, A.M. and Bender, C.L. (1996). Use of translational fusions to the maltose-binding protein to produce and purify proteins in *Pseudomonas syringae* and assess their activity *in vivo*. *Mol. Plant-Microbe Interact.* **9:** 637-641.

Peña-Cortés, H., Fisahn, J. and Willmitzer, L. (1995). Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. *Proc. Natl. Acad. Sci. USA* **92:** 4106-4113.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J.-P., Manners, J.M. and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* **8:** 2309-2323.

Peumans, W.J. and Van Damme, E.J.M. (1995). The role of lectins in plant defence. *Histochem. J.* **27:** 253-271.

Pieretti, M., Zhang, F., Fu, Y.-H., Warren, S.T., Oostra, B.A., Caskey, C.T. and Nelson, D.L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* **66:** 817-822.

Pieterse, C.J.M. and Van Loon, L.C. (1999). Salicylic acid-independent plant defence pathways. *Trends in Plant Sci.* **4:** 52-58.

Pieterse, C.M.J., Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A. and Van Loon, L.C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8:** 1225-1237.

Pirhonen, M.U. et al. (1996). Phenotypic expression of *Pseudomonas syringae avr* genes in *E. coli* is linked to the activities of the *hrp*-encoded secretion system. *Mol. Plant-Microbe Interact.* **9:** 252-260.

Pollard, T.D. and Cooper, J.A. (1986). Actins and actin binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* **55:** 987-1035.

Popham, P., Pike, S. and Novacky, A. (1995). The effect of harpin from *Erwinia amylovora* on the plasmalemma of suspension-cultured tobacco cells. *Physiol. Mol. Plant Pathol.* **47:** 39-50.

Preston, G., Deng, W.-L., Huang, H.-C. and Collmer, A. (1998). Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* **180:** 4532-4537.

Preston, G., Huang, H.-C., He, S.Y. and Collmer, A. (1995). The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. *Mol. Plant- Microbe Interact.* **8:** 717-732.

Promega Corporation (1996a). Promega technical manual no. 042: pGEM®-T vector system. Promega Corporation, Madison, WI.

Promega Corporation (1996b). Promega technical manual no. 117: Wizard DNA miniprep system. Promega Corporation, Madison, WI.

Puri, N., Jenner, C., Bennet, M., Stewart, R., Mansfield, J., Lyons, N. and Taylor, J. (1997). Expression of *avrPphB*, an avirulence gene from *Pseudomonas syringae* pv. *phaseolicola*, and the delivery of signals causing the hypersensitive reaction in bean. *Mol. Plant-Microbe Interact.* **10:** 247-256.

Qui, D., Wei, Z.-M., Bauer, D.W. and Beer, S.V. (1997). Treatment of tomato seed with harpin enhances germination and growth and induces resistance to *Ralstonia solanacearum*. *Phytopathology* **87:** S80.

Raff, M.C. (1992). Social controls on cell survival and cell death. *Nature* **356:** 397-400.

Raggi, V. (1998). Hydroxyproline-rich glycoprotein accumulation in TMV-infected tobacco showing systemic acquired resistance to powdery mildew. *J. Phytopathology* **146:** 321-325.

Ramsay, G. (1998). DNA chips: state-of-the-art. *Nature Biotechnology* **16:** 40-44.

Reymond, P. and Farmer, E.E. (1998). Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1:** 404-411.

Ries, G.F. de W., Appel, M. and Bellstedt, D.U. (1994). *Fusarium solani* infection induces the formation of chitinases and β-1,3-glucanases in potato tubers. Poster presented at the 12th congress of the South African Biochemical Society. Stellenbosch, South Africa, January 1994.

Ririe, K., Rasmussen, R.P. and Wittwer, C.T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* **245:** 154-160.

Roche Diagnostics (1999). Roche Molecular Diagnostics PCR Applications Manual, 2nd ed. Roche Diagnostics, Mannheim.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romantschuk, M. and He, S.Y. (1997). Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA* **94:** 3459-3464.

Romeis, T., Piedras, P. and Jones, J.D.G. (2000). Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell* **12:** 803-815.

Roos, I.M.M. and Hattingh, M.J. (1983a). Bacterial canker of stone fruit in South Africa. *Deciduous Fruit Grower* **3:** 405-409.

Roos, I.M.M. and Hattingh, M.J. (1983b). Fluorescent pseudomonads associated with bacterial canker of stone fruit in South Africa. *Plant Disease* **67:** 1267-1269.

