Plant defence genes expressed in tobacco and yeast

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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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8 February 2002

Date

SUMMARY

Pathogen devastation of food products has been the topic of extensive research efforts worldwide. Fungal infections are foremost amongst these pests, contributing not only to losses in product yield, but also significantly affecting the quality thereof. It is not surprising then that producers of these foodstuffs and their derived products continually strive towards the highest possible product quality. Therefore, it remains imperative that satisfactory methods are implemented to control these fungal pathogens. The current strategies are all hampered by drawbacks, and severe crop losses are still experienced.

New technologies are being explored; one such technology is the genetic transformation of plant species. This method has enabled scientists to introduce foreign genes, with known functions and predictable outcomes, into plants. Genes identified to be involved in disease resistance have become the focus of numerous research efforts concerned with the improvement of the plant's innate defence response. This study aimed to enhance disease resistance to fungal pathogens by means of the genetic transformation of two genes previously shown to be involved in disease resistance. These genes encode polygalacturonase-inhibiting proteins (PGIPs) from *Phaseolus vulgaris* and resveratrol synthase from *Vitis vinifera*. PGIPs specifically inhibit the action of fungal polygalacturonases (PGs), which are enzymes responsible for the hydrolytic breakdown of plant cell walls. These enzymes were also found to be the first enzymes that are secreted by fungal pathogens during infection of the host plant. Additionally, PGIP-PG interaction results in the existence of molecules involved in the activation of plant defence responses. Resveratrol, the product of resveratrol synthase, exerts its antifungal action by destruction of the microbial cellular membranes. These mentioned genes were transformed alone, and in combination, into *Nicotiana tabacum* and the resultant transgenic lines were evaluated for enhanced disease resistance and for possible synergistic effects between the transgenes.

Several independent transgenic lines were regenerated with genes integrated into the tobacco genome. Almost all the plants harbouring only *pgip* or *vst1* genes also expressed these genes at a high frequency. Some non-expressing lines were identified from the transgenic plants that had integrated both genes, but several lines were obtained expressing both transgenes. Good correlations were observed between transgene product activity and enhanced resistance to the fungus *Botrytis cinerea* in an antifungal *in planta* assay. Lines showing the highest PGIP activity and resveratrol levels were more resistant to the pathogen, leading to disease resistance of up to 80% seven days after inoculation in comparison to an untransformed control. These lines maintained their strong inhibition, even three weeks post-inoculation, showing a complete halt in disease development and fungal growth. These results provide good indications of the efficacy of these transgenes in the upregulation of plant defence. However, the study will have to be expanded to

include even more transgenic lines to elucidate the possible synergistic effects of these genes.

In an additional pilot study, genes encoding for precursors and for the formation of resveratrol were introduced into the yeast *Saccharomyces cerevisiae*. The resultant recombinant yeast strains were evaluated for their ability to produce the phenolic substance, resveratrol. This compound has been implicated in beneficial aspects relating to human health, including positive effects on atherosclerosis and platelet aggregation as a direct result of its antioxidant and anti-inflammatory activities.

Recombinant yeast strains were constructed that expressed genes coding for coenzyme A ligase and resveratrol synthase. These strains were shown to be able to produce the phenolic compound resveratrol from the precursors present in the yeast as well as from the products introduced with the transformation. The resveratrol was complexed with an added glucose moiety. These results are extremely positive, considering the possibility of manipulating wine yeasts to produce resveratrol during the wine fermentation, thereby adding to the health aspects of both red and white wine. This is the first report of the production of this compound by the introduction of genes necessary for its biosynthesis in a foreign host.

This study has confirmed the importance of PGIPs and resveratrol in the effort to enhance disease resistance in plants through genetic transformation technology. It has also shown that the health benefits of resveratrol could be exploited more optimally in the wine industry, by producing wine yeasts with the ability to synthesise this important antioxidant.

OPSOMMING

Patogeen-geïnduseerde skade aan belangrike voedselsoorte vorm reeds geruime tyd 'n integrale deel van wêreldwye navorsingspogings. Die vernaamste organismes verantwoordelik wat tot dié skade bydra is swamme. Hierdie patogene kan beide die produkopbrengs, sowel as die kwaliteit daarvan beduidend beïnvloed. Produsente van voedselprodukte moet deurlopend streef na die hoogste kwaliteit; die noodsaaklikheid om effektiewe metodes te vind om hierdie siektes te beheer word dus al hoe belangriker. Huidige beheermaatreëls het egter almal nadele, wat daartoe lei dat produsente steeds noemenswaardige opbrengsverliese lei.

Nuwe beheermetodes word tans ondersoek en die genetiese transformasie van plantspesies is een van daardie tegnologieë. Hierdie metode het aan wetenskaplikes die vermoë gegee om heteroloë gene, met bekende funksies en dus voorspelbare resultate, in plante te transformeer. Sulke gene, met bevestigde funksies wat by die siekteweerstand van plante betrokke is, het dus toenemend die fokus van plantverbeteringsprogramme geword. Hierdie studie het ten doel gehad om die plant se intrinsieke weerstand teen swampatogene te verhoog. Twee gene met bewese betrokkenheid by plantweerstand is deur middel van genetiese transformasie in plante geïnkorporeer. Hierdie gene kodeer vir poligalakturonase-inhiberende proteïene (PGIPs) van *Phaseolus vulgaris* en resveratrol sintase (*vst1*) van *Vitis vinifera*. Poligalakturonases (PGs), ensieme wat verantwoordelik is vir die hidrolitiese afbraak van plantselwande, word spesifiek deur PGIPs geïnhibeer. Dit is ook bevind dat PGs die eerste ensieme is wat tydens swaminfeksie van die gasheerplant afgeskei word. Verdere bewyse vir die rol van PGIPs in plantweerstand is gevind toe bewys is dat die interaksie van PGIP en PG lei tot die verlengde teenwoordigheid van molekules wat die vermoë het om die plant se weerstandsrespons aan te skakel. Resveratrol, wat die produk van die proteïen resveratrol sintase is, het antifungiese aktiwiteit wat op die afbraak van mikrobiese sellulêre membrane gebaseer is. Hierdie gene is in *Nicotiana tabacum* getransformeer, beide alleen en in kombinasie, en die gevolglike transgeniese plante is vir verhoogde weerstand teen swampatogene geëvalueer. Die moontlike sinergistiese effekte van die twee transgene is ook geëvalueer.

Verskeie onafhanklike transgeniese tabakplante, met die heteroloë gene in die genoom geïntegreer, is gegenereer. Plante wat slegs die *pgip*- of *vst*-gene in die genoom geïntegreer het, het hierdie gene teen hoë frekwensie uitgedruk. Verskeie lyne van transgeniese plante met beide gene geïntegreerd is geregenereer, maar heelwat is ook gevind waar integrasie nie plaasgevind het nie. Goeie korrelasies tussen transgeenprodukaktiwiteit en verhoogde weerstand teen die swam *Botrytis cinerea* is na 'n antifungiese *in planta* toets verkry. Lyne met die hoogste PGIP-aktiwiteit en dié met die hoogste vlakke van resveratrol was ook meer weerstandbiedend teen die swampatogeen. In vergelyking met 'n ongetransformeerde swampatogeen, is siekteweerstandsvlakke van tot 80% in sommige lyne na inokulasie en inkubasie vir sewe dae met die patogeen verkry.

Hierdie lyne het voorts hul sterk antifungiese aktiwiteit na drie weke van inkubasie behou en sodoende swamontwikkeling en -verspreiding totaal gestuit. Hierdie resultate is 'n goeie weerspieëling van die doeltreffendheid van die gebruik van hierdie gene met die oog om die plant se swambestandheid te verhoog. Die studie sal egter uitgebrei moet word om meer transgeniese lyne in te sluit om die moontlike sinergistiese effek tussen die transgene te evalueer.

Gene wat vir die voorlopers en produksie van resveratrol kodeer, is in 'n addisionele voorloperstudie tot die gis *Saccharomyces cerevisiae* toegevoeg. Die gevolglike rekombinante gisrasse is getoets vir hulle vermoë om die fenoliese molekuul, resveratrol, te produseer. Hierdie molekuul word met voordelige aspekte van menslike gesondheid geassosieer. Die antioksiderende- en anti-inflammatoriese aktiwiteit daarvan veroorsaak positiewe effekte op beide aterosklerose en bloedplaatjieversameling.

Rekombinante gisrasse wat die enkoderende gene vir koënsiem-A ligase en resveratrol sintase uitdruk, is gemaak. Hierdie rasse het die vermoë om die molekuul, resveratrol, vanaf die voorlopers wat teenwoordig is en ook tot die medium toegevoeg is, te produseer. Die resveratrol is bevind om in 'n kompleks met 'n glukosemolekuul te wees. Hierdie resultate is uiters belowend vir die moontlike manipulasie van wyngiste om resveratrol tydens wynfermentasie te vorm, wat uiteraard 'n direkte bydrae tot gesondheidsaspekte wat met wit- sowel as rooiwyn geassosieer is, te maak. Hierdie is die eerste aanduiding van die produksie van resveratrol deur transformasie van 'n heteroloë gasheer.

Hierdie studie het die belangrikheid van PGIPs en resveratrol in plantsiekteweerstand beklemtoon. Voorts sal dit moontlik wees om die gesondheidsaspekte van resveratrol meer optimaal in die wynindustrie te benut deur wyngiste te maak met die vermoë om hierdie belangrike antioksidant tydens die fermentasieproses te produseer.

This thesis is dedicated to my parents.
Hierdie tesis word opgedra aan my ouers.

BIOGRAPHICAL SKETCH

John Becker was born in Cape Town, South Africa, on 23 January 1975. He attended Eversdal Primary and matriculated from Roodepoort High School in 1993. John enrolled at the Rand Afrikaans University in 1995 and obtained the BSc degree in Biochemistry and Botany in 1997. In 1998 he received the degree BScHons from the Institute for Wine Biotechnology, Stellenbosch University. In 1999 he enrolled at the same Institute for an MSc degree.

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journals to which the manuscripts (Chapters 3 and 4) will be submitted for publication.

Chapter 1 **General Introduction and Specific Project Aims**

Chapter 2 **Literature Review**
Upregulation of plant genes involved in disease resistance

Chapter 3 **Research Results**
"Evaluation of bean polygalacturonase-inhibiting proteins (PGIPs), alone and in combination with resveratrol synthase from *Vitis*, in transgenic tobacco as an antifungal strategy" will be submitted to *Molecular Plant-Microbe Interactions* in modified format

Chapter 4 **Research Results**
"The production of resveratrol by *Saccharomyces cerevisiae*" will be submitted to *FEMS Yeast Research* in modified format

Chapter 5 **General Discussion and Conclusions**

I hereby declare that I was the primary contributor with respect to the experimental data presented on the multi-author manuscript presented in Chapter 3. My supervisors, Prof. IS Pretorius and Dr. MA Vivier, were involved in the conceptual development and continuous critical evaluation of the study. Gareth Armstrong, along with the supervisors of the study as outlined in Chapter 4, was responsible for the conceptual development thereof. Plasmids containing genes *vst1* and *CL216* were provided by these authors. Yeast transformation and liquid chromatography mass spectrometrical assays were done by myself.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND SPECIFIC PROJECT AIMS

GENERAL INTRODUCTION AND SPECIFIC PROJECT AIMS

1.1 INTRODUCTION

The grapevine and its derived products form an integral part of the global as well as the South African agricultural portfolio. Efforts to improve both the crop and its derived products have become commonplace, with producers continually aiming to provide better quality grapes and wine for consumption. Factors considered detrimental to these efforts have enjoyed much attention, with pathogens and pests being foremost among these. Research efforts dealing with these factors should be applauded, but the reality remains that grapevine crops still suffer extensive losses associated with pathogen attack. Apart from the much-employed chemical control strategies, other possible solutions associated with pathogen control include biological control and breeding for disease resistance. Biological control efforts have met with a degree of success, but high inocula of the antagonistic agent are required for success. The effects of the introduction of foreign species at high concentrations in the vineyard and the consequences for the grape microflora, and ultimately the wine quality, have also not been investigated fully. Moreover, the efficacy of pathogen control by antagonistic microorganisms has been shown to be highly dependent on the prevailing climatic conditions.

Traditional breeding methods involving woody plant species such as *Vitis vinifera* remain time consuming, a direct result of the long regeneration time associated with these woody plants. Also, most of the characteristics that are desirable in grapevine are inherited polygenically, leading to extensive backcrossings to amplify the specific phenotype bred for. Although these mentioned initiatives are extremely important in the improvement of grapevine plant material, newer technologies are increasingly incorporated into these approaches or are implemented as stand-alone technological applications. One such technology is the genetic transformation of plant species. The ability to introduce a gene or genes of interest, from potentially any source, into the plant genome has revolutionised plant sciences. For a number of years, manipulated disease resistance has been one of the major focuses of the genetic transformation of plant species (Shah, 1997; Salmeron and Vernooij, 1998; Rommens and Kishore, 2000). This was because it has been shown that disease resistance or susceptibility is often under the control of a single gene (Agrios, 1997). It is not surprising therefore that huge efforts have been initiated in several laboratories to study the effects of genes known to confer resistance to pathogens. Genetic transformation technology, however, also is an extremely useful and powerful tool to elucidate complex phenomena in plants and has led to a vast amount of new knowledge being generated on the topic of plant disease resistance.

Several proteins and their encoding genes that have been confirmed to be involved in disease resistance or pathogenicity have been isolated from plants and pathogens respectively. The study of these proteins and genes, as well as their interactions, has shed light on the complex and dynamic interaction between plant hosts and their respective pathogens. Two of these well-established disease resistance genes have been the subject of this thesis, within the broad framework of genetic transformation technology of plant species to upregulate the plant's endogenous disease-resistance mechanism. The substances under investigation were the polygalacturonase-inhibiting proteins (PGIPs) from bean and the phenolic stress metabolite, resveratrol, both of which are associated with pathogen limitation.

1.2 SPECIFIC AIMS

The first, and overriding goal of this thesis was to evaluate the PGIP-encoding genes from bean (*pgip1* and *pgip2*) alone, or in combination with the resveratrol synthase (*vst1*) gene from *V. vinifera,* for their individual effectiveness or possible synergism against fungal pathogens in a transgenic plant environment. These specific genes and their encoded products were chosen due to their established antimicrobial activities (Hain et al., 1993; Powell et al., 2000). The second goal was to manipulate the yeast *Saccharomyces cerevisiae* to produce active resveratrol by introducing the necessary biosynthetic genes into the yeast on episomal plasmids.

The PGIPs have been shown to inhibit the action of fungal polygalacturonases (PGs) (Cervone et al., 1993), which are the first enzymes secreted by fungal pathogens during the early stages of colonisation and infection (Jones et al., 1972). These PGs are responsible for the breakdown of plant cell wall homogalacturonans and, as such, are important pathogenicity factors of fungi. They also serve as potential avirulence factors, releasing molecules with the ability to activate plant defence responses from the plant cell walls. Ultimately, PGIP favours the prolonged existence of these molecules, thereby contributing not only to the activation of other defence responses, but also to the inhibition of cell-wall degrading enzymes (Cervone et al., 1993).

Resveratrol synthase (also known as stilbene synthase) catalyses the formation of the stilbene, resveratrol. Stilbenes are secondary plant products with phytoalexin properties. These molecules may be modified in several ways in the plant, including by polymerisation, methylation or the addition of glucose moieties (Schröder et al., 1990). Stilbenes exert their antifungal activities by destructing the cellular membranes (Pont and Pezet, 1990). The specific aims of this study included:

i) The construction of plant expression cassettes harbouring the *pgip1* and the *pgip2* genes from *Phaseolus vulgaris* and the *vst1* gene from *V. vinifera.* All constructs were transformed into *Nicotiana tabacum* cv. Petit Havana SR1 on their own, or in combination in co-transformation events. Plantlets were regenerated and tested for gene integration and expression.

ii) The analysis of the various transgenic lines for the presence of active PGIPs and/or resveratrol.
iii) The analysis of the various transgenic lines for their ability to inhibit and curb fungal infection and disease development in an *in planta* antifungal assay against *Botrytis cinerea*.

These experiments and results are presented in Chapter 3 of this thesis and form part of our aim to upregulate the grapevine's endogenous disease resistance against fungal pathogens. Tobacco was used merely as a convenient model system to test the various genes and their antifungal activities alone or synergistically in a transgenic plant environment.

In addition to these specific aims regarding the mentioned antifungal genes and their overexpression in a plant system, another route for resveratrol production from yeast formed a small part of this study in a collaborative effort with yeast molecular biologists from our Institute. The overriding aim of this project was the construction of wine yeast strains with the ability to produce resveratrol. Resveratrol has increasingly been linked to the improvement of certain human health aspects. This is mainly due to its strong antioxidant and anti-inflammatory activities (Huang, 1997). These activities might inhibit, amongst others, biochemical changes involved in tumor development (Huang, 1997), platelet aggregation (Kopp, 1998) and certain aspects of the development of atherosclerosis (Steinberg, 1991). Several reports have claimed that the resveratrol present in red wine specifically could be beneficial for these health aspects within a framework of moderate wine comsumption and a healthy diet and life-style. The ability to increase the resveratrol content of both red and white wines through the production of resveratrol by the yeast performing the wine fermentation could be an attractive option.

A study describing the genetic constructs prepared to this end formed part of the MSc thesis of Mr G Armstrong, but the production of resveratrol could not be detected in that study. With the methodology used in our plant analysis for resveratrol, this subject was revisited with the following aims:

i) The transformation of a laboratory yeast strain of *S. cerevisiae* with yeast expression cassettes harbouring the 4CL216 coenzyme A ligase encoding gene and the Vst1 resveratrol synthase encoding gene and the confirmation of the expression of these genes with Northern blot analysis.
ii) The preparation of intracellular yeast extracts for the analysis of resveratrol content. The use of dual mass spectrometrical analysis of samples before and after treatment with a β-glucosidase to quantify the resveratrol content of the various samples.

This is a first report of the reconstruction of a biochemical pathway in a heterologous host to produce the important antioxidant, resveratrol, which might contribute to the improvement of the health benefits of wines and other foodstuffs. This work is presented in Chapter 4 of this thesis.

1.3 LITERATURE CITED

Agrios, G.N. 1997. Plant Pathology, 4th edition. Academic Press, San Diego.

Cervone, F., De Lorenzo, G., Caprari, C., Clark, A.J., Desiderio, A., Devoto, A., Leckie, F., Nuss, L., Salvi, G. and Toubart, P. 1993. The interaction between fungal endopolygalacturonase and plant cell wall PGIP (Polygalacturonase-inhibiting protein). Pages 64-67 in: Mechanisms of Plant Defense Response; ed by B. Fritig and M. Legrand, Kluwer Academic Publishers.

Hain, R., Reif, H., Krause, E., Langebartels, R., Kindl, H., Vorman, B., Wiese, W., Schmelzer, E., Shreier, P.H., Stöcker, R. H. and Stenzl, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153-156.

Huang, M-T. 1997. Diet for cancer prevention. Food Sci. 24: 713-727.

Jones, T.M., Anderson, A.J. and Albersheim, P. 1972. Host-pathogen interaction. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Path. 2: 153-166.

Kopp, P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur. J. Endocrinol. 138: 619-620.

Pont, V. and Pezet, R. 1990. Relation between the chemical structure and biological activity of hydroxystilbenes against *Botrytis cinerea*. J. Phytopath. 130: 1-8.

Powell, A.L.T., Van Kan, J., Ten Have, A., Visser, J., Greve, L.C., Bennett, A.B. and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13: 942-950.

Rommens, C.M. and Kishore, G.M. 2000. Exploiting the full potential of disease-resistance genes for agricultural use. Curr. Opin. Biotechnol. 11: 120-125.

Salmeron, J.M. and Vernooij, B. 1998. Transgenic approaches to microbial disease resistance in crop plants. Curr. Opin. Biotechnol. 1: 347-352.

Schröder, J., Lanz, T. and Schröder, G. 1990. Genes for biosynthesis of stilbene-type phytoalexins. Plant Gene Transfer. 311-318.

Shah, D.M. 1997. Genetic engineering for fungal and bacterial diseases. Curr. Opin. Biotechnol. 8: 208-214.

Steinberg, D. 1991. Antioxidants and artherosclerosis: a current assessment. Circulation 84: 1420-1425.

CHAPTER 2

LITERATURE REVIEW

Upregulation of plant genes involved in disease resistance

LITERATURE REVIEW

2.1 INTRODUCTION

The continual co-evolution of plant and pathogen in a bid for survival has led to intricate interactions when they encounter one another. Plants better suited for resisting micro-organisms might not be ideally suited to the production of quality crops, and vice versa. Considerable effort has been invested in endeavours attempting the improvement of plant resistance concerning single traits. Such efforts have been rewarding and targeted applications for the improvement of specific aspects of plant metabolism have proven successful to an extent. However, it has become obvious that multifaceted approaches, as well as those leading to the activation of even more defence responses, are needed for pathogen control. Aspects of plant defence are discussed in order to identify suitable target genes that, when upregulated, should provide extensive protection against plant pathogenic fungi. These genes all form part of the active, induced defence and have been proven effective against pathogens. The genes that will be discussed encode polygalacturonase-inhibiting proteins and resveratrol synthase respectively, both of which have been shown to be important factors in fungal-host interactions. *In vitro* results have shown that these gene products are effective at a high concentration, and these levels are attainable when constitutive overexpression is utilised. The upregulation of these genes furthermore will involve the abolishment of the time delay associated with induced defence. Finally, three methods for the delivery of multiple genes into plants and the general considerations associated with these methods in the context of transformation technology are discussed. Included in this section are discussions regarding high regeneration frequency, high transformation efficiency and the importance of genetic linkage.

2.2 GENERAL PLANT DEFENCE

2.2.1 Preformed defence

Plants have evolved to respond to a barrage of pathogens in mainly two ways. The intrinsic structural characteristics of the plant cells prohibit the entrance and spread of the pathogen within the plant, whereas chemicals and substances that are either lethal to pathogens or unfavourable for their growth are formed in preparation of attack (Agrios, 1997). Preformed defence structures may include particular characteristics of the epidermis and its wax or cuticle, stomata and lenticel shape, size and location and the presence of thick-walled cells. Although these preformed defence structures are important

and effective in defence, it is interesting to note that certain pathogens are not able to colonise particular plant species, despite a lack of physical barriers. In these cases pathogens are controlled by chemical substances or inhibitors (Agrios, 1997). These inhibitors might be present within the plant cell, and typically include phenolic compounds, tannins and fatty acid substances, such as dienes. Other proteins shown to be involved include certain lectins and chitinase and glucanase hydrolases. Alternatively, the cell might excrete inhibitors. The phenolic compounds, protocathechuic acid and catechin, are known to interact with moisture in the soil or on leaves, thereby inhibiting the germination of fungal conidia that are present. Finally, the absence of certain factors can also provide defence. A lack of essential factors, including a lack of recognition between pathogen and host, a lack of receptors and insensitivity to pathogen toxins, as well as the absence of essential substances required by the pathogen (Agrios, 1997), are powerful defence strategies in plants.

2.2.2 Induced defence

Once the preformed defence has been breached, induced cellular defences prevent the further spread of pathogens. These are termed active defences, since the host's metabolism is involved and upregulated following invasion by the pathogen. Induced defences in plants can be activated by a wide range of microorganisms. Incompatibility between the host and pathogen may comprise three classes of active defence: the primary response is localised to the cells in contact or in close association with the pathogen; the outcome thereof in these cells frequently is programmed cell death. Secondary responses are induced in adjacent cells responding to elicitors or products of the initial response. Systemic acquired resistance throughout the plant represents the third class of response and is hormonally induced, involving complex signal transduction mechanisms (Hutcheson, 1998).

The above-mentioned responses are triggered by recognition of the avirulence (*avr*) gene products contained in the pathogen by the matching resistance (*R*) gene products in the host (Dempsey et al., 1998). A lack of either will result in pathogen colonisation of the host. Once the *avr* gene product has been recognised, the cell responds by several local metabolic alterations to the site of infection. Firstly, cell wall reinforcements occur by means of the deposition and crosslinking of polysaccharides, proteins, glycoproteins and phenolics. Secondly, activation of metabolic pathways occurs, leading to secondary product formation, specifically products with antimicrobial activity (phytoalexins), but also including salicylic acid, ethylene and other defence regulators. Lastly, an accumulation of antimicrobial peptides and proteins takes place (Fritig et al., 1998).

Critical to the recognition process and preceding the activation of signal-specific responses, are the generation, inter- and intracellular conversion as well as transduction of signal compounds (Ebel, 1998). Such elicitors may comprise the products of pathogen avirulence genes, plant cell wall or pathogen-released compounds, or pathogen-secreted

substances. Oligosaccharide elicitors of fungal and plant origin have been characterised (Fig. 1). Oligoglucan, oligochitin and oligochitosan from fungi and oligogalacturonide from plants have been studied in detail in this regard (Ebel, 1998).

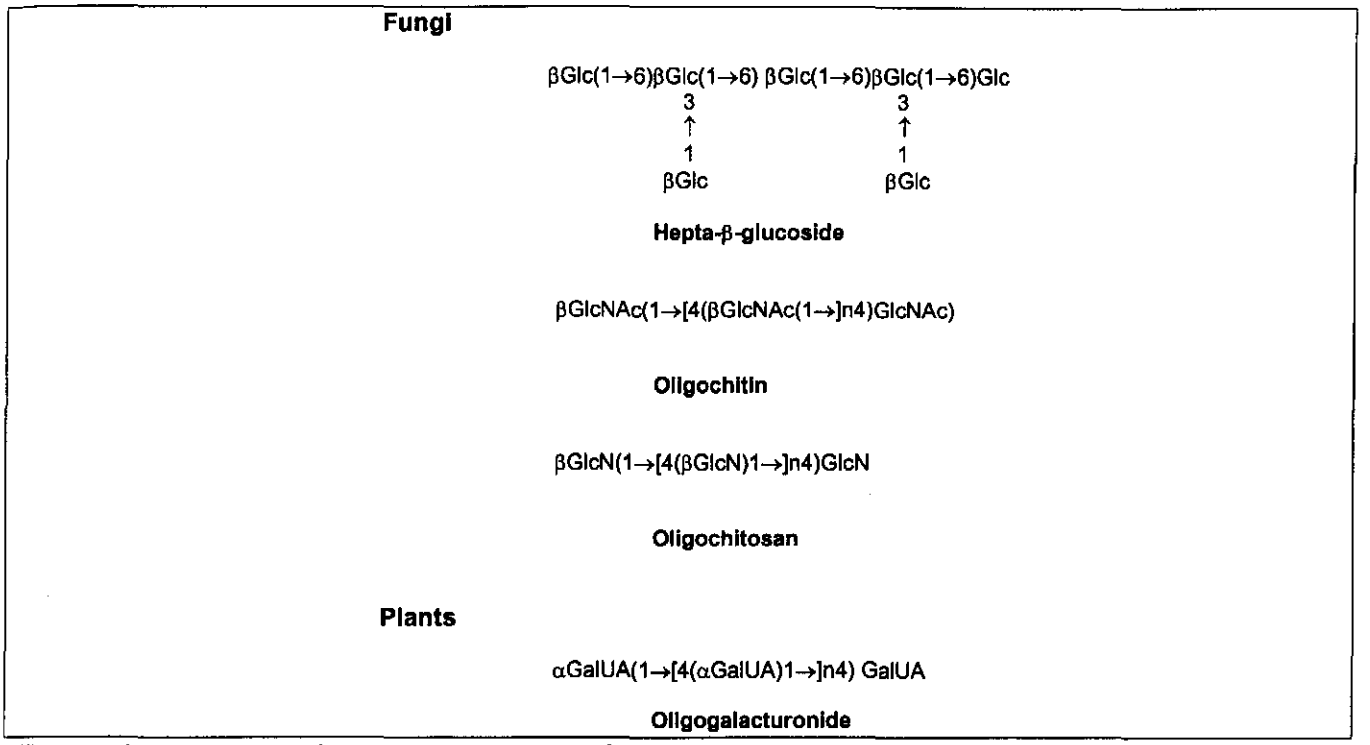

Figure 1. Structures of the major classes of elicitor-active oligosaccharides. βGlc, β glucan residue; βGlcNAc, β-D-*N*-acetyl-glucosaminyl residue; βGlcN, de-*N*-acetylated derivative of βGlcNAc; αGalUA, α-(1-4)-linked galactosyluronic acid residue. Adapted from Ebel, 1998.

Regardless of the origin of the elicitor, signal perception mechanisms seem to rely on receptors in the plant cell. This perception might be on the cell surface, for fungal elicitors, or intracellular, for certain bacterial elicitors, resulting in signalling processes that in turn activate defence responses (Ebel, 1998).

The early events following pathogen recognition (Fig. 2) include the opening of ion channels and the formation of reactive oxygen species, such as O_2^-, H_2O_2 and OH^-. This is hypothesised to be the result of the plasma membrane-associated NAD(P)H oxidases and/or apoplastic-localised peroxidases (Somssich and Hahlbrock, 1998). These transient reactions form part of the prerequisites for further events concerning signal transduction, that might lead to a concerted signalling network required for triggering the total host defence response. Included in this network are changes in metabolic activity, membrane polarity, cytosolic Ca^{2+} and H^+ concentrations, and an array of regulated enzymes, including NAD(P)H oxidases, phospholipases, phosphatases and protein kinases. Various metabolic pathways are believed to be affected by these rapid changes; phospholipases might liberate linolenic acid, a substrate for the production of jasmonic acid, methyl jasmonate and related molecules via several enzymatic steps (Somssich and Hahlbrock,

1998). These compounds have been shown to serve as signal molecules during pathogen attack (Fig. 2).

The generation of oyxgen intermediates results in oxidative stress in the plant cells, but also acts to limit tissue damage as a result of pathogen attack. High concentrations of these reactive oxygen intermediates may serve to kill both the pathogen and the affected plant cell (hypersensitive cell death). However, in low doses they may act as signal molecules for the induction of detoxification mechanisms. These may include the activation of defences in adjacent cells and the induction of superoxide dismutases and glutathione-*S*-methyltransferases. Furthermore, the crosslinking of structural proteins in the cell wall is driven by a burst of H_2O_2, further reinforcing the physical barrier.

O_2^- may also act further downstream in the activation of nuclear genes (Somssich and Hahlbrock, 1998). Altered gene expression patterns in the nucleus are also the result of signal-transduction chains differing in both length and complexity. The rapidly induced proteinaceous compounds involved are those including proteins that catalyse phytoalexin synthesis, pathogenesis-related (PR) proteins, inhibitors of fungal macerating enzymes (particularly soft-rot fungi) and antimicrobial peptides. PR proteins act directly on microbial cell walls, whereas inhibition proteins lead to the prolonged existence of elicitors of the active, induced defence response. These elicitors, in turn, will result in the upregulation of the transcription of genes sensitive to these molecules, thereby strengthening the host's innate defence system.

Just as important as the generation and transduction of signal molecules in the defence response of plants is the activation and rendering of antimicrobial substances to inhibit/eliminate the invading pathogen. Phytoalexins are excellent examples of antimicrobial substances in plants and have been studied widely. Phytoalexins are small, broad-spectrum antimicrobial compounds that are produced rapidly upon microbial challenge (Dempsey et al., 1998). Plants that are phytoalexin biosynthetic mutants have been shown to be more susceptible to fungal pathogens (Maleck and Lawton, 1998). Conversely, increased resistance to *Botrytis* has been achieved in transgenic plants expressing the stilbene synthase gene producing the phytoalexin, resveratrol (Hain et al., 1993). The resultant increased resistance can be assumed to be a function of the presence of the novel stilbene accumulating in the transgenic plant, since the plants lacked the genes coding for the phytoalexin prior to transformation. The accumulation of phytoalexin mRNAs might take minutes to several hours to commence, particularly for those cells adjacent to the infection site, and even longer for genes responding systemically (Somssich and Hahlbrock, 1998).

When considering the most optimal scenarios to increase disease resistance in plants, the approach most likely to result in increased resistance would incorporate the upregulation of several aspects of the active defence response. From that perspective, this review will emphasise only the importance of two proteins in possible transgenic approaches to improve disease resistance. Firstly, the importance of polygalacturonase-inhibiting proteins (PGIPs) as an example of proteins involved in elicitor recognition and

stabilisation, as well as their role in defence signal transduction, will be discussed. PGIPs and higher levels thereof specifically inhibit the macerating enzymes of fungal origin, while simultaneously activating even more defence responses. The second protein under discussion is resveratrol synthase as an example of a phytoalexin-producing protein and its role in the active defence system of plants.

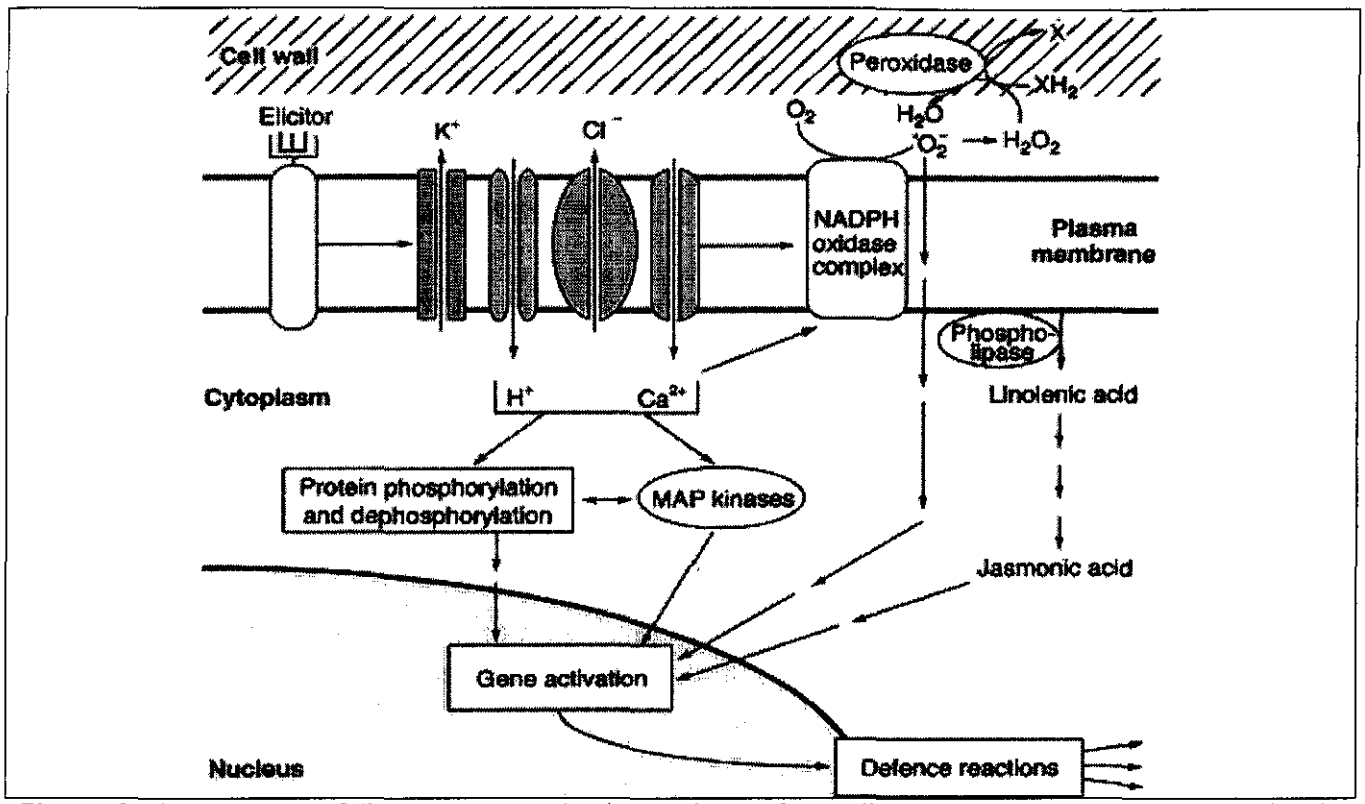

Figure 2. Components of the signal-transduction pathway, from elicitor perception to gene activation in cultured parsley cells. Large and transient ion fluxes result from the recognition of an elicitor by its receptor, a plasma-membrane bound protein. These fluxes activate specific MAP-kinases and an NAD(P)H oxidase, which produce oxygen intermediates. MAP-kinase activation results in its translocation into the nucleus. Elicitor-dependent protein phosphorylation/dephosphorylation, the generation of reactive oxygen species required for peroxidase-mediated crosslinking of cell wall components, as well as the activation of genes involved in defence are observed. Binding of the elicitor to its receptor also leads to the generation of jasmonic acid via phospholipase and the activation of a subset of elicitor-responsive genes (Somssich and Hahlbrock, 1998).

2.3 POLYGALACTURONASE-INHIBITING PROTEINS (PGIPs)

2.3.1 Introduction

The rapid and extensive breakdown of host cell walls by microbial polygalacturonases (PGs) originating from invading pathogens render these hydrolytic enzymes important pathogenicity factors. Not only do they provide microbes with a nutrient growth source, but they facilitate the further breakdown of the host cell walls by other pectin-degrading enzymes. This was confirmed when it was found that endo-PGs were the first polysaccharide-degrading enzymes secreted by cell wall-cultured pathogens (Jones et al.,

1972). Most dicotyledonous plants, however, contain cell wall-bound inhibitory proteins that are specific against these endo-PGs. It appears that plants have evolved this mechanism to regulate the activity of these enzymes, while simultaneously exploiting the liberated cell wall products as elicitors to intensify the defence responses, as discussed in the previous section. The endo-PG-PGIP interaction therefore provides a dynamic and useful mechanism to study host-pathogen interactions and the elicitation of defence responses.

2.3.2 PGIPs isolated from plant species

Among the PGIPs characterised from plants are those from bean (Cervone et al., 1987), raspberry (Johnston et al., 1993), pear (Stotz et al., 1993), soybean (Favaron et al., 1994), tomato (Stotz et al., 1994), leek (Favaron et al., 1997), apple (Yao et al., 1999), and grapevine (De Ascensoa et al., 2002). All *pgip* genes share a high sequence homology. Similarly, with the exception of apple, all PGIPs possess high isoelectric points. Although this is thought to facilitate the binding of PGIP to negatively charged pectin in the cell wall (Johnston et al., 1993), Favaron et al. (1997) found cell wall-bound and soluble PGIP in leek with similar isoelectric points. Further similarities include the presence of multiple copies of *pgip* in the plant genome- up to three were noted in leek (Favaron et al., 1997), and the possibility exists of multiple copies in tomato, pear and apple. This might be the first indication of the binding specificity of each of the members toward fungal PGs of diverse origin.

PGIPs have very specific inhibition spectra, and vary considerably among the isolated proteins. Leek PGIPs were unable to inhibit PGs from *Fusarium moniliforme* and *Sclerotium cepivorum* (Favaron et al., 1997). However, two PGs from *Sclerotinia sclerotiorum* and one from *B. aclada* were inhibited almost completely. Endo-PGs exhibiting greater sensitivity to PGIP *in vitro* were inactive or close to inactive on leek tissue, and those not inhibited showed greater activity on the plant tissue, even at lower levels. Two isozymes of *S. sclerotiorum* endopolygalacturonases were inhibited differentially by soybean PGIP. Raspberry PGIP inhibited endo-PGs from *B. cinerea* to a similar extent, and those from *Aspergillus* to a lesser extent. Conversely, PG was poorly inhibited by tomato PGIP. Abu-Goukh et al. (1983) found that the most abundant form of PGIP in pear fruits had little effect on *A. niger* PG and inhibited *B. cinerea* polygalacturonase competitively (i.e. binding of PGIP to PG active site). Yet, Sharrock and Labavitch (1994) found that this PGIP had no detectable effect on prolonging the presence of elicitor-active oligogalacturonides by the inhibition of *Botrytis* PG. Only the favouring of di- rather than monogalacturonides was observed. Thus, PGIPs differ in their abilities to inhibit several fungal PGs. Differential inhibition will lead to varying abilities of these PGIPs to activate the host defence response via the formation of elicitor-active molecules.

PGIP levels are controlled developmentally. Furthermore, wounding and pathogen challenge increase the levels of these proteins, as they form part of the active, induced

defence. Raspberry *pgip* transcript levels varied in fruits of differing maturity, as did those of apple. The latter were expressed constitutively throughout fruit development. Pear *pgip* mRNA accumulates preferentially in the fruit, with lower levels detected in the flowers. PGIP is absent from the leaves. Furthermore, wounding strongly elicited PGIP accumulation in soybean and apple plants. Transcript levels were similarly increased upon *Penicillium expansum* and *B. cinerea* challenge and cold storage in apple fruits, although the latter had a limited ability for induction compared to immature and ripe fruit. Pathogen-induced transcript levels were considerably higher than those observed after wounding following the same incubations (24 h).

From the above investigations it became quite clear that PGIPs possess differing specificities towards fungal PGs of diverse origin. The fact that they are inducible by both pathogen challenge and wounding provides a clear indication for their role in defence. The observation that the ability of pathogens to colonise raspberries was inversely correlated with the PGIP level in maturing fruit further substantiates this role. Similarly, grapevine cultivars that were the most resistant to *B. cinerea* infection were those retaining a high level of PGIP activity throughout maturity (Johnston et al., 1993).

The *pgip* gene family of bean has been studied extensively and will be used in the following section to illustrate some of the characteristics associated with *pgip* gene families, their encoded products and their structure/function relationship in host-pathogen interactions.

2.3.3 *Phaseolus vulgaris* PGIPS

On the basis of the affinity that PGIP exhibits for polygalacturonase, Cervone et al. (1987) were able to isolate and purify the first polygalacturonase-inhibiting protein from the hypocotyls of *Phaseolus vulgaris*. The studies conducted on this isolated PGIP showed that the protein inhibits the action of fungal endo-PG on polypectate, resulting in the formation of polymerised oligogalacturonides that specifically favour the elicitation of plant defence responses.

A gene encoding a single, continuous open reading frame spanning 1026 nucleotides (342 amino acids), and containing two in-frame start codons, was subsequently isolated from *P. vulgaris* cv. Saxa genomic DNA and cv. Pinto cDNA (Toubart et al., 1992, Frediani et al., 1993). On the basis of hybridisation analysis, it was shown that a small gene family encodes PGIP in the genome of bean. Western blot analysis performed with PGIP-specific antibodies indicated the presence of at least three different reacting polypeptides. However, only one species of mRNA was detected following Northern blot analysis, either indicating differential expression of the *pgip* genes, or the fact that several, but different, gene transcripts may be of similar size. The presence of multiple genes was confirmed by Desiderio et al. (1997) after expression of the cloned *pgip* gene in tomato under the control of the constitutive cauliflower mosaic virus promoter. Purified PGIP from transgenic plants possessed a different spectrum of inhibition towards fungal pathogens in comparison to

bulk PGIP isolated from *P. vulgaris*. Multiple gene existence was also supported by the fact that *P. vulgaris* possesses a PGIP with the ability to interact with *F. moniliforme* PG and by the existence of a second PGIP in the same tissue without this ability, confirming the high degree of specificity associated with these gene products.

It was established that PGIP is synthesised as a precursor and undergoes posttranslational modification to yield a mature polypeptide of 34 kDa. Additionally, a region of high hydrophobicity corresponded to a signal peptide. A high occurrence of lysine and arginine residues in the protein confers a high isoelectric point, indicating possible interactions with acidic pectins in the cell wall (Toubart et al., 1992). Further analysis (De Lorenzo et al., 1994) revealed the internal structure of bean PGIP. A region spanning from amino acid 69-326 exhibits tandem repeated units originating from the modification of a peptide consisting of 24 amino acids. The 258 amino acid domain, termed the leucine-rich repeat, comprises 10.5 tandem repeats, with regularly spaced leucine residues (Table 1).

In-situ hybridisation (Frediani et al., 1993) placed the bean *pgip* gene family in the region of the pericentromeric heterochromatin of chromosome pair X. In *Phaseolus*, no discrepancies exist between the transcriptional activity of the euchromatic and heterochromatic regions. Additionally, numerous functional *Phaseolus* genes reside in the heterochromatic region. Developmental analyses on bean *pgip* expression were performed by Devoto et al. (1997) and mRNA levels were studied in different organs, including the leaves, stems, flowers, pods and seeds at different developmental stages. Light-grown seedlings and adult plants, as well as etiolated seedlings, were also analysed. From the experiments, *pgip* transcripts were found in all light-grown bean plants in all the tissues examined. Transition zones between the elongating and mature regions of young hypocotyls and the basal areas of the stem tissue of adult bean plants were shown to contain elevated levels of transcripts, whereas the bean pods and etiolated elongating hypocotyls contained the highest levels. Light-grown hypocotyls revealed much lower levels (five- to six-fold), indicating a light-dependent regulation of bean *pgip* expression.

PGIP induction was studied in relation to defence substances in bean (Bergmann et al., 1994) by monitoring mRNA levels following the addition of either fungal glucan, or oligogalacturonides in the active elicitor state. It was found that *pgip* mRNA accumulates in suspension cultures of *P. vulgaris* following these inductions. Experiments confirmed the presence of a basal level of PGIP in the cell walls of both uninoculated resistant and susceptible bean plants. Western and Northern blot analyses following the challenge of bean hypocotyls with *Colletotrichum lindemuthianum* also demonstrated the preferential increase of PGIP in the cells proximal to the infection site. Similar increases were noted when the hypocotyls were wounded, or after application of salicylic acid. The involvement of bean PGIP in disease resistance is further stressed by the fact that the highest accumulation of PGIP occurred in the cells of the epidermis that provides the first structural barrier encountered by the pathogen in the bean plant. Thus, by promoting the formation of elicitor-active oligogalacturonides, which are capable of activating defence

responses, and by serving as an early biochemical barrier, PGIP protects the plant from pectin-degrading fungi.

Time-course induction experiments (Nuss et al., 1996) on the elicitation of PGIP correlated well with susceptible and resistant near-isogenic lines of *P. vulgaris*. mRNA levels were studied following *C. lindemuthianum* infection. An increase of up to 20-fold over the basal level was observed in the incompatible interaction, leading to resistance, whereas an increase of only eight-fold was seen in the compatible reaction, leading to disease. Moreover, a rapid increase in transcript levels was observed in the resistant interaction, whereas a delayed and slight increase in transcript levels coincided with lesion formation (Fig. 3).

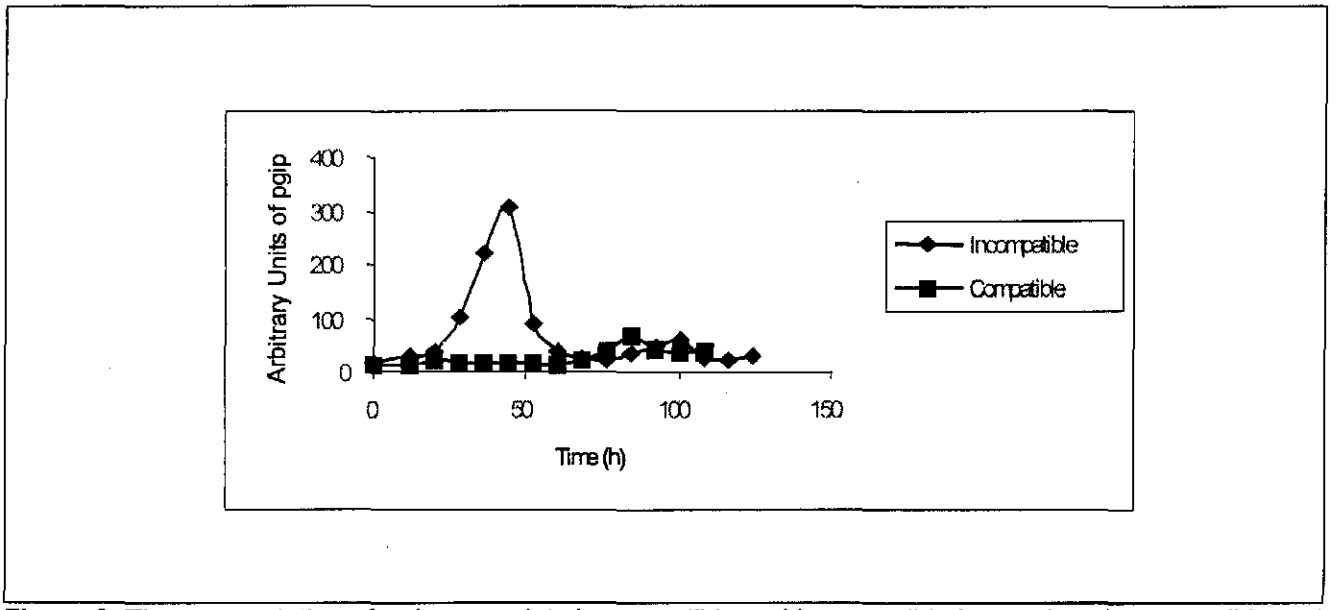

Figure 3. The accumulation of *pgip* transcripts in compatible and incompatible interactions in susceptible and resistant bean lines, respectively. In the incompatible interaction, *pgip* mRNA levels increased 20 h after infection, whereas no significant differences were noted within the first 68 h in the compatible interaction. Maximal levels in the former were reached after 36 to 40 h, compared to only a slight increase in mRNA levels after 84 h, by which time fungal hyphae were well established and associated with lesion formation in the plant tissue. Representation of densitometric readings of Northern blot autoradiographs normalised to a constitutively expressed bean cDNA clone are shown (Adapted from Nuss et al., 1996).

The microbial and plant pectic enzymes that are inhibited by *Phaseolus* PGIPs have been determined (Cervone et al., 1990). Several fungal endo-PGs (*A. niger, C. lindemuthianum, F. moniliforme* and *A. japonicus*) and isoforms thereof were inhibited completely, whereas endo-PG from the bacterium *Erwinia carotovora* was not inhibited. Interestingly, bean PGIPs also inhibited an exo-PG of plant origin, specifically that of corn. Cervone et al. (1990) thus observed that PGIP has to recognise and interact with motifs or structures contained in fungal endo-PGs that are not present in functionally similar cell wall-degrading enzymes. Since the exo-PG from corn was inhibited, it was decided to determine whether the antibodies raised against *A. niger* PG could react with the plant PG. Although they did not react with tomato PG (which bean PGIP did not inhibit), the antibodies did react with both *Aspergillus* and corn PGs, indicating that the latter might

have a common epitope recognisable by *Phaseolus* PGIP, most likely interacting with the leucine-rich repeats in the latter protein.

The strong similarity of PGIP to the structures of a range of widely divergent proteins is quite striking. This similarity is particularly significant in an area consisting of a high number of leucine residues (Table 1). The modular structure of PGIP provides a basis for its role as a receptor component of the cell-surface signaling involved in the recognition events concerning pathogen and plant. This is based on the high number of leucine residues and the extensive similarity to other leucine-rich proteins that are involved mainly in signal-transduction pathways (De Lorenzo et al., 1994). Interestingly, two leucine-rich repeat proteins, RPS2 from *Arabidopsis* and N from tobacco, mediate resistance to different pathogens. In the former, resistance is mediated against a bacterial pathogen, and in the latter case, against a viral pathogen. This suggests a similar signal transduction pathway leading to the activation of resistance responses in the plant to both bacterial and viral pathogens (Kobe and Deisenhofer, 1995).

Table 1. Amino acid sequence alignment of the bean PGIP LRR repeat with those from several leucine-rich proteins. Most of the included proteins are involved in signal transduction pathways (De Lorenzo et al., 1994).

Comparison of Leucine-rich Proteins

Protein	Organism	Consensus sequence
PGIP	Bean	G I P L LKnL LdLs N L
RLK5	*Arabidopsis*	G I P L L L LdLs N LS
sds22$^+$	Yeast	L L L L L N I I
Adenylate cyclase	Yeast	P L L L L L N L L
LRG	Human	P LL L L L L N L L
GPIb	Human	P GLL LP L L L SN LTTL
Toll	Fly	P PF H NL L L N L L
Chaoptin	Fly	P F L L LDLS N L I
PG40	Human	G F LK L L L NN IS V

2.3.4 Comparison of *Phaseolus pgip*-1 and *pgip*-2

The *pgip* genes in *Phaseolus* are organised into multigene families, consisting of at least five, and possibly up to 15 members (Frediani et al., 1993). It is known that *pgip* genes exhibit similar biochemical properties, but that they differ in their ability to recognise and interact with PG genes from different fungi.

Recently, Leckie et al. (1999) isolated a second member of the bean *pgip* family (*pgip*-2) from a cDNA library of *P. vulgaris* cv. Pinto. The previously characterised bean *pgip* was renamed *pgip-1,* and the newly isolated *pgip-2* gene differed by only 26 nucleotides from the *pgip-1* gene. These changes resulted in 10 amino acid changes (Fig. 4). Both these genes were introduced into *Nicotiana benthamiana* via the potato virus X transformation system (Chapman et al., 1992), allowing the purification and characterisation of single *pgip* gene products.

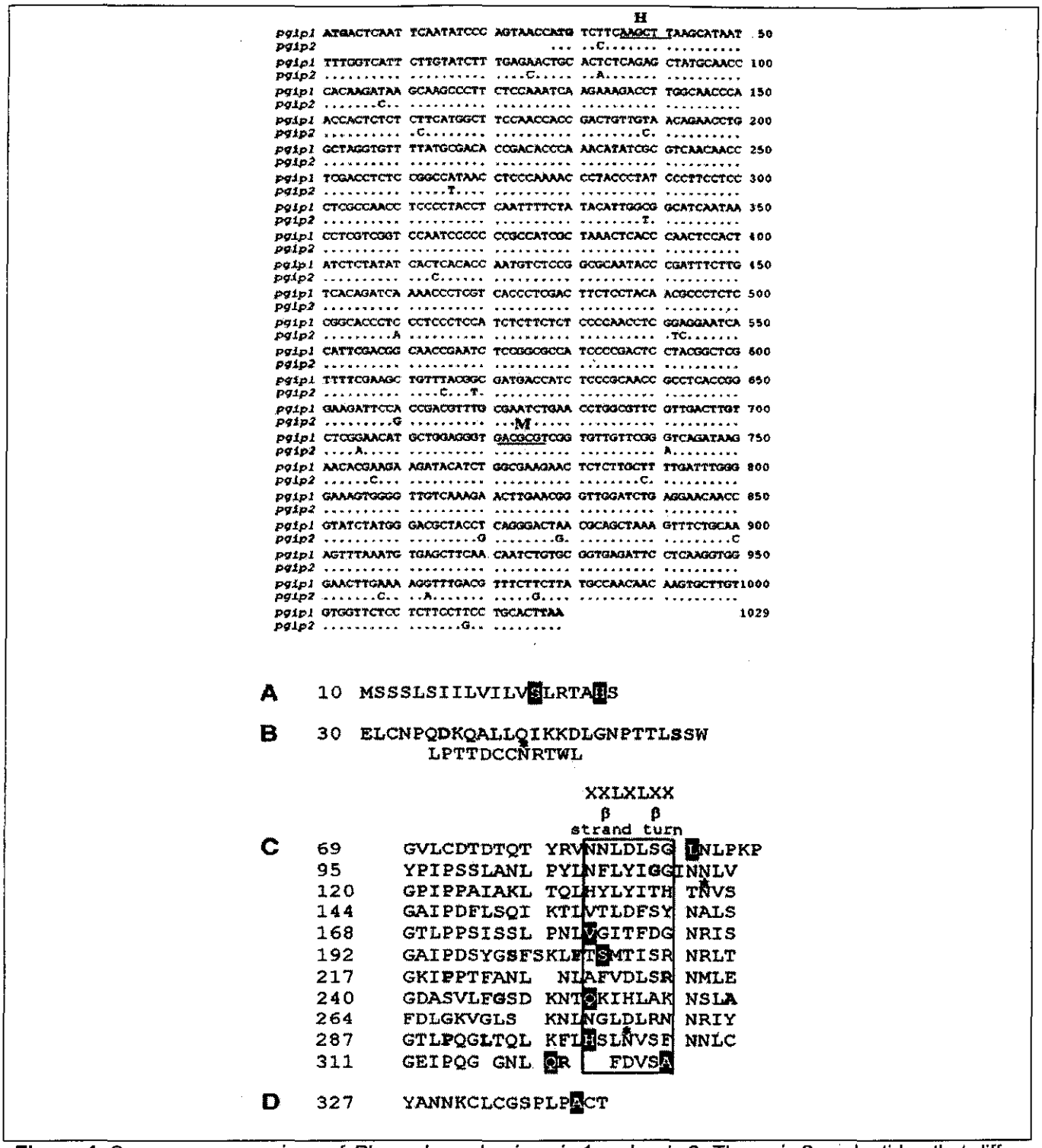

Figure 4. Sequence comparison of *Phaseolus vulgaris pgip*-1 and *pgip*-2. The *pgip*-2 nucleotides that differ from *pgip*-1 are indicated. The deduced amino acid sequence of PGIP-2 is presented in A-D: A, signal peptide; B, presumed N terminus of mature protein; C, 10.5 LRR structure; D, C terminus. The boxed area is predicted to form the β-sheet/β-turn structural motif (Adapted from Leckie et al., 1999).

The individual contribution of each PGIP to the bulk of the bean PGIP activity was subsequently determined (Leckie et al., 1999). Equal amounts of PGIP-1 and PGIP-2 were tested for inhibition spectra against *Aspergillus*, two *Fusarium* species and *Botrytis*. It was found that PGIP-2 almost completely inhibited PGs from all the fungi. However, only about 60% inhibition by PGIP-2 was observed with PGs from *F. oxysporum* f. sp. *lycopersici*.

PGIP-1 did not inhibit PGs from *Fusarium moniliforme*, and had a reduced ability to inhibit crude preparations of PG from *F. oxysporum* f. sp. *lycopersici* and *B. cinerea*. Interestingly, PG from *A. niger* was inhibited completely by PGIP-1. PGIP-2 thus exhibits a broader spectrum of inhibition than PGIP-1 against the above pathogens, and has the ability to recognise and interact with *F. moniliforme* PG, a feature absent from PGIP-1 (Leckie et al., 1999). Further analysis of the two near-identical proteins was carried out using a combination of site-directed mutagenesis and surface plasmon resonance. These studies confirmed that a single glutamine residue, at position 253 (Fig. 5), is responsible for the interaction with *F. moniliforme* PG. Mutation of the lysine at position 253 to glutamine conferred on it the ability to recognise and interact with *Fusarium* PG. Since this mutation is not likely to confer major conformational changes, it is thought that glutamine might form hydrogen bonds in its interaction with *F. moniliforme* PG (Leckie et al., 1999). The alanine residue at position 326 was also a significant contributor to the increased binding energy in the PG-PGIP complex. A comparison of the *pgip* sequences confirmed that amino acid variability occurs preferentially within or close to the xxLxLxx motif (Fig. 4). Therefore, the β-sheet/β-turn region of PGIP might be considered as a region of probable high variability, conferring different ligand recognition specificities (Leckie et al., 1999). Additionally, the alignment of several PGIP sequences (Mattei et al., 2001) reveal that cysteine residues are highly conserved in all PGIPs. Disulphide bridges resulting from these residues are indicative of conserved structural features present in all PGIPs. A consideration of the similarity in biological function between proteins with leucine-rich repeats suggests that these features might be important in signal transduction, a feature shared by proteins of diverse origin. While retaining specificity towards a range of fungal PGs, the aforementioned features presumably allow pathways to be activated that are directly associated with the upregulation of the host's defence response.

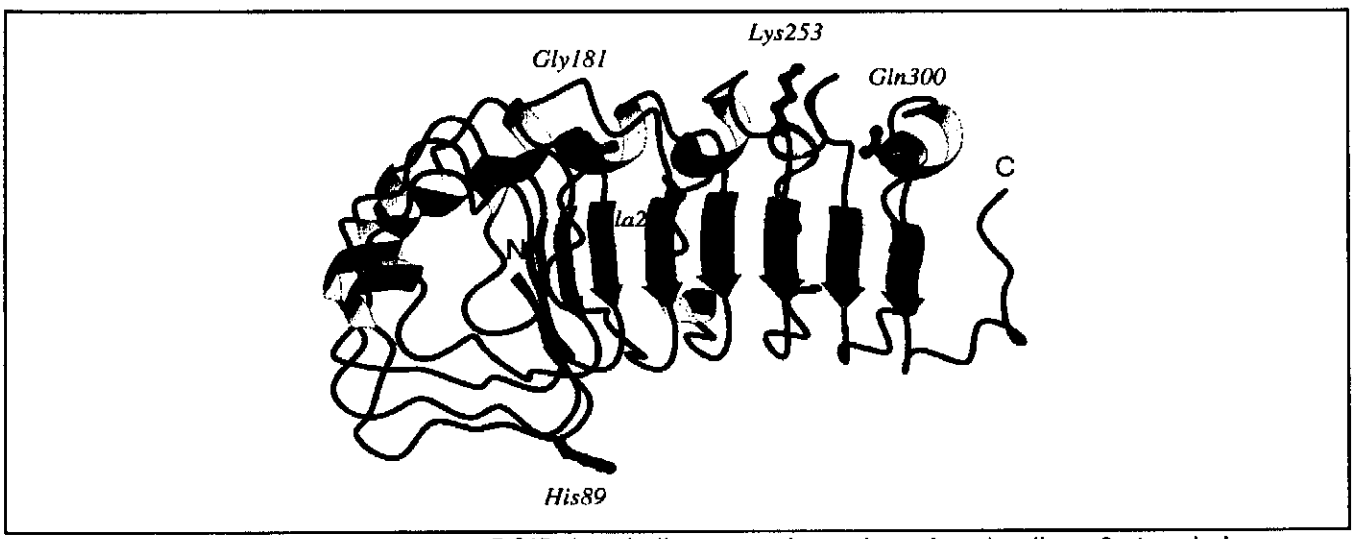

Figure 5. Folding of modelled bean PGIP-1. α-helices are shown in red and yellow, β-strands in green. Amino acid differences occurring between bean PGIP-1 and PGIP-2 within the LRR repeat are indicated by the numbered amino acids (Adapted from Leckie et al., 1999).

2.3.5 Proposed Interaction of fungal endo-PGs and PGIP

Fungal PGs catalyse the breakdown of plant cell wall homogalacturonans, aiding in the colonisation of plant tissue and thereby liberating nutrients for pathogen growth, rendering PGs an important pathogenicity factor of fungi. Alternatively, PGs are potential avirulence factors that can activate defence responses by the formation of elicitor-active oligogalacturonides from host cell wall homogalacturonans (Fig. 6). This will result in the synthesis and accumulation of phytoalexins, chitinases, glucanases and other defence-related proteins and compounds. However, prolonged activity of PG on these galacturonides results in the formation of lesser-polymerised molecules that are incapable of eliciting defence responses. The inhibition of PG by means of PGIP-PG interaction favours the prolonged existence of oligogalacturonides that are capable of eliciting defence responses and confirms the role of PGIP in plant resistance and the elicitation of the defence mechanism (Cervone et al., 1993).

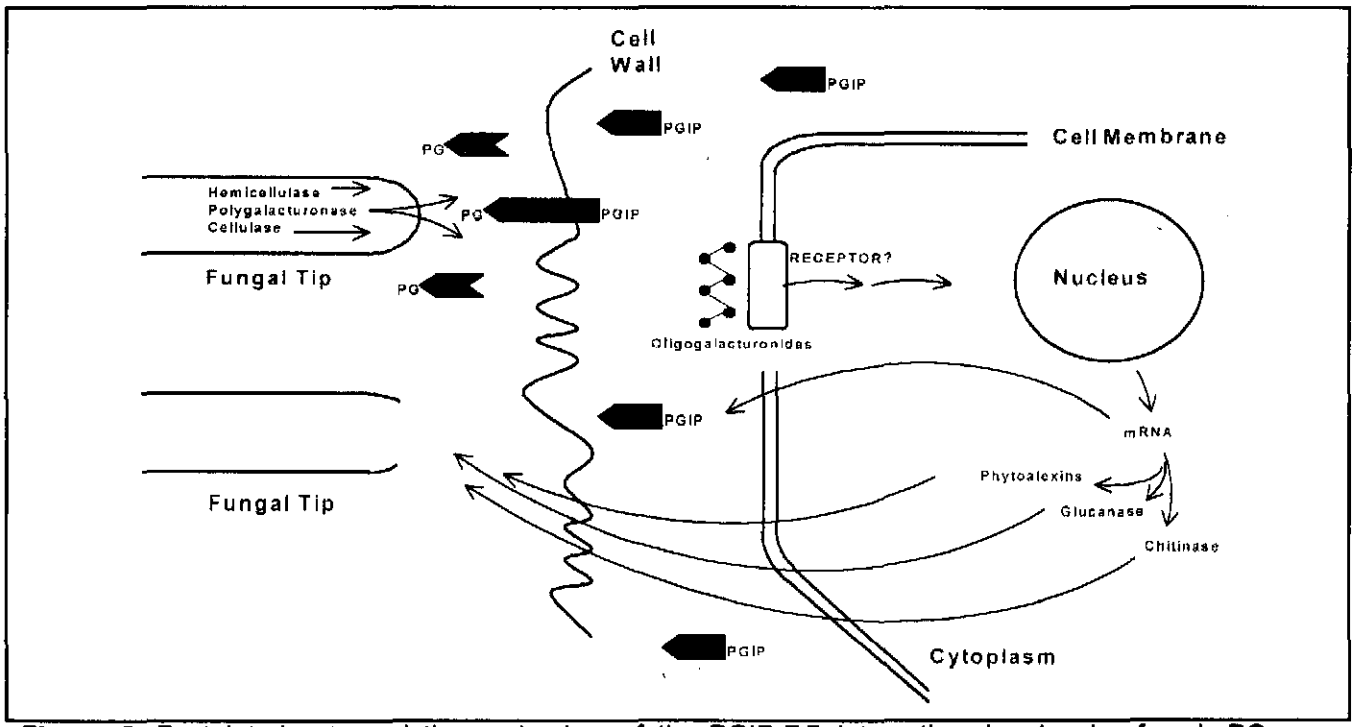

Figure 6. Postulated autocatalytic mechanism of the PGIP-PG interaction: low levels of endo-PGs are present in the initial infection and interact with basal levels of PGIP in the plant cell walls. Oligogalacturonides resulting from this interaction elicit plant defence responses, including phytoalexin synthesis and the accumulation of still more PGIP in the cells surrounding the infection site. This increased level of PGIP might further inhibit the added levels of PG produced by the fungus, resulting in the continuous production of elicitor molecules (Cervone et al., 1993).

2.4 RESVERATROL SYNTHASE

2.4.1 Introduction

Stilbenes, which are present in a limited number of plant species, are secondary plant products that exhibit phytoalexin properties. They are present either constitutively, or are

induced by stress conditions, such as pathogen ingress. Phenylalanine-ammonialyase, chalcone synthase and resveratrol synthase are key enzymes in this process, since they catalyse the formation of the stilbene backbone. Stilbenes share a common diphenylethylene backbone, which renders the basic molecule modifiable in several ways. Included in these modifications are: methylation of hydroxyl groups, attachment of sugars, C-alkylation of isoprenyl groups, or polymerisation (e.g. viniferins) (Schröder et al., 1990). The biological effects of the aforementioned derivatives are related to the electronic character, the lipophility of the substances and their molecular volume. The interaction of these molecules with known pathogens, such as *B. cinerea,* takes place via the destruction of cellular membranes and possible interference with proteins of the electron transport systems in the pathogen (Pont and Pezet, 1990). In this section, resveratrol synthase and its product, as one of the stilbenes, will be discussed.

2.4.2 Molecular analysis

The gene encoding for resveratrol synthase (RS), a homodimeric polyketide synthase, is present as part of a large gene family, possibly with more than seven members (Sparvoli et al., 1994; Wiese et al., 1994) in plants. The gene encoding RS in *V. vinifera* (*vst1*) translates into a polypeptide of 392 amino acids, with a molecular mass of 41 kDa. A single 358 nucleotide intron is present in the coding region (Wiese et al., 1994). The encoded enzyme performs sequential condensation of three acetate units from malonyl-CoA to a coumaroyl residue, yielding an enzyme-bound tetraketide intermediate in the resveratrol biosynthetic pathway (Fig. 7). This reaction is almost identical to that of chalcone synthase and other fatty acid and polyketide synthases. Tetraketide intermediates are then folded into new aromatic ring systems, these being specific to the different polyketide synthases. The final step involves a decarboxylation reaction to yield the stilbene, resveratrol (Tropf et al., 1995). This reaction is typically not performed by chalcone synthase, an enzyme also utilising the aforementioned precursors.

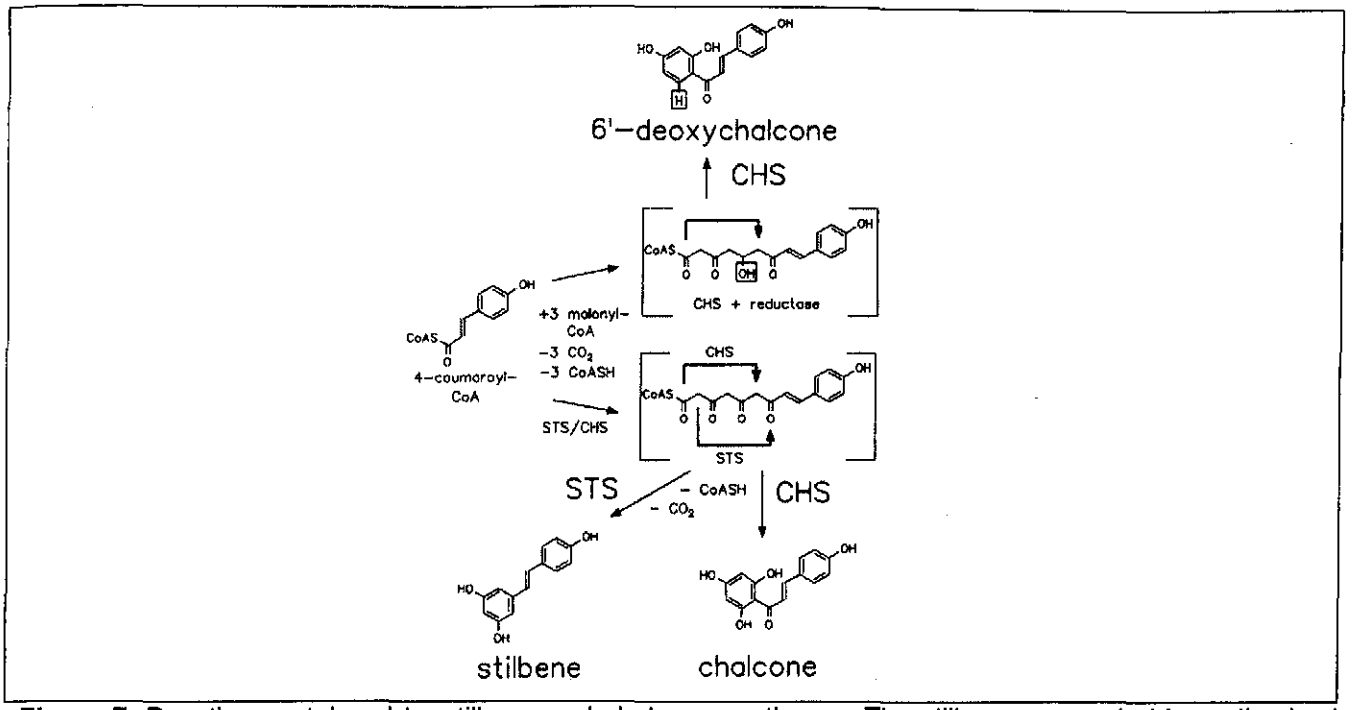

Figure 7. Reactions catalysed by stilbene and chalcone synthases. The stilbene resveratrol is synthesised from 4-coumaroyl-CoA and three molecules of malonyl CoA. STS, stilbene synthase; CHS, chalcone synthase (Adapted from Tropf et al., 1995).

2.4.3 Fungal elicitation

Resveratrol synthase is elicited by biotic signals in numerous plant species, such as peanut (Vornam et al., 1988), pine (Gehlert et al., 1990), and, of relevance to this study, grape (Liswidowati et al., 1990). Conversely, spruce and rhubarb, both of which contain stilbene, do not show increased *de novo* synthesis of the substance upon biotic elicitation (Melchior and Kindl, 1991).

The elicitation effect of stilbenes by pathogen attack was elegantly confirmed by Langcake and Pryce (1976), when they reported that the trihydroxy stilbene, *trans*-resveratrol, is the major phenolic component responsible for bright blue fluorescence under long wavelength ultraviolet (UV) light after the inoculation of detached grapevine leaves with *B. cinerea*. Leaves infected with downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*) similarly produced elevated levels of the stilbene.

Phytoalexin accumulation in berries at different developmental stages was investigated following *B. cinerea* inoculation of two *Vitis vinifera* L. cultivars, Castor and Huxelrebe (Bavaresco et al., 1997). The berries of the fungal-resistant cultivar, Castor, synthesised more phytoalexins in the form of *trans*-resveratrol and ε-viniferin than the susceptible Huxelrebe. However, pterostilbene levels generally were much lower than those reported for the other stilbenes. The accumulation of the latter was enhanced up to the fourth day following infection. The levels of *trans*-resveratrol exceeded those of ε-viniferin until after the second day following inoculation, whereafter less *trans*-resveratrol than ε-viniferin was found. This confirms the role of trans-resveratrol in acting as a precursor of the viniferins (Bavaresco et al., 1997). It seems that these compounds are

stable, since appreciable levels of stilbenes were still present 16 days after the inoculation of *véraison* stage and ripe berries.

The phytoalexin content of healthy and diseased grapes was also analysed. Chardonnay, Pinot Noir and Gamay grapes, either healthy or roughly 10% affected by *B. cinerea,* were harvested and assayed for resveratrol content (Jeandet et al., 1995). The levels were highest in the skin surrounding the infection site of the non-infected fruits. Peripheral berries in the same bunch also exhibited levels equal and higher to that of the primary infection site. This could either be the result of microlesions caused by the fungus, on apparently uninjured berries, or systemic signals that might reach distal host areas and induce defence responses, including phytoalexin synthesis (Jeandet et al., 1995).

Phytoalexin synthesis has also been induced successfully by fungal elicitors in cultured grapevine cells (Liswidowati et al., 1990). A soluble glucan elicitor was prepared from *Botrytis* cell walls and added to the culture. Proteins with the highest *de novo* rate of synthesis were stilbene synthase and phenylalanine ammonia-lyase (PAL), indicating a strong metabolic shift towards defence. The temporal expression of PAL and stilbene synthase was measured in an analogous culture, utilising *Phytophtora cambivora* fungal mycelium as the elicitor (Melchior and Kindl, 1991). In these experiments, stationary mRNA levels reached a maximum six hours after the onset of elicitor treatment. A comparison of mRNA levels and enzyme activities revealed that the appearance of PAL and stilbene synthase mRNAs were closely co-ordinated, although the profiles of the enzyme activities were not. Additionally, chalcone synthase levels were one twentieth that of stilbene synthase, further confirming the shift towards resveratrol synthesis.

2.4.4 UV elicitation of resveratrol synthesis

Following a 10-minute exposure to short wavelength (254 nm) UV light, detached grapevine leaves emitted bright blue fluorescence (366 nm) that was not detectable in healthy, unirradiated leaves (Langcake and Pryce, 1976). The characterisation of this fluorescent compound revealed that it was a simple hydroxylated stilbene, with further chemical analyses confirming it to be the trihydroxy stilbene *trans*-resveratrol. This elicitation was reported for four of the 11 genera of the Vitaceae family. Berries from several *V. vinifera* cultivars were subjected to an identical treatment (Sarig et al., 1997), indicating maximum resveratrol levels four- to eight-fold higher than that of pterostilbene, with the elicitation of the former always preceding that of pterostilbene (18 h compared to 40 h). However, after reaching peak values, the resveratrol content declined rapidly after 50 h, whereas the pterostilbene content remained steady. Only trace amounts of either stilbene were found in unirradiated grapes. Berries at different developmental stages were also irradiated and analysed. In all the cultivars, the amount of phytoalexin synthesised declined with increasing maturity, indicating developmental regulation of the *vst1*-encoding gene. Although UV elicitation generally produced higher levels of stilbene, the fact remains

that fungal elicitation maintains significant levels due to the continued presence of inoculum on the berries.

2.4.5 Resveratrol oxidation by *Botrytis* laccase

Typical of the dynamic interaction between host and pathogen is the presence of substances in the pathogen that have the ability to hinder or inhibit the defence strategies in the plant. To this end, it has been shown that several grapevine *Botrytis* isolates produce a *p*-diphenol:oxygen oxidoreductase (laccase), which can cause the degradation of stilbenes (Pezet et al., 1991, Sbaghi et al., 1996). This ability, however, is not directly linked to the pathogenicity of the pathogen, since, from seven *Botrytis* isolates, five had detectable laccase activity and only three of these were considered to be more than moderately pathogenic towards grapevine (Sbaghi et al., 1996) (Fig. 8).

Two signals are required for maximal laccase production in the fungal pathogen: the first being phenolic compounds present in plants and the second being pectin fragments that indicate breakdown of the host tissues. *Botrytis* apparently has the ability to adjust the molecular structure of its laccase to the host pH and the nature of the phenolics contained therein. This would clarify the heterogeneity of laccases purified from *B. cinerea* cultures (Marbach et al., 1985).

Evidently, *Botrytis* can create a suitable environment for its growth in ripening berries by the production and secretion of laccase from the cytoplasm of mycelial cells (Hodson et al., 1987). This process, however, does not occur in unripe berries of *Vitis* spp. It has also been shown that oxidation of resveratrol also takes place in the cytoplasm of fungal conidia (Adrian et al., 1998), but not during conidial germination (Jeandet et al., 1993). This implies that constitutive laccase activity is absent from dormant conidia.

When considering the defence-related phytoalexin accumulation pattern in response to pathogen elicitation, it is evident that it is a rapid response that renders significant levels of antimicrobial substances within 24 h of induction. The expression of the stilbene-degrading enzymes of the pathogen, in contrast, is delayed for two days following germination of the conidia. This temporal difference in expression of the degrading enzymes favours defence in the host, leading to limited infection and/or lesions. Stilbene oxidation, although a major factor in compatibility, therefore should not be considered as the primary determinant of disease.

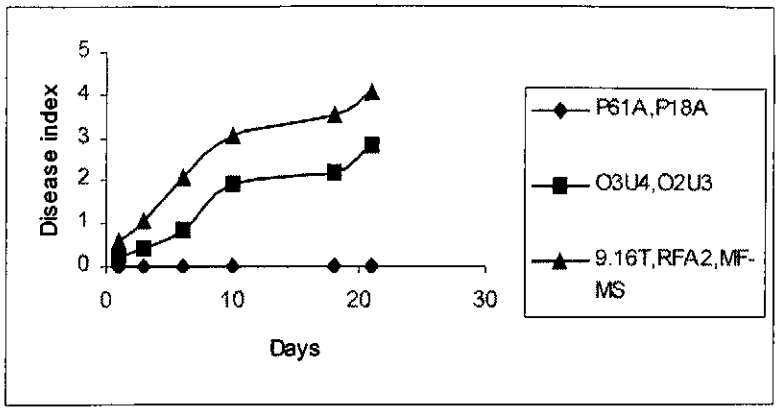

Figure 8. Pathogenicity of eight *B. cinerea* isolates (P61A, P18A, O3U4, O2U3, 9.16T, RFA2 and MF-MS) on 13-month-old grapevine plantlets. Disease rating: 0 = no symptoms to 4 = severe necrosis and withering (Adapted from Sbaghi et al., 1996).

2.5 PGIPs AND RESVERATROL: BIOTECHNOLOGICAL APPLICATIONS

2.5.1 Introduction

Genetic transformation techniques involving single genes have become commonplace and seldom present difficulty for the obtainment of transgenic lines. Early transformation procedures involving *Agrobacterium*-mediated transformation have shown that transgene incorporation in the host genome and the expression of such foreign genetic material can be done with relative ease. Reporter gene transformations, for example, have led the way in establishing optimal procedures for genetic manipulation and have resulted in observable, direct effects of transgenics. It soon became apparent that genes of interest may be used for targeted applications, including the increased yield of economically important crops, higher tolerance of plants associated with suboptimal growth conditions and, most importantly, strengthening of the plant's resistance to its natural pathogens. A wealth of recent information has become available concerning plant defence responses and plant-pathogen interactions, thus increasing the genetic engineer's efficacy with regard to the upregulation of certain aspects of the defence response.

2.5.2 Transgenically expressed PGIPs

The heterologous overexpression of bean and pear PGIPs in tomatoes has confirmed the efficacy of these inhibitors against several fungal pathogens. Table 2 clearly illustrates both PGIP specificity towards fungal PGs of diverse origin and the fact that heterologous overexpression is utilisable as a viable tool in enhancing resistance to fungal pathogens. Notably, *Aspergillus* crude PG extracts were inhibited to the same extent as by bulk PGIP, showing that PGIP-1 interaction was almost solely responsible for PG inhibition of the fungi. The ability of bulk PGIP to inhibit a wide range of fungal pathogens might be the result of the collective abilities of individual PGIPs to confer a broad range of inhibitory

activities on *P. vulgaris* (Desiderio et al., 1997). Appreciable levels of PGIP and its associated specificity may be used as a powerful tool in promoting plant health, as rendered by specific fungal PG catalysing host cell wall breakdown.

Table 2. *pgip* genes of different origin expressed in transgenic tomato plants. The resultant inhibition of fungal PGs is indicated.

pgip origin	PG tested against	Resultant PG-PGIP interaction	Reference
P. vulgaris pgip1	*F. moniliforme*	No PG inhibition	Desiderio et al., 1997.
P. vulgaris pgip1	*F. oxysporum* f.sp. *lycopersici*	10% PG inhibition in comparison to bulk PGIP activity	Desiderio et al., 1997.
P. vulgaris pgip1	*A. solani*	10% PG inhibition in comparison to bulk PGIP activity	Desiderio et al., 1997.
P. vulgaris pgip1	*A. niger*	100% PG inhibition in comparison to bulk PGIP activity	Desiderio et al., 1997.
P. vulgaris pgip1	*B. cinerea*	30% PG inhibition in comparison to bulk PGIP activity	Desiderio et al., 1997.
P. vulgaris pgip1	*Stenocarpella maydis*	30% PG inhibition	Berger et al., 2000
Pyrus communis pgip1	*B. cinerea*	25% reduction of symptoms of fungal growth	Powell et al., 2000

2.5.3 Successes obtained with resveratrol

Phytoalexin-mediated resistance in plants is highly dependent on the rapid production of appreciable levels of the fungitoxic substance (Hain et al., 1993). Theoretically, it should therefore be possible to upregulate a plant's resistance by overexpressing the phytoalexin-encoding genes. This has been shown in experiments in which a significant increase in host resistance was observed when RS was expressed (Table 3). For example, in *Nicotiana tabacum* cv. Petit Havana SR1, this strategy proved to be very effective in generating transgenic lines resistant to *Botrytis* infections. These transgenic lines and others were evaluated and, from an analysis of the stilbene accumulation and the kinetics thereof (Fig. 9 A,B), it was found that up to 400 μg phytoalexin per gram fresh weight was present in the leaves. Phytoalexins of this concentration have been shown to inhibit *Botrytis* growth *in vitro*, making this a feasible strategy to upregulate plant disease resistance. Confirmation of this notion came when a clear correlation was found between resveratrol content extracted from leaves and disease incidence in transgenic tobacco expressing resveratrol synthase (Fig. 9 C).

Table 3. Plant species transformed with resveratrol synthase-encoding genes from either groundnut or grapevine.

Plant species	Promoter	RS Origin	Resultant interaction	Reference
Nicotiana tabacum cv. Petit Havana SR1 (tobacco)	native	*Arachis hypogaea* (groundnut)	Inducible by *Phytophtora megasperma* elicitor preparation and UV light	Hain et al., 1990
Nicotiana tabacum cv. Petit Havana SR1	native	*Vitis vinifera*	Inducible by *P. megasperma* elicitor, lines obtained reproducibly more resistant to *B. cinerea* infection	Hain et al., 1993
Oryza sativa cv. Nipponbare (rice)	native	*V. vinifera*	Inducible by wounding, elicitor, UV irradiation; preliminary results indicate enhanced resistance to fungus *Pyricularia oryzae*	Stark-Lorenzen et al., 1997
Hordeum vulgare cv. Igri (barley)	native +CaMV35s enhancer sequences	*V. vinifera*	Preliminary results indicate enhanced resistance to *B. cinerea*	Leckband and Lörz, 1998
Triticum aestivum cvs. Veery #5 and Florida (wheat)	native +CaMV35s enhancer sequences	*V. vinifera*	None noted	Leckband and Lörz, 1998
Medicago sativa (alfalfa)	enhanced CaMV35s promoter	*A. hypogaea*	Significant increase in resistance to fungus *Phoma medicaginis*	Hipskind and Paiva, 2000

The resultant interaction of pathogen challenge on the transformed plants is noted. A significant increase in host resistance was obtained when resveratrol synthase was overexpressed. The introduction of the novel phytoalexin into these plants under its native promoter also showed increased resistance, but to a lesser extent (CaMV35s = cauliflower 35s promoter).

2.5.4 Co-transformation techniques

2.5.4.1 Rationale

Since information has become available on previously analysed transgenic lines, it has become apparent that the synergistic and additive effects of such transgenes would be regarded as a direct benefit to the host's defence arsenal. In this study, two aspects of the defence response come to the fore: the effect of overexpression of a phytoalexin-producing gene, namely resveratrol, and of the gene encoding PGIP. These genes, their products and the upregulation thereof present the tools used in this study for a concerted resistance effort.

The basic processes underlying the *Agrobacterium*-mediated transformation of plants have been reviewed extensively (Klee et al., 1987; Zambryski, 1988, 1992; Hooykaas and Schilperoort, 1992; Perl et al., 1996; Birch, 1997 and Gelvin, 1998). In the following sections, the technology associated with multiple gene delivery to plants will be discussed,

specifically simultaneous transformation, sequential transformation and particle bombardment.

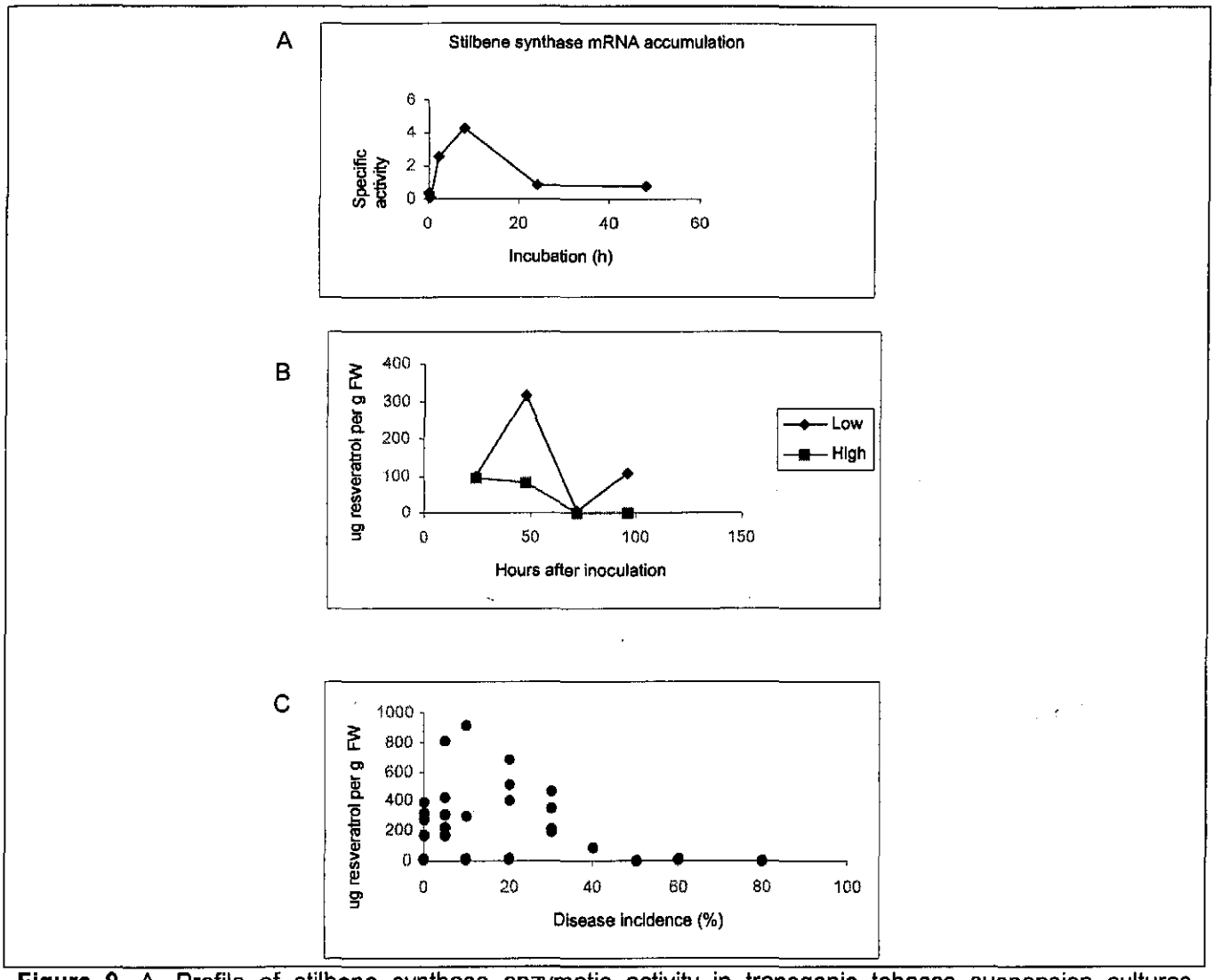

Figure 9. A, Profile of stilbene synthase enzymatic activity in transgenic tobacco suspension cultures following addition of the elicitor. B, Comparison of kinetics of resveratrol accumulation in leaves with high and low disease incidence. Resveratrol content was analysed after inoculation with *Botrytis*. Leaves accumulating high levels of resveratrol 48 h after inoculation were more resistant to *Botrytis*. C, Correlation of resveratrol production in leaves and susceptibility to *B. cinerea* infection. Plants were inoculated, half were analysed at 24 h intervals, the remaining half after 120 h (Adapted from Hain et al., 1993).

2.5.4.2 Co-transformation technologies

Multigