REGULATION OF THE *VITIS VINIFERA PGIP1* GENE ENCODING A POLYGALACTURONASE-INHIBITING PROTEIN

by

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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.

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SUMMARY

Plant-pathogen interactions have been intensively investigated in the last decade. This major drive towards understanding the fundamental aspects involved in plant disease resistance is propelled by the obvious agricultural and economical benefits that are intrinsically linked to disease and stress resistant plants. It is, therefore, not surprising that fundamental research in this area is not just restricted to model organisms, such as *Arabidopsis* and tobacco, but also extends to more traditional crop plants, such as maize, bean, soybean, apples, grapevine etc. In grapevine for instance, several genes involved in disease resistance have been isolated. One of these genes, encoding for a polygalacturonase inhibiting protein (PGIP), has been studied extensively. PGIPs are cell wall bound, contain leucine rich repeats (LRR) and are found in all dicotyledonous plants so far examined. In most cases, *pgip* genes occur in small multigene families and expression is often tissue specific and developmentally regulated. Up-regulation of PGIP-encoding genes typically occurs upon pathogen infection, treatment with elicitors, salicylic acid (SA), jasmonic acid (JA), cold treatment and wounding. Differential regulation and specificity have been shown to occur between members of the same multigene family. Differential regulation even extends to the utilization of separate pathways to induce *pgip* genes from the same family in response to a single stress stimulus. PGIPs interact with cell wall macerating polygalacturonases (PGs) that are secreted by pathogenic fungi during the infection process. The antifungal action of PGIPs is thought to depend on a dual action. The physical interaction of PGIP with PGs has an inhibitionary effect, resulting in (i) a slower fungal infection rate and (ii) the prolonged existence of long chain oligogalacturonides (OGs). These oligosaccharides are able to elicit a general plant defense response, enabling the plant to further retard or curb the spread of infection.

The main objective of this study was to investigate the regulatory aspects underlying PGIP expression in grapevine. Unlike most characterized PGIP encoding genes from other dicotyledonous plant species, no evidence to support the existence of a *V. vinifera* PGIP multigene family could be found from either genetic or biochemical analyses. Recently, a genomic DNA fragment from *Vitis vinifera* cv Pinotage was

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isolated in our laboratory containing a PGIP encoding open reading frame (ORF) (*Vvpgip1*) and putative 5' upstream regulatory sequences. The spatial and temporal expression pattern of the gene was investigated, as well as the effect of several environmental and pathogenic related stress stimuli on *Vvpgip1* expression. Regulatory mechanisms of *Vvpgip1* include specific *in planta* developmental cues, with environmental stress and pathogen signals superimposed on them. Accordingly, mRNA transcripts of *Vvpgip1* were limited to root and berry tissues and levels varied between berry developmental stages. Cumulative expression was observed in *veráison* berries upon wounding and osmotic stress. Tissue specificity of PGIP expression was also abolished in leaves in response to *Botrytis cinerea* infection, wounding, osmotic stress, auxin [indole acetic acid (IAA)], as well as SA. In addition, expression is down-regulated by a staurosporine-sensitive protein kinase, suggesting the involvement of protein phosphorylation in the signal transduction cascade that leads to PGIP expression. The induced expression profile of *Vvpgip1* in grapevine leaves was also mirrored in transgenic tobacco transformed with *Vvpgip1* regulated by its native promoter. PG inhibition assays using membrane proteins isolated from induced grapevine leaves furthermore conformed to the observed inhibition profile of the *Vvpgip1* gene.

Expression results from the transgenic tobacco plants confirmed that the promoter of the *Vvpgip1* gene was responsible for the observed PGIP expression pattern in grapevine. *In silico* analysis of the promoter area revealed the presence of several defense and stress associated *cis*-acting elements within the 5' upstream region. The core promoter and transcriptional start site was subsequently determined. Transient expression analyses identified several regions involved in stimuli-related inductions. Positionally, these regions correspond well with the predicted *cis*-acting elements and could provide the basis for further studies regarding *Vvpgip1* regulation.

With this study it has been shown for the first time that grapevine PGIP is regulated by environmental factors that can be related to temporal developmental conditions within the plant. The data obtained also reinforces the role of PGIP in plant defense responses and contributes specifically to the rapidly expanding field of plant-

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pathogen interactions with regards to the fundamental processes underlying defense gene regulation.

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OPSOMMING

Die ooglopende voordele wat, vanuit 'n landboukundige én ekonomiese oogpunt, uit siekte- en stresbestande plante spruit, het gedurende die laaste dekade aanleiding gegee tot die ontwikkeling van plantpatogeen-interaksies as 'n baie belangrike studieveld. Dit was dus ook te verwagte dat fundamentele navorsing in hierdie area nie net beperk gebly het tot modelorganismes soos *Arabidopsis* en tabak (ook natuurlik van landboukundige belang) nie, maar ook na meer tradisionele landbougewasse soos mielies, boontjies, sojaboontjies, appels, druiwe, ens. oorgevloei het. Verskeie siekteweerstands-verwante gene is byvoorbeeld al vanuit wingerd geïsoleer. Een só 'n geen wat vir 'n poligalakturonase-inhiberende proteïen (PGIP) kodeer, vorm deel van hierdie groep gene. Die funksie en regulering van PGIP's is baie goed bestudeer. Hierdie proteïene word normaalweg in die selwande van die meeste dikotiele plante aangetref. Leusienryke herhalings is algemeen in PGIP's en hierdie tipe van herhalings is kenmerkend van proteïene betrokke by proteïen-proteïen-interaksies. Verder word *pgip*-gene gewoonlik in klein multigeenfamilies aangetref, waar in die meeste gevalle die uitdrukking weefselspesifiek en die regulering spesifiek ten opsigte van die ontwikkelingsfase is. Verskeie faktore kan tot die induksie van *pgip*-gene lei, soos onder andere patogeen-infeksie, elisitoor-, salisiensuur-, jasmoonsuur- en koue-behandeling, asook verwonding. Differensiële regulering word in baie gevalle tussen lede van dieselfde multigeenfamilie aangetref. Hierdie differensiële regulering kan selfs bemiddel word deur onafhanklike reguleringsweë in reaksie op dieselfde induksiestimulus. PGIP's is in staat om te reageer met poligalakturonases (PGs), wat selwande afbreek en wat gedurende die infeksieproses deur swamme of fungi afgeskei word. Die effek van hierdie interaksie is tweeledig: (i) Die fisiese interaksie tussen PGIP en PG moduleer die aktiwiteit van die PG deur die ensiemaksie te inhibeer, en (ii) PG-inhibisie lei tot die verhoogde stabiliteit van langketting-oligogalakturonades, molekules wat daartoe in staat is om die weerstandsrespons van plante te ontlok. Die inhibisie van die patogeen-PG's, tesame met die geïnduseerde weerstandrespons, stel die plant dan in staat om verdere infeksie te vertraag of te verhoed.

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Die doel van hierdie studie was om die onderliggende aspekte van PGIP-regulering in wingerd te bestudeer. In teenstelling met die meeste plantspesies waar *pgip*-gene in klein multigeenfamilies aangetref word, is daar nie 'n *pgip*-multigeenfamilie in wingerd nie. Veelvuldige kopieë van 'n enkele *pgip*-geen word egter in die wingerdgenoom aangetref. Daar is onlangs in ons laboratorium 'n genoom-DNA-fragment vanaf *Vitis vinifera* cv Pinotage geïsoleer wat die oopleesraam en 5'-stroomopsekwense van 'n PGIP-enkoderende geen (*Vvpgip1*) bevat. In hierdie studie is die uitdrukkingspatroon van *Vvpgip1* ten opsigte van weefselspesifisiteit, korrelontwikkelingsfase, asook die effek van verskeie omgewings en patogeenverwante stres-stimuli ontleed. Die regulatoriese meganismes van *Vvpgip1* bevat spesifieke *in planta*-ontwikkelingsfaseseine wat verder deur spesifieke faktore, insluitende omgewings- en patogeenstres, gereguleer word. In lyn hiermee is mRNS-transkripte van *Vvpgip1* tot wortel- en korrelweefsels beperk, terwyl die mRNS-vlakke ook tussen verskillende korrelontwikkelingsfases wissel. Kumulatiewe uitdrukking kon waargeneem word in *veráison*-korrels in reaksie op verwonding en osmotiese stres. Die weefselspesifieke uitdrukkingspatroon tipies van wingerd-PGIP is in blare opgehef in reaksie op *Botrytis cinerea*-infeksie, verwonding, osmotiese stres, ouksien (indoolasynsuur) en salisiensuur. PGIP-uitdrukking word ook onderdruk deur 'n staurosporien-sensitiewe proteïenkinase, wat 'n goeie aanduiding is van die betrokkenheid van proteïenfosforilasie in die seintransduksiekaskade wat tot PGIP-uitdrukking aanleiding gee. Die geïnduseerde PGIP-uitdrukkingsprofiel in wingerdblare kan ook nageboots word in tabak wat met die *Vvpgip1*-geen en –promotor getransformeer is. PG-inhibisie-eksperimente met membraan-geassosieerde proteïen-ekstrakte van geïnduseerde wingerdblare het ook dieselfde profiel getoon as dié van PGIP wat deur die *Vvpgip1*-geen geënkodeer is.

Die uitdrukkingsprofiel van PGIP in die transgeniese tabakplante het ook bewys dat die promotor van die *Vvpgip1*-geen vir die geïnduseerde PGIP-uitdrukkingsprofiel in wingerdblare verantwoordelik is. *In silico*-analise van die promotorarea dui op die teenwoordigheid van verskeie *cis*-werkende elemente. Die kernpromotor en transkripsie-aanvangsgedeelte is gevolglik eksperimenteel bepaal. Verder het uitdrukkingseksperimente met promotorfragmente verskeie dele van die promotor

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geïdentifiseer wat by stimulis-geassosieerde uitdrukking betrokke is. Posisioneel is hierdie fragmente in goeie konteks met die voorspelde *cis*-werkende elemente en kan dus die basis vorm vir verdere studies oor *Vvpgip*-regulering.

Met hierdie studie word die eerste data verskaf waar die regulering van PGIP deur omgewingsverwante faktore verbind kan word met onwikkelingspesifieke toestande in die plant. Verder verskaf die resultate verdere bewyse vir die rol van PGIP in plant-patogeen-interaksies en lewer spesifieke bydraes tot die onderliggende prosesse wat by die regulering van siekteweerstandverwante gene betrokke is.

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This dissertation is dedicated to Sterna Brand.

Hierdie proefskrif is opgedra aan Sterna Brand.

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BIOGRAPHICAL SKETCH

Dirk Albert Joubert was born in Pretoria, South Africa on the 18th of May 1973. He matriculated at Huguenot High School, Wellington in 1991. Albert enrolled at Stellenbosch University in 1993 and obtained a BSc degree, majoring in Microbiology, Biochemistry and Genetics, in 1996. The degrees HonsBSc (Wine Biotechnology, 1997) and MSc (Wine Biotechnology, *cum laude*, 2000) were subsequently awarded to him. His masters' thesis was entitled, "Development of an *Agrobacterium vitis* transformation system for grapevine". He visited the laboratory of Profs Felice Cervone and Giulia de Lorenzo (Dipartimento di Biológia Vegetale, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy) from July 2001 to December 2001 to complete part of his doctoral studies.

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PREFACE

This dissertation is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of *Plant Physiology*, chapters three and four will be submitted for publication.

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Chapter 2 **Literature review**
Plant disease resistance: an overview

Chapter 3 **Research Results**
The transcriptional regulation of a polygalacturonase inhibiting protein (PGIP) from grapevine (*Vitis vinifera* L.)

Chapter 4 **Research Results**
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CHAPTER 1

INTRODUCTION AND PROJECT AIMS

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1.1. INTRODUCTION

Plants are sessile organisms and use unique mechanisms of perception and adaptation to survive in a rapidly changing environment. The speed of perception and reaction often relates to survival or death. In this regard, the lack of mammalian-like circulatory systems complicates signal perception as well as defense against invading pathogens (Bohnert et al. 1995; Baker et al. 1997; Blumwald et al. 1998). Plants compensate for their lack of mobility by utilizing a complex array of signal perception and transduction cascades that often result in a typical response, termed the hypersensitive response (HR), characterized by a rapid oxidative burst and resulting in localized cell death (Bolwell 1999; Delledonne et al. 2001). The HR in turn triggers an array of other defense-related signaling cascades that ultimately lead to systemic acquired resistance (SAR), characterized by elevated general pathogen resistance throughout the plant (Ryals et al. 1996).

All plant-microbe interactions do not necessarily result in disease. Some pathogens simply lack the ability to infect specific plants, termed non-host plants. Pathogen interactions with host plants (defined by a specific genotype within susceptible plant species) can further be defined as compatible (disease forming) and incompatible (resistance) reactions (Veronese et al. 2003). Compatibility of host-pathogen interactions is defined by the gene-for-gene hypothesis (Flor, 1956). The interaction of pathogen associated avirulence (*avr*) gene products with host resistance (R) genes, direct or indirectly, determines whether a pathogen attack is successful or not (Shirasu et al. 1996; Cook 1998). In most cases this interaction marks the start of massive transcriptional reprofiling and *de novo* gene expression to limit disease and stress related damage (Cook 1998; Bonas & Van den Ackerveken 1999).

1.2. POLYGALACTURONASE INHIBITING PROTEINS (PGIPs) IN PLANT DEFENSE

PGIPs are leucine rich, cell wall bound proteins that have been shown to play an active role in plant defense against pathogenic fungi (De Lorenzo et al. 2001). PGIPs recognize and interact with the active cleft of endo-α-1,4-polygalacturonases (endoPGs)

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secreted by phytopathogenic fungi (Mattei et al. 2001). EndoPGs cleave the α-1,4 linkages of galacturonic acid residues within the homogalacturonan domain of the plant cell wall pectic matrix (Esquerre-Tugaye et al. 2000). The degradation products that result due to the action of endoPGs consist of intermediate long-chain oligogalacturonides that are subsequently degraded to single galacturonides. However, the PGIP:PG interaction inhibits endoPGs, thereby prolonging the existence of long-chain oligogalacturonides (De Lorenzo et al. 1994). Oligogalacturonides (specifically those between 10 and 14 residues in length) are endowed with a host of biological properties, including the ability to elicit plant defense responses (Reymond et al. 1995). The action of PGIPs in disease resistance can, therefore, be considered as two-fold: restricting the spread of the pathogen by inhibiting cell wall macerating PGs, as well as facilitating the induction of plant defense responses. The direct involvement of PGIP in plant defense has been demonstrated by the reduction of disease symptoms in transgenic tomato, *Arabidopsis thaliana* as well as tobacco plants over-expressing PGIP encoding genes (De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002).

PGIPs normally occur in multigene families and members from the same multigene family often display differential regulation and recognition abilities (De Lorenzo & Ferrari 2002). In most cases, the expression of PGIPs is spatially and temporally regulated and various stress stimuli, including pathogen infection, cold treatment, wounding, salicylic acid (SA), jasmonate and osmotic stress, induce the expression of *pgip* gene families (Bergmann et al. 1994; Stotz et al. 1994; Bergey et al. 1996; Devoto et al. 1998; Mahalingam et al. 1999; De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002). Also, differential regulation between PGIP family members is often observed. In *A. thaliana* for instance, two members of the PGIP family are induced via separate pathways in response to *Botrytis cinerea* infection (Ferrari et al. 2003). To counteract PGs from various fungal pathogens, PGIPs from the same multigene family also display differential specificities. The five-member PGIP family from *Phaselous vulgaris* recognizes PGs from different fungal sources, but this ability is not reflected in the specificity profiles of individual family members (Desiderio et al. 1997). Differential regulation and specificity among PGIP family members is thought to give plants an added advantage to counteract diversity among fungal pathogens (De Lorenzo et al.

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2001). Recently a genomic DNA fragment from *Vitis vinifera* cv Pinotage containing the open reading frame (ORF) and upstream regulatory sequences of a PGIP encoding gene (*Vvpgip1*) was isolated in our laboratory. The gene was found to be expressed specifically in developing berries and the protein encoded by the *Vvpgip1* gene was furthermore shown to inhibit among others, PGs from the necrotrophic fungus, *Botrytis cinerea*. When transgenic tobacco plants over-expressing the *Vvpgip1* gene, was challenged with *B. cinerea*, these plants also exhibited reduced infection rates and lesion sizes (De Ascensao 2001).

1.3. SPECIFIC AIMS

The role of PGIPs in plant defense has been well characterized and the recently isolated *pgip1* gene from grapevine presented an ideal opportunity to characterize the molecular basis of host-pathogen responses in grapevine. Although PGIPs share high structural and functional homology, the regulation of PGIP expression have been found to differ significantly between species (Desiderio et al. 1997; Leckie et al. 1999; Ferrari et al. 2003). This study aimed at elucidating the regulation of the *Vvpgip1* in response to specific biotic and abiotic stimuli as well as at analyzing the specific role of the putative promoter within this context. This work forms part of a multidisciplinary research objective in the Institute for Wine Biotechnology, which apart from the gaining of fundamental knowledge, also has as focus the genetic improvement of grapevine cultivars. Within the goal of improved disease resistance, a clear understanding of the fundamental processes involved during stress responses, including pathogen attack is imperative. Within this context the PGIP:PG model provides an useful tool to elucidate these processes.

Specific aims of this study included:

1. Elucidation of the regulation of polygalacturonase inhibiting proteins (PGIPs) in grapevine (*V. vinifera* L.).

   i) The determination of tissue specific expression patterns of *Vvpgip1 as well as* the expression profile of *Vvpgip1* in response to several stress stimuli;

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ii) The determination of the induced inhibition profile of *Vvpgip1* in grapevine as well as in a heterologous host.

2. Promoter analysis of the *Vvpgip1* gene from *Vitis vinifera* L. that encodes a PGIP with high activity against *Botrytis cinerea* polygalacturonase.

   i) Verification of *Vvpgip1* promoter activity and delimiting the core promoter;

   iii) Determination of the transcription initiation site of *Vvpgip1*;

   iv) Quantitative promoter activity analysis of internal and sequential deletion constructs to identify promoter regions involved in specific induction related expression patterns *of Vvpgip1*.

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CHAPTER 2

LITERATURE REVIEW

Plant disease resistance: an overview

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2.1. INTRODUCTION

The ability of plants to adapt to biotic and abiotic stresses has been extensively studied and is well documented (Ryan 1994; Ryan & Jagendorf 1995; Halterman & Martin 1997; Dong 1998; Genoud & Metraux 1999; Jouannic et al. 1999; Martin 1999; Klessig et al. 2000; Keen 2000; McDowell & Dangl 2000; Delledonne et al. 2001; Nurnberger & Scheel 2001). At the same time, major advances have been made towards unraveling the basic mechanisms involved in eukaryotic transcription and the regulation thereof (Perez-Martin & De Lorenzo 1997; Fiedler & Marc Timmers 2000; Lee & Young 2000; Dvir et al. 2001; Gill 2001). The elucidation of signaling events between the perception of non-self and the *de novo* transcription of defense-related genes, however have fallen short of giving a clear and concise picture of the complex interaction between plant and pathogen. The diverse nature of pathogens has largely thwarted efforts to establish a common model to describe the molecular interactions that occur between plants and invading pathogens. To further complicate efforts, plants have developed unique strategies for responding to the constant changes in the surrounding environmental conditions. The characteristic defense response of plants is termed the hypersensitive response (HR), often resulting in localized cell- and tissue death at the site of infection, thereby limiting the further spread of the infection. Cell death is an important physiological process in plants and is achieved through a genetically conserved process (Pontier et al. 1998). Cell death genes are encoded by the plant genome and regulated by pathogen-related signals. Activation of cell death genes may result in an oxidative burst, or rapid production of active oxygen species (AOS) such as superoxide anions (O^{-2}_2), hydroxy radicals (OH^-) and hydrogen peroxide (H_2O_2). The production of AOS is one of the earliest detectable responses of plants treated with pathogen elicitors and precedes cell death (Lam et al. 1999; Delledonne et al. 2001).

The HR in turn, often triggers a battery of non-specific defense-related processes throughout the plant, collectively known as systemic acquired resistance (SAR). The products of these processes include among others, 11 classes of pathogenesis related (PR) proteins, e.g. PR-1 (antifungal), PR-2 (acidic and basic β-1,3-glucanases), PR-3 (chitinase), PR-4 (antifungal), PR-5 (thaumatin-like protein) and PR-8 (acidic and basic

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class III chitinases), as well as defensins, cyclophilin-like proteins, ribosome-inactivating proteins (RIPs) etc. and provide resistance to a wide range of pathogens for several days (Shirasu et al. 1996; Baker et al. 1997; Cordeiro et al. 1998; Selitrennikoff 2001). Whether a specific bacterial infection leads to plant disease or to the HR and subsequent responses, is determined by the initial recognition events between host and pathogen, the genetic basis of which is known as the gene-for-gene hypothesis (Shirasu et al. 1996; Cook 1998). Signal perception usually occurs through various receptors and normally results in the expression of a host of target genes to adjust metabolic systems in order to maintain homeostasis (Pastori & Foyer 2002). A graphical representation of the general events during plant-pathogen interactions is presented in Fig. 1.

Signal perception and transduction almost invariably results in altered gene expression. The regulation of *de novo* gene expression is complex, but mostly occurs at the level of transcription. The huge number of genes involved underwrites the importance of this aspect. In *Arabidopsis thaliana*, 25% of the 25 498 genes encoding proteins from 11 000 families are involved in transcription, signal transduction, and the control of cell fate. On chromosome 4 alone, approximately 15% of the sequenced genes are involved in the regulation and mechanistic aspects of transcription (Bevan et al. 1998).

The proteins involved in eukaryotic transcription can be classified into four different functional groups; (i) the basic transcription apparatus and intrinsic associated factors, or general transcription factors (GTFs); (ii) large multi-subunit coactivators and other cofactors; (iii) sequence specific DNA binding transcription factors and (iv) chromatin-related proteins (Riechmann 2002). Of specific interest here are the signaling pathways involved in the regulation of sequence specific DNA binding transcription factors during plant defense responses.

The majority of plant-microbe interactions do not result in disease. From the perspective of a potential pathogen, plants can be categorized into two broad classes, hosts (defined by a specific genotype within susceptible plant species) and non-hosts (defined by species level resistance) (Veronese et al. 2003). Generally, pathogen recognition and the subsequent activation of disease resistance responses occur at

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both the host and non-host levels. Host specific resistance generally conforms to the gene-for-gene hypothesis and is genetically determined by complementary pairs of pathogen-encoded avirulence (*avr*) and plant resistance (*R*) genes (Nurnberger & Brunner 2002). In this review, a general overview of the series of events involved during plant-pathogen interactions in host plants will be provided. These aspects that will be covered include: pathogen recognition, ion influx, the alkalinization of extracellular spaces, the accumulation of reactive oxygen (ROS) and reactive nitrogen species (RNS), protein phosphorylation cascades, the roles of plant hormones abscisic acid (ABA) jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) in disease as well as defense-related transcription factors.

2.2. PLANT-PATHOGEN INTERACTIONS

2.2.1. Pathogen recognition: Gene-for-gene interactions

Models describing specific host-pathogen interactions have been largely based on the gene-for-gene interactions originally reported by Flor (1956). Pathogen recognition and subsequent activation of defense responses are conditional on the presence of complimentary pairs of *R* genes in the host and *avr* genes in the invading microbe (Fritig et al. 1998; McDowell & Dangl 2000). Defense responses are autonomous in the sense that every cell can sense and respond to microbial attack. In most cases *avr* genes confer a selective advantage to the pathogen in the absence of the corresponding *R* gene. It is, therefore, possible that *avr* genes primarily act as virulence factors that, in the course of plant-pathogen co-evolution were recognized by plant *R* genes (Bonas & Lahaye 2002). This view is further supported by the huge amount of diversity found between *avr* genes from different classes of pathogens (Fritig et al. 1998).

During plant pathogen interactions, *R* genes fulfill at least two functions, the recognition of pathogen-derived signals and initiation of the plant defense response (Bonas & Lahaye 2002). Unlike the diverse group of *avr*-encoded proteins, the majority of R proteins are structurally related and their functional requirements typically are reflected in their architectural structure.

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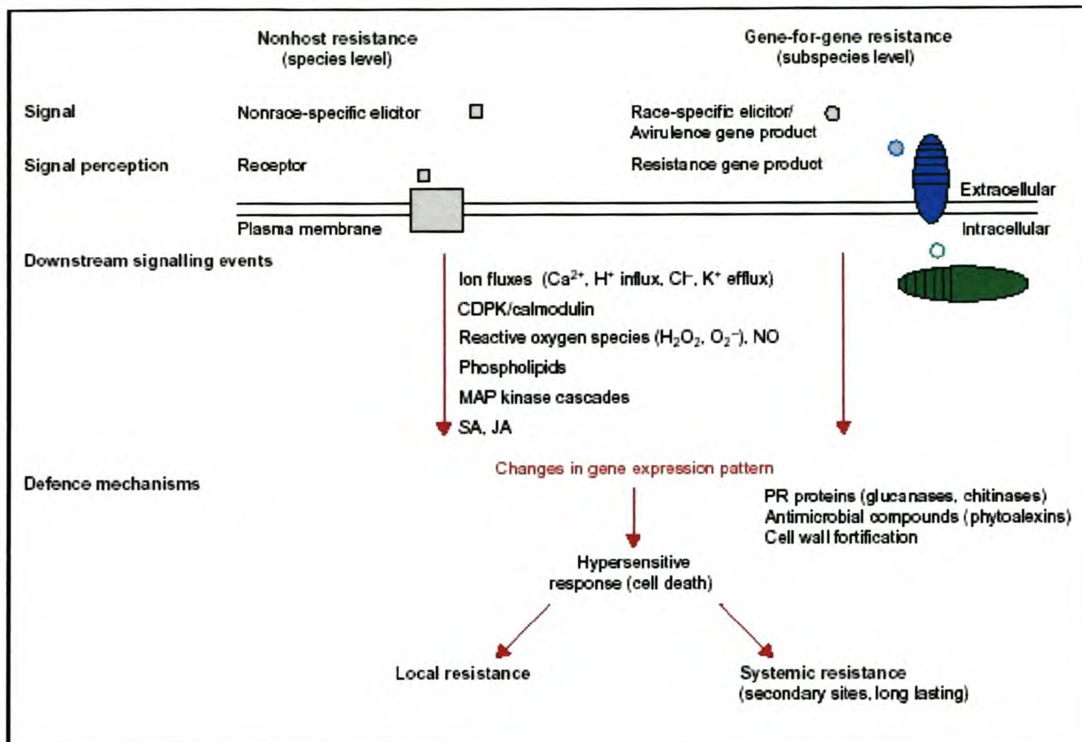

Figure 1. A general overview of plant defense responses after pathogen attack. Plant membrane-bound receptors, or extracellular and intracellular resistance (*R*)-gene products perceive the invading pathogen, downstream signaling events become initiated, resulting in altered gene expression and the activation of defense mechanisms. CDPK, calcium dependent protein kinase; JA, jasmonic acid; MAP, mitogen activated kinase; PR, pathogen related; SA, salicylic acid (Romeis 2001)

The majority of *R* genes products contain predicted extracellular or intracellular leucine-rich repeat (LRR) motifs (Dangl & Jones 2001). These LRR domains have been implicated in protein-protein interactions and their presence in R proteins are indicative of the proposed receptor function of R proteins (De Lorenzo & Ferrari 2002). A putative nucleotide-binding (NB) domain that is associated either with an amino-terminal Toll/Interleukin-1-receptor (TIR) homologous region, or a coiled-coil (CC) domain, can also be found in R proteins that contain intracellular LRRs. Consistent with their function in signal perception and transduction, the structure of R proteins is modular (Bonas & Lahaye 2002).

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At least four models have been postulated to describe the function of R proteins in plants (Fig. 2). These include, the direct interaction between an Avr protein and a matching R protein, the binding of the Avr protein to a high affinity co-receptor, which, in turn interacts with the R protein, the guard model, in which the R protein safeguards a matching pathogenicity target or, giving the fact that several bacterial, fungal and viral *avr* genes are predicted to encode proteases, it is possible that proteolytically processed host proteins trigger plant defenses. These models are not mutually exclusive and any combination of the four could theoretically play a role during host-pathogen interactions (Veronese et al. 2003). This could explain several conflicting observations including, (i) specific Avr proteins could be associated with a seemingly inappropriate R protein (Nishiuchi et al. 1999); (ii) a single R protein can recognize two different effectors (Nishiuchi et al. 1999); (iii) R proteins can interfere functionally with one another (Ritter & Dangl 1996) and; (iv) direct interaction between R/Avr proteins cannot always be demonstrated (Nimchuk et al. 2001).

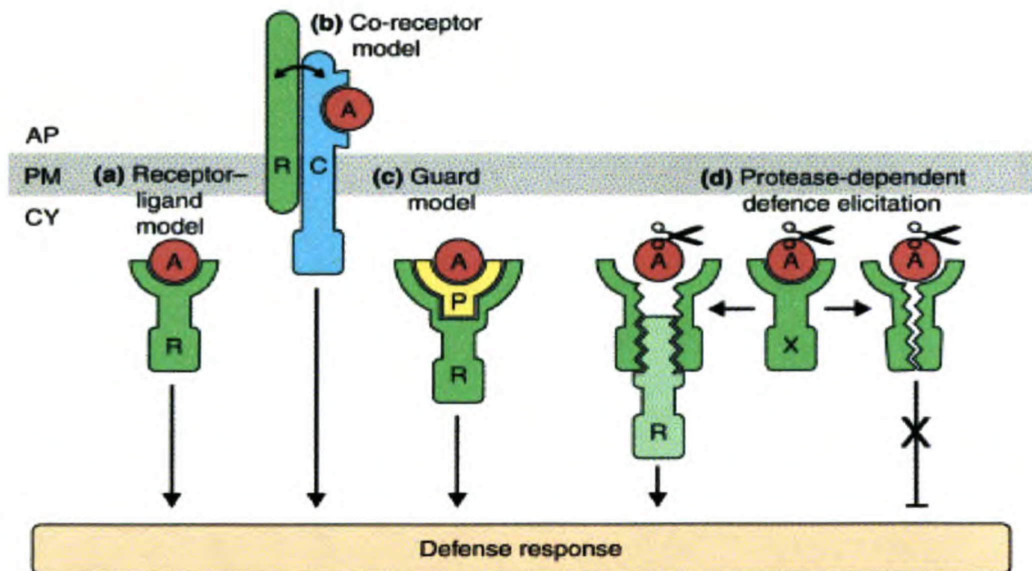

Figure 2. A graphical representation of the four proposed models for the biochemical basis for the gene-for-gene model. (a) The classical receptor-ligand model. (b) The co-receptor model. (c) The guard model and (d) protease-dependent defense activation. R, R protein, X, protease target, A, Avr protein (red); C, co-receptor (blue); AP, apoplast; CY, cytoplasm; P (yellow), matching pathogenicity target, PM, plasma membrane (Bonas & Lahaye 2002).

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For a more detailed review on the structure and function of *avr* and *R* genes, the reader is directed to Bonas & Van den Ackerveken (1999); Nurnberger & Scheel (2001); Dangl & Jones (2001); Nimchuk et al. (2001); Bonas & Lahaye (2002) and Veronese et al. (2003).

2.2.2. Ion fluxes and alkalinization of extracellular spaces

Pathogen recognition is followed by rapid responses within the host cells. The earliest of these responses are changes in the permeability of the plasma membrane that allows the development of ion fluxes across the membrane, including the influx of H^{+} and Ca^{2+} as well as the efflux of Cl^{-} and K^{+} (Fritig et al. 1998). Specific K^{+} channels and elicitor responsive Ca^{2+} channels of the plasma membrane are involved in the regulation of the K^{+} efflux and increase in cytosolic Ca^{2+} levels. Furthermore, elicitor stimulated increases in cytosolic Ca^{2+} are sensitive to protein kinase inhibitors, suggesting a role for protein phosphorylation in receptor-mediated regulation of Ca^{2+} channels. *R*-genes products and receptors mediate regulation of membrane permeability and the subsequent ion flux is essential for the activation of a variety of defense-related genes (Blumwald et al. 1998). The blocking of anion-channels indicates a position of anion flux upstream of the Ca^{2+} flux and pharmacological studies showed that an influx of extracellular Ca^{2+} is needed to increase the level of cytosolic Ca^{2+} (Zimmermann et al. 1997; Nurnberger & Scheel 2001).

The importance of Ca^{2+} in these early events is well documented and many downstream events require a sustained transient increase in cytosolic Ca^{2+} (Grant et al. 2000b; Lee & Rudd 2002). Some of the earliest elicitor-responsive downstream targets of cytosolic Ca^{2+} have been identified as calcium dependent protein kinases (CDPKs), as well as calmodulin, a universal Ca^{2+} binding signal. Both CDPKs and calmodulin have been indicated in the regulation of downstream defense responses (Nurnberger & Scheel 2001; Romeis 2001).

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2.2.3. The role of ROS and RNS in plant defense responses

A direct consequence of the increase in cytosolic Ca^{2+}, is the production of ROS (such as superoxide [$O_2{\cdot}^-$] and hydrogen peroxide [H_2O_2]) as well as RNS (in particular nitrogen oxide [NO]) (Durner & Klessig 1995; Durner et al. 1998; Delledonne et al. 2001; Delledonne et al. 2002; Hancock et al. 2002). These molecules play a central role in plant stress responses; ROS and NO are produced in reaction to biotic (pathogen related) as well as abiotic (temperature, UV, dehydration etc.) stresses. They act as direct toxins to pathogens, catalyze early reinforcement of physical barriers and are involved in signaling events eliciting later defense reactions (Scheel 1998). The ubiquitous nature of these reactive molecules is characteristic of common signaling factors in plant stress responses and evidence suggests that ROS and NO function as key signaling molecules during stress responses (Halterman & Martin 1997; Delledonne et al. 1998; Durner et al. 1998; Bolwell 1999; Durner & Klessig 1999; McDowell & Dangl 2000).

Although the generation of ROS and NO in plant tissues during plant-pathogen interactions has been well established, the mechanisms involved in particularly NO, and to a more limited extent ROS synthesis, still largely remain unclear (Hancock et al. 2002). Parallels for ROS production can be found in human phagocytes where the enzymatic generation of superoxide ions is mediated by the enzyme NAPDH oxidase. Similar to mammalian systems, superoxide ions are the first ROS generated and are then rapidly converted to H_2O_2 and O_2 in plants (Jabs et al. 1997). Homologs of the catalytic subunit of human NAPDH oxidase (gp91) have also been isolated from various plants, including *Arabidopsis* and parsley (Scheel 1998). Interestingly, all plant homologs isolated to date contains an EF-hand motif, indicative of Ca^{2+} regulation (Nurnberger & Scheel 2001). Other mechanisms and enzymes involved in ROS generation include germin/oxalate oxidase as well as cell wall peroxidases (Allan & Fluhr 1997; Bolwell 1999). Considering the many potential sources for ROS and the importance of these molecules in stress related signaling, it seems logical that a clearer understanding of the regulation of ROS production could significantly contribute to the elucidation of several stress-related signaling pathways in plants.

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The mechanisms involved during NO production are less clear; nitric oxide synthase (NOS) seems the most likely candidate to catalyze NOS formation, but appears not to be present in all plant species. Elicitor induced NO generation has been observed in both tobacco as well as *A. thaliana* tissues, but to date, no gene sequences for NOS have been identified in *A. thaliana* (Hancock et al. 2002). However, NOS-like proteins, have been identified in several plants and a partial NOS clone was recently obtained from a pea (*Pisum sativum*) cDNA library (Corpas et al. 2001). Furthermore, evidence shows that NO generation in plants can be inhibited by mammalian NOS enzyme inhibitors, suggesting that at least some plants do contain NOS-like enzymes (Barroso et al. 1999; Ribeiro et al. 1999; Corpas et al. 2001). Nitrate reductase and xanthine oxidoreductase potentially provides two additional enzymatic sources of NO in plants, but very little evidence exist to supporting this hypothesis (Hancock et al. 2002).

The most profound effect of ROS and NO production in plant cells is undoubtedly the induction of programmed cell death (PCD). The generation of ROS, and specifically H_2O_2, after elicitor challenge leads to PCD, an active process that involves the *de novo* transcription and translation of various defense-related genes (Levine et al. 1994; Levine et al. 1996; Desikan et al. 1998). In addition to PCD, ROS and NO also have less dramatic effects, in particular their role as important signaling molecules for the induction of downstream defense responses. H_2O_2, for instance, induces the expression of phenyl ammonia-lyase (PAL) and glutathione *S*-transferase (GST) in *A. thaliana* suspension cultures (Desikan et al. 1998). PAL has been shown to be involved in various defense-related responses. The over-expression of PAL in tobacco for instance, led to a SA dependent, marked reduction in susceptibility to *Cercospora nicotianae* (Shadle et al. 2003). GST functions as a family of cellular detoxification enzymes, involved in the removal of H_2O_2. Regulation of GST by H_2O_2 has also been demonstrated (Desikan et al. 1998; Barroso et al. 1999; Lederer & Boger 2003). *A. thaliana* microarray analysis showed that over a hundred genes were up-regulated by H_2O_2 treatment, including signaling enzymes, stress-related proteins and transcription factors, while over sixty were down-regulated (Hancock et al. 2002). NO have also been shown to be involved in the up-regulation of defense-related genes such as *PR-1*, *PAL* and *GST* (Delledonne et al. 1998; Durner et al. 1998).

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Alternative targets for both ROS and NO include mitogen-activated protein kinase (MAPK) pathways. MAPK pathways are highly conserved phosphorylation cascades that are activated in response to biotic as well as abiotic stress and transduce extracellular signals to nuclear or cytoplasmic targets (Zhang & Klessig 2001). MAPK activation in plants by both H_2O_2 and NO have been reported (Desikan et al. 1999; Clarke et al. 2000; Grant et al. 2000a; Kovtun et al. 2000b). Cyclic guanosine monophosphate (cGMP) are also implicated in NO signaling, (Durner et al. 1998), but no source for cGMP has yet been identified in plants (Hancock et al. 2002).

2.2.4. Protein phosphorylation cascades

Protein phosphorylation cascades function both during pathogen perception, as well as downstream of the recognition of the pathogen or pathogen derived signals. The phosphorylation of key proteins to transduce signals is a vital, but complex component of many signaling pathways, most of which fall outside the scope of this review. For recent reviews about the role of protein phosphorylation in signaling pathways, the reader is directed to Cheng et al. (2002) and Lohrmann & Harter (2002). Only the role of protein kinases in downstream protein phosphorylation cascades, in particular, CDPKs, and MAPKs will be discussed in this review.

The superfamily of CDPKs is comprised of four types of protein kinases. These include; kinases regulated by the binding of Ca^{2+} (CDPKs), kinases regulated by the binding of Ca^{2+}/calmodulin (calmodulin-dependent kinases [CaMKs]), a combination of both (calcium and calmodulin-dependent protein kinases [CCaMks]), or neither (Cdpk-related protein kinases [CRKs]) (Harmon et al. 2000). CDPKs are well characterized in plants and comprise a family of multifunctional serine/threonine protein kinases (Romeis 2001). Structurally, CDPKs contains four domains, an N-terminal domain of variable length and sequence, a protein kinase catalytic domain, an auto-inhibitory junction domain containing a calmodulin (CaM)-binding domain, and a C-terminal Ca^{2+} binding domain (Zhang & Lu 2003). Recently the regulatory calcium-binding domain of CDPKs has been linked directly to the kinase catalytic domain, enabling CDPKs to translate

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changes in intracellular calcium concentration into kinase activity, thereby facilitating signal transduction (Harmon et al. 2000; Romeis 2001).

Evidence to corroborate the role of CDPKs as signaling mediators stems from experiments where elicitor treatments led to the accumulation of transcripts of a CDPK from tobacco (*NtCDPK1*). Also, a putative CDPK was biochemically characterized from elicitor treated French bean cells (Allwood et al. 1999; Yoon et al. 1999). Virus-induced gene silencing (VIGS) of a CDPK subfamily in transgenic tobacco plants expressing the *Cf-9* resistance gene from tomato, resulted in an inability of these plants to induce a Cf/Avr-specific HR (Romeis 2001). Although the role of CDPKs in defense signaling is undisputed, significantly more research is still needed to elucidate the roles of specific CDPK isoforms in stimulus-response pathways.

MAPK cascades are important components downstream of the receptors for extracellular stimuli. Several of these kinases have been reported to be activated during plant responses to elicitors or pathogens (Meskiene & Hirt 2000; Romeis 2001; Zhang & Klessig 2001). The basic assembly of a MAPK cascade is conserved in all eukaryotes and consists of a three-kinase module, of which MAPK is the last kinase in the cascade. Plant MAPKs can be classified into six subfamilies, all of which contains a Thr-Glu-Tyr or Thr-Asp-Tyr activation motif (Zhang & Klessig 2001). MAPK is activated by dual phosphorylation of the Thr and Tyr residues in the activation motif. This phosphorylation is mediated by a MAPK kinase (MAPKK or MEK), which in turn, is activated by a MAPKK kinase (MAPKKK or MEKK) (Fig. 3) (Romeis 2001).

MAPK encoding genes from various plant species have been characterized; these include two orthologous groups comprising wound induced protein kinase (*WIPK*), salt induced MAPK (*SIMK*), *A. thaliana* MAPK3 (*AtMPK3*) and extracellular signal-regulated MAPK (*ERMK*) from tobacco, alfalfa, *A. thaliana* and parsley, respectively, and salicylate-induced MAPK (*SIPK)*, stress activated MAPK (*SAMK*) and *AtMPK6* from tobacco, alfalfa and *A. thaliana* (Seo et al. 1995; Ligterink et al. 1997; Zhang & Klessig 1997; Cardinale et al. 2000; Nuhse et al. 2000). MAPK-mediated signaling is complex, not only are specific isoforms activated by race- and nonrace-specific pathogen-related stimuli, but several MAPK pathways are utilized in parallel upon elicitation by a single stimulus.

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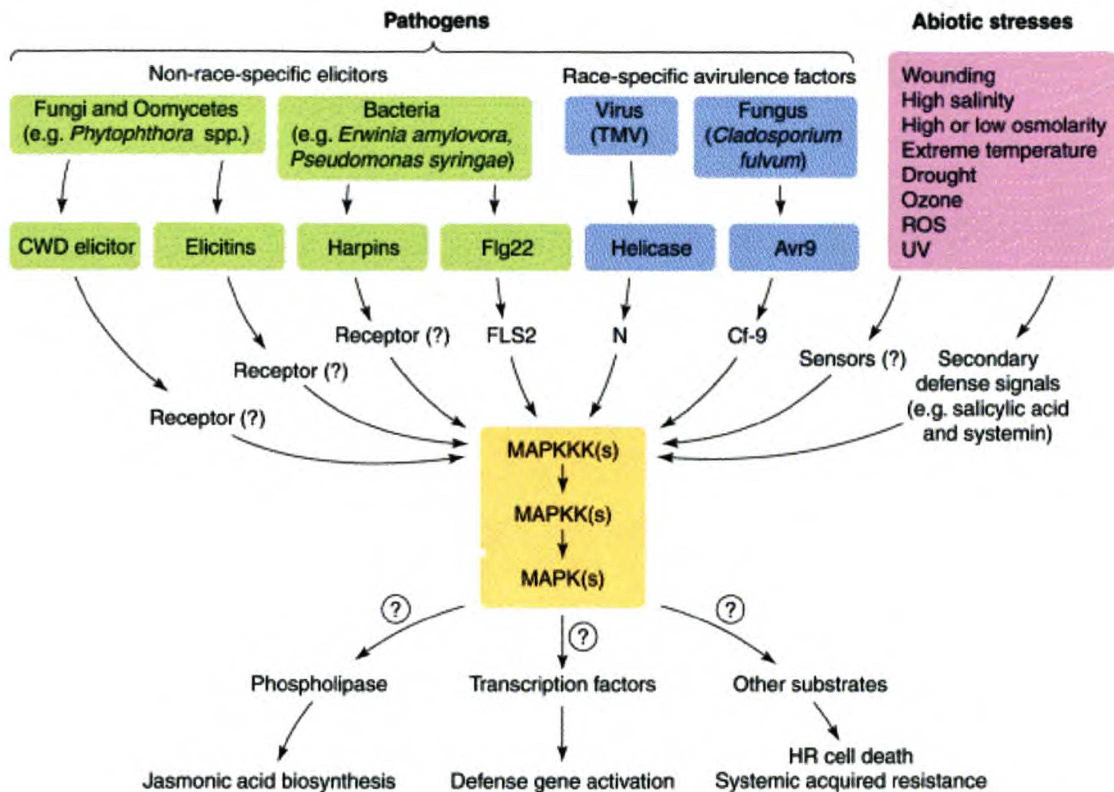

Figure 3. Graphical representation of the convergence of various stress-stimuli onto MAPK cascades. MAPK cascades are activated in response to several abiotic and biotic stresses, including pathogen invasion, wounding, high salinity, high or low osmolarity, extreme temperature, drought, reactive oxygen species (ROS), ozone and ultraviolet (UV) radiation). MAPKs are also activated by secondary defense signaling molecules such as salicylic acid and systemin (Zhang & Klessig 2001).

Furthermore, gene families exist for MAPKKKs, MAPKKs and MAPKs, all of which play distinct roles within elicitor specific signal mediation (Romeis 2001; Droillard et al. 2002). MAPK cascades have been implicated in oxidative stresses, wound responses, cell death induction and defense gene activation and repression (Fig. 3) (Lamb & Dixon 1997; Ligterink et al. 1997; Somssich & Hahlbrock 1998; Romeis et al. 1999; Suzuki et al. 1999; Zhang & Klessig 2001). Inhibitor studies with broad-spectrum kinase inhibitors that blocked the oxidative burst, also suppressed *SIPK* and *WIPK* activation. Specific inhibition of MAPKK, however, did not suppress H_2O_2 production, suggesting that the oxidative burst did not require MAPK activation. H_2O_2 furthermore activates a specific group of *A. thaliana* MAPKKKs [*A. thaliana* NPK1-like protein

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kinases, in which NPK is a *Nicotiana* protein kinase (*ANP1*), *ANP2 and ANP3*]. These results suggests that H_2O_2 itself acts as a stress, rather than a signal to activate MAPKs (Lamb & Dixon 1997; Zhang et al. 1998; Romeis et al. 1999; Kovtun et al. 2000a). Direct evidence for the role of SIPK and WIPK in the HR response stems from gain-of-function experiments with NtMEK2, the upstream MAPKK of SIPK and WIPK. Expression of ntMEK^{DD}, a constitutively active mutant of ntMEK under control of a steroid-inducible promoter in tobacco, resulted in the activation of both SIPK and WIPK. Shortly after SIPK and WIPK activation, HR-like cell death was visible (Zhang & Klessig 2001). The activation of SIPK and WIPK furthermore led to the induction of PAL and 3-hydroxy-3-methylglutaryl coenzyme A reductase, both of which are associated with the plant defense response (Yang et al. 2001). The MAPK involved during the wounding response is thought to be WIPK, due to its transcriptional activation in response to wounding (Droillard et al. 2000). Recent evidence suggests, however, that the major kinase activated is in fact SIPK, not WIPK. The precise function of SIPK during wounding still remains unclear (Seo et al. 1995; Romeis et al. 1999).

MAPK are also involved in the negative regulation of plant defense responses. The *A. thaliana* mutants *mpk4* and *edr1* have recently been identified in screens for loss-of-function mutants (Petersen et al. 2000; Frye et al. 2001). Interestingly, the *mpk4* mutant exhibits constitutive SAR, with elevated salicylate levels, increased resistance to virulent pathogens and constitutive *PR* gene expression, suggesting that wild-type MPK4 suppresses SAR (Petersen et al. 2000). In contrast, the *edr1* mutant shows elevated resistance to several pathogens, but displays neither elevated levels of salicylate, nor constitutive expression of *PR* genes (Frye et al. 2001). Since the elevated resistance in the *edr1* mutant still depends on salicylate and NPR1, it seems that the absence of functional EDR1 enhances the responsiveness of plants to pathogen infection (Zhang & Klessig 2001).

The precise role of MAPK during plant defense responses still remains largely unelucidated. MAPKs are regulated post-transcriptionally by phosphorylation, and their function depends on the kinetics and amplitude of their activation. Efforts to identify specific MAPK cascades have, therefore, been largely unsuccessful. Furthermore, the function of MAPK cascades in cells is frequently pleiotropic, and disruption of single

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MAPK genes generates unspecific effects that further complicate data. Further experimentation combining biochemical and genetic studies is needed to provide a clearer picture of MAPK in plant defense responses (Zhang & Klessig 2001).

2.2.5. Plant hormones in defense

Early signaling events are likely to induce a number of defense responses, including cell wall fortification, alterations in metabolism, and the generation of signals that regulate defense gene expression. Typically these signals include plant hormones such as ABA, JA, ET and SA, all of which have been shown to either influence defense gene expression directly, or are required for the development of full defense responses (Audenaert et al. 2002; Lorenzo et al. 2003; Spoel et al. 2003).

2.2.5.1. ABA

ABA is typically not associated with the plant defense response, but rather in developmental programs, such as seed dormancy, root geotropism, opening closing of stomata through stomatal guard cells and dormancy of buds (Leung & Giraudat 1998). ABA, however, is associated with the wound response that is activated upon insect feeding (Birkenmeier & Ryan 1998). Evidence for the involvement of ABA during plant-pathogen interactions is mainly based on indirect observations. Plants show increased levels of ABA upon infection with viruses, bacteria and fungi, but the application of exogenous ABA increases the susceptibility of plants to fungal pathogens (Audenaert et al. 2002). ABA also seemed to have a negative effect on pathogen-associated plant defense by suppressing PAL activity, as well as transcription of *PAL* mRNA in hypocotyls of soybean (*Glycine max*) inoculated with *Pythophthora megasperma* f.sp. *glycina* (Ward et al. 1989).

Furthermore, physiological ABA concentrations down-regulate β-1,3-glucanase at the level of transcription in tobacco (Rezzonico et al. 1998). Audenaert et al. (2002) used ABA deficient *sitiens* tomato mutants (plants that only have a residual ABA level of 8% in comparison to the wild type plants and are unable to increase their ABA levels

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upon elicitation by wounding, heat or electrical current) to study the potential cross-talk between ABA- and SA-associated plant defenses. These plants showed decreased susceptibility to *B. cinerea* infection and displayed hypersensitivity to the SA analogue benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH). Their results suggest a negative regulatory interaction between ABA and NPR1, one of the first characterized proteins downstream of SA-dependent defense signaling (Spoel et al. 2003). These results, however, cannot exclude the involvement of other plant hormones and, therefore, still reveals a rather fragmented picture. A significant amount of research is still needed to elucidate the role of ABA in plant defense responses.

2.2.5.2. JA, SA and ET in defense responses

In addition to localized defense responses, plants also have evolved mechanisms of systemic immunity in which local defenses establish a state of heightened resistance throughout the plant. Two variants of this phenomenon are found in plants; SA-dependent, SAR and SA-independent, induced systemic resistance (ISR) (Feys & Parker 2000). Although JA, SA and ET have all been individually shown to contribute to defense responses in plants, it is very seldom that a specific hormone acts in isolation to induce specific defense responses. Recent studies revealed that induced defenses against microbial pathogens and herbivorous insects are regulated by a network of interconnecting signaling pathways in which JA, SA and ET play a dominant role (Fig. 4) (Klessig et al. 1994; Dong 1998; Reymond & Farmer 1998; Maleck & Dietrich 1999).

SAR induction in plants has long been associated with SA accumulation (Reymond & Farmer 1998; Spoel et al. 2003). SA has been shown to accumulate in pathogen-infected plant tissues and increased levels of SA correlate with both increased resistance, and the induction of SAR marker genes (Kunkel & Brooks 2002). Furthermore, *A. thaliana* plants expressing the salicylate hydroxylase (*NahG*) gene, a

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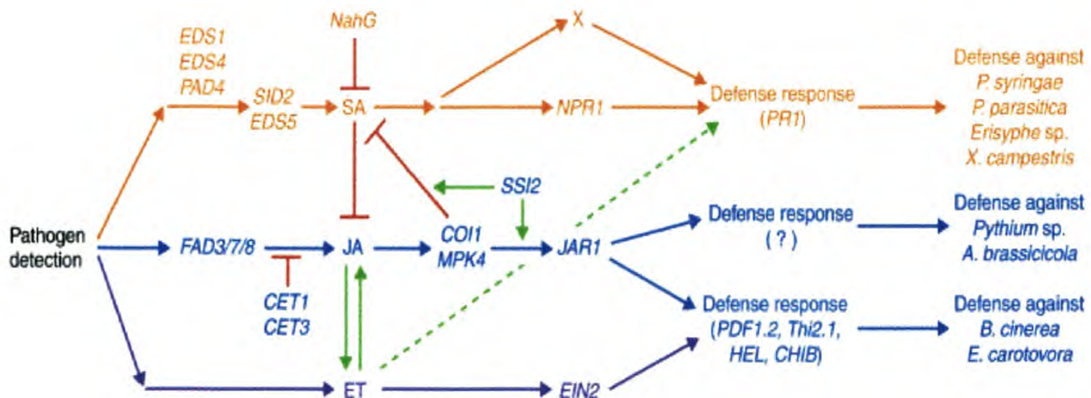

Figure 4. Summary of the interactions between signaling pathways involving the plant hormones JA, ET and SA during pathogen attack in *Arabidopsis*. Positive regulatory interactions between these signaling pathways are indicated by green arrows, antagonistic interactions by red lines. The hypothesis that *SSI2* modulates cross talk between the JA and SA pathways is indicated by the short green arrows. The dashed green arrow indicates potential positive interactions between the ET and SA pathways. Putative positive interactions between the SA and JA pathways, and potential negative interactions between the ET and SA pathways are not shown. *CET*, constitutive expressor of thionin; *CHIB*, chitinaseB; *COI1*, coronatine insensitive1; eds1, enhanced disease susceptibility1; ein2, ethylene insensitive2; ET, ethylene; *FAD*, fatty acid desaturase; *HEL*, hevein-like protein; JA, jasmonic acid; *JAR1*, jasmonic acid resistant1; *MPK4*, mitogen-activated protein kinase4; *NahG*, salicylate hydroxylase; *PAD4*, phytoalexin deficient4; *PDF1.2*, PLANT DEFENSIN1.2; *PR*, pathogenesis-related; SA, salicylic acid; *SID2*, SA induction deficient2; *SSI2*, suppressor of SA insensitivity2; *Thi2.1*, thionin2.1 (Kunkel & Brooks 2002).

SA-metabolizing enzyme from *Pseudomonas putida* that converts SA to the biologically inactive catechol, do not express SAR marker genes and are unable to elicit SAR (Ryals et al. 1996). Several *A. thaliana* genes have been identified by mutational analyses that are involved in SA accumulation or the induction of SAR. These include genes such as *enhanced disease susceptibility1* (*eds1*), *eds4*, *eds5*, *phytoalexin deficient4* (*pad4*), *NON-EXPRESSOR OF PR-1* (*NPR1*), *SALICYLIC-ACID-INDUCTION DEFICIENT1* (*sid1*) and *sid2* (Glazebrook et al. 1996; Jirage et al. 1999; Li et al. 1999; Nawrath & Metraux 1999). All of these mutants showed suppressed or partially suppressed *R*-gene mediated responses, leading to enhanced disease susceptibility to

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a range of virulent pathogens. NPR1 has been found to be central to SAR (Cao et al. 1997). A closer examination of the *NPR1*, revealed that NPR1 contains a functional important ankyrin-repeat domain that may be involved in protein-protein interaction. NPR1 clearly functions downstream of SA and is localized to the nucleus during SAR where it interacts with members of the TGA family of basic leucine zipper protein (bZIP) transcription factors (Dong 1998; Thomma et al. 2001). Recent results also suggests a separation of *NPR1* from SA-dependent processes. *PR-1* expression (a reliable SAR marker) for example, was blocked in a NahG background, but only partially compromised in an *npr1* mutant. These results suggest an alternative, *NPR1* independent, but SA-dependent pathway (Kunkel & Brooks 2002). Furthermore, the phenotypic similarities between *pad4*, *sid1/eds5* and *sid2*, combined with defects in SA accumulation, suggest the involvement of numerous genes in SA-dependent plant defenses (Feys & Parker 2000). SA have also been found to interact with NO during pathogen elicited defense responses in a synergistic fashion, but the extent of this interaction is not yet clear (Klessig et al. 2000).

The fatty acid derivative, JA, has been indicated in several aspects of plant biology, including pollen and seed development, defense against wounding, ozone, insect pests and microbial pathogens (Creelman & Mullet 1997; Reymond & Farmer 1998; Klessig et al. 2000; Li et al. 2001). Several *A. thaliana* lines exist that are either impaired in JA production e.g. *fatty acid desaturase* triple mutants (*fad3/fad4/fad8*), or impaired in JA perception e.g. *coronatine insenstive1* (*coi1*) and *jasmonic acid resistance1* (*jar1*). These mutants all exhibit enhanced susceptibility to a variety of necrotrophic pathogens (Kunkel & Brooks 2002). Several mutants that exhibit enhanced or constitutive JA responses, such as *constitutive expression of VSP1* (*cev1*), *cex1*, and several constitutive *expressor of thionin* (*cet1*) and *jasmonate overexpressing* (*joe*) mutants, have been identified, but to date only the *cev1* mutant has been analyzed for enhanced disease resistance (Ellis & Turner 2001; Hilpert et al. 2001; Jensen et al. 2002; Xu et al. 2001). Surprisingly, these mutants exhibit increased resistance against *Erisyphe* sp. (Ellis & Turner 2001). It seems likely, however, that other constitutive JA-signaling mutants would exhibit enhanced resistance against necrotrophic pathogens that are normally controlled by the JA-pathway.

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Although ethylene has been shown to promote disease resistance in some interactions (Thomma et al. 1999; Norman-Setterblad et al. 2000), it promotes disease production in others (Lund et al. 1998). The *ethylene insensitive2* (*ein2*) mutant of *A. thaliana* for example, shows increased susceptibility to *B. cinerea* infection, but decreased symptoms when infected with *Pseudomonas syringue* or *Xanthomonas campestris* pv. *campestris* (Bent et al. 1992). Interestingly, the pattern of altered pathogen responses in the *ein2* ET-signaling mutant is remarkably similar to that observed for the *coi1* and *jar1* JA signaling mutants; the expression of several JA-dependent defense genes also requires *EIN2*. Furthermore, JA and ET signaling pathways are also required for induced systemic resistance (ISR), a form of resistance that is triggered by the root-colonizing bacterium *P. fluorescence* (Pieterse & van Loon 1999). These results led to the establishment of oversimplified models in which ET and JA are placed together in a single pathway. These models, however, do not take into account the modulating effect of the separate JA and ET pathways on each other (Kunkel & Brooks 2002).

2.2.5.3. Crosstalk among JA, SA and ET pathogen defense signaling pathways

Ample evidence exist that SA-, JA-, and ET-dependent defense pathways function in an interdependent fashion. SA and JA control the expression of mostly non-overlapping sets of responses, and a number of studies have revealed antagonistic effects of SA application on wound- and/or JA-induced gene expression (Doares et al. 1995). These observations are substantiated by recent observations in tobacco plants that showed an inverse relationship between the level of phenylpropanoid compounds, including SA, and the induction of systemic resistance to insect feeding mediated by JA (Felton et al. 1999). This inhibition of wound-induced gene expression can be partially overcome by exogenous application of JA and ET. The interaction between SAR and the wound response, at least in *A. thaliana*, however, seems to be more complex than just direct inhibition. The activity of allene oxide synthase (AOS), a key enzyme in JA biosynthesis, is up-regulated upon treatment with SA (Laudert & Weiler 1998). A possible explanation for this apparent contradiction is that SA stimulates the formation

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of a JA precursor, oxophytodienoic acid (ODPA), which might be involved in pathogen defense. Simultaneously, SA inhibits the final step in the biosynthesis of JA, suppressing the wound response (Maleck & Dietrich 1999).

JA and ET co-regulate a subset of *PR* genes in *A. thaliana* encompassing amongst others, the *PR*-3, *PR*-4, *PR*-12 (*PDF1.2*) defense genes. The precise mechanism of regulation is still obscure, at least *PR-12* requires the concomitant activation of both JA and ET response pathways (Penninckx et al. 1998; Thomma et al. 2001). The regulation of these genes also is distinct from the SA-dependent genes such as *PR-1*, *PR-2* and *PR-5*, because it does not depend on NPR1/NIM1 (Penninckx et al. 1998; Thomma et al. 1998). JA insensitive mutants such as *coi1*, which are more susceptible to *B. cinerea*, do not display enhanced sensitivity towards the fungus *P. parasitica* (Thomma et al. 1999). This observation further corroborates the existence of at least two separate signal-transduction pathways in *A. thaliana* essential for resistance against different pathogens, a SA-dependent and JA/ET-dependent pathway (Thomma et al. 2001).

Multiple defense mechanisms provide the plant with defense mechanisms against challenges from different groups of pathogens. The HR effectively restricts growth of biotrophic pathogens, but the cell death mediated by the HR, might in fact benefit necrotrophic pathogens. Evidence for this stems from the fact that growth of the necrotrophic fungi, *Botrytis* and *Sclerotina* is reduced in HR deficient plants such as the *A. thaliana* mutant *dnd1* (Govrin & Levine 2002). Thus, the evolution of JA/ET defense regulated pathways, with an antagonistic effect on SA-induced cell death, could be an adaptation to operate against necrotrophic fungi. Recent evidence suggests, however, that the employment of two general pathways, controlled by different signaling molecules, is an oversimplification. The two polygalacturonase inhibiting proteins genes (*Atpgip1* and *Atpgip2*) of *A. thaliana* for instance, are both strongly up-regulated by *B. cinerea* infection. The up-regulation of *Atpgip2* upon infection, however, is mediated via a jasmonic acid dependent pathway, whereas *Atpgip1* is up-regulated independently of JA, SA or ET (Ferrari et al. 2003). These results clearly demonstrate the complexity of signaling events during induced plant disease resistance.

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2.3. TRANSCRIPTIONAL CONTROL OF DISEASE RESISTANCE

Pathogen recognition and the subsequent induced signaling cascades almost invariably results in the induction of stress and defense-related genes. Stress gene induction primarily occurs at the level of transcription and a large portion of the genome capacity of plants is devoted to transcription, with the *A. thaliana* genome encoding in excess of 1500 transcription factors (Riechmann et al. 2000). The ensuing part of the review will give an overview of recent developments within the field of transcription, as well as discuss the role of transcription factors in plant defense responses.

2.3.1. Initiation of eukaryotic transcription: a basic overview

The process of transcription is central to cellular function and metabolic regulation. Functional diversity and specificity of cell function are determined by the correct temporal and spatial transcription of specific genes. This complex regulation of transcription enables cells to adapt to environmental cues, such as water stress, or a lack of nutrients. Improper regulation of transcription is often associated with serious developmental abnormalities, disease or even death. Basically, transcription by DNA-dependent RNA polymerases is a cyclic process composed of four steps: (i) promoter binding and activation, (ii) RNA chain initiation and promoter escape, (iii) RNA transcript elongation, and (iv) RNA transcript termination. Regulation of transcription can occur at any of these four steps (Uptain et al. 1997).

2.3.2. RNA polymerases

Purification of nuclear RNA polymerases in 1960 provided the basis for elucidating eukaryotic gene expression (Lee & Young 2000). Three distinct RNA polymerase are found in eukaryotic cells, protein encoding genes are transcribed by RNA polymerase II (pol II) to yield messenger RNAs (mRNAs), ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are transcribed by RNA polymerase I and III respectively (Table 1). All three enzymes are complex units, consisting of 8 – 14 different subunits each, yet still have

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several features in common (Butler & Kadonaga 2002, Schramm & Hernandez 2002). The two largest subunits of each of the three eukaryotic enzymes resemble the β and β' subunits of the single *Escherichia coli* enzyme. Furthermore, five subunits of the eukaryotic enzymes are found in all three enzymes (Zawel & Reinberg 1995). For the purpose of this review, only the RNA polymerase II machinery will be discussed.

Eukaryotic RNA pol II functions as a 12-unit, DNA-dependent RNA polymerase (Table 2) and is responsible for transcribing genes encoding mRNAs as well as several small nuclear RNAs (Nikolov & Burley 1997). Recently a ~3.5 Å resolution structure of a transcriptionally active 10 subunit form of *Saccharomyces cerevisiae* pol II was obtained by X-ray crystallography (Cramer et al. 2000). Along with the polymerase structure, a lower resolution structure of an actively transcribing pol II complex with associated DNA template and an 11 nucleotide nascent RNA transcript was obtained by two-dimensional electron crystallography. These structures provided considerable insight into the overall architecture of the enzyme, the nature of the DNA template and transcribed RNA within the enzyme's catalytic site. Conformational changes resulting in increased stability of the interaction between the enzyme and the DNA template during the transition from the pre-initiation to the elongation stage of transcription could also be elucidated (Dvir et al. 2001).

Table 1. Classes of genes transcribed by eukaryotic RNA polymerases

Type of RNA synthezised	RNA polymerase
Nuclear genes	
mRNA	II
tRNA	III
rRNA	
5.8S, 18S, 28S	I
5S	III
snRNA and scRNA	II and III[a]
Mitochondrial genes	Mitochondrial[b]
Chloroplast genes	Chloroplast[b]

[a] Some small nuclear (sn) and small cytoplasmic (sc) RNAs are transcribed by polymerase II and others by polymerase III.
[b] The mitochondrial and chloroplast RNA polymerases are similar to bacterial enzymes.

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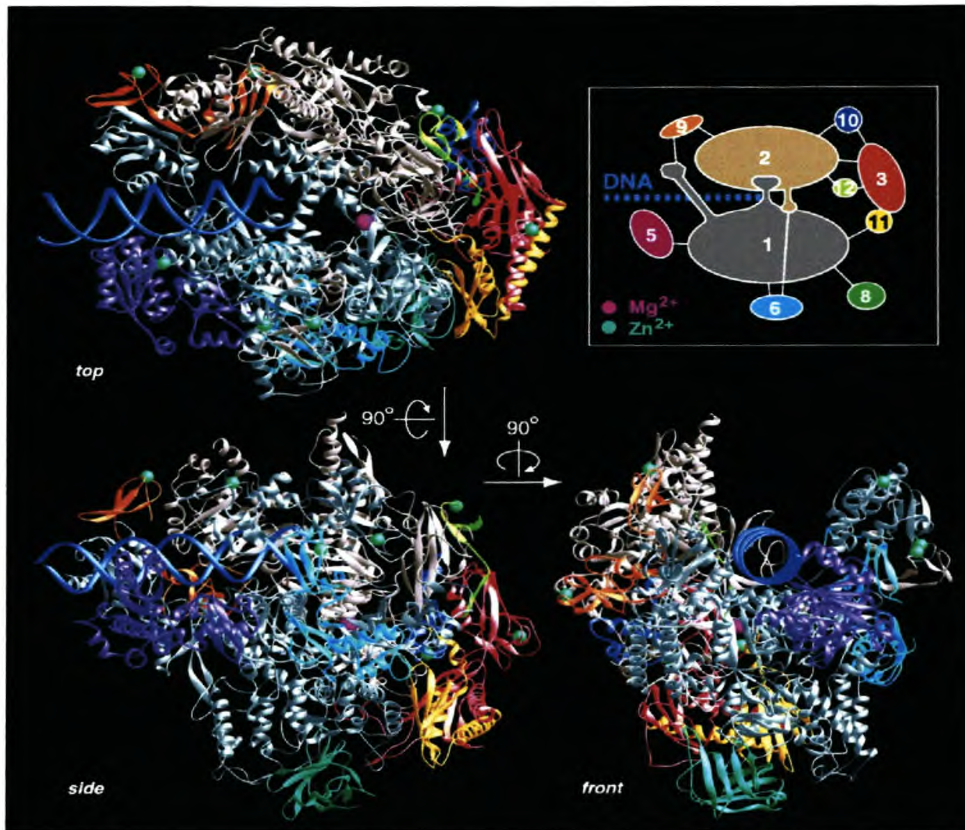

Figure 5. Architecture of yeast RNA polymerase II. Backbone models for the 10 subunits are shown as ribbon diagrams. The three views are related by 90° rotations as indicated. Downstream DNA, is placed onto the ribbon models as 20 base pairs of canonical B-DNA (blue) in the location previously indicated by electron crystallographic studies. Eight zinc atoms (blue spheres) and the active site magnesium (pink sphere) are shown. The box (upper right) contains a key to the subunit (Rpb1-10) color code and an interaction diagram (Cramer et al. 2000).

The core of the pol II enzyme is formed by two large subunits, Rpb1 and Rpb2, with a deep cleft between them and the remaining subunits occupying positions on their surfaces (Fig. 5). Each of these subunits occurs in a single copy and the structure is strutted by elements of Rbp1 and Rbp2 that traverse the cleft. The cleft is bridged by an Rpb1 helix, whereas the COOH region of Rpb2 extends to the opposite side. A subassembly of Rpb3, Rpb10, Rpb11 and Rpb12 anchors the Rpb1-Rpb2 complex on one end (Cramer et al. 2000).The polymerase catalytic site is situated at the end of the cleft. This part of the cleft is sufficient to bind nine base pairs of the RNA-DNA-hybrid,

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and the binding occurs in such a way that the 3'-hydroxyl end of the nascent RNA transcript is aligned properly with the catalytic Mg^{2+} ion located at the bottom of the cleft. Entry for ribonucleoside triphosphates to the catalytic site is facilitated through a "pore" located directly beneath the catalytic Mg^{2+} ion. The cleft between the Rbp1 and Rbp2 subunits downstream of the catalytic site is of sufficient size to accommodate ~20 bp of the DNA template (Dvir et al. 2001).

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Table 2. Yeast RNA pol II subunits (Cramer et al. 2000)

Subunit	Mass (kD)	Residues in sequence	Identity to human (%)
Rpb1	191.6	1733	52
Rpb2	138.8	1224	61
Rpb3	35.3	318	46
Rpb4	25.4	221	30
Rpb5	25.1	215	45
Rpb6	17.9	155	59
Rpb7	19.1	171	61
Rpb8	16.5	146	43
Rpb9	14.3	122	37
Rpb10	8.3	70	73
Rpb11	13.6	120	50
Rpb12	7.7	70	43

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The clamp forms on one side of the Rbp1-Rpb2 cleft from where it may interact with the DNA from the active site to about 15 residues downstream. The interaction adds increased stability to the interaction of the polymerase with DNA after the open complex has formed and the DNA template is bound in the catalytic site (Cramer et al. 2000). The term sliding clamp originated from the inferred importance of this binding site for the stability of a transcribing complex and the processivity of transcription. Once pol II have initiated transcription and synthesized a 10-20 nucleotide RNA transcript, one of a pair of potential RNA-binding "grooves" is in position to provide the pol II elongation complex with maximum stability by binding tightly to the nascent RNA transcript (Dvir er al. 2001).

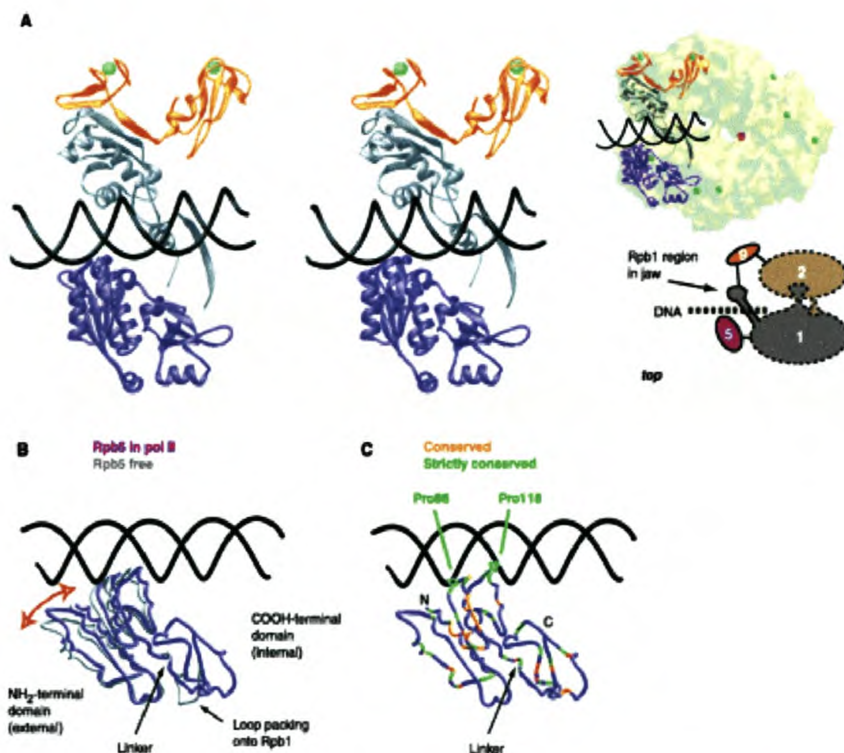

Figure 6. Jaws. (**A**) Stereoview of structural elements constituting the jaws (left) and the location of these elements within pol II (right). (**B**) Mobility of the larger, NH_2-terminal domain of Rpb5. Backbone models of free Rpb5 (gray) and Rpb5 in pol II (pink) are shown with their smaller, COOH-terminal domains superimposed. (**C**) Conservation of amino acid residues of Rpb5 (Cramer et al. 2000).

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Although pol II is the core of the transcription machinery and can, unwind the DNA double helix on its own, polymerize RNA and proofread the nascent transcript, it needs additional proteins to function efficiently (Armache et al 2003). The assembly of large initiation and elongation complexes, capable of promoter recognition and response to regulatory signals, also requires additional proteins (Shilatifard 1998). A regulated transcription initiation complex comprises pol II, five general transcription factors and a multi-protein Mediator. Initiation complexes can contain some 60 proteins, with a total molecular mass of 3.5 MD (Nikolov & Burley 1997) and the assembly of this complex on the chromatin template comprises the first step in eukaryotic transcription (Beckett 2001).

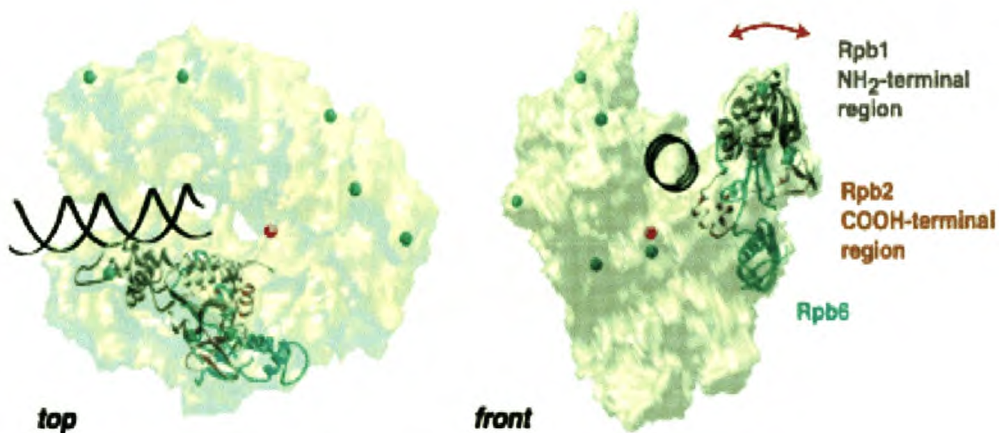

Figure 7. Structural elements constituting the clamp and their location in pol II are shown. The COOH-terminal region of Rpb2 and the NH_2-terminal region of Rpb1 bind one and two zinc ions, respectively (blue spheres). The NH_2-terminal tail region of Rpb6 extends from its main body (at the bottom in the front view) into the clamp. The direction of movement of the clamp revealed by comparison with electron crystal structures is indicated (double-headed red arrow) (Cramer et al. 2000).

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2.3.3. Promoter anatomy

Eukaryotic class II nuclear gene promoters share at least three common features; these include core or basal promoter elements, promoter proximal elements and distal enhancer elements (Nikolov & Burley 1997) (Fig. 8). The average core promoter encompasses approximately 100 bp and usually consists of a transcription initiation site and a TATA box. Core promoters are sufficient for transcription initiation by the basal transcription machinery (Lee & Young 2000). The TATA box is an AT rich sequence located at about 25 to 30 bp upstream of the start site in higher eukaryotes and 40 to 120 bp upstream in yeasts (Struhl 1995). The TATA box functions as a binding site for the TATA box binding protein (TBP). The sequence motive for these proteins are very loosely defined and they can function at a broad range of sequences, making it very difficult to identify genuine TBP-binding sites from sequence alone (Hoffmann et al. 1997; Zhao & Herr 2002; Zhao et al. 2003).

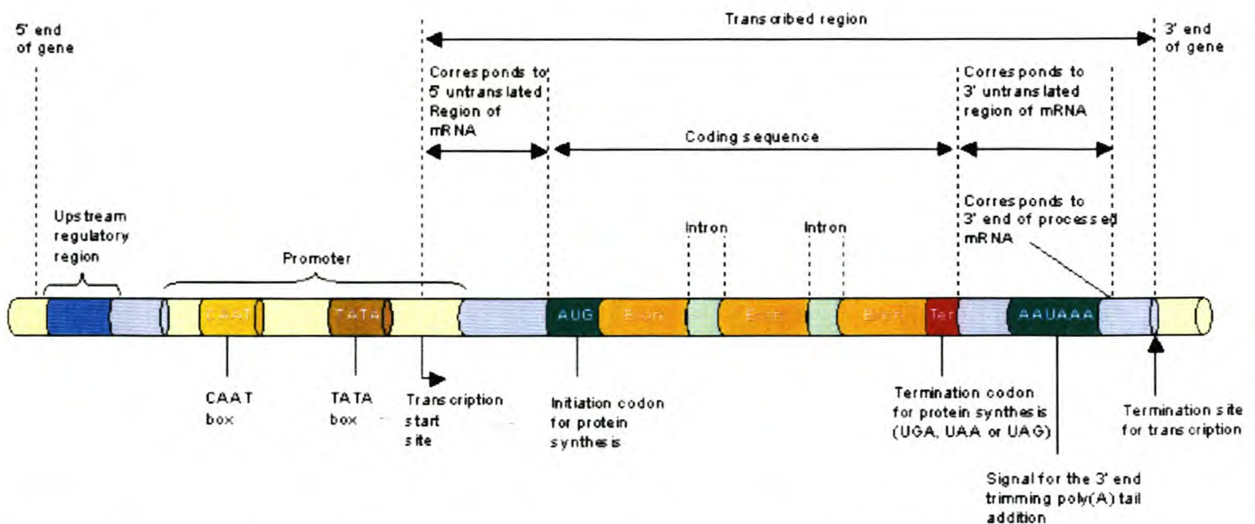

Figure 8. Typical eukaryotic nuclear type II gene. The basic promoter structure is shown in context with the open reading frame of the gene (ORF). Conserved protein binding elements are shown and the start of transcription is indicated. The actual sequence, size and position of these elements vary greatly among different promoters. Adapted from Ferl & Paul (2000).

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In some cases an additional element, the initiator (Inr) element encompassing the transcription start site, is present as well. This element is also capable of binding regulatory factors that may facilitate recruitment of the transcription apparatus (Kaufmann & Smale 1994; Roy et al. 1997). It is interesting to note that core promoters can contain TATA and Inr elements (composite), either element alone (TATA- or Inr-directed), or neither element (null) (Novina & Roy 1996). The core promoter context and sequence, although fundamental for binding general transcription apparatus, not only determines the basal, unregulated level of transcription, but can also contribute to the regulation of gene expression (Ohtsuki et al. 1998).

Although capable of accurate transcriptional initiation *in vitro*, most eukaryotic core promoters are virtually inactive in eukaryotic cells. This means that transcription in essentially all eukaryotic genes requires activators (Struhl 1999). Interaction of these activators with the DNA template and/or pol II machinery is facilitated through sequences normally located a few hundred base pairs upstream of the core promoter, termed upstream activating sequences (UAS) and enhancers (Lee & Young 2000). UASs typically describes elements bound by activators that influences transcription from nearby start sites, whereas enhancers are clusters of transcriptional regulator binding sites that function independent of orientation and at greater distances (Blackwood & Kadonaga 1998). Upstream repressing sequences (URSs), like UASs, facilitate the binding of sequence specific transcriptional regulatory proteins. In this case, however, the proteins act to repress, rather than activate transcription. Repression can be achieved through various mechanisms, including interfering with the binding of the activator, preventing recruitment of the transcription apparatus by the activator, and modifying chromatin structure (Hanna-Rose & Hansen 1996; Knoepfler & Eisenman 1999; Maldonado et al. 1999).

Other elements that are commonly found in eukaryotic promoter structures include silencers and locus control regions (LCRs). Classical silencers are defined that can repress promoter activity in an orientation- and position-independent fashion (Ogbourne & Antalis 1998). LCRs function as complex arrangements of multiple regulatory elements. They differ from classical enhancers in the sense that unlike enhancers, LCRs stimulate transcription independent of their site of integration,

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although their effects are limited by orientation and distance (Fraser & Grosveld 1998; Bulger & Groudine 1999; Engel & Tanimoto 2000).

Conserved elements in promoters, in addition with numerous other DNA elements, function to increase or decrease expression of multiple genes within regions of the genome, effectively subdividing the chromosomes into active and inactive regions.

2.3.4. Initiation of transcription and formation of the preinitiation complex

RNA pol II alone is not sufficient to initiate eukaryotic transcription and the enzyme requires a number of additional factors to specifically initiate transcription (Gill 2001). Accurate transcription is dependent on assembling pol II and a minimum of five transcription factors (TFs), namely IID, IIB, IIF, IIE and IIH (Fig. 9) into a preinitiation complex (PIC) (Nikolov & Burley 1997). Together with the polymerase, the complex comprises >30 distinct polypeptides with an aggregate molecular mass of nearly 2 megadaltons (Dvir et al. 2001). Of the five general TFs, only TFIID binds specifically and independently to the core promoter. TFIID subunits includes more than a dozen distinct polypeptides, ranging in mass from 15 to 250 kDa and these include the TBP and about ten TBP-associated factors (TAFs) (Burley & Roeder 1996; Woychik & Hampsey 2002). The formation of the PIC still remains controversial; a more conservative school of thought perpetuates the idea that formation of the complex occurs in a stepwise fashion (Roeder 1996). First, TFIID binds through the TBP and TAF_{II}s to the TATA element in the promoter. The three-dimensional structure of TBP is very similar to that of a saddle; the concave underside of the saddle is responsible for DNA binding, whereas the convex upper side of the saddle binds various components of the transcription machinery. The TAFIIs are thought to make additional DNA contacts (i.e. binding to the Inr element) and may also play a role in core promoter selectivity (Verrijzer & Tjian 1996). These contacts are, however, not essential since the TBP binding alone can facilitate basal transcription (Fiedler & Marc Timmers 2000).

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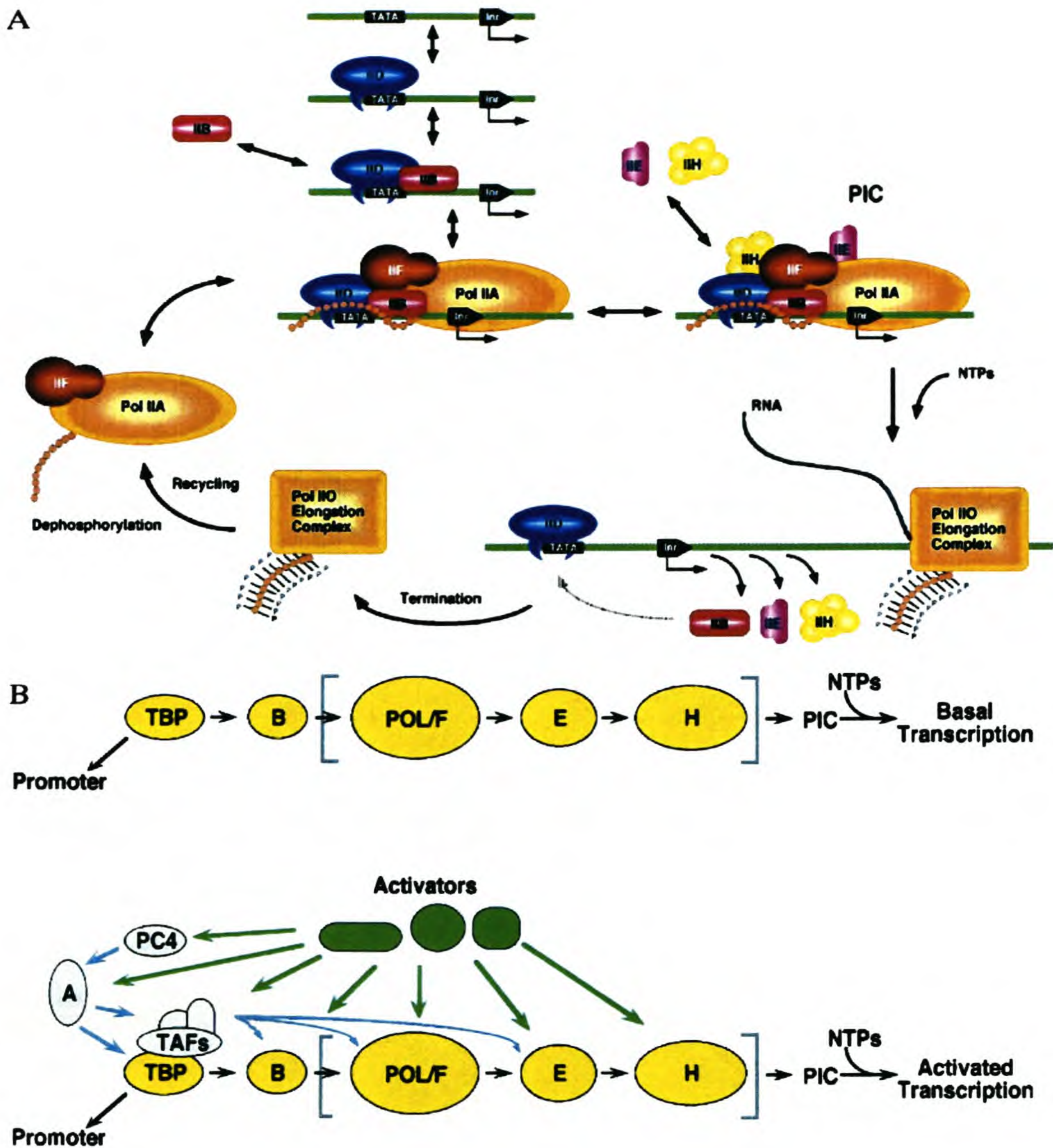

Figure 9. (A) Preinitiation complex (PIC) assembly begins with TFIID recognizing the TATA element, followed by coordinated accretion of TFIIB, the nonphosphorylated form of pol II (pol IIA) plus TFIIF, TFIIE, and TFIIH. Before elongation pol II is phosphorylated (pol IIO). Following termination, a phosphatase recycles pol II to its nonphosphorylated form, allowing the enzyme to reinitiate transcription in vitro. TBP (and TFIID) binding to the TATA box is an intrinsically slow step, yielding a long-lived protein-DNA complex. Efficient reinitiation of transcription can be achieved if recycled pol II reenters the preinitiation complex before TFIID dissociates from the core promoter. (B) Schematic representation of functional interactions that modulate basal (Upper) and activator-dependent transcription (Lower). The basal factors TBP, TFIIB, TFIIF, TFIIE, and TFIIH and pol II are denoted by yellow symbols, with the general initiation factor contents of a "pol II holoenzyme" enclosed by square brackets. TAFII and non-TAFII coactivators (purple) and transcriptional activators (green) are shown interacting with their targets in the PIC (Nikolov & Burley 1997).

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TBP binding can also be influenced by interaction of TFIIB with the DNA just 5' of the TATA box, but TFIIB binding can only occur when TBP is already bound. TFIIB is also the next protein to bind; the TFIIB-TFIID-DNA platform in turn is recognized by a complex of pol II and TFIIF, followed by TFIIE and TFIIH (Verrijzer & Tjian 1996) (Fig. 10). TFIIB binding stabilizes the TBP-DNA complex and may also play a role in determining the polarity of TATA element recognition. This is important because if TBP were to bind to the TATA box in such a way that the N-terminal half of the molecular saddle were to interact with the 5' end of the TATA element, the basic/hydrophobic surface of the N-terminal stirrup would make unfavorable electrostatic interactions with the basic cleft of TFIIB (Nikolov & Burley 1997).

The binding of TFIIF follows the formation of the TFIIB-TFIID-DNA platform and is a prerequisite for the entry of TFIIE and TFIIH into the PIC. TFIIF is the only transcription factor to form a very stable bond with the pol II, referred to as pol/F (Woychik & Hampsey 2002). This association prevents pol II binding to nonpromoter sites and stabilizes binding of pol II to the trimeric (TFIIB-TFIID-DNA) complex (Fiedler & Marc Timmers 2000). TFIIF is critical for tight wrapping of DNA around the PIC, possibly inducing torsional strain in the DNA and thereby facilitating promoter melting. TFIIF is also capable of suppressing transient pauses during transcription, possibly through its wrapping action or with association with known elongation factors and, therefore, stimulating polymerase elongation (Lee & Young 2000). TFIIE acts to recruit TFIIH and is also responsible for the subsequent regulation of TFIIH activity by stimulating the CTD and ATPase activities of TFIIH. Both TFIIE and TFIIH are required for the formation of an open complex prior to the formation of the first phosphodiester bond. In addition, TFIIF plays an important role in preventing arrest of early pol II elongation intermediates by functioning as an adapter that links TFIIE and TFIIH to the transcribing polymerase (Dvir et al. 2001).

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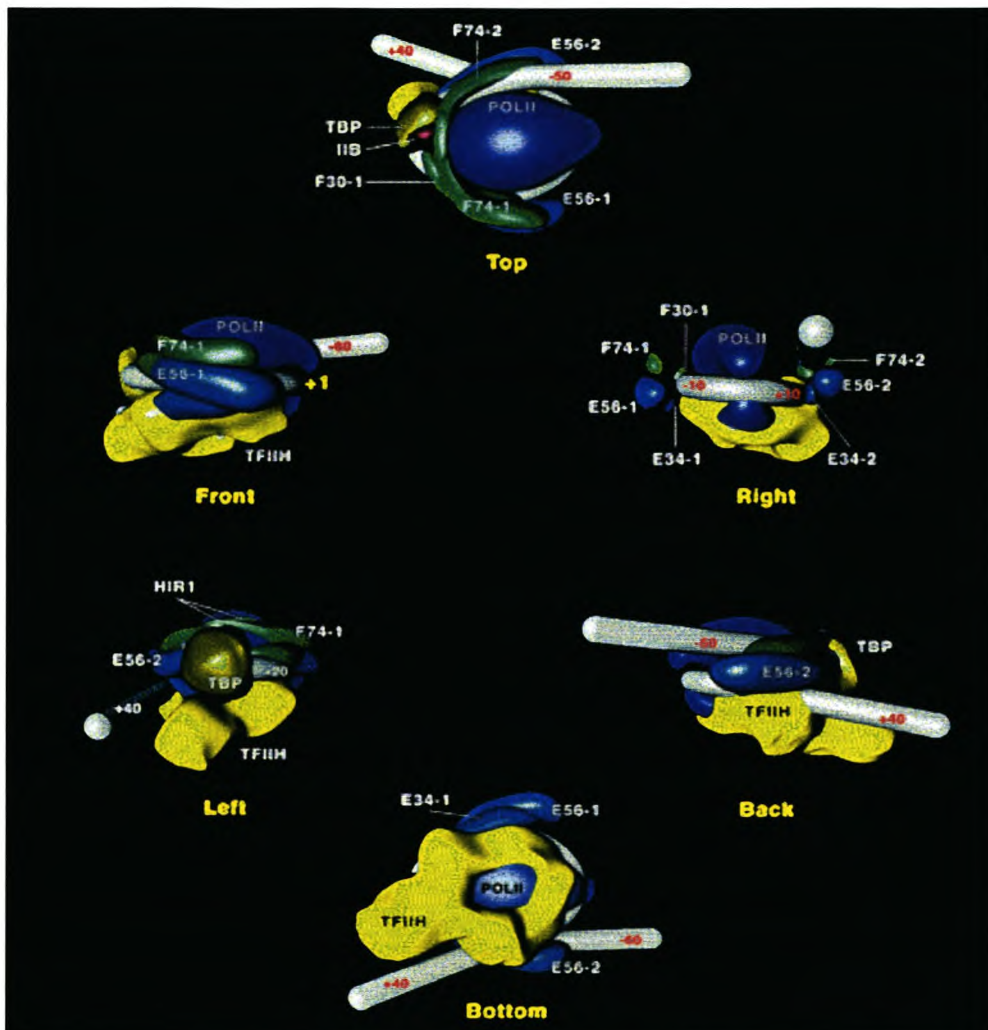

Figure 10. Topological organization of pol II, the general initiation factors, and DNA in the pol II preinitiation complex. The model includes two copies of TFIIE and TFIIF. IIB, TFIIB; F74, RAP74 subunit of TFIIF; F30, RAP30 subunit of TFIIF; E56, large subunit of TFIIE; E34, small subunit of TFIIE. (Dvir et al. 2001)

Cooperation between TFIIE, TFIIF and TFIIH further suppresses arrest of very early pol II elongation that requires the DNA helicase activity of TFIIH (Dvir et al. 1997; Kugel & Goodrich 1998). TFIIH consists of nine subunits ranging in mass from 39 – 89 kDa (Drapkin & Reinberg 1994). It is the only general transcription factor capable of enzymatic activity, including two ATP-dependent DNA helicases with opposite polarity (XPB and XPD), DNA-dependent ATPase as well as a serine/threonine kinase that is

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regulated by the cyclin H subunit and is capable of phosphorylating the C-terminal domain of the large subunit of pol II (cdk-activating kinase complex) (Nikolov & Burley 1997; Woychik & Hampsey 2002). TFIIH not only plays a role during the assembly of the PIC and the formation of the open complex, but the XPB and XPD proteins are essential components of the nucleotide-repair (NER) mechanism (Weeda et al. 1998). Within the context of PIC formation, TFIIH is responsible for the destabilization of the dsDNA structure (Fiedler & Marc Timmers 2000).

Recent studies have suggested that the core pol II enzyme is already complexed to a subset of basal transcription factors within the cell, forming a pol II holoenzyme. This holoenzyme is also associated with accessory factors such as SRB (suppressor response-element-binding protein) as well as proteins from the yeast mediator complex. The holoenzyme form of pol II, however, still requires the same set of basal factors, whether they assemble via a stepwise procedure or in a single step, have little effect on the roles of each component within the PIC (Lee & Young 2000).

2.3.5. Transcription factors in plant defense responses

Transcriptional regulation of defense-related genes depends on the integration of various signals to change the rate of transcription of specific target genes. Signal integration is in part governed by transcriptional activators and repressors, many of which bind DNA in a sequence specific manner (Cowell 1994). DNA binding domains of transcriptional activators and repressors are highly conserved, allowing classification into several classes in which DNA-binding specificity is brought about by subtle changes in the amino acid sequence (Schwechheimer & Bevan 1998). Unlike the DNA-binding domains, the amino acid sequences governing activation and repression are not conserved, and consensus sequences have yet to be derived from many eukaryotic activation domains (Triezenberg 1995). In recent years significant progress has been made to link specific transcription factors with plant stress responses. In particular, members from the ethylene-responsive-element-binding factors (ERF), the bZIP proteins and WRKY proteins have been studied intensively and this review will focus mainly on these factors.

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2.3.5.1. ERF transcription factors

ERF proteins are unique to plants and belong to a subfamily of the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) transcription factor family. They are fairly abundant in *A. thaliana* with approximately 124 ERF family members (Riechmann et al. 2000). Similarity between ERF proteins is confined to the ERF-domain, consisting of 58-59 conserved amino acids. These domains can bind two *cis*-acting elements found in plant promoters, the GCC box, which is found in several *PR* gene promoters where it confers ET responsiveness, and the C-repeat (CRT)/dehydration-responsive element (DRE), which is involved in the expression of dehydration- and low-temperature-responsive genes (Singh et al. 2002). ERF proteins are involved in several important processes and the large size of the ERF family is an indication of the large variety of stresses to which family members have been linked. In *A. thaliana*, ERF expression is regulated by dehydration, salt and cold stress (Stockinger et al. 1997; Fujimoto et al. 2000; Park et al. 2001), abscisic acid (Finkelstein et al. 1998), pathogen infection, wounding, ET, SA, and JA (Buttner & Singh 1997; Onate-Sanchez & Singh 2002; Singh et al. 2002). Timing of gene induction also seems to be important since several *A. thaliana ERF* genes for example, are induced in response to pathogen infection with different, but overlapping kinetics, which may help to orchestrate the correct temporal defense response (Onate-Sanchez & Singh 2002; Singh et al. 2002). Regulation also occurs on a post-translational level; the tomato *Pseudomonas* Tomato resistance (PTO) kinase, interacts with and phosphorylates an ERF protein (PTO-Interacting4 [PTO4]) and thereby increases the DNA-binding activity of PTI4 to the GCC box (Gu et al. 2000). ERF proteins typically activate transcription, although some observations support a transcriptional repressive function (Fujimoto et al. 2000). This observation was further supported by domain swapping studies and mutational analysis that showed a conserved seven-amino-acid motif that was sufficient to repress activation. It also was shown that a single amino acid substitution could abolish this activity (Ohta et al. 2001).

To date, no loss of function mutants have been isolated for any *ERF* genes, but several *ERF* genes have been over-expressed in plants. Typically GCC or CRT/DRE

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motif-containing genes are up-regulated in these plants and many exhibited enhanced resistance to specific stresses, as well as growth defects in response to ERF over-expression (Liu et al. 1998; He et al. 2001; Berrocal-Lobo et al. 2002; Wu et al. 2002). Although the functionality of ERF proteins across species has been shown to increase stress tolerance of plants, over-expression often has harmful effects. Stress-inducible promoters offer a solution to this problem and this approach has been used successfully for the DRE-binding factor *DREB1A*. Controlled over-expression in *A. thaliana* resulted in enhanced protection against freezing, drought and high salinity with no deleterious side effects (Kasuga et al. 1999).

2.3.5.2. bZIP transcription factors

Proteins with bZIP domains are present in all eukaryotes analyzed to date. Structurally, bZIP proteins have a basic region that binds DNA and a leucine zipper dimerization motif (Jakoby et al. 2002). A very large family of bZIP proteins is found in *A. thaliana*, between 75 and 81 putative genes are present, which is approximately four times as much as in yeast, worm or human (Riechmann et al. 2000). Many bZIP transcription factors involved during stress responses have been reported (Seki et al. 2003). Among these is one class of bZIP proteins that comprise the TGA/*octopine synthase* (*ocs*)-element-binding factor (OBF) proteins. These proteins bind to the *activation sequence-1* (*as-1*)/*ocs* element which regulates the expression of some stress-responsive genes such as the *PR-1* and *GLUTATHIONE-S-TRANSFERASE6* (*GST6*) genes (Lebel et al. 1998). In *A. thaliana*, this family comprises seven members and they are involved in key responses during plant defense, xenobiotic stress responses and development (Singh et al. 2002). More interestingly, these proteins have been shown to interact with NPR1, which, as discussed, functions as a key component of SA-dependent and independent defense signaling cascades (Zhang et al. 1999; Despres et al. 2000). NPR1 have been shown to bind specifically to transcription factors, enhancing their specificity and DNA-binding activity, resulting in increased PR protein expression (Despres et al. 2000).

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The TGA/OBF family of bZIP proteins is not the only bZIP proteins involved during stress responses, various others have been implicated during stress conditions such as UV light, and salt/drought (Jakoby et al. 2002). In vegetative tissues, ABA and abiotic stresses induce gene expression through *cis*-elements that include the ABA response element (ABRE). *In vitro* and *in vivo* protein/DNA binding analyses indicates that the ABRE binding factor (ABF) and ABA-responsive element (AREB) proteins can bind to different ABRE containing promoters (Choi et al. 2000; Uno et al. 2000). Transcriptional regulation of the ABF/AREB group of bZIP proteins occurs via ABA or abiotic stresses, whereas post-translational regulation is mediated through ABA (Jakoby et al. 2002; Singh et al. 2002). Over-expression of the ABF3 or ABF4 encoding genes in *A. thaliana* resulted in altered expression of ABA/stress regulated genes, ABA hypersensitivity and other ABA-associated phenotypes, as well as reduced transpiration and enhanced drought tolerance (Kang et al. 2002). Transcriptional regulation of bZIP proteins, however, is complex. This is clearly demonstrated by observations that pathogen responses override UV protection through an inversely related ACGT-containing element (ACE) promoter element (Logemann & Hahlbrock 2002). Furthermore, the small amount of mutational data available regarding bZIP proteins prohibit confident predictions about common functions for structurally different bZIP proteins (Jakoby et al. 2002). Further research elucidating bZIP expression analysis within specific families is needed to fully understand bZIP relationships within defined functional groups.

2.3.5.3. WRKY transcription factors

WRKY proteins are a novel family of transcription factors that are unique to plants and form a large family with 74 members in *A. thaliana* (Singh et al. 2002). These proteins contain the WRKY domain, a 60 amino acid region that includes the amino acid sequence WRKYGQK and a zinc-finger-like-motif, which are highly conserved among family members. Increasing evidence indicates that WRKY proteins are regulatory transcription factors, with a binding preference for a DNA sequence motif (T)(T)TGAC(C/T) called the W box (Ishiguro & Nakamura 1994; Eulgem et al. 2000).

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WRKY proteins can be classified with regard to their WRKY domains, both on the number of WRKY domains and the features of their zinc-finger-like-motif. WRKY proteins with two WRKY domains belong to group I, whereas proteins with one WRKY domain belong to group II. A few WRKY proteins with a distinct single finger motif have recently been assigned to a group III. WRKY proteins nevertheless bind in a sequence-specific manner to W-boxes, irrespective of their group classification (Eulgem et al. 2000). WRKY proteins exhibited up-regulated expression patterns upon induction by pathogens, defense signals and wounding (Eulgem et al. 1999). Up-regulation is typically extremely rapid, transient and independent of the *de novo* synthesis of regulatory factors (Eulgem et al. 2000). Furthermore, expression profiling in *A. thaliana* revealed that 49 of the 72 WRKY genes were differentially regulated in response to SA treatment or bacterial pathogen infection (Dong et al. 2003).

Several target genes have been identified for WRKY factors, including many plant defense genes (Maleck et al. 2000) as well as regulatory genes such as receptor protein kinases and *npr1* (Du & Chen 2000; Yu et al. 2001; Robatzek & Somssich 2002). Mutation of the W-boxes in the promoter of the *NPR1* gene resulted in the inability of this gene to complement an *npr1* mutant for disease resistance (Yu et al. 2001). Interestingly, proteins regulated by WRKY proteins include WRKY proteins themselves. The promoters of many pathogen-and/or SA-regulated *AtWRKY* genes are rich in W boxes (Dong et al. 2003). W-boxes are typically described as positive *cis*-acting elements, but negative regulation also has been shown in defense genes such as the *A. thaliana PR1* gene. In this case, the basal and SA-induced expression levels might be negatively regulated by W-boxes (Lebel et al. 1998). Another example of negative regulation can be found in the case of the AtWRKY encoding gene. W-boxes in the promoter of the AtWRKY18 encoding gene may prevent over-expression during a defense response, minimizing the detrimental effects of this gene on plant growth (Chen & Chen 2002).

Although WRKY proteins have only recently been identified as a new family of transcription factors, a remarkable amount of information has been amassed regarding both their function and regulation. Current information clearly indicates a key role for

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these proteins in regulating not only the pathogen-induced defense program but in a variety of regulatory processes (Eulgem et al. 2000).

2.4. PGIP AS A DEFENSE GENE MODEL

In the previous sections of the review, a concise overview of the processes involved from pathogen perception to defense gene induction was given. Here we would like to give a brief overview of the function and regulation of a typical defense-related protein, specifically the PGIP family of proteins. PGIPs have been isolated from a large number of dicotyledonous plants, including alfalfa, apple, bean, chestnut, grape, green pepper, leek, *Lupinus albus* (Bird's foot trefoil), orange, and many more (De Lorenzo et al. 2001). In recent years a large amount of data has emerged regarding the regulation, structure and function of PGIPs. For recent reviews, see (De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002).

To understand the role of PGIPs in plant defense, it is important to consider the role of microbial pectinases, most notably polygalacturonase (PGs) during plant-pathogen interactions. PGs are among the first enzymes secreted by a number of fungal- as well as bacterial pathogens when breaching plant cell walls (Herlache et al. 1997; De Lorenzo et al. 2001; Idnurm & Howlett 2001). The interaction between plant cell walls and PGs has been well studied and their contribution to the molecular dialogue between host and pathogen is well established (Esquerre-Tugaye et al. 2000). Fungal pathogens such as *B. cinerea*, *A. flavus* and *Altenaria citri* are all dependent on PG genes to maintain full virulence on their respective hosts (Ishiguro & Nakamura 1994; Shieh et al. 1997; ten Have et al. 1998). The structure, specific activity, pH optimum, substrate preference and mode of action vary considerably among PGs (De Lorenzo & Ferrari 2002). Furthermore, the family size of PG-encoding genes also seems to be reflective of the specificity of the interaction. Pathogens with a broad host range, like *Botrytis* and *Sclerotinia*, have large PG-encoding gene families, whereas pathogens with a restricted host range, such as *Colletotrichum lindemuthianum*, contain only two endopolygalacturonase (endoPG) encoding genes (Esquerre-Tugaye et al. 2000).

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EndoPGs cleave the α-1,4 linkages of galacturonic acid residues not esterified in the C_2, C_3 or C_6 positions within the homogalacturon domain (Esquerre-Tugaye et al. 2000). PGIPs specifically inhibit this reaction by interacting with residues within the active cleft of the PG, thereby inhibiting binding of the PG to its substrate while simultaneously blocking the active site (Federici et al. 2001; De Lorenzo & Ferrari 2002). Typical of proteins involved in protein-protein interactions, PGIPs contain a consensus sequence for leucine-rich repeats (LRRs), GxIPxxLGxLxxLxxLxLxxNxLT/S, that has been shown to be involved in the PGIP:PG interaction (Bergmann et al. 1994; Leckie et al. 1999; Kobe & Kajava 2001). The PGIP:PG interaction results in the prolonged resistance of long-chain oligogalacturonides (OGs) that are endowed with biological activities. (De Lorenzo et al. 1994; Navazio et al. 2002). Among others, OGs are capable of eliciting plant defense responses; even at very low concentrations they have the ability to induce defense systems as efficiently as in response to pathogens (Reymond et al. 1995; Esquerre-Tugaye et al. 2000). PGIPs, therefore, acts not only to hamper the fungal invasion process by inhibiting cell wall degrading PGs, but also enhance the defense response by prolonging the existence of long-chain oligogalacturonides (De Lorenzo & Ferrari 2002).

The regulation of PGIP encoding genes correlates well with their role in defense. PGIPs from *Phaseolus vulgaris* are induced by pathogen infection, wounding, salicylic acid, as well as elicitors (Bergmann et al. 1994). Pathogen infection also induces PGIPs from apple fruits and soybean (Komjanc et al. 1999; Mahalingam et al. 1999). Multiple signaling pathways are involved in the regulation of multigene PGIP families. In *P. vulgaris* for instance, a multigene family of at least five *pgip* genes are found that are differentially regulated in response to pathogen attack as well as stress stimuli (Devoto et al. 1998; De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002). Similarly, the two *A. thaliana pgip* genes exhibit differential expression that is most probably mediated by distinct signal transduction pathways involving jasmonate and OGs (Ferrari et al. 2003).

The importance of PGIPs in defense against fungal pathogens is further demonstrated by the over-expression of various PGIP encoding genes in native as well as heterologous hosts. These include the over-expression pear *pgip* in tomato (Powell et al. 2000), the over-expression of bean *pgip* in tobacco (De Lorenzo & Ferrari 2002)

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as well as the over-expresssion of *A. thaliana pgip* genes in *A. thaliana* (Ferrari et al. 2003). In all cases a reduction in disease symptoms were reported when transgenic plants were infected with *B. cinerea*. Similar results were obtained when the *Vvpgip1* gene was over-expressed in tobacco. VvPGIP1 was shown to, among others, inhibit PGs from *B. cinerea*. Transgenic tobacco plants that were shown to contain high levels of *Vvpgip1* transcripts were also less susceptible to *B. cinerea* infection, with reductions in both infection tempo and lesion size (De Ascensao 2001). The importance of PGIPs in plant defense is, therefore, difficult to ignore. Not only do they provide direct protection against tissue damage by fungal invaders, but they also act to mediate defense response signaling, enabling the plant to successfully curb fungal infection.

2.5. CONCLUDING REMARKS

In recent years vast amounts of data have been obtained regarding the molecular basis of plant-pathogen interactions. It has become clear that a previously unimaginable complex system of interactions between the various components comprising the detection, signaling and reaction mechanisms exist in plants. Undoubtedly, with modern techniques and the ability to analyze whole transcriptomes and/or proteosomes, the amounts of available data will become exponentially more. The challenge regarding plant-pathogen interactions in the near future will, therefore, not be to obtain the necessary data, but rather to obtain a comprehensive representation of the flow of events involved in plant-pathogen interactions across plant species.

This being said, the available picture regarding these events is currently, at best still fragmented. Current data often point to seemingly conflicting results, again underlying the complexity of these events. Before a clear picture regarding plant-pathogen interactions can be obtained, additional experimentation highlighting the interactions between the various components involved is still required.

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CHAPTER 3

RESEARCH RESULTS

THE TRANSCRIPTIONAL REGULATION OF A POLYGALACTURONASE INHIBITING PROTEIN (PGIP) FROM GRAPEVINE (*VITIS VINIFERA* L.)

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The transcriptional regulation of a polygalacturonase inhibiting protein (PGIP) from grapevine (*Vitis vinifera* L.)

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Abstract

Polygalacturonase inhibiting proteins (PGIPs) are cell wall proteins that specifically inhibit various fungal polygalacturonases (PGs) and are involved in plant defense responses. PGIPs are encoded by small gene families, and the inhibition profile of each individual PGIP within a family is often unique. The expression of PGIPs is regulated by various stimuli, including, physiological, environmental and pathogen related factors. Recently a PGIP encoding gene from grapevine (*Vvpgip1*) has been cloned in our laboratory. Expression of PGIP in grapevine is developmentally regulated, is berry-specific and is induced in a tissue-independent manner by wounding, osmotic stress, *Botrytis cinerea* infection, indole acetic acid and salicylic acid. In addition, expression is down regulated by a staurosporine-sensitive protein kinase, suggesting the involvement of protein phosphorylation in the signal transduction cascade that leads to PGIP expression. PGIP induced by *B. cinerea* infection, wounding and osmotic stress in leaves displayed the same PG inhibition spectrum as that of the product of cloned *Vvpgip1* gene. The mRNA induction profile of grapevine PGIP was mimicked in transgenic tobacco expressing the cloned *Vvpgip1* gene under control of its own promoter, indicating that regulatory mechanisms for PGIP expression are conserved in tobacco and grapevine.

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3.1. INTRODUCTION

Grapevine (*Vitis vinifera*) is one of the most versatile and widely cultivated crops worldwide and is used for the production of fresh and dried fruit, juice concentrate, wine, and distilled liquors. Due to this versatility and the historical importance of wine, it has become one of the world's leading most important crops (Coombe, 1989). In spite of this apparent economical importance, the process of grape maturation (Fillion et al. 1999) is still poorly understood. Grape ripening is nonclimacteric, characterized by three distinct stages of berry growth that follow a double-sigmoid curve (Coombe, 1973). Stage I, the first period of berry growth, directly follows flowering and is characterized by increases in the pericarp and seed cell number. During stage II, approximately 7 to 10 weeks post-flowering, very little change in berry size occurs, but significant metabolic reorganization occurs, as well as high levels of *de novo* gene expression during this period (Davies and Robinson, 2000). Stage III, characterized by a second period of berry growth, starts with berry softening, rapid accumulation of sugars and amino acids, as well as a decrease in acidity and expansion of the flesh cells. The end of stage II and inception of stage III is termed *veráison* (Coombe, 1992) and denotes the physiological stage in the development of a grape berry when it begins to ripen as indicated by a softening of the fruit and a change in color (red for dark varieties and translucent for white varieties). Typically during grape berry maturation, the developmental-stage-specific increase in hexoses is accompanied by an accumulation of numerous developmentally regulated defense-associated proteins. These include acidic chitinases, thaumitin-like proteins and lipid-transfer proteins (Salzman et al. 1998, Davies and Robinson, 2000).

Plants rely on both passive and active defense mechanisms to defend against invading pathogens. Preformed defenses include structural barriers, such as a waxy cuticle, or strategically positioned antimicrobial compounds to prevent tissue colonization (Osborne, 1996). Induced active defense responses are characteristic features of incompatible plant-pathogen-interactions associated with disease resistance (Hutcheson et al. 1998). During pathogenesis, cell walls act as the first line of defense against phytopathogens. To overcome this barrier, plant-pathogenic bacteria and fungi

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have evolved a battery of pectic enzymes, consisting primarily of endo-polygalacturonases (endo-PGs) (Mahalingam et al. 1999). Plants on the other hand, have evolved specific methods to restrict pectic enzyme damage; one of these involves the polygalacturonase inhibiting protein (PGIP) gene family found in many plant species. These proteins are typically cell wall bound, tissue-specific, developmentally regulated and inducible by various stimuli, including pathogen attack (Stotz et al. 1993, Bergman et al. 1994, Devoto et al. 1998). Furthermore, PGIPs belong to a large family of leucine-rich repeat (LRR) proteins, which are normally associated with protein-protein interactions and have been indicated to have a role in the defense response (Cervone et al. 1997). A large body of evidence indicates that PGIPs can inhibit the activity of fungal PGs and this PG:PGIP interaction favors the accumulation of elicitor-active oligogalacturonides *in vitro* (Cervone et al. 1987). These proteins therefore, can be considered to be part of the preformed as well as the primary defense responses in plants. Similar to many resistance (R) genes, *pgip* genes are organized into complex multigene families. The individual PGIP encoding genes within such a family is often differentially regulated. Moreover, the expression and specificity profiles of a single member of the family do not necessarily mirror that of the whole family (Desiderio et al. 1997, Devoto et al. 1998, Leckie et al. 1999).

Recently, a PGIP-encoding gene (*Vvpgip1*) from *V. vinifera* was cloned in our laboratory (Genbank Ac: AF499451). Contrary to most characterized PGIP encoding genes from other dicotyledonous plant species, no evidence to support the existence of a *V. vinifera* PGIP multigene family could be found from either genetic (De Ascensao et al. manuscript in preparation) or biochemical analyses (this study). Here we report that the expression of the *Vvpgip1* gene is tissue specific with the highest levels of transcripts occurring in *veráison* berries. Tissue independent expression was found in response to abscisic acid (ABA), indole acetic acid (IAA), salicylic acid (SA), osmotic stress, pathogen infection and wounding as inducing agents. The induction by osmotic stress is novel to the *pgip* genes currently known and was specifically investigated in relation with the accumulation of certain hexoses. Furthermore, the broad-range serine/threonine protein kinase inhibitor, staurosporine, induced *pgip* expression in grapevine leaves, but had no inhibitory effect on any of the inducing treatments.

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The *Vvpgip1* gene under control of its own promoter was subsequently integrated into *Nicotiana tabacum* and the response to osmotic stresses, *Botrytis cinerea* infection, hormonal treatments and wounding was assayed. The specificity profile of grapevine PGIP induced by osmotic stress, pathogen infection and wounding was also determined against the polygalacturonases from various pathogenic fungi and compared to that of the *Vvpgip1* gene transiently expressed in *N. benthamiana* with the Potato virus X (PVX) expression system (Baulcombe et al. 1995). In both the stable and transient heterologous expression systems, the regulation and specificity of the *Vvpgip1* gene mirrored that of the native grapevine PGIP.

3.2. MATERIALS AND METHODS

3.2.1. Plant growth and light conditions

In vitro tobacco (*Nicotiana tabacum* L.) and grapevine (*Vitis vinifera* cv. Chardonnay) plantlets were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and incubated at 26°C in a 16-h light 8-h dark photoperiod. Tobacco plants were germinated from seed in soil and maintained in a greenhouse at 26°C. *V. vinifera* cv. Superior Seedless plantlets were clonally propagated and maintained in a greenhouse at 26°C

3.2.2. Constructs and probe preparation

Standard techniques for DNA cloning and manipulation were performed according to Sambrook et al. (1989). A 7.2 kb *Eco*RV fragment from pSK(Pgip1), a pBluescript based genomic clone containing the *Vvpgip1* gene as well as the putative 5' upstream regulatory regions (De Ascensao 2001), was cloned into the blunt-ended *Hind*III/*Eco*RI sites of pBI101 (obtained from Clontech, California) to yield pBI(*gpgip1*). This plasmid was mobilized into *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) by tri-parental mating with the use of *Escherichia coli* HB101 containing the helper plasmid pRK2013 (Armitage, 1988) and used for the stable transformation of tobacco. For

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transient expression analysis, the *Vvpgip1* open reading frame (ORF) was obtained by the polymerase chain reaction (PCR) amplification from plasmid pSK(Pgip1) using the primers 5'-ATCGATGGAGACTTCAAAACTT-3' and 5'-GTCGACTCACTTGCA-GCTCTG-3'. The PCR product was digested with *Cla*I and *Sal*I and cloned into the corresponding sites of PVX201 (Baulcombe et al. 1995) to obtain *PVX.Vvpgip1*. Nucleic acid hybridization experiments were carried out using the DIG system for filter hybridization from Roche Molecular Biochemicals. Specific probes for the *V. vinifera pgip* transcripts were obtained and labeled by PCR amplification of the ORF of a cloned genomic copy of the *Vvpgip1* gene according to the manufacturers specifications. The primers used were grapePGIP5' (5'-AGGACAGAGAAATGGA-GACTTCAAC-3') and grapePGIP3' (5'-AGTC-AGATCTGAGCCGTCACTTGC-3').

3.2.3. Plant transformation and PVX-mediated expression

For stable transformation, leaf discs of *N. tabacum* cv. Petit Havana SR1 were transformed with pBI(*gpgip1*) according to the method of Gallios and Marinho (1995) and regenerated under kanamycin selection. In all subsequent manipulations, independent transformed T1 progeny plant lines were used that were able to grow on selective MS media, supplemented with 100 $mg.l^{-1}$ kanamycin.

Direct inoculation of *N. benthamiana* plants with PVX201 DNA alone and PVX201. *Vvpgip1* DNA was done as previously described (Baulcombe et al. 1995). Leaves from infected plants were harvested 20 days after inoculation, weighed, flash frozen in liquid nitrogen and stored at -20°C until further analysis.

3.2.4. RNA extraction and Northern blot analysis

Total RNA from tobacco plants was extracted as follows: tobacco tissue (100 mg) was ground in liquid nitrogen and added to 1 ml of extraction buffer consisting of 300 µl of phenol (buffered at pH 8.0) and 700 µl of extraction solution (0.1 M Tris/HCl pH 8.0, 1.5% [w/v] SDS, 300 mM LiCl, 10 mM Na_2EDTA pH 8.0, 1% (w/v) Na-deoxycholate, 1% Igepal CA-630 [w/v], and 5 mM Thiourea and 1% [w/v] Na-Metabisulfate added after

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autoclaving). The homogenate was incubated for 5 min at room temperature, followed by the addition of 200 μl of chloroform. The solution was vortexed briefly, incubated for 5 min at room temperature and centrifuged for 10 min at 12 500 x g at room temperature. Nucleic acids were precipitated from the supernatant with one volume of isopropanol. Excess genomic DNA was removed by pipetting before subsequent centrifugation steps.

The protocol for RNA extraction from *in vitro* and field grown grapevine plants was adapted from Davies and Robinson (1996), with the following modifications: grapevine tissue was ground in liquid nitrogen and 2 g were added to 10 ml of extraction buffer (5 M sodium perchlorate, 0.3 M Tris/HCl pH 8.3, 8.5% [w/v] insoluble polyvinylpolypyrrolidone, 2% [w/v] PEG 4000, 1% [w/v] SDS, 1% [w/v] β-mercaptoethanol) and further homogenized in a commercially available polytron homogenizer. The homogenate was centrifuged at 12500 x g for 10 min at room temperature and the supernatant filtered through a 10 ml syringe stuffed with cotton wool. The filtered homogenate was extracted at least 3 times with an equal volume of phenol:chloroform (1:1) followed by an equal volume of chloroform. Nucleic acids were precipitated with 1 volume isopropanol. Unless otherwise stated, RNA extractions were carried out after 0 h, 24 h and 48 h, respectively. RNA from *B. cinerea* infected tissues was extracted after 0 h, 48 h and 72 h, respectively. The RNA was fractionated in formaldehyde-agarose gels and was blotted onto Hybond-N filters (Amersham Biosciences). All hybridizations reactions were performed overnight at 50°C in DIG easy hybridization buffer from Roche Molecular Biochemicals. Image acquisition and signal normalization was done using an Alpha Imager system from Alpha Innotech and the Alpha Ease software package using the 25S ribosomal RNA as normalization standard.

3.2.5. Leaf and berry explant infections and induction treatments

Leaf, stem, and root tissue was harvested from greenhouse grown *V. vinifera* cv Superior seedless plants. Inflorescences and berries (as individual berries and/or as mini berry clusters containing approximately four sub-laterals of the cluster rachis) in the

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green, *veraìson* and ripe stages were harvested from a Pinotage vineyard in the Stellenbosch area of South Africa. All berry material was stored at 4°C.

For induction experiments, plant tissues were infected with *B. cinerea* spores, treated with ABA, IAA, SA, wounded and osmotically treated in individual experiments. All induction experiments were independently repeated at least once. Except if stated otherwise, all uninduced controls were incubated for equivalent time periods as the longest treatments in a specific experiment. *B. cinerea* spores were provided by Prof. G. Holtz, Department of Plant Pathology, Stellenbosch University; spores were resuspended in sterile grape juice to a final concentration of 1 x 10^6 spores.ml^{-1}. *In vitro* Chardonnay leaves were briefly submerged in the spore suspension, placed on wet sterile filter paper and incubated in the dark at room temperature. Control leaves were submerged in sterile grape juice and incubated as described.

For wounding, *in vitro* Chardonnay leaves were placed on wet sterile filter paper and wounded uniformly with sterile forceps before being incubated in the dark at room temperature.

For treatments with ABA, IAA and SA, Pinotage leaves were placed upright in a 100 μM solution of each hormone solution so that only the petioles were covered (non-submersive); sterile water was used as control. In a separate experiment leaves were instead submerged in the solutions containing the hormone as well as in sterile water; untreated leaves were also included in the experiment.

For osmotic treatments, Pinotage leaves were submerged in sterile water as well as in solutions containing 0.1 M, 0.2 M, 0.4 M and 0.6 M sucrose, glucose, fructose and NaCl respectively. RNA was isolated 24 h after treatments. Berries were submerged in pure water as well as 0.2X, 0.4X, 0.6X, 0.8X, 1.0X, 1.2X, 1.4X, 1.6X, 1.8X and 2X sugar solution (1X = 72 g.l^{-1} glucose, 72 g.l^{-1} fructose and 68 g.l^{-1} sucrose). At least four Pinotage *veráison* berries from the same sublateral in a cluster were selected for each treatment based on berry size and colour. The berries were weighed before and after treatments. RNA was extracted after 24 h for the sterile distilled water, 0.4X, 0.6X, 0.8X and 1.6X sugar submersions. In an additional experiment, mini berry clusters were cut from the main cluster and also submerged in sterile distilled water.

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Inductions with the broad range serine/threonine protein kinase inhibitor were done by incubating the petioles of leaves from *V. vinifera* cv Superior seedless in a 20 μM staurosporine solution. RNA was extracted after 24 h, 48 h and 72 h. Inductions were done as described on leaves pre-incubated in a 20 μM staurosporine solution for 3 h and RNA extracted after 24 h.

To investigate the regulation of the *Vvpgip1* gene in transgenic tobacco plants, leaves from T1 progeny plants were subjected to *B. cinerea* infection, osmotic stress facilitated by pure water or a 0.1 M sucrose solution, wounding, IAA and SA treatments as described.

The effect of the inducing stimuli on PGIP activity was analyzed in leaves from field grown Trebiana Toscana plants from a commercial vineyard near Rome, Italy. Leaves were infected with *B. cinerea* spores, wounded and osmotically induced as described. *B. cinerea* spores were suspended in sterile grape juice to a final concentration of 1 x 10^6 spores.ml^{-1}. Leaves were infected with 1 x 10^4 spores by applying 10 μl of the spore solution on the leaf surface. Leaves were uniformly wounded with sterile forceps and for the osmotic induction, submerged in sterile distilled water. For all treatments, the leaves were incubated for 72 h at 26°C for a 16-h light, 8-h dark photoperiod. All inductions were verified with Northern blot analysis (results not shown). Total proteins were extracted and used for inhibition assays as described below.

3.2.6. Protein extraction and enzyme assays

Tobacco leaves were frozen in liquid nitrogen and ground with a mortar and pestle to a fine powder. Extraction buffer (20 mM Na acetate pH 4.7, 1 M NaCl) was added to a final ratio of 2 ml buffer per 1 g starting tissue. Tissue was further ground in extraction buffer to a fine homogenate and incubated on ice for one hour with gentle shaking. The mixture was centrifuged at 15 000 x g for 25 min at 4°C and the supernatant filtered through one layer of miracloth, aliquoted and stored at –20°C. Grapevine leaves were frozen in liquid nitrogen and ground. Isolation buffer (50 mM Na acetate pH 6.0, 1% [w/v] insoluble polyvinylpolypyrrolidone, 10 mM β–mercaptoethanol) was added to a

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final ratio of 2 ml buffer per 1 g starting material. Tissue was further ground in isolation buffer to a fine homogenate. The mixture was centrifuged at 15 000 x g for 10 min at 4°C and the supernatant discarded. The pellet was resuspended in 1 ml extraction buffer (50 mM Na acetate, 1 M NaCl, 1% [v/v] Triton X-100, 0.1% [w/v] Na-lauroylsarcosine) per 1 g of starting material, and incubated on ice with gentle shaking for one hour. The suspension was centrifuged again at 15 000 x g for 25 min at 4°C and the supernatant filtered through one layer of miracloth, aliquoted and stored at -20°C. Protein concentration was determined according to Bradford (1976) using a Biorad protein assay kit and bovine serum albumin (BSA) as a standard. Inhibition of polygalacturonase (PG) activity was determined as previously described in an agarose diffusion plate assay (Taylor and Secor, 1988).

3.2.7. Preparation of fungal PGs

Crude PG preparations from *B. cinerea*, *Colletotrichum acutatum*, *Alternaria alternata, Aspergillus niger, and Stenocarpella maydis* were prepared by culturing the fungi in citrate phosphate buffer (pH 6.0), supplemented with 1% (w/v) citrus pectin, 2 mM $MgSO_4.7H_2O$, 0.6 µM $MnSO_4.H_2O$, 25 mM KNO_3, 30 µM $ZnSO_4.7H_2O$, 0.9 µM $CuSO_4$ and 65 µM $FeSO_4$ for 10 days at 22°C. The cultures were filtered through Whatman No. 1 paper and precipitated overnight with 80% $(NH_4)_2SO_4$ at 4°C. Proteins were recovered by centrifugation at 10 000 x g for 20 min at 4°C and resuspended in 40 mM Na acetate (pH 5.0). PG from *Fusarium monoliforme* was prepared according to De Lorenzo et al. (1987). PG activity was determined using an agarose diffusion assay (Taylor and Secor, 1988). Small holes were punched in agarose plates containing 1% (w/v) Type II agarose (Sigma), 0.5 % (w/v) polygalacturonase (sodium salt) and 100 mM Na acetate, pH 5.2. Crude PGIP-containing membrane extracts were mix with PG preparations, added to the holes and incubated at 30°C for 16 h. The plates were clarified with 0.5 N HCl to observe and measure the zones around the holes. Complete inhibition was expressed as 100% and correlated to no observed zones.

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3.3. RESULTS

3.3.1. Expression analyses of the *Vitis vinifera* PGIP encoding gene (*Vvpgip1*)

In an effort to determine the expression profile of the PGIP gene in grapevine, tissue specificity as well as the response to abiotic and biotic factors was investigated. The recently isolated *Vvpgip1* gene was used as a probe in nucleic acid hybridization experiments to determine *pgip* transcript levels in leaves, stems, roots, inflorescences, and in green, as well as *veráison* stage and ripe berries. *Pgip* transcripts could not be detected in leaves, stems or inflorescences, but were detected in roots and in green, *veráison*- and ripe berries, Levels in *veráison* berries were approximately two-fold higher than those in roots, green or ripe berries (Fig. 1A). This is consistent with previous results obtained in our laboratory that showed a two- to three-fold increase in *pgip* transcript levels in *veráison* berries compared to levels in green-and ripe berries (De Ascensao 2001).

The effect of *B. cinerea* infection, osmotic stress, wounding as well as non-submersive hormonal treatments that included ABA, IAA and SA on *Vvpgip* transcript levels was analyzed (Fig. 1A to C). Treatments with hormones were performed also with leaves submerged in solutions containing the individual hormones as well as in sterile water (submersive treatments) (Fig. 1D). To eliminate the possibility that small changes in *pgip* transcript levels might be masked in tissues already expressing PGIP, grapevine leaves showing no basal expression were selected to investigate the induction profile of *Vvpgip1*. Accumulation of transcripts occurred after *B. cinerea* infection, wounding, IAA and SA and osmotic treatments, but was not observed upon treatment with ABA (Fig. 1A to C). Wounding provoked an approximately 10 times higher induction than *B. cinerea* infection (Fig. 1B). Severe tissue necrosis was, however, observed in *B. cinerea* infected tissues. In non-submersive treatments, IAA provoked an induction approximately 10 times and five times higher than ABA and SA treatments respectively (Fig. 1C). All submersive treatments, including that with sterile water, resulted in induction with no appreciable differences in transcript levels (Fig. 1D). Solutions of sucrose and fructose at a concentration of 0.1 M, induced *Vvpgip1* transcript levels two

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to four times higher than those observed with pure water; however this effect was lost at higher concentrations. The effect of NaCl at 0.1 M had a less pronounced effect, but the induction pattern seemed similar to that of fructose and sucrose. (Fig 2A to C). Glucose (0.1 M) had neither any apparent inducing effect above that of pure water, nor any repressive effect on *pgip* expression at higher concentrations (Fig. 2D).

Grapevine *veráison* berries were also subjected to cold stress (4°C storage) as well as osmotic stresses induced by pure water and various concentrations of a solution of sucrose, glucose and fructose. No induction was observed in response to cold treatment (results not shown). In agreement with the results obtained with leaves, accumulation of *Vvpgip1* transcript was observed in berries in response to osmotic stress facilitated by water. However, in contrast with the leaf experiments, no further differences in transcript levels were observed in response to osmotic stress facilitated by the sugar solution (Fig. 3A). Changes in berry water potential were verified by weighing berries before and after treatments (Fig. 3C). Results from this experiment clearly indicated water flow into berries under hypo-osmotic stress and water flow from berries under hyperosmotic stress conditions. To determine whether *Vvpgip1* expression is indeed induced by osmotic stress and not by other conditions created as a result of the submersion, direct water flow to and from the berries was retarded. This was done by comparing the levels of *Vvpgip1* transcripts in non-submerged and submerged berry clusters with *Vvpgip1* levels in non-submerged berries. No appreciable differences between transcript levels of the submersive and non-submersive treatments were observed under these conditions (Fig. 3B); effectively ruling out hypoxia or any other factors associated with the submersion.

The involvement of protein kinases in the regulation of *Vvpgip1* was investigated with the use of the broad range serine/threonine protein kinase inhibitor. Staurosporine induced PGIP in grapevine leaves at a concentration of 20 μM (Fig. 4A). No apparent inducing or inhibitory effect by staurosporine was observed when leaves were induced by pathogen infection, osmotic stresses, wounding or hormonal treatments respectively (Fig. 4B).

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All in all, our studies have revealed that several biotic and abiotic factors influence the expression of *pgip* in grapevine, including osmotic stress, pathogen infection, wounding and IAA and SA treatments.

3.3.2. Expression of the *Vvpgip1* gene in tobacco under its native promoter

To determine whether the *Vvpgip1* gene maintains its regulation features in plants other than *V. vinifera*, a 7.2 kb genomic fragment containing the *Vvpgip1* gene including its promoter was introduced into tobacco by *Agrobacterium*-mediated transformation. Six independent transgenic lines were selected for further analysis. Leaves of T1 progeny plants that were able to grow on 100 $\mu g.ml^{-1}$ kanamycin were subjected to osmotic stress, wounding, *B. cinerea* infection, IAA and SA treatment. *Vvpgip* transcripts were detected in five of the six independent lines. Absolute transcript levels varied significantly between the lines, but the lines all showed induction in response to osmotic stress, wounding, *B. cinerea* infection, or treatment with IAA or SA (Fig. 5).

3.3.3. Specificity profile of grapevine PGIPs

Crude total protein extracts from grapevine leaf tissue induced by hypo-osmotic stress, *B. cinerea* infection and wounding were tested against PGs from various fungi. The inhibition profile of each sample was compared to that of the *Vvpgip1* gene product heterologously expressed in *N. benthamiana* using the PVX system. Crude protein extracts from PVX.*Vvpgip1*-infected *N. benthamiana*, and from hypo-osmotically induced, wounded and *B. cinerea* infected grapevine leaves all exhibited similar inhibition profiles (Fig. 6). Inhibition was obtained against crude extracts from *B. cinerea*, *C. acutatum* and S. *maydis*. No inhibition could be detected against extracts from *A. niger*, *F. monoliforme* and *A. alternata*.

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3.4. DISCUSSION

In this study the effect of several biotic and abiotic factors on the expression of PGIP in grapevine with specific reference to the recently isolated *Vvpgip1* gene was investigated. PGIPs have been shown to be tissue-specific as well as developmentally regulated. Up-regulation of PGIP typically occurs upon pathogen infection, treatment with elicitors, SA, Jasmonic acid (JA), cold treatment and wounding (Stotz et al. 1993, Bergman et al. 1994, Devoto et al. 1998, Yao et al. 1999). Devoto et al. (1998) furthermore hypothesized that the regulatory mechanism of *pgip* families must include specific *in planta* developmental cues with environmental stress and pathogen signals superimposed on them. During ripening, grapevine berries are subjected to significant physiological changes, including a rapid increase in volume, increased berry softness, development of pigments as well as an accumulation of hexoses and other metabolic components (Davies and Robinson, 1996). Berry ripening represents a significant stress condition in the grapevine berries, mainly due to severe changes in osmotic potential. Berries store very high levels (up to 20% [w/w] hexoses when fully ripe) and may, therefore, be especially vulnerable to a reduction in water activity due to high sugar osmolarity (Lott and Barrett, 1967). As expected, a dramatic change in mRNA profile occurs during berry ripening and many of the up-regulated proteins are involved in stress responses (Davies and Robinson, 2000). The ripening process furthermore leads to increased vulnerability to fungal attack, which also necessitates the up-regulation of defense-related genes within the berry.

Within this context it is not surprising that the induction profile of grapevine PGIPs is closely associated with various factors associated with berry development as well as environmental and pathogen related stress conditions. The induction stimuli tested included ABA (developmental and stress responses), IAA (developmental and stress responses), SA (defense responses), osmotic (developmental and stress responses), wounding (defense- and stress responses) and *B. cinerea* infection (defense responses). In grapevine, PGIP expression is found exclusively in roots and ripening berries with transcript levels peaking in *veráison* berries. This tissue specificity is easily overcome in leaves (and one can probably assume in other plant tissues as

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well) if stress conditions associated with berry ripening are emulated, as well as with wounding and pathogen infection. This is clearly demonstrated by the induction of PGIP transcripts in grapevine leaves upon treatment with ABA, IAA, SA, *B. cinerea* infection, wounding, as well as osmotic stresses (Figs. 1B to C and 2A to D). When the hormonal treatments were combined with osmotic stress (submersive conditions), no clear induction above that of pure water could be observed (Fig 1D).

It remains unclear if ABA is directly involved in the regulation of *pgip* transcription. ABA is very effective in causing stomatal closure and its accumulation plays an important role in the reduction of water loss by transpiration under water stress conditions (Taiz and Zeigler, 1998). In our experiments involving ABA treatments, leaves were not exposed to water stress, the resulting increase in ABA levels in the leaves, however, still affected stomatal closure, resulting in a loss of transpiration and a consequent increase in water potential in the leaves (results not shown). We have, furthermore, shown that a decrease in *pgip* transcript levels occurs in post *veráison* berries. The decrease in *pgip* transcript levels is accompanied by a sharp increase in ABA levels during this phase of berry development (Coombe 1992). It is, therefore, very probable that the low levels of *pgip* transcripts observed in these experiments are in fact due to osmotic stress and are not a direct result of ABA (Fig 1B). The effects of IAA are more pronounced and an increase in IAA levels in leaves results in high levels of *pgip* transcripts (Fig 1B). IAA is involved in almost every process in plant growth and growth regulation and has different effects at different concentrations. Recently, it was shown that IAA levels increased in wounded potato tubers (Fabbri et al. 2000). Contrary to the findings of Cheong et al. (2002), who showed that IAA responsive genes are down-regulated by wounding, grapevine PGIP is up regulated by both wounding and IAA. From our data it still remains unclear what the exact function of IAA in PGIP induction is, but a role under stress conditions such as wounding is not excluded. The fact that grapevine PGIP is induced by both fungal infection and SA, reinforces the hypothesis that PGIP functions in the resistance of plants against fungal attack.

Osmotic stress is associated with berry ripening (Lott and Barrett, 1967), wounding and pathogen attack (Cheong et al. 2002). When leaves were submerged in 0.1 M solutions of sucrose and fructose, expression levels above that induced by water

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were observed. Although the effect seemed less pronounced for the 0.1 M NaCl solution, the general induction profile still followed the same pattern as that of sucrose and fructose. The induction seems to be due to osmotic conditions, and not sugar specific as indicated by the similar induction profile of NaCl solutions. At higher concentrations, expression levels dropped significantly (Fig 2A to C). Unlike sucrose, fructose and to an extent NaCl, a 0.1 M solution of glucose did not induce PGIP expression above that of pure water, and higher concentrations did not seem to have any repressive effect either (Fig 2D). It seems, therefore, that glucose can overcome the repressive effect of hyperosmotic stress on PGIP expression. To further elucidate the relationship between osmotic stress, hexose accumulation and PGIP expression in grapevine berries, *veráison* berries were treated with a solution consisting of sucrose, fructose and glucose (Fig. 3A). Transcript levels were highest in berries submerged in pure water, but no clear-cut relationship between an increase in hexoses and PGIP expression could be observed. When direct water flow to submerged berries was retarded, no significant induction of PGIP could be observed (Fig. 3B), indicating that the induction is not due to hypoxia nor other factors associated with the submersion. Although PGIP expression decreases in solutions with lower water potential, this effect is clearly masked by glucose (Fig 2D). The change in water potential in grapevine berries during ripening could offer an explanation for the gradual induction of PGIP up to *veráison*, but the reason for the post-*veráison* decrease in transcript levels still needs to be elucidated. This is to our knowledge the first report of a *pgip* gene responding to osmotic stress.

Protein phosphorylation is a key component of many defense-related signaling pathways (Lee and Rudd, 2002). Our results indicate that a serine/threonine protein kinase is involved in the negative regulation of PGIP expression in grapevine (Fig. 4A). Inhibition of this kinase led to PGIP expression in grapevine leaves, but neither cumulative induction, nor inhibition of PGIP expression was observed when leaves were subjected to induction stimuli in the presence of staurosporine (Fig. 4B). Ferrari et al. (2003) recently showed that one of the two *pgip* genes in *Arabidopsis* is upregulated after *B. cinerea* infection and that this upregulation is mediated via the jasmonic acid pathway. Rojo et al. (1998) furthermore showed the involvement of a staurosporine

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sensitive protein kinase in a jasmonic acid dependent wound signal transduction pathway. It therefore is not impossible that a serine/threonine protein kinase plays a role in pathogen and stress induced signaling pathways resulting in the induction of grapevine PGIP.

To investigate the effect of the environmental and pathogen- related factors on *Vvpgip1* expression in a heterologous system, the gene was introduced under the control of its native promoter into tobacco. Six independent transformed lines from the F1 generation were subjected to osmotic stress, *B. cinerea* infection, wounding as well as IAA and SA treatments and the levels of *Vvpgip1* transcripts determined. Induction upon wounding, *B. cinerea* infection osmotic stress as well as IAA and SA treatment is reproducible in transgenic tobacco plants (Fig. 5) indicating that the factors involved in the transduction of the induction signals are conserved in grapevine and tobacco. Low levels of *Vvpgip1* transcripts could also be detected in some of the uninduced tobacco leaves (results not shown), indicating a loss of tissue specificity in transgenic tobacco. We also compared the fungal PG inhibition profiles of crude protein extracts from induced grapevine leaf tissue (osmotic, wounding and *B. cinerea* infection stimuli) with that of VvPGIP1 obtained by transient expression in *N. benthamiana* plants (Fig. 6). The inhibition profile of the induced extracts matched that of the protein encoded by the *Vvpgip1* gene, further corroborating the observed transcriptional regulation of the *Vvpgip1* gene. The transcription profile of the grapevine PGIP suggests that the regulatory aspects of the protein conform to the hypothesis of Devoto et al. 1998 regarding the regulatory mechanism of *pgip* families. The specific factors and their interactions that are responsible for the tissue specificity and developmental regulation in *V. vinifera*, however, still remain unclear and need to be further elucidated.

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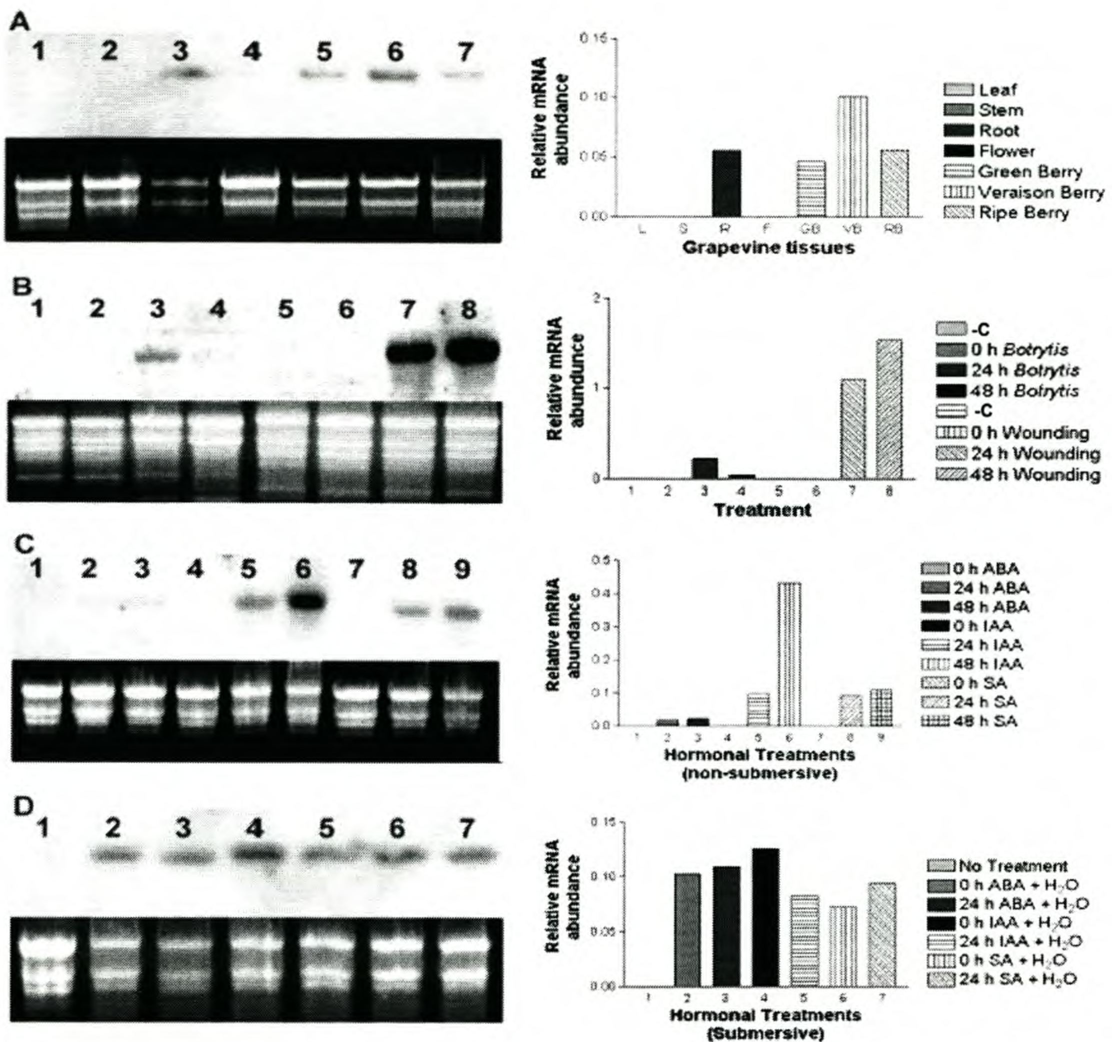

Figure 1. The expression profile of *V. vinifera* PGIP. Total RNA was extracted from **A**: *V. vinifera* cv Superior seedless leaves (Lane 1), stems (Lane 2), roots (Lane 3) and inflorescence (Lane 4) as well as *V. vinifera* cv Pinotage green berries (Lane 5), *veráison* berries (Lane 6) and ripe berries (Lane 7); **B**: *V. vinifera* cv Chardonnay leaves, untreated (Lanes 1 and 5), infected with *Botrytis cinerea* at 0 h, 48 h and 72 h post-inoculation (Lanes 2 - 4) and at 0 h, 24 h, 48 h after wounding (Lanes 6 - 8); **C**: *V. vinifera* cv Pinotage leaves treated with 100 μM ABA for 0 h, 24 h, and 48 h (Lanes 1 – 3), with 100 μM IAA for 0 h, 24 h and 48 h (Lanes 4 – 6) with 100 μM SA for 0 h, 24 h and 48 h; **D**: Untreated Pinotage leaves (Lane 1), Pinotage leaves submerged in pure water (Lanes 2, 4 and 6), Pinotage leaves submerged in 100 μM ABA (Lane 3), 100 μM IAA (Lane 5) and 100 μM SA (Lane 7). RNA was probed with the *Vvpgip1* gene and for every hybridization experiment transcript levels were normalized against total 25 S RNA to yield the quantitative bar graphs next to each blot.

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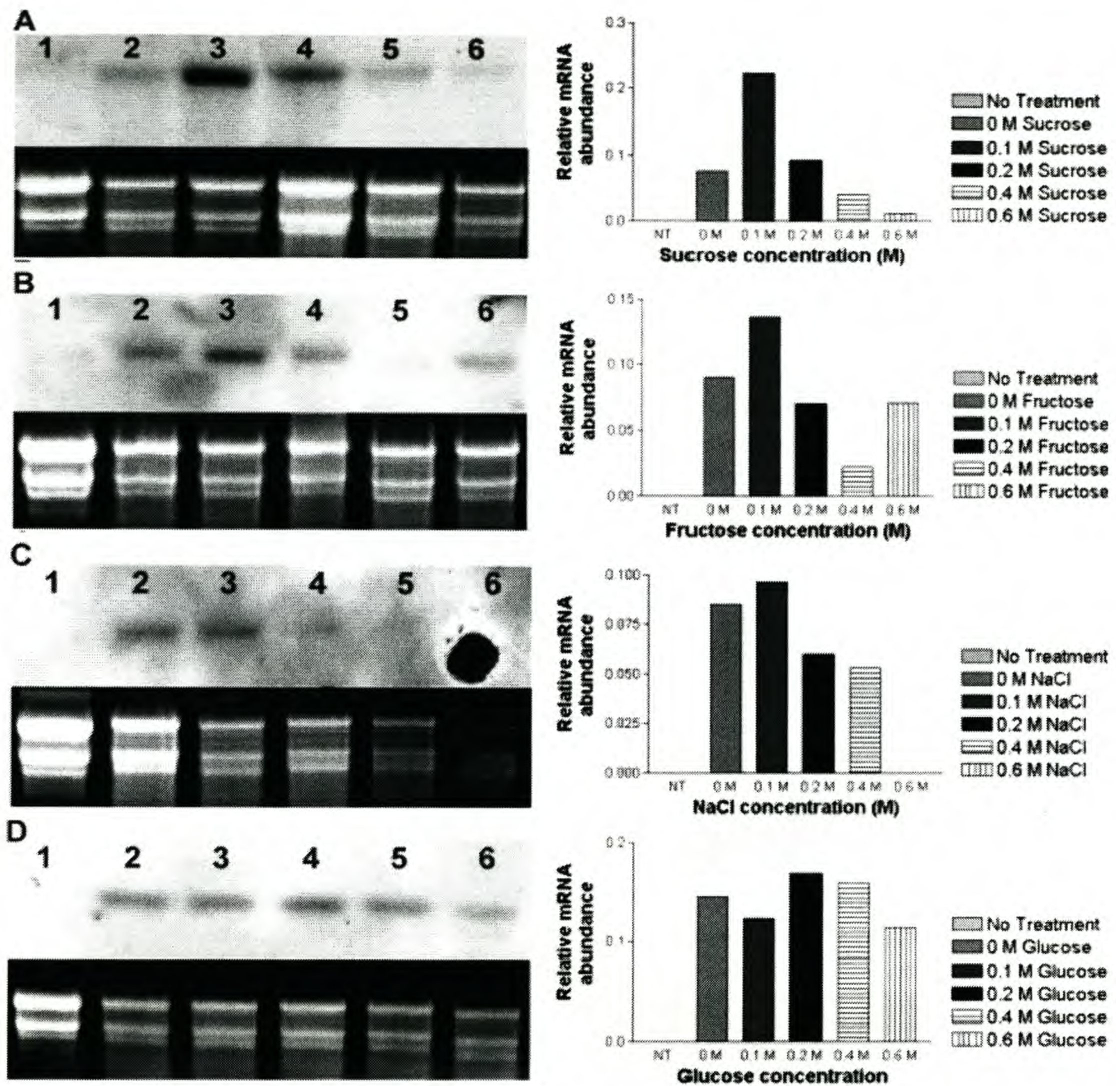

Figure 2. The effect of osmotic stress on PGIP expression in *V. vinifera* cv Pinotage leaves. Total RNA was extracted from **A**: Untreated leaves (Lane 1), leaves submerged in 0 M (water), 0.1 M, 0.2 M, 0.4 M and 0.6 M sucrose (Lanes 2 – 6); **B**: Untreated leaves (Lane 1), leaves submerged in 0 M, 0.1 M, 0.2 M, 0.4 M and 0.6 M fructose (Lanes 2 – 6); **C**: Untreated leaves (Lane 1), leaves submerged in 0 M (water), 0.1 M, 0.2 M, 0.4 M and 0.6 M NaCl (Lanes 2 – 6); **D**: Untreated leaves (Lane 1), leaves submerged in 0 M (water), 0.1 M, 0.2 M, 0.4 M and 0.6 M glucose (Lanes 2 – 6). RNA was probed with the *Vvpgip1* gene and for every hybridization experiment transcript levels were normalized against total 25 S RNA to yield the quantitative bar graphs next to each blot.

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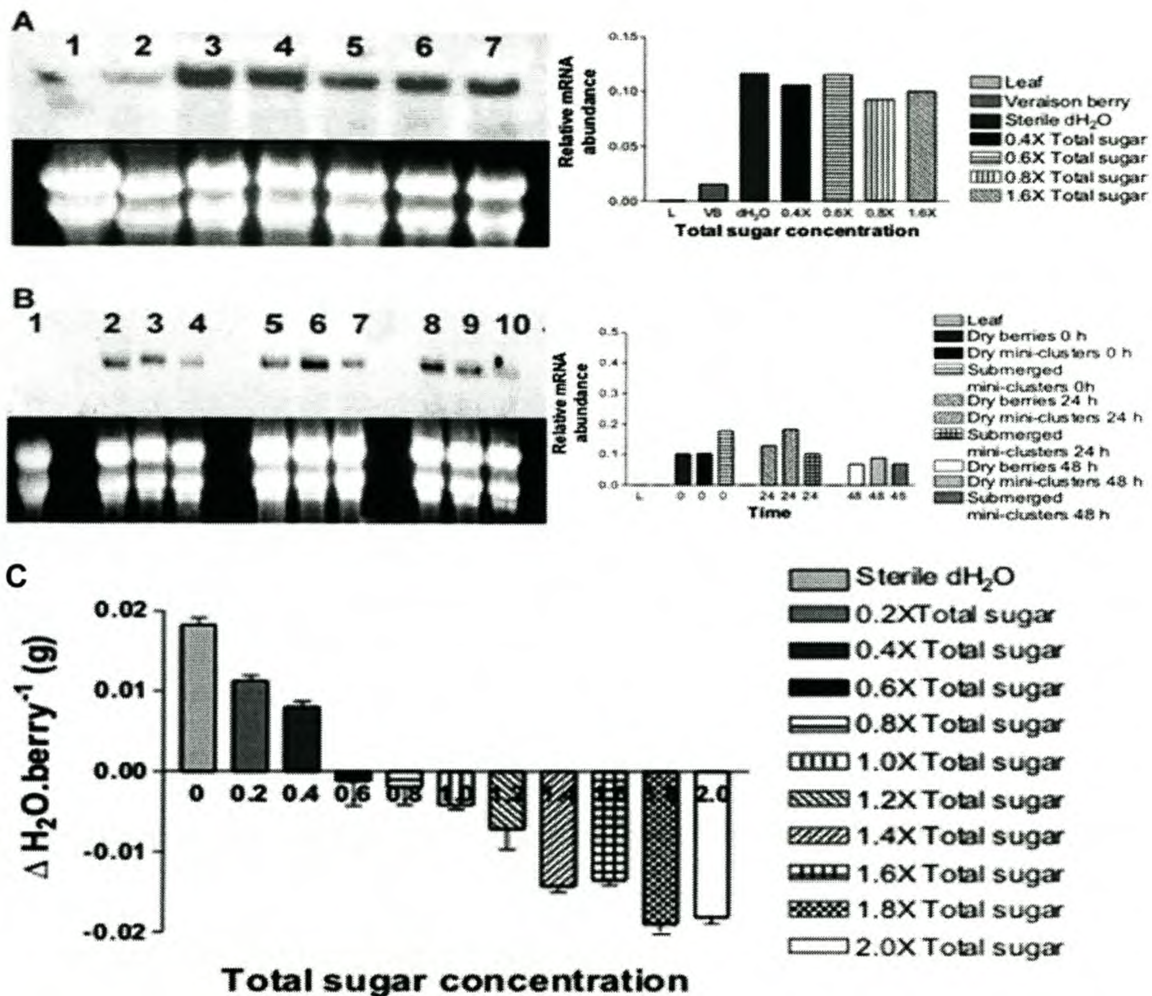

Figure 3. The effect of osmotic stress on PGIP expression in *V. vinifera* cv Pinotage *veráison* berries (A and B) and confirmation of movement in and out of berries (C) causing osmotic stress. Total RNA was extracted from **A:** Untreated leaves (Lane 1), *veráison* berries (untreated berries), berries submerged in pure water (Lane 2), 0.4X, 0.6X, 0.8X and 1.6X of a sugar solution (1X solution = 72 g/l glucose, 72 g/l fructose and 68 g/l sucrose) (Lanes 2 – 7); **B**: Untreated leaves (Lane 1), non-submerged berries after 0 h, 24 h and 48 h (Lanes 2 – 4), non-submerged mini berry clusters after 0 h, 24 h and 48 h (Lanes 5 – 7) and submerged mini clusters after 0 h, 24 h, and 48 h (Lanes 8 – 10). RNA was probed with the *Vvpgip1* gene and for every hybridization experiment; transcript levels were normalized against total 25 S RNA to yield the quantitative bar graphs next to each blot. **C**: Average change in berry weight of *veráison* berries subjected to a hexose concentration range. Berries were submerged in increasing hexose concentrations. Individual berries (at least four for each hexose concentration) were weighed before submersion in the sugar solution, incubated for 24 h hours in the solution, washed and weighed. Each lane represents the average change in berry weight per 0.2 X sugar increment.

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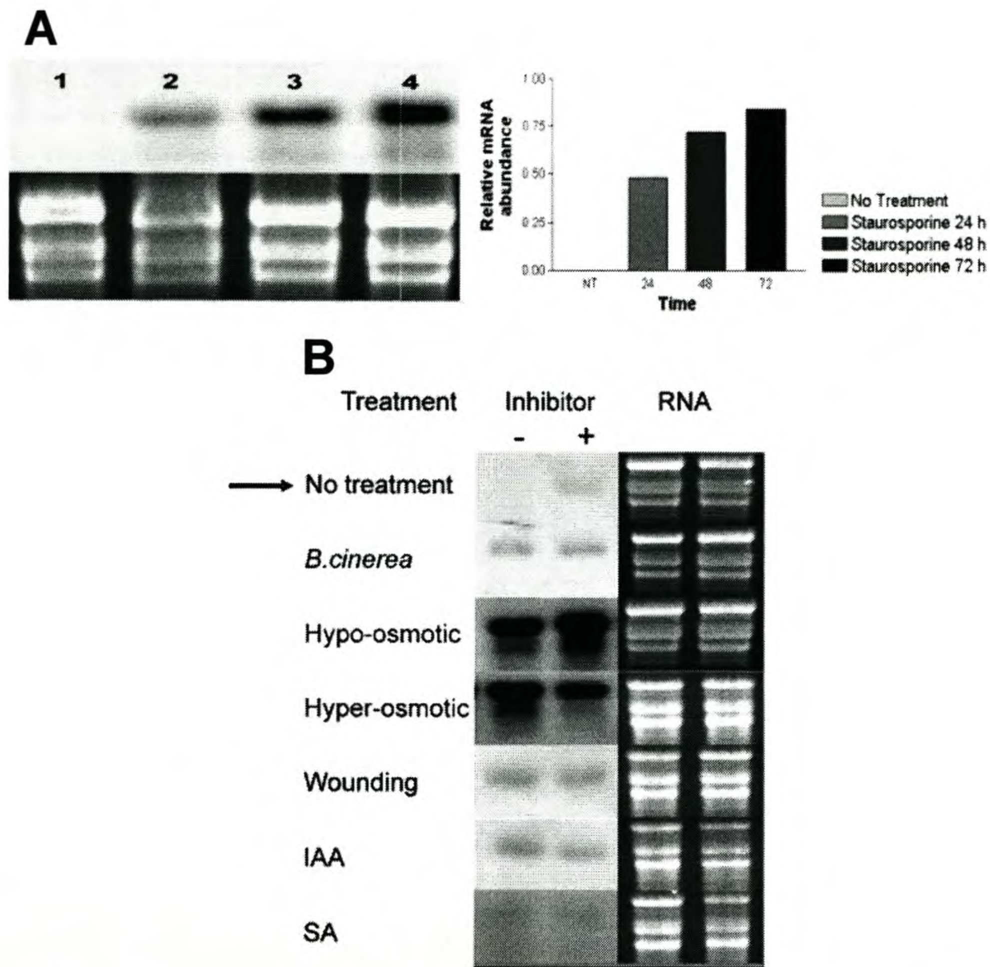

Figure 4. The effect of the broad range serine/threonine kinase inhibitor, staurosporine, on PGIP expression in *V. vinifera* cv Superior seedless. A. Detached leaves were placed in pure water (negative control) and a 20 mM staurosporine solution in such a way that only the petioles were covered. RNA was extracted after 24 h, 48 h and 72 h (Lanes 2 - 4) for leaves placed in the staurosporine solution and after 72 h for leaves placed in pure water (Lane 1). RNA was probed with the *Vvpgip1* gene and for every hybridization experiment; transcript levels were normalized against total 25 S RNA to yield the quantitative bar graph. B. Detached leaves were induced as described in the text in the presence or absence of 20 mM staurosporine. RNA was extracted after 24 h and probed as described.

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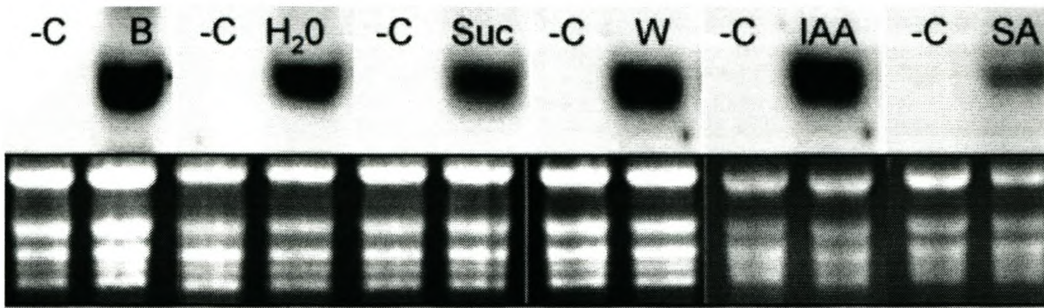

Figure 5. The effect of *B. cinerea* infection (B), osmotic stress facilitated by pure water (H_2O), osmotic stress facilitated by 0.1 M sucrose (Suc), wounding (W), 0.2 μM indole acetic acid (IAA), 0.2 μM salicylic acid (SA) on transgenic tobacco plants expressing the *Vvpgip1* gene under control of its native promoter. RNA was isolated after 24 h. RNA was probed with the *Vvpgip1* gene. Experiments were carried out on six independent transgenic lines and representative results relative to three of these lines are shown.

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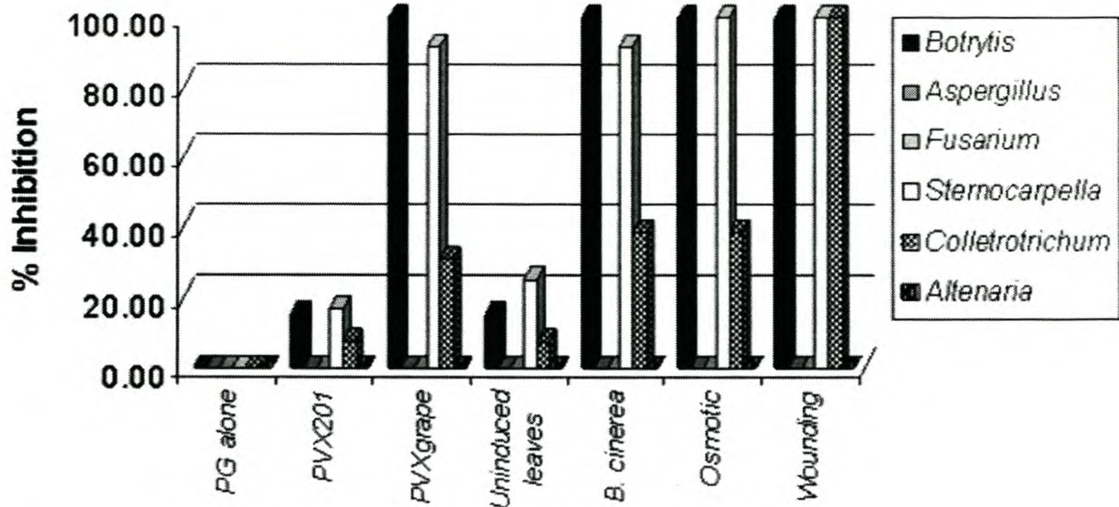

Figure 6. The inhibition spectra of PGIP in grapevine leaves induced by hypo-osmotic stress, wounding and pathogen infection compared to that of VvPGIP1 transiently expressed in *Nicotiana benthamiana*. Crude protein extracts were prepared from grapevine leaves 72 h after each induction. *N. benthamiana* plants were infected with a modified PVX201 (Baulcombe et al. 1995) harboring the *Vvpgip1* gene and crude protein extracts were collected 21 - 25 days after infection. Two μg of each extract were assayed against crude PG preparations from *Botrytis*, *Aspergillus*, *Fusarium*, *Stenocarpella*, *Colletrotrichum* and *Altenaria* using an agarose diffusion assay (Taylor and Secor, 1988). The degree of inhibition was determined by measuring the resulting zone sizes and comparing it to zones produced by PG's alone (0% inhibition).

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CHAPTER 4

RESEARCH RESULTS

PROMOTER ANALYSIS OF THE *VvPGIP1* GENE FROM *VITIS VINIFERA* L. THAT ENCODES A PGIP WITH HIGH ACTIVITY AGAINST *BOTRYTIS CINEREA* POLYGALACTURONASE

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Promoter analysis of the *Vvpgip1* gene from *Vitis vinifera* L. that encodes a PGIP with high activity against *Botrytis cinerea* polygalacturonase.

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Abstract

Regulation of defense is a complex process mediated by various signaling pathways, the products of which normally converge at the promoter of a specific gene. Promoter analysis of specific defense-related genes is useful to elucidate some aspects of the signaling pathways involved in regulation. To this end, the promoter of the polygalacturonase inhibiting protein (PGIP) encoding gene from *Vitis vinifera* (*Vvpgip1*) was isolated and analyzed. Here we analyzed the *Vvpgip1* promoter with regard to putative regulatory elements and core promoter size, and determined the start of transcription. Using a modified RACE technique, the start of transcription was mapped to 17 bp upstream of the putative ATG. Association of DNA binding proteins with this area was confirmed with preliminary gelshift analyses. The core promoter was, furthermore, mapped to the area between 137 bp and 100 bp upstream of the putative ATG. Promoter areas involved in auxin- and *Botrytis* responsiveness were mapped to the area between positions –3.1 kb and –1.5 kb. Responses to osmotic stress (novel to *Vvpgip1*) involve the area between positions –1.1 kb and –0.4 kb while wound responses are mediated by the area between positions –0.4 kb and –0.1 kb. *In silico* analyses, furthermore, revealed *cis*-acting elements in these areas that corresponds well to the induction stimuli tested.

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4.1. INTRODUCTION

The ability to respond to internal and environmental cues is central to the survival of all living organisms. Survival often depends on rapid and effective recognition of, and response to stress related stimuli (Hetherington & Waterhouse 2002). This recognition and response ability is regulated by various signaling cascades that typically culminate in *de novo* gene expression. Significant overlaps exist between the patterns of gene expression that are induced in plants in response to stress stimuli and crosstalk or coordinate transcriptional control between the signaling pathways occurs frequently to modulate an effective response (Genoud & Metraux 1999; Kunkel & Brooks 2002). Ample evidence exist to show that plant stress responses, including the salicyclic- (SA), jasmonic acid- (JA), and ethylene (ET)-dependent defense pathways, function in an interdependent fashion (Shirasu et al. 1996; Seki et al. 2003; Spoel et al. 2003). SA and JA control the expression of mostly non-overlapping sets of responses, and a number of studies have revealed antagonistic effects of SA application on wound- and/or JA-induced gene expression (Doares et al. 1995).

Temporal and spatial expression is an important part of the plant stress response and is primarily regulated at the level of transcription. Transcriptional regulation is in most cases determined by the presence or absence of transcription factors, both activators or suppressors (Aarts & Fiers 2003). Transcription factors in turn recognize specific promoter elements and in recent years a large amount of these binding sites have been identified in plant promoters (Higo et al. 1999). Transcription factors typically involved in defense response include ERF transcription factors, the DOF family of transcription factors, the bZIP family as well as the WRKY family (Kim et al. 1997; Eulgem et al. 2000; Beckett 2001; Latchman 2001; Singh et al. 2002; Yanagisawa 2002).

The extensive studies done to identify transcription factors involved in plant-pathogen responses extend to the promoters of individual defense gene promoters. Promoter analysis studies have identified a large number of conserved transcription factor binding sites in plant defense promoters, leading to the development of various binding site databases, such as the database for Plant Cis-acting Regulatory DNA

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Elements (PLACE) for the analysis of putative binding sites in promoters (Higo et al. 1999; Pedersen et al. 1999; Werner 1999).

The induction of defense signaling cascades by either pathogen perception or environmental stress stimuli, invariably leads to the induction of defense and/or stress associated genes. Among these, the polygalacturonase inhibiting protein (PGIP) gene family found in many plant species (De Lorenzo et al. 2001) is one of the best characterized. In recent years a large amount of data has been amassed regarding the regulation, structure and function of PGIPs. These proteins are typically cell wall bound, tissue-specific, developmentally regulated and inducible by various stimuli, including pathogen attack, wounding, SA, JA, oligogalacturonides (OGs), and cold treatment (Bergmann et al. 1994; Stotz et al. 1994; Desiderio et al. 1997; Devoto et al. 1998; Komjanc et al. 1999; Mahalingam et al. 1999; De Lorenzo et al. 2001; Ferrari et al. 2003). The most widely accepted model for PGIP mediated defense suggests a dual function for PGIPs (De Lorenzo et al. 2001). PGIP inhibits the action of fungal endopolygalacturonases (PGs) by binding to the active cleft of the PG, thereby preventing binding of the ligand to the enzyme (Federici et al. 2001). The inhibition of PGs in turn results in the prolonged existence of long-chained OGs that can subsequently activate multiple plant defense responses (Reymond et al. 1995). The inhibition of fungal PGs by PGIP, therefore, slows down fungal infection by limiting cell wall maceration, as well as activating multiple defense responses to curb the spread of infection.

Although much data are available regarding the regulation of PGIP expression, surprisingly little data correlates expression to promoter elements. No experimental data are available yet that links *pgip* promoter elements to the signaling cascades involved. The promoter of the bean *pgip1* gene was analyzed using *in silico* techniques to identify putative promoter elements. Functional analysis of this promoter in transgenic tobacco plants suggested a differential regulation among the individual members of the bean *pgip* family (Devoto et al. 1998). To date, however, no *pgip* gene promoter has been analyzed in detail, nor has any promoter elements been identified that plays a role in *pgip* gene regulation.

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Recently, a genomic DNA fragment from *Vitis vinifera* cv Pinotage containing a PGIP encoding gene (*Vvpgip1*) (Genbank Ac: AF499451) was cloned in our laboratory (De Ascensao 2001). The gene was analyzed with regard to regulation and functionality and shown to be induced by indole acetic acid (IAA), SA, hyper- and hypo-osmotic stress, pathogen infection and wounding. To date, osmotic induction is unique to grapevine PGIP and could play a role in the observed developmental regulation of *Vvpgip1* (De Ascensao 2001; Chapter 3, this dissertation). The induced expression profile of the *Vvpgip1* gene in grapevine could also be confirmed in transgenic tobacco expressing the gene under control of the native *Vvpgip1* promoter.

In this study, a 4.9 kb region upstream of the putative *Vvpgip1* ATG was analyzed to determine putative promoter elements by *in silico* analysis, core promoter size and start of transcription. Using a transient expression system, quantitative analyses of promoter activity were also performed by using sequential and internal promoter deletion constructs. The first 100 bp upstream of the putative ATG were shown to be crucial for promoter activity. The association of the transcription machinery with this area was confirmed by gelshift analyses. The analysis of the promoter under inducing conditions was facilitated by *Agrobacterium* infiltration-mediated transient expression and resulted in the identification of regions in the promoter putatively involved in regulation by *B. cinerea* infection, IAA, SA, wounding and osmotic stresses. *In silico* analysis also revealed corresponding *cis*-acting elements within these regions that could play a role in *Vvpgip1* regulation.

4.2. MATERIALS AND METHODS

4.2.1. Plant growth and culture conditions

Nicotiana tabacum var Xanthi plants were grown in a peet moss:vermiculite mixture (3:1) in a glasshouse at 26°C in a 16 h light/8 h dark photoperiod. *In vitro* tobacco (*N. tabacum* var Petite Havana) and grapevine (*Vitis vinifera* cv Chardonnay) plants were cultivated on Murashige and Skoog (MS) media (Murashige & Skoog 1962) at 26°C in a 16h light, 8h dark photoperiod. Somatic embryos from *V. vinifera* cv Sultana

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were obtained from immature anther filaments based on a protocol described by Franks et al. (1998) and maintained in the dark at 26°C.

4.2.2. Bacterial strains and culture conditions

All bacterial strains and plasmids used are listed in Table 1. *Agrobacterium tumefaciens* EHA105 strains were routinely cultured at 28°C in Luria Bertani (LB) (Sambrook et al 1989) medium supplemented with 0.1% (w/v) glucose and 30 μg/ml rifampicin unless otherwise stated. *Escherichia coli* strains were grown at 37°C in LB media or LB supplemented with 100 $\mu g.ml^{-1}$ ampicilin or 50 $\mu g.ml^{-1}$ kanamycin for the selection of transformants.

4.2.3. DNA manipulations

Standard techniques for DNA cloning and mapping were performed according to Sambrook et al (1989). Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics and used according to the supplier's recommendations. Sequencing was done by the Central DNA Sequencing Facility, Department of Genetics, Stellenbosch University using an ABI Prism 3100 automated DNA sequencer from PE Biosystems.

PCR amplifications were done using an Expand high fidelity DNA polymerase from Roche Diagnostics. All PCR primers and their applications are listed in Table 2. PCR reactions were performed in 50 μl reactions mixtures typically consisting of 1 x Expand high fidelity PCR buffer without $MgCl_2$, 200 μM dNTP's, 200 nM of each primer, 5 – 10 ng template DNA, and $MgCl_2$ added to optimal concentration. Typical amplification conditions included an initial DNA denaturation step at 95°C for 2 minutes, followed by cycles of denaturation at 95°C for 10 seconds, primer annealing according to the specific primer melting temperatures, and elongation at 72°C, allowing 40 seconds per 1 kb amplified. Reactions were allowed to proceed for 30 cycles.

A 4902 bp putative promoter fragment was isolated by PCR using the T7 primer in combination with the Pgip(-1)as primer from a cloned 7.2 kb fragment containing the

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5' upstream region of the grapevine *pgip* gene [pSK(gPgip)] (De Ascensao 2001). This fragment was cloned into the pGEM-T-Easy vector to create pGEM(-4902/-1) and completely sequenced in both directions. The -4335/-1, -3793/-1 and -3114/-1 putative promoter fragments were subsequently isolated by PCR using pGEM(-4902/-1) as template and the Pgip(-4335)s, Pgip(-3793)s, Pgip(-3114)s and Pgip(-1)as primers, respectively. The fragments were subsequently cloned into pGEM-T-Easy vector to yield pGEM(-4335/-1), pGEM(-3793/-1) and pGEM(-3114/-1) respectively.

A 2262 bp *Hinc* II fragment from pSK(Pgip) was mobilized into the *Hinc* II sites of pGEM 3zf^{+} from Promega yielding pGEM(2260). This construct contains a stretch of DNA 729 bp downstream and 1531 bp upstream of the putative ATG of the *Vvpgip1* gene. This construct was used as template in all subsequent PCR reactions to amplify putative promoter fragments. Promoter fragments were generated as follows: The -1534/-137, -1534/-1 and -1543/+51 fragments were amplified using the Sp6 primer as sense primer in combination with the Pgip(-137)as, Pgip(-1)as and Pgip(+51)as primers respectively. The -1133/-1 fragment was amplified using the Pgip(-1133)s primer in combination with the Pgip(-1)as primer. The -747/-137, -747/-1 and -747/+51 fragments were amplified using the Pgip(-747)s primer in combination the Pgip(-137)as, Pgip(-1)as and Pgip(+51)as primers respectively. The -464/-1 fragment was amplified using the Pgip(-464)s primer in combination with the Pgip(-1)as primer. The -137/+51 and -137/-1 fragments were amplified using the Pgip(-137)s primer in combination with the Pgip(-1)as and Pgip(+51)as primers. All promoter fragments generated by PCR were subsequently subcloned into the pGEM-T-Easy vector from Promega.

All the putative promoter fragments were excised from the pGEM-T-Easy vector using *Pst*I and *Nco*I before subcloning it into the corresponding sites of pCAMBIA1301 (obtained from the Center for the Application of Molecular Biology International Agriculture [CAMBIA]). The latter contained the β-glucuronidase (GUS)-gene (Jefferson et al. 1987) for expression analysis from the promoter. The -101/-1, -65/-1 and -18/-1 fragments were obtained by digesting pGEM(-1534/-1) with the following enzyme combinations: for -101/-1, *Pvu*II and *Nco*I; for -65/-1 *Msc*I and *Nco*I and for -18/-1, *Sna*BI and *Nco*I. All three fragments were cloned into the *Ecl*136I and *Nco*I sites of pCAMBIA1301 to yield pCAMBIA(-101/-1), pCAMBIA(-65/-1) and pCAMBIA(-18/-1)

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respectively. The second 35S CaMV promoter was removed from all pCAMBIA based promoter constructs used in infiltration experiments (Table 3) by digestion with *Xho*I and *Sal*I and subsequent religation. To construct a negative control for transient expression experiments, the *Bcpg1* gene from *B. cinerea* was isolated by PCR using the BcPG1(s) and BcPG1(as) primers and cloned into pGEM-T-Easy vector to yield pGEM(BcPG1). The gene was excised from pGEM-T-Easy with *Nco*I and *Sal*I and cloned into the *Nco* I and *Xho*I sites of pCAMBIA1301, effectively removing all the 35S CaMV promoters but keeping the GUS gene intact. All pCAMBIA based constructs were mobilized into *A. tumefaciens* strains EHA105 (Hood et al. 1993) by electroporation.

4.2.4. Plant transformations

In vitro leaves from *N. tabacum* var Petite Havana, *V. vinifera* cv Chardonnay, as well as somatic embryos from *V. vinifera* cv Sultana were used as target tissues in biolistic bombardment experiments to determine the minimum functional promoter length (core promoter). Promoter fragments used are listed in Table 3. Tissues were placed on 1% agarose plates and bombarded using a biolistic PDS-1000/He particle delivery system (BioRad) with 1100 p.s.i. rupture discs and the application of 80 kPa vacuum in the chamber. The microcarrier (1.0 mm gold particles) preparation and subsequent DNA-coating were performed as described by the supplier (BioRad) using 5 μg of DNA. The macrocarrier was spaced 6 mm from the stopping screen and the samples were placed 9 cm from the macrocarrier. All bombardments were done in triplicate and repeated independently. Bombarded tissues were incubated in the dark for 4 days before staining for GUS activity. GUS staining was performed according to Jefferson et al. (1987).

Tobacco infiltration and fluorometric assays were done as described by Yang et al. (2000) using the *A. tumefaciens* strain EHA105 to infiltrate the third and fourth leaves of 6-week-old tobacco (*N. tabacum* var Xanthi) plants. Constructs used for infiltration experiments are listed in Table 3. All infiltration experiments were done in triplicate and repeated independently. Infiltrations were allowed to proceed for 48 h before leaves were excised and induced. Protein extractions and GUS assays were performed

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according to Yang et al. (2000). Total protein concentration was determined using the method described by Bradford et al. (1976).

4.2.5. Leaf infections and induction treatments

For induction experiments, infiltrated tobacco leaves were excised and infected with *B. cinerea* spores, treated with absisic acid (ABA), indole acetic acid (IAA), salicylic acid (SA), wounded and osmotically treated in individual experiments. All inductions were allowed to proceed for 48 h in a glasshouse at 26°C before the leaves were flash frozen in liquid nitrogen and stored at -80°C. *B. cinerea* spores were provided by Prof. Holtz, Department of Plant Pathology, Stellenbosch University. The spores were resuspended in sterile grape juice to a final concentration of 1 x 10^6 spores.ml^{-1}. Leaves were briefly submerged in the solution and placed upright in water so that only the petiole was covered. Inoculated leaves were covered with a translucent plastic container on a wet filter paper base to provide a humid environment.

Leaves were treated with IAA and SA by placing the leaves upright in a 100 μM solution of each hormone solution so that only the petioles were covered. Sterile water was used as control. For wounding inductions, leaves were placed upright in water in such a way that only the petiole was covered and wounded uniformly with sterile forceps. For osmotic treatments, leaves were submerged in sterile water and in a separate experiment, submerged in a 0.1 M fructose solution.

4.2.6. Determination of the transcription start site

V. vinifera cv. Pinotage *veráison* berries were collected from a commercial vineyard in the Stellenbosch area, South Africa. The protocol for RNA extraction from field grown grapevine plants was adapted from Davies and Robinson (1996), with the following modifications: grapevine tissue was ground in liquid nitrogen and 2 g were added to 10 ml of extraction buffer (5 M sodium perchlorate, 0.3 M Tris/HCl pH 8.3, 8.5% [w/v] insoluble polyvinylpolypyrrolidone, 2% [w/v] PEG 4000, 1% [w/v] SDS, 1% [w/v] β-mercaptoethanol) and further homogenized in a commercially available polytron

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homogenizer. The homogenate was centrifuged at 12500 x g for 10 min at room temperature and the supernatant filtered through a 10 ml syringe stuffed with cotton wool. The filtered homogenate was extracted at least three times with an equal volume of phenol:chloroform (25:25) followed by an equal volume of chloroform. Nucleic acids were precipitated with 0.6 volumes isopropanol. First strand cDNA was synthesized based on a modification of the protocol from Schmidt and Mueller (1999). Superscript^{TM} II Rnase H^- reverse transcriptase from Invitrogen was used in combination with the *Not* I oligo(dT) and cDNA(dG) primers according to the manufacturers instructions. cDNA ends were amplified using the cDNA(amp) and Pgip(+280)as primers. Two independent amplifications were done and PCR products were cloned into the pGEM-T-Easy vector. Five clones from each independent amplification were sequenced. Clones containing sequences upstream of the putative ATG were aligned with the *pgip* gene and promoter sequence to determine the putative start of transcription.

4.2.7. Electrophoretic mobility shift assays (EMSAs)

Crude nuclear proteins were extracted from glasshouse grown tobacco (*N. tabacum* var Petite Havana) according to the method of Escobar et al. (2001). Probe labeling and EMSA analysis was done using the DIG gelshift kit from Roche Diagnostics according to the manufacturer's instructions. The -137/+51 promoter fragment was used as probe to detect DNA:protein interactions. Unlabeled probe was used as specific competitor and was added in 50x, 100x and 150x excesses. The binding reaction was done with 10, 15 and 20 μg of crude nuclear extract respectively, and allowed to proceed for 15 min at room temperature. Protein:DNA complexes were separated on a 6% polyacrylamide gel and electroblotted onto positively charged nylon membranes from Roche Diagnostics. Signals were detected according to the manufacturer's instructions.

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4.3. RESULTS

4.3.1. *In silico* promoter analysis

A 4902 bp sequence containing the *Vvpgip1* promoter was analyzed using the PLACE database (Higo et al. 1999). Several putative elements were identified, but only those that could be related to the observed expression pattern of the promoter were considered. Putative *cis*-acting regulatory elements are listed in Table 4. Analysis of the nucleotide sequence immediately to the 5' side of the coding region revealed the presence of a putative CAAT box at position -129 and a putative TATA box at position –48 in good sequence context.

4.3.2. Core promoter analysis

To verify promoter activity and identify the core promoter, several PCR-generated deletions were made of the 4.9 kb upstream region and the resulting fragments cloned upstream of the GUS *(UidA)* reporter gene. These constructs were analyzed by microbombardment experiments to determine their ability to drive GUS expression. Different tissues, including *in vitro* tobacco leaves, *in vitro* grapevine leaves, as well as grapevine somatic embryos were used in these experiments. Results are summarized in Table 5.

Fragments containing regions -1534/+51 or -1534/-1 showed comparable activity, indicating that sequences from +51 to -1 do not play an important part in transcription initiation. Activity was also comparable in different tissues, with no appreciable difference in spot frequency between leaves and somatic embryos of grapevine and tobacco. Constructs containing regions -1534/-119 and -747/-119 gave no GUS expression. Also, promoter fragments -100/-1 or -70/-1 showed significantly reduced activity, while fragment 20/-1 showed no activity, indicating a crucial role for the -100/-1 promoter area in the initiation of transcription.

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4.3.3. Determination of the start of transcription

The putative start of transcription of the *Vvpgip1* gene was determined using a modification of the Rapid Amplification of cDNA Ends (RACE) approach. Complementary DNA was synthesized from *veráison* berries and the 5' ends were amplified. The products were cloned, sequenced and aligned with the *Vvpgip1* gene and promoter. The alignments are depicted in Fig 1. The longest cDNA sequences obtained with this approached corresponded to 17 bp upstream of the putative ATG.

4.3.4. Gel shift analysis

The association of proteins from the transcription machinery with a DNA fragment corresponding to the -137/+51 promoter region was verified using a gelshift assay. Crude nuclear proteins from tobacco leaves were isolated and increasing amounts were allowed to associate with the fragment end-labeled with DIG. Associations were allowed to proceed in the presence or absence of specific competitor. The fragments were separated on a polyacrylamide gel and protein:DNA complexes were identified by a shift in mobility of labeled fragments. The gel is depicted in Fig. 2. Protein interactions could be detected with the addition of 10 μg, 15 μg and 20 μg of nuclear protein. No increase in protein:DNA complexes could be seen with increasing nuclear protein concentrations. This interaction was also completely abolished by the addition of 50X specific competitor.

4.3.5. Quantitative promoter analysis

Agrobacterium infiltration was also used as a transient expression system to determine promoter activity, and the induction profiles of a series of promoter deletions were determined using quantitative GUS assays. Infiltrated leaves were infected with *B. cinerea*, treated osmotically (submerged in distilled water and, in a separate experiment, 0.1 M fructose), wounded, treated with 0.1 mM IAA or 0.1 mM SA. Induction profiles are shown in Fig. 3 and the relative expression levels determined by

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each promoter fragment are shown in Table 6. Although expression levels varied significantly among treatments and promoter fragments, essential promoter areas involved in stimuli-related induction could be identified. Activation upon *B. cinerea* infection and IAA treatment was lost in fragments shorter than 3.1 kb, whereas induction in distilled water (osmotic) treatments could be observed for fragments up to 1.1 kb. Induction in response to treatments with fructose could not be abolished. Induction by wounding was lost in fragments shorter than 0.4 kb. SA induced GUS expression was inconsistent among promoter fragments and induction could only be observed in the 4.3 kb and 1.1 kb fragments. Overall, the osmotic (dH_20 and fructose) treatments yielded the highest relative inductions (up to 21 fold) whereas SA treatments resulted in the lowest induction (1.45 fold).

4.4. DISCUSSION

In most plant species, *pgip* genes exist as small multi-gene families (De Lorenzo et al. 1994). In grapevine, however, this seems not to be case (A de Ascensao et al [in preparation]). The observed induced expression profile of PGIP in grapevine is, furthermore, mirrored exactly by *Vvpgip1* under the control of its own promoter in transgenic tobacco plants (Chapter 3, this dissertation). Not only does this indicate conserved regulatory aspects involving defense-related genes in tobacco and grapevine, but clearly demonstrates that the promoter of the *Vvpgip1* gene is solely responsible for the observed induced expression profile of grapevine PGIP. *In silico* analysis of a 4905 kb region upstream of the putative ATG revealed a putative TATA element at position –42 and a putative CAAT box at position –129. The importance of this region is illustrated in promoter deletion experiments. No blue spots were observed in tissues bombarded with promoter fragments fusions shorter than 20 bp, containing neither CAAT-, nor TATA boxes, fused to *GUS*. Bombardments with fragments shorter than 100 bp, containing a putative TATA box, but no putative CAAT box, fused to GUS (Table 3), resulted in spot frequencies of less than 1 per cm^3, indicating an important role for the region containing the putative CAAT box in basal expression. The crucial role of the first 137 bp upstream of the putative ATG was further demonstrated by the

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lack of promoter activity of fragments fused to GUS lacking this area in all bombarded tissues (Table 5).

The role of the first 137 bp upstream of the putative ATG in *Vvpgip1* transcription initiation was confirmed by the determination of the putative start of transcription analysis and preliminary DNA:protein interaction analysis. Using a modified RACE technique, we mapped the putative start of transcription to position –17 relative to the predicted translational start site (Fig. 1). This is in excellent context with the putative TATA box, considering that the TATA box elements serve to recruit the transcription initiation machinery and that the eukaryotic RNA polymerase II (pol II) complex spans approximately 20 bp (Dvir et al. 2001). The position of the putative transcriptional start site also provides an explanation for the lack of promoter activity observed in fragments fused to GUS lacking the first 137 bp of the promoter, as well as the 20 bp promoter fragment. Preliminary gelshift analysis using the -137 bp/+51 bp) promoter fragment was used to show DNA:protein interactions within this region (Fig. 2), further corroborating the association of the pol II machinery with this region. Protein interaction could be detected using relatively low levels of crude nuclear extracts from tobacco. No apparent increase in protein interaction could, however, be detected with an increase in nuclear protein concentration. The interaction was also abolished by the addition of 50X specific competitor. Together these preliminary results may indicate a weak interaction or could be indicative of sub-optimal experimental conditions that could further be optimized (i.e. buffer- and/or association conditions). These results might also indicate that the promoter needs additional elements to facilitate the formation of a stable pol II-DNA complex and the 137 bp fragment is too short to facilitate binding of these elements. This observation is also reflected in the relatively low basal expression levels of the -137 bp/ -1 bp promoter fragment compared to levels obtained for longer promoter fragments in quantitative non-inducing experiments (Fig 3).

Transient expression analyses of promoter fragments indicated higher expression levels for larger promoter fragments (4.3 kb and 3.1 kb) with lower expression levels for smaller fragments (1.5 kb, 1.1 kb, 0.4 kb and 0.1 kb). The very low expression levels for the 3.7 kb fragment is surprising. A possible explanation could be the presence of point mutations in areas crucial for the initiation of transcription

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(Fig. 3 and Table 6). The induction profile of this fragment was, however, unaffected and corresponded well to profiles of similar length fragments.

The transient expression analysis also confirmed that the promoter of the *Vvpgip1* gene is activated upon *B. cinerea* infection, wounding, auxin (IAA), SA as well as osmotic stress (Fig. 3). *In silico* analysis revealed several putative *cis*-acting elements present in the 4905 bp promoter fragment that could potentially impact on *Vvpgip1* promoter mediated expression (Table 4). The positions of most of these *cis*-acting elements correlated well with promoter areas identified by transient expression analysis to be involved in stimulus specific induction. From our data it seems that the area responsible for induction upon *B. cinerea* infection is situated between 4.3 kb and 3.1 kb. The relatively high expression levels obtained for the 1.1 kb fragment in this regard cannot be explained with the current data set, but could possible reflect on more complex regulation. Several W-box elements have been predicted in this area (Table 4). W-box elements have been shown to be actively involved in defense gene induction (Eulgem et al. 2000; Chen et al. 2002) and contain the core TTGAC(C/T) sequence that are recognized by WRKY proteins, a recently identified class of DNA-binding proteins. WRKY proteins are, among others, induced upon wounding, pathogen infection and SA. Recent results also suggest that defense-related expression of WRKY proteins involves extensive activation and repression by its own family (Dong et al. 2003).

The same area seems to be involved in IAA- mediated activation and, consistent with this observation, is the predicted presence of the CATATGGMSAUR, ASF1MOTIFCAMV and ARFAT elements that have been shown to be involved in auxin responsiveness (Lam et al. 1989; Xu et al. 1997; Guilfoyle et al. 1998; Ulmasov et al. 1999). Promoter activation by SA seems to be inconsistent; possible induction can be seen in the 4.3 kb and 1.1 kb promoter fragments, but the low levels observed in the 3.7 kb fragment cannot be explained (Table 6). The presence of several predicted W-boxes involved in SA induction in the promoter region between 4.3 kb and 1.1 kb, however, provides a theoretical basis for SA mediated induction. Interestingly, in both IAA and SA mediated induction; repression seems to occur in the shorter promoter fragments, possibly suggesting a dual role for these hormones in PGIP regulation.

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Promoter activation by distilled water seems to be mediated by the area between 1.1 kb and 0.4 kb. Analysis of this area did not render known *cis*-acting elements specific for osmotic induction, suggesting the presence of a novel osmotic-responsive element. Activation by 0.1 M fructose could not be abolished in our experiments. The reasons for this could be two-fold, (i) a promoter area shorter than 137 bp is involved in mediating this type of response or, (ii) fructose influences the GUS assay, resulting in higher expression levels (i.e. an artifact). In our opinion the latter seems to be more plausible explanation. Leckie et al. (1994) found that if the GUS extraction buffer is modified by adding enzyme-stabilizing factors, such as BSA and glycerol, much higher GUS activity levels can be obtained. Also, previous experiments using sucrose instead of fructose gave similar results (results not shown) and it seems as if the small amounts of sugar could have a stabilizing effect on the enzyme. Finally, the area responsible for wound-mediated induction seems to be situated between 0.4 kb and 0.1 kb. No relevant *cis*-acting elements could be identified in this area, but this does not rule out the possibility that the several W-box elements found throughout the promoter (Table 4) could play a role in this regard.

In this study we present the first in depth analyses of a *pgip* promoter. Previous work regarding *pgip* promoter analyses focused on highlighting differential regulation between different members of the *pgip* families from *Phaseolus vulgaris* and *A. thaliana* (Devoto et al. 1998; Ferrari et al. 2003). For the first time we were able to identify *pgip* promoter regions involved in response to *Botrytis* infection, auxin, osmotic stress and wounding. These results are, at this stage, however, still preliminary and the identified promoter areas too large to be useful in the elucidation of signaling cascades involved in *Vvpgip1* regulation (the ultimate goal of this work). These areas remain to be analyzed further in order to pinpoint specific *cis*-acting elements and identify the respective transcription factors involved. This study provided an important fundamental and technical basis for these studies.

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Table 1. Strains and plasmids used in this study

Strain or plasmid(s)	Relevant features or insert	Source or reference
E. coli strains DH5α	supE44lacU169[*ϕ80lac*ZM15hsdR17recA1gyr A96*thi*-1relA1]	Life Technologies (GIBCO/BRL)
A. tumefaciens strains		
EHA105	Disarmed, succinomopine strain	Hood et al. 1993
Plasmids		
pBlueskript SK$^+$	Cloning vector	Stratagene
pSK(gPgip)	~7.2 kb Pinotage genomic fragment containing the *pgip* gene and upstream regulatory sequences cloned into pBlueskript SK$^+$	De Ascensao 2001
pGEM-T-Easy	pGEM5Zf(+) based PCR cloning vector	Promega
pGEM3Zf(+)	Cloning vector	Promega
pGEM(BcPG1)	BcPG1 gene from *B. cinerea* cloned into the pGEM-T-Easy vector	This study
pGEM(-4902/-1)	-4902/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-4335/-1)	-4335/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-379/,-1)	-3793/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-3114/-1)	-3114/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(2263)	2263 bp *Hinc* II fragment upstream of the *V.vinifera pgip1* gene cloned in *Hinc* II site of pGEM3Zf(+)	This study
pGEM(-1531/+51)	-1534/+51 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-1531/-1)	-1534/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-1531/-137)	-1534/-137 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-1133/-1)	-1133/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-747/+51)	-747/+51 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-747/-1)	-747/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-747/-137)	-747/-137 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-464/-1)	-464/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-137/+51)	-137/+51 promoter fragment cloned in pGEM-T-Easy vector	This study
pGEM(-137/-1)	-137/-1 promoter fragment cloned in pGEM-T-Easy vector	This study
pCAMBIA(-4335/-1)infil	-4335/-1 promoter fragment cloned in *Pst*I/*Nco*I sites of pCAMBIA1301 with no 35SCAMV promoters	This study
pCAMBIA(-3793/-1)infil	-3793/-1 promoter fragment cloned in *Pst*I/*Nco*I sites of pCAMBIA1301 with no 35SCAMV promoters	This study
pCAMBIA(-3114/-1)infil	-3114/-1 promoter fragment cloned in *Pst*I/*Nco*I sites of pCAMBIA1301 with no 35SCAMV promoters	This study

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pCAMBIA(-1531/+51)	-1534/+51 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-1531/-1)	-1534/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-1531/-1)infil	-1531/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301 with no 35SCAMV promoters	This study
pCAMBIA(-747/+51)	-747/+51 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-747/-1)	-747/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-747/-137)	-747/-137 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-464/-1)	-464/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-464/-1)infil	-464/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301 with no 35SCAMV promoters	This study
pCAMBIA(-137/+51)	-137/+51 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-137/-1)	-137/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-137/-1)infil	-137/-1 promoter fragment cloned in *PstI*/*NcoI* sites of pCAMBIA1301 with no 35SCAMV promoters	This study
pCAMBIA(-101/-1)	-100/-1 promoter fragment cloned in *Ecl*136II/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-65/-1)	-70/-1 promoter fragment cloned in *Ecl*136II/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(-18/-1)	-20/-1 promoter fragment cloned in *Ecl*136II/*NcoI* sites of pCAMBIA1301	This study
pCAMBIA(BcPG1)	pCAMBIA1301; 35S CaMV promoters have been replaced by the BcPG1 gene from *B. cinerea*	This study

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Table 2. Primer pairs and sequences used in this study

Primer	Sequence	Paired with	Template	Product
Sp6	ATTTAGGTGACACTATAG	Pgip(-137)as, Pgip(-1)as, Pgip(+51)as	pGEM(2263)	-1534/+51 -1534/-1 -1534/-119 -1534/-747
T7	CGCGCGTAATACGACTCACTATAG	Pgip(-1)	pSK(gPgip)	-4902/-1
BcPG1(s)	ATGGTTCAACTTCTCTCAATGG	BcPG1(as)	*B. cinerea* genomic DNA	*Bcpg1* gene from *B. cinerea*
BcPG1(as)	TAAGATGTTTAACACTTGACACCAG	BcPG1(s)	*B. cinerea* genomic DNA	*Bcpg1* gene from *B. cinerea*
Pgip(-4335)s	CGAAATAAAGAAAAAGACAGAGAAAGG	Pgip(-1)	pSK(gPgip)	-4335/-1
Pgip(-3793)	GTCACTTTTATAGAAGTATGTTTTGGAG	Pgip(-1)	pSK(gPgip)	-3793/-1
Pgip(-3114)	GAATGAATTAAAGTAAGTTAATATTTTTTATG	Pgip(-1)	pSK(gPgip)	-3114/-1
Pgip(-1133)s	TGGTGGGAATAGATTTGAAAGCC	Pgip(-1)as	pGEM(2263)	-1133/-1
Pgip(-747)s	CGTAGGATCCCCTATGATTAAATCATTTGAG	Pgip(-137)as, Pgip(-1)as, Pgip(+51)as	pGEM(2263)	-747/+51 -747/-1 -747/-119
Pgip(-747)as	CTCAAATGATTTAATTCATAGGG	Sp6	pGEM(2263)	-1534/-1
Pgip(-464)s	GTATTTTGAAAATGTCTTTTAAAT	Pgip(-1)as	pGEM(2263)	-464/-1
Pgip(-137)s	CGTAGGATCCCCAAATAAGCCCTCAAGG	Pgip(-1)as, Pgip(+51)as	pGEM(2263)	-137/+51 -137/-1
Pgip(-137)as	CCTTGAGGGCTTATTTGG	Sp6, Pgip(-747)s	pGEM(2263)	-1534/-119 -747/-1
Pgip(-1)as	CGTAGGATCCTTCTCTGAATTTGGCTACGT	Sp6, Pgip(-1133)s, Pgip(-747)s, Pgip(-464)s, Pgip(-137)s	pGEM(2263)	-1534/-1 -1133/-1 -747/-1 -464/-1 -137/-1
Pgip(+51)as	CGTAGGATCCTAAGAGTAGGAGGAGAGAGGA	Sp6, Pgip(-747)s, Pgip(-137)s	pGEM(2263)	-1534/+51 -747/-1 -137/+51
Not I – oligo(dT)	ATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTT(N)		*Veráison* berry RNA	1st strand cDNA
cDNA(dG)	GGACCGGCCGGATCCGGAGGGG		*Veráison* berry RNA	1st strand cDNA
cDNA(amp)	GGACCGGCCGGATCC	Pgip(+230)as	*Veráison* berry cDNA	-17/+230
Pgip(+230)as	GGTTAGCGAGTTGATGC	cDNA(amp)	*Veráison* berry cDNA	-17/+230

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Table 3. Constructs used in this study for biolistic bombardments and *Agrobacterium*-infiltration experiments

Constructs used for Biolistic bombardment	Constructs used for *Agrobacterium* infiltration
pCAMBIA(-1531/+51)	pCAMBIA(-4335/-1)infil
pCAMBIA(-1531/-1)	pCAMBIA(-3793/-1)infil
pCAMBIA(-1531/-137)	pCAMBIA(-3114/-1)infil
pCAMBIA(-747/+51)	pCAMBIA(-1531/-1)infil
pCAMBIA(-747/-1)	pCAMBIA(-1133/-1)infil
pCAMBIA(-747/-137)	PCAMBIA(-464/-1)infil
pCAMBIA(-137/+51)	pCAMBIA(-137/-1)infil
pCAMBIA(-137/-1)	pCAMBIA(BcPG1)
pCAMBIA(-101/-1)	
pCAMBIA(-65/-1)	
pCAMBIA(-18/-1)	
pCAMBIA(BcPG1)	

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Table 4. *Cis*-acting promoter elements identified by PLACE in the *Vvpgip1* promoter

Element (nomenclature as in the PLACE database)	Position(s)*	Consensus sequence	Reference
ACGTOSGLUB1 "ACGT motif" found in the GluB-1 gene in rice. Required for endosperm-specific expression, conserved in the 5'-flanking region of glutelin genes	-3658 (-)	GTACGTG	(Washida et al. 1999)
ARFAT "ARF (auxin response factor)" binding site found in the promoters of primary/early auxin response genes of *A. thaliana*	-4305 (+) -2095 (-)	TGTCTC	(Guilfoyle et al. 1998; Ulmasov et al. 1999)
ASF1MOTIFCAMV "ASF-1 binding site" in CaMV 35S promoter; ASF-1 binds to two TGACG motifs, found in HBP-1 binding site of wheat histone H3 gene, TGACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin and/or salicylic acid	-4856 (+)	TGACG	(Lam et al. 1989)
CANBNNAPA Core of "(CA)n element" in storage protein genes in *Brasica napus*, embryo- and endosperm-specific transcription of napin (storage protein) gene	-4102 (+)	CNAACAC	(Ellerstrom et al. 1996)
CATATGGMSAUR Sequence found in NDE element in soybean SAUR (Small Auxin-Up RNA) 15A gene promoter; involved in auxin responsiveness	-3597 (+) -3597 (-)	CATATG	(Xu et al. 1997)
ELRECOREPCRP1 ElRE (Elicitor Responsive Element) core of parsley PR1 genes; consensus sequence of elements W1 and W2 of parsley PR1-1 and PR1-2 promoters; Box W1 and W2 are the binding site of WRKY1 and WRKY2, respectively	-967 (+) -1468 (-)	TTGACC	(Chen & Chen, 2000; Rushton et al. 2002)
SURE2STPAT21 Sucrose Responsive Element 2 (SURE2), a motif conserved among genes regulated by sucrose	-2259 (-) -465 (-)	AATACTAAT	(Grierson et al. 1994)
WBBOXPCWRKY1 "WB box" found in the Parsley WRKY1 gene promoter, also conserved in WRKY3 gene promoter, required for elicitor responsiveness	-1010 (+) -1020 (-)	TTTGACT	(Eulgem et al. 2000)
WBOXATNPR1 "W-box" found in promoter of *A. thaliana* NPR1 gene, located between +70 and +79 in tandem; they were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins;	-4729 (+) -4089 (+) -4033 (+) -1533 (+) -1527 (+) -4823 (-) -2904 (-) -1586 (-) -1467 (-) -1019 (-)	TTGAC	(Chen et al. 2002)

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Table 5. Results of the biolistic bombardment of various tissues with *Vvpgip1* promoter fragments fused to GUS

Promoter fragment	Tobacco leafdiscs	Grapevine leafdiscs	Grapevine somatic embryos
(-1534/+51)-GUS	++	++	++
(-1534/-1)-GUS	++	++	++
(-1534/-119)-GUS	-	-	-
(-747/+51)-GUS	++	++	++
(-747/-1)-GUS	++	++	++
(-747/-119)-GUS	-	-	-
(-137/-+51)-GUS	++	++	++
(-137/-1)-GUS	++	++	++
(-100/-1)-GUS	+	+	+
(-70/-1)-GUS	+	+	+
(-20/-1)-GUS	-	-	-
GUS	-	-	-

-, no spots
+, < 1 spot/cm^3
++, > 10 spots/cm^3

```
CLUSTAL X (1.81) multiple sequence alignment

1                                GGACCGGCCGGATCCGGAGGGGGATCCAAATTCATASAAGWGRAKAC
2                                GGACCGGCCGGATCCGGAGGGGCAGCCAAATTCARARAAAKGGARAC
3                                GGACCGGCCGGATCCGGAGGGGGAGCCAAATTCAGAGAAATGGAGAC
4                                GGACCGGCCGGATCCGGAGGGGTAGCCAAATTCAGAGAAATGGAGAC
5                                GGACCGGCCGGATCCGGAGGGG-AGCCAAATTCAGAGAAATGGAGAC
6                                GGACCGGCCGGATCCGGAGGGG---------------------AGAC
7                                GGACCGGCCGGATCCGGAGGGGTAGCCAAATTCAGAGAAATGGAGAC
vvpgip1                                                TAGCCAAATTCAGAGAAATGGAGAC
                                 **********************                     ****

1                   TTCAAAACTTTTTCTACTCTCCTCMTCTCMTCCTCCTACTCTTACTCRCCACTCGTCCAT
2                   TTCAAAACTTTTTCTACTCTCCTCCTCTS-KCCKCCTACTCTTACTCGCCACTCGTCCAT
3                   TTCAAAACTTTTTCTACTCTCCTCCTCTC-TCCTCCTACTCTTACTCGCCACTCGTCCAT
4                   TTCAAAACTTTTTCTACTCTCCTCCTCTC-TCCTCCTACTCTTACTCGCCACTCGTCCAT
5                   TTCAAAACTTTTTCTTCTCTCCTCCTCTC-TCCTCCTAGTCTTACTCGCCACTCGTCCAT
6                   TTCAAAACTTTTTCTTCTCTCCTCCTCTC-TCCTCCTAGTCTTACTCGCCACTCGTCCAT
7                   TTCAAAACTTTTTCTACTCTCCTCCTCTC-TCCTCCTAGTCTTACTCGCCACTCGTCCAT
vvpgip1             TTCAAAACTTTTTCTACTCTCCTCCTCTC-TCCTCCTACTCTTACTCGCCACTCGTCCAT
                    *************** ******** ***   ** **** ******** ************
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Figure 1. Clustal X alignment of the cDNA 5' ends of *Vvpgip1* transcripts. cDNA ends were amplified using a modified RACE technique. Five clones from 2 independent reactions were sequenced; sequences of clones containing short cDNA fragments were discarded while the others were aligned with the sequences immediately up- and downstream of the putative *Vvpgip1* ATG. The putative ATG is shown in red, while the putative start of transcription is shown in bold blue.

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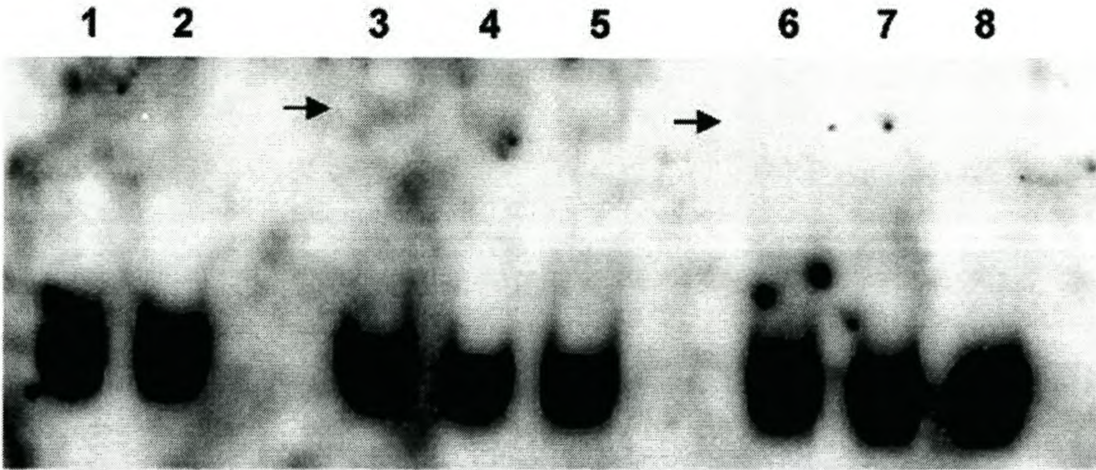

Figure 2. Gelshift analysis of the interaction between crude nuclear extracts (NEs) from tobacco leaves and the -137/+51 *pgip* promoter fragment using the DIG gelshift kit from Roche Molecular diagnostics. Lane 1. Probe alone; lane 2, probe + BSA; lane 3 probe + 10 μg NE; lane 4, probe + 15 μg NE; lane 5, probe + 20 μg NE; lanes 6 – 8 probe + 10 μg NE and 50X, 100X and 150X specific inhibitor respectively. Complexes were separated on a 6% polyacrylamide gel, electroblotted to a Nylon membrane and developed according to the manufacturers instructions. The DNA:protein interactions in lane 3 – 5, as well as the effect of a specific competitor (lanes 6 – 8) on the interactions are depicted by black arrows.

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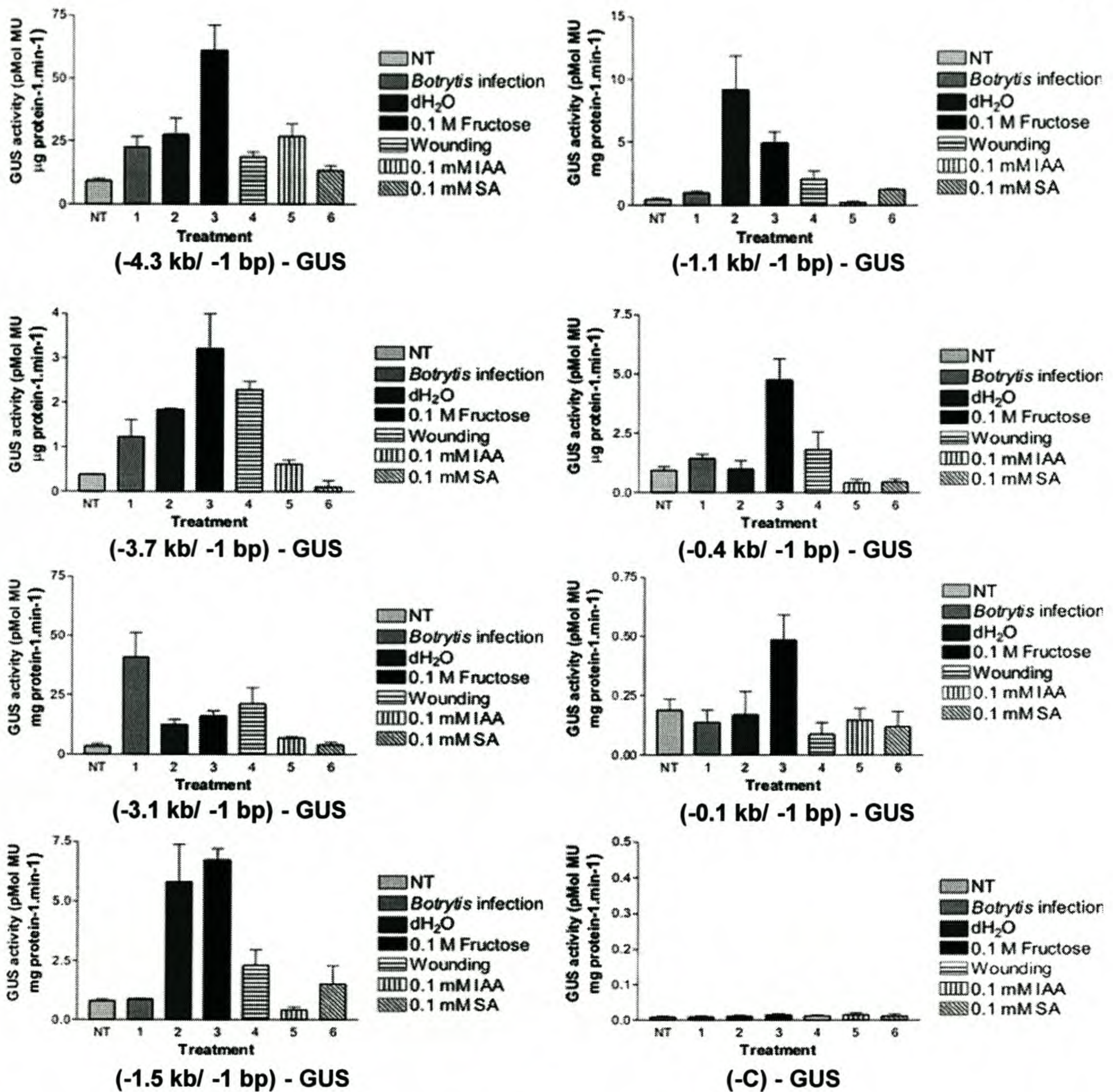

Figure 3. Quantitative analysis of GUS activity mediated by various fragments of the *Vvpgip1* promoter. Promoter fragments were generated by PCR and cloned in front of the GUS gene in a pCAMBIA-based plasmid background. Promoter constructs were introduced into *A. tumefaciens*, which were subsequently infiltrated into *N. tabacum* var Xanthi leaves. Leaves were induced by the various inducing agents, total proteins isolated, and GUS activity measured using a fluorometrical protocol described by Yang et al. (2000). Promoter fragments used are depicted underneath each graph and the inductions comprised: NT – No Treatment; *Botrytis* – Leaves infected with *B. cinerea*; distilled water – Leaves submerged in sterile distilled water; 0.1 M Fructose – Leaves submerged in 0.1 M fructose; Wounding – Leaves uniformly wounded with sterile forceps; 0.1 mM IAA – Leaves treated with 0.1 mM indole acetic acid; 0.1 mM SA – Leaves treated with 0.1 mM salicylic acid.

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Table 6. GUS activity levels in tobacco leaves infiltrated with *Vvpgip1* promoter fragments – GUS constructs (Table 3) in response to various induction stimuli relative to uninduced controls.

	4.3 kb	3.7 kb	3.1 kb	1.5 kb	1.1 kb	0.4 kb	0.1 kb	-C
NT	1	1	1	1	1	1	1	1
Botrytis	1.85	3.26	12.22	1.09	2.19	1.28	0.77	1.06
dH_2O	3.01	4.88	3.72	7.31	21.04	1.07	0.90	1.23
0.1 M Fructose	6.71	8.51	4.77	8.45	11.29	5.16	2.60	1.80
Wounding	2.03	6.07	6.33	2.91	4.66	1.96	0.47	1.67
0.1 mM IAA	2.93	1.64	2.05	0.51	0.43	0.46	0.79	1.03
0.1 mM SA	1.45	0.26	1.14	1.04	2.21	0.48	0.64	1.07

Shaded areas represent values that is significantly higher than that of the respective treatment control

NT – No Treatment

Botrytis – Leaves infected with *B. cinerea*

dH_2O – Leaves submerged in sterile distilled water

0.1 M Fructose – Leaves submerged in 0.1 M fructose

Wounding – Leaves uniformly wounded with sterile forceps

0.1 mM IAA – Leaves treated with 0.1 mM indole acetic acid

0.1 mM SA – Leaves treated with 0.1 mM salicylic acid

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

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5.1. GENERAL DISCUSSION AND CONCLUSION

The face of global agriculture is changing at a rapid rate. Not only does a changing trend in consumer lifestyle demand the implementation of environmentally friendly cultivation practices, but world population statistics also suggest that agricultural yields should significantly increase to sustain the increasing demands of consumers (Haddad & Martorell 2002). The practices of monoculture (such as is widely employed in modern agriculture) demands huge amounts of chemical pesticides and fertilizers to facilitate consistent production of high quality and quantity crops. Furthermore, considering available arable land, these solutions do not provide for sustainable crop production in some inhabited areas (Hinrichsen 1998). Within this context, it is not surprising that in an effort to find alternative solutions, an increasing amount of work has been focused on improving disease and stress resistance traits in crop plants. This of course precipitated an increasing need for fundamental knowledge regarding the processes involved during plant stress- and disease responses. Traditionally, in pursuit of fundamental knowledge regarding any process, model systems are utilized to systematically dissect and reassemble the processes involved. Within an agricultural perspective, however, the obvious economical benefits intrinsic of disease and stress resistant plants extended this research from model organisms, such as *Arabidopsis thaliana* and tobacco to more traditional crop plants, such as maize, bean, soybean, apples, and grapevine.

In an effort to try and understand some of the basic processes underlying disease resistance in grapevine, we have isolated a well studied and characterized defense associated gene, the polygalacturonase inhibiting protein (PGIP), from grapevine (De Ascensao 2001). PGIPs are cell wall bound proteins that interacts with, and inhibit fungal polygalacturonases (PGs) (De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002). The contribution of the inhibitory action of PGIPs to the plants defense response is thought to be two-fold; (i) the PGIP:PG interaction physically inhibits the action of cell wall macerating PGs that are important for fungal infection (Ten Have et al. 1998; Isshiki et al. 2001) and (ii) the inhibition of fungal PGs results in the prolonged existence of long-chain oligogalacturonides, the latter being biologically active

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molecules that have been shown to elicit plant defense responses (Reymond et al. 1995; De Lorenzo et al. 2001; De Lorenzo & Ferrari 2002). The objective of this study was to try and elucidate some of the underlying mechanisms involved in the regulation of the grapevine *pgip* gene (*Vvpgip1*).

Within the context of disease resistance, PGIP proteins are probably some of the most extensively studied proteins across plant species. PGIP encoding genes have been isolated from various plant species, including alfalfa, apple, bean, chestnut, grape, green pepper, leek, *Lupinus albus* (Bird's foot trefoil) and orange (De Lorenzo et al. 2001). Typically, PGIPs are tissue specific, developmentally regulated and, consistent with their role in plant defense, are up regulated in response to pathogen infection, treatment with elicitors, salicylic acid (SA), jasmonic acid (JA), cold treatment and wounding (Stotz et al. 1994; Bergmann et al. 1994; Devoto et al. 1998; Mahalingam et al. 1999). These observations have led to the hypothesis by Devoto et al. (1998) that the regulatory mechanisms of PGIP must include *in planta* developmental cues with environmental stress and pathogen signals superimposed on them. Grapevine PGIP seems to conform very well to this hypothesis. PGIP expression is found exclusively in roots and ripening berries with transcript levels peaking in *veráison* berries. No basal expression levels could be detected in leaves, but this apparent tissue specificity is easily overcome by several biotic (pathogen infection) and abiotic (wounding, osmotic stresses and hormonal [auxin and SA] treatments) stress stimuli. Interestingly, apart from pathogen infection, stress conditions that resulted in PGIP expression, corresponded well with physiological conditions under which PGIP is expressed in berries and roots. During ripening, berries are subjected to significant stresses, mainly due to changes in osmotic potential (Lott and Barrett, 1967). Other physiological changes include a rapid increase in volume, increased berry softness, development of pigments as well as an accumulation of metabolic components such as hexoses (Davies and Robinson, 1996). Furthermore, physiological conditions in roots also represent tissue specific altered osmotic states and increased auxin concentrations (Sabatini et al. 1999; Ranathunge et al. 2003). It seems likely, therefore, that the developmental cue for grapevine PGIP expression could be related to temporal physiological conditions, most likely an altered osmotic state.

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Higher sugar concentrations, accompanied by the thinning of cell walls to accommodate volume increase, as well as increased osmolarity in the berries, represent a state of increased vulnerability with regard to fungal attack. It is not surprising then, that significant transcriptional reprogramming occurs during this phase, of which many genes are involved in the plant stress response (Davies and Robinson, 2000). PGIP expression during this phase would lead to "priming" of the berry in anticipation of pathogen attack, resulting in increased resistance. Induction experiments conducted with leaves confirmed that grapevine PGIP is also up-regulated in response to *Botrytis cinerea* infection, wounding and SA. This expression profile is typical of defense and stress related genes and reinforces the hypothesis that PGIP is involved in the resistance of plants against fungal attack.

Our hypothesis, however, cannot explain the decline in PGIP transcript levels in post *veráison* berries. From an evolutionary perspective, however, ripe grapevine berries serves to attract birds etc. in order to spread the seeds (already fully formed in post *veráison* berries) (Coombe 1992). There is no further need for fungal protection at this stage and the plant can afford to "discard" these precautions. The molecular basis underlying these events, specifically with regard to *Vvpgip1* repression are, however, still unclear. In grapevine leaves, high levels of sucrose, fructose and NaCl repressed PGIP transcript levels, but this phenomenon was not reproduced in grapevine berries. The involvement of additional regulatory aspects involved in tissue specificity and developmental regulation should, therefore, also be considered. To this end, protein phosphorylation, which has been shown to be an important component of many signaling pathways (Lee & Rudd 2002), was investigated. A broad range serine/threonine kinase inhibitor, staurosporine, was used to show PGIP expression is down regulated by a serine/threonine protein kinase, suggesting the involvement of protein phosphorylation in the signal transduction cascade that leads to PGIP expression. A staurosporine sensitive protein kinase was recently shown to be involved in a JA dependent wound induced signaling pathway (Rojo et al. 1998). Ferrari et al. (2003) furthermore showed that a JA dependent pathway mediates the regulation of PGIP expression in *A. thaliana*. Combined, these observations provide the fundamental basis to elucidate specific signaling cascades involved in *Vvpgip1* regulation.

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We have also investigated whether the signaling cascades responsible for the induction of grapevine PGIP was conserved in other plant species. The *Vvpgip1* gene under control of its own promoter was subsequently transformed into tobacco and the resulting transformants analyzed with regard to PGIP expression. The *Vvpgip1* gene exhibited the same induction profile in the heterologous system as in grapevine. This clearly illustrates some extent of conservation between disease resistance associated regulatory pathways in tobacco and grapevine with regard to PGIP regulation. This observation was further corroborated by a comparison of the induced activity profile of grapevine PGIP with the activity profile of *Vvpgip1* over-expressed in *Nicotiana benthamiana*. PGIP induced by *B. cinerea* infection, wounding and osmotic stress in grapevine leaves, displayed the same PG inhibition spectrum as PGIPs obtained from heterologous over-expression of the cloned *Vvpgip1* gene.

The regulation of grapevine PGIP was further investigated by analyzing the promoter of the *Vvpgip1* gene. The heterologous induction profile of the *Vvpgip1* gene controlled by the native promoter confirmed that the *Vvpgip1* promoter is activated in response to *B. cinerea* infection, osmotic stresses, wounding, IAA and SA. *In silico* analysis of the *Vvpgip1* promoter revealed a putative TATA box element at position –42 and a putative CAAT box at posisiton –129 relative to the putative ATG. The importance of these elements in *Vvpgip1* transcription was illustrated by promoter deletion experiments. GUS-expression driven by promoter fragments lacking the putative CAAT box was significantly lower than levels obtained using longer promoter fragments. No expression was detected using promoter fragments lacking the putative TATA box. We were also able to map the start of transcripion of the *Vvpgip1* gene to 17 bp upstream of the putative ATG. This is in excellent sequence context to the putative TATA box, considering that the pol II transcription initiation complex spans approximately 20 bp (Dvir et al. 2001). Preliminary gelshift analysis confirmed the association of proteins with this promoter area, reinforcing our transient expression data. Several *cis*-acting elements that could potentially be involved in mediating the observed expression profile of the *Vvpgip1* gene were identified by *in silico* analysis in the 4902 bp upstream region. The most abundant of these elements are the W-boxes (Eulgem et al. 2000; Chen et al. 2002) as well as elements involved in auxin and SA

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responsiveness (Lam et al. 1989; Xu et al. 1997; Guilfoyle et al. 1998; Ulmasov et al. 1999), sucrose responsiveness (Grierson et al. 1994) and tissue specific expression (Ellerstrom et al. 1996; Washida et al. 1999). Positionally, most of these elements corresponded well to the promoter areas that were experimentally identified by transient expression analysis to be involved in stimulus specific induction. A functional representation of the *Vvpgip1* promoter is presented in Fig. 1, illustrating the positions of stimuli-responsive areas.

The promoter area involved in mediating *Botrytis*, IAA and to some extent SA induction, could be mapped to the 3.1 kb – 1.5 kb region, osmotic induction could be linked to the 1.1 kb – 0.4 kb region and induction mediated by wounding to the 0.4 kb – 0.1 kb region. From our data it was not possible to identify regions involved in fructose mediated induction. Osmotic treatments with fructose resulted in observed inductions GUS activity for all the promoter fragments tested. Experiments using sucrose instead of fructose gave similar results (data not shown) and it is tempting to speculate that these sugars have a positive influence on GUS activity, rendering the observed inductions as possible artifacts.

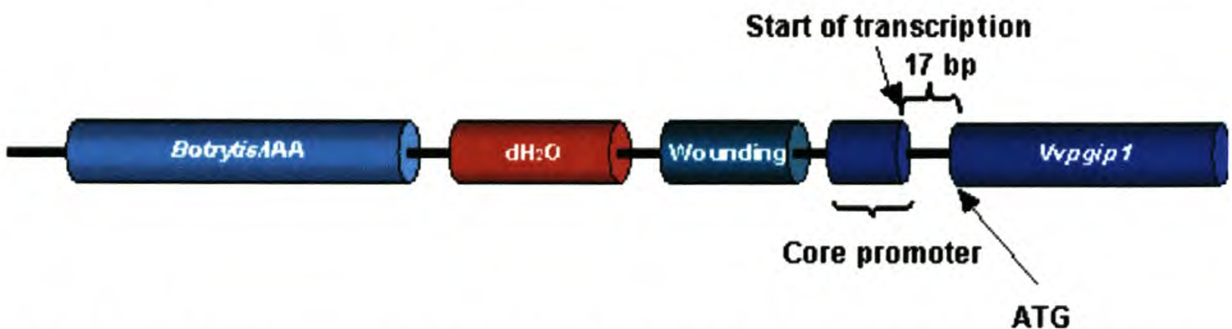

Figure 1. Functional representation of the *Vvpgip1* promoter (not to scale). The promoter sequence is represented by the thick black line, the putative ATG and start of transcription are indicated by arrows and the core promoter, as well as areas involved in promoter activation, is represented by colored boxes. The start of transcription was mapped 17 bp upstream of the putative ATG and the first 137 bp of the promoter was found to be crucial for transcriptional initiation (core promoter). The light blue box represents the area between positions –3.1 kb and –1.5 kb that is involved in *Botrytis*, indole acetic acid (IAA) and to some extent, salicylic acid (SA), responsiveness. The red and green boxes represent areas between positions –1.1 kb and –0.4 kb and –0.4 kb and –0.1 kb that are involved in osmotic and wound responsiveness respectively.

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Experiments utilizing "non-inducing" sugars like glucose should verify whether this is indeed the case. Further studies are also needed to confirm the involvement of the identified *cis*-acting elements, since these elements could potentially provide the basis to identify transcription factors and the elucidation of signaling pathways involved in grapevine PGIP regulation.

Fundamental knowledge regarding the processes involved in plant defense responses enables scientists to further elucidate and, ultimately, safely manipulate certain traits within the context of specific identified areas such as enhanced pathogen perception or improved signal transduction. To this end, we have identified specific aspects such as temporal and spatial expression patterns that provide an important basis for further research regarding the regulation of grapevine PGIP. Several environmental- and pathogenic factors were identified that contribute to grapevine PGIP regulation and we were able to map the effect of most of these stimuli to specific areas of the promoter of the *Vvpgip1* gene. This data will be utilized to ultimately create a better understanding of the interactions involved during grapevine-pathogen (specifically fungal) interactions that will lead to obvious advantages for the grapevine industry, both from an ecological and commercial perspective.

5.2. LITERATURE CITED

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