Roos, I.M.M. and Hattingh, M.J. (1987). Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. *Phytopathology* **77:** 900-908.

Roos, I.M.M., Mansvelt, E.L. and Hattingh, M.J. (1993). Pathological anatomy of infection and systemic invasion of deciduous fruit trees by bacterial pathogens. In: Biggs, A.R. (ed.). Handbook of cytology, histology and histochemistry of fruit tree diseases, pp. 281-298. CRC Press, Boca Ranton.

Ross, A.F. (1961a). Localized acquired resistance to plant virus infection in hypersensitive hosts. *Virology* **14:** 329-339.

Ross, A.F. (1961b). Systemic acquired resistance induced by localized virus infections in plants. *Virology* **14:** 340-358.

Roy, S. and Nicholson, D.W. (2000). Programmed cell-death regulation: basic mechanisms and therapeutic opportunities. *Mol. Med. Today* **6:** 264-266.

Rusnak, F., Sakaitani, M., Drueckhammer, D., Reichert, J. and Walsh, C.T. (1991). Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. *Biochemistry* **30:** 2916-2927.

Ryals, J., Lawton, K.A., Delaney, T., Friedrich, L., Kessmann, H., Neuenschwander, U., Uknes, S., Vernooij, B. and Weyman, K. (1995). Signal transduction in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA* **92:** 4202-4205.

Ryals, J., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y. and Hunt, M.D. (1996). Systemic acquired resistance. *Plant Cell* **8:** 1809-1819.

Ryals, J.A., Uknes, S. and Ward, E. (1994). Systemic acquired resistance. *Plant Physiol.* **104:** 1109-1112.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.-Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P. and Uknes, S. (1997). The *Arabidopsis NIM1* protein shows homology to the mammalian transcription factor inhibitor IκB. *Plant Cell* **9:** 425-439.

Salmond, G.P.C. (1994). Secretion of extracellular virulence factors by plant pathogenic bacteria. *Annu. Rev. Phytopathol.* **32:** 181-200.

Salmond, G.P.C. and Reeves, P.J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. *Trends in Biol. Sci.* **18:** 7-12.

Sargent, T. (1987). Isolation of differentially expressed genes. *Methods Enzymol.* **152:** 423-432.

Sauer, N., Corbin, D.R., Keller, B. and Lamb, C.J. (1990). Cloning and characterization of a wound-specific hydroxyproline-rich glycoprotein in *Phaseolus vulgaris*. *Plant Cell. Environ.* **13:** 257-266.

Scheel, D. and Parker. J.E. (1990). Elicitor recognition and signal transduction in plant defense gene activation. *Z. Naturforsch* **45:** 569-575.

Schneider, M., Schweizer, P., Meuwly, P. and Métraux, J.P. (1996). Systemic acquired resistance in plants. *Intl. Rev. Cytol.* **168:** 303-340.

Schroth, M.N., Hildebrand, D.C. and Panopoulos, N.J. (1992). Phytopathogenic pseudomonads and related plant-associated pseudomonads. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H. (eds.). The Prokaryotes (2nd ed.), vol. 3, pp. 3104-3131. Springer-Verlag, New York.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W. and Staskawicz, B.J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274:** 2063-2065.

Seehaus, K. and Tenhaken, R. (1998). Cloning of genes by mRNA differential display induced during the hypersensitive reaction of soybean after inoculation with *Pseudomonas syringae* pv. *glycinea*. *Plant Mol. Biol.* **38:** 1225-1234.

Shah, D.M., Hightower, R.C. and Meagher, R.B. (1983). Genes encoding actin in higher plants: intron positions are highly conserved but the coding sequences are not. *J. Mol. Appl. Genet.* **2:** 111-126.

Shaner, G., Stromber, E.L., Lacy, G.H., Barker, K.R. and Pirone, T.P. (1992). Nomenclature and concepts of pathogenicity and virulence. *Annu. Rev. Phytopathol. 30:* 47-66.

Sheen, J. (1996). Ca^{2+}-dependent protein kinase and stress signal transduction. *Science* **274:** 1900-1902.

Sheng, J., D'Ovidio, R. and Mehdy, M.C. (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. *Plant J.* **3:** 345-354.

Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E. and Lamb, C.J. (1985). Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. *Proc. Natl. Acad. Sci. USA* **82:** 6551-6555.

Shuman, H.A., Silhavy, T.J. and Beckwith, J.R. (1980). Labeling of proteins with β-galactosidase by gene fusion. *J. Biol. Chem.* **255:** 168-174.

Sigee, D.C. (1993). Bacterial plant pathology: cell and molecular aspects. Cambridge University Press, Cambridge.

Smith, M.C., Furman, T.C., Ingolia, T.D., Pidgeon, C. (1988). Chelating peptide-immobilized metal ion affinity chromatography. *J. Biol. Chem.* **263:** 7211-7215.

Smith, D.B. and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. *Gene* **67:** 31-40.

Staiger, C.J. and Shliwa, M. (1987). Actin localization and function in higher plants. *Protoplasma* **141:** 1-12.

Stakman, E.C. (1915). Relation between *Puccia graminis* and plants highly resistant to its attack. *J. Agric. Res.* **4:** 193-299.

Staskawicz, B.J., Dahlbeck, D. and Keen, N.T. (1984). Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. *Proc. Natl. Acad. Sci. USA* **81:** 6024-6028.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267:** 1445-1449.

Sticher, L., Mauch-Mani, B. and Métraux, J.P. (1997). Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35:** 235-270.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. and Fritig, B. (1993). Plant pathogenesis-related proteins and their role in defense against pathogens. *Biochimie* **75:** 687-706.

Strobel, N.E., Ji, C., Gopolan, S., Kuc, J.A. and He, S.Y. (1996). Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein. *Plant J.* **9:** 431-439.

Suggs, S.V., Hirose, T., Myake, E.H., Kawashima, M.J., Johnson, K.I. and Wallace, R.B. (1991). Using purified genes. *ICN-UCLA Symp. Mol. Cell. Biol.* **23:** 683-693.

Sutherland, M.W. (1991). The generation of oxygen radicals during host plant responses to infection. *Physiol. Mol. Plant Pathol.* **39:** 79-93.

Suzuki, K., Suzuki, N., Ohme-Takagi, M. and Shinshi, H. (1998). Immediate early induction of mRNAs for ethylene-responsive transcription factors in tobacco leaf strips after cutting. *Plant J.* **15:** 657-665.

Svetic, A., Finkelman, F.D., Jian, Y.C., Dieffenbach, C.W., Scott, D.E., McCarthy, K.F., Steinberg, A.D. and Gause, W.C. (1991). Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. *J. Immunol.* **147:** 22391-2397.

Szczyglowski, K., Hamburger, D., Kapranov, P. and De Bruijn, F. (1997). Construction of a *Lotus japonicus* late nodulin expressed sequence tag library and identification of novel nodule-specific genes. *Plant Physiol.* **114:** 1335-1346.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **17:** 1105-1109.

Taiz, L. and Zeiger, E. (1991). Plant physiology. The Benjamin/Cummings Publishing Company, Redwood city.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y. and Martin, G.B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274:** 2060-2063.

Taylor, C.B., Bariola, P.A., delCardaré, S.B., Raines, R.T. and Green, P.J. (1993). RNS2: a senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. *Proc. Natl. Acad. Sci. USA* **90:** 5118-5122.

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. (1995). Function of the oxidative burst in hypersensitive disease resistance. *Proc. Natl. Acad. Sci USA* **92:** 4158-4163.

Tsuji, J., Jackson, E., Gage, D., Hammerschmidt, R., Sommerville, S.C. (1992). Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive response to *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* **98:** 1304-1309.

Uhlén, M. and Moks, T. (1990). Gene fusions for purpose of expression: an introduction. *Methods Enzymol.* **185:** 129-143.

Uknes, S., Lawton, K., Ward, E., Gaffney, T., Friedrich, L., Alexander, D., Goodman, R., Métraux, J.-P., Kessmann, H., Goy, P.A., Rella, M.G. and Ryals, J. (1993). The molecular biology of systemic acquired resistance. In: Gresshoff, P.M. (ed.) Plant responses to the environment, pp. 1-9. CRC Press, Boca Ranton.

Ullmann, A. (1984). One-step purification of hybrid proteins which have β-galactosidase activity. *Gene* **29:** 27-31.

Van der Biezen, E.A. and Jones, J.D.G. (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. *Curr. Biol.* **8:** 226-227.

Van Gijsegem, F., Genin, S. and Boucher, C.A. (1993). Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. *Trends in Microbiol.* **1:** 175-180.

Van Loon, L.C. (1985). Pathogenesis-related proteins. *Plant Mol. Biol.* **4:** 111-116.

Van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994). Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Rep.* **12:** 245-264.

Van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van't Westende, Y.A.M., Hartog, F. and Van Loon, L.C. (1997). Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* **10:** 761-768.

Vance, C.P., Kirk, T.K. and Sherwood, R.T. (1980). Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopathol.* **18:** 259-288.

VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994). Two classes for plant antibiotics: phytoalexins versus "phytoanticipins". *Plant Cell* **6:** 1191-1192.

Vivian, A. and Gibbon, M.J. (1997). Avirulence genes in plant-pathogenic bacteria: signals or weapons? *Microbiology* **143:** 693-704.

Wan, J.S., Sharp, S.J., Poirier, G.M.-C., Wagaman, P., Chambers, J., Pyati, J., Hom, Y.-L., Galindo, J.E., Huvar, A., Peterson, P.A., Jackson, M.R. and Erlander, M.G. (1996). Cloning differentially expressed mRMAs. *Nature Biotechnology* **14:** 1685-1691.

Wang, W.-C. and Liu, Z.-H. (1999). $Harpin_{Pss}$-induced peroxidase and lignin accumulation in tobacco during the hypersensitive response. *Aust. J. Plant Physiol.* **26:** 265-272.

Wattiau, P., Bernier, B., Deslee, P., Michiels, T., Cornelis, G.R. (1994). Individual chaperones required for Yop secretion by *Yersinia*. *Proc. Natl. Acad. Sci. USA* **91:** 10493-10497.

Wei, W., Plovanich-Jones, A., Deng, W.-L., Jin, Q.-L., Collmer, A., Huang, H.-C. and He, S.Y. (2000). The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*. *Proc. Natl. Acad. Sci. USA* **97:** 2247-2252.

Wei, Z.-M. and Beer, S.V. (1996). Harpin from *Erwinia amylovora* induces plant resistance. *Acta Hortic.* **411:** 223-225.

Wei, Z.-M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A. and Beer, S.V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257:** 85-88.

Welsh, J., Chada, K., Dalal, S.S., Cheng, R., Ralph, D. and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucl. Acids Res.* **20:** 4965-4870.

White, T.J., Burns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds.) PCR protocols, pp. 315-322. Academic Press, London.

White, F.F., Yang, B. and Johnson, L.B. (2000). Prospects for understanding avirulence gene function. *Curr. Opin. Plant Biol.* **3:** 291-298.

Willis, D.K., Rich, J.J. and Hrabak, E.M. (1991). *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* **4:** 132-138.

Wittwer, C.T., Herrmann, M.G., Moss, A.A. and Rasmussen, R.P. (1997). Continuous fluorescence monitoring of rapid cycle DNA amplifications. *BioTechniques* **22:** 130-138.

Xie, Z. and Chen, Z. (2000). Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. *Mol. Plant-Microbe Interact.* **13:** 183-190.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.* **36:** 1251-1275.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. and Nishiuchi, Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: Proposal of *Ralstonia pickettii* (Ralson, Palleroni and

Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol. Immunol.* **39:** 897-904.

Yamamoto, S., Suzuki, K. and Shinshi, H. (1999). Elicitor-responsive, ethylene-independent activation of GCC box-mediated transcription that is regulated by both protein phosphorylation and dephosphorylation in cultured tobacco cells. *Plant J.* **20:** 571-579.

Yang, Y. and Gabriel, D.W. (1996). *Xanthomonas* avirulence/pathogenicity gene family encodes functional nuclear targeting signals. *Mol. Plant-Microbe Interact.* **8:** 627-631.

Yang, Y. and Klessig, D.F. (1996). Isolation and characterization of a tobacco mosaic virus-inducible *myb* oncogene homolog from tobacco. *Proc. Natl. Acad. Sci. USA* **93:** 14972-14977.

Yoshikawa, M., Yamaoka, N. and Takeuchi, Y. (1993). Elicitors: their significance and primary modes of action in the induction of plant defense reactions. *Plant Cell Physiol.* **34:** 1163-1173.

Young, N.D. (2000). The genetic architecture of resistance. *Curr. Opin. Plant Biol.* **3:** 285-290.

Yuan, J. and He, S.Y. (1996). The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J. Bacteriol.* **178:** 6399-64022.

Zhao, J. and Last, R.L. (1996). Co-ordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis*. *Plant Cell* **8:** 2235-2244.

Zhou, J., Loh, Y.-T., Bressan, R.A. and Martin, G.B. (1995). The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell* **83:** 925-935.

Zhou, J., Tang, X. and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO J.* **16:** 3207-3218.

Zook, M. and Hammerschmidt, R. (1997). Origin of the thiazole ring of camalexin, a phytoalexin from *Arabidopsis thaliana*. *Plant Physiol.* **113:** 463-468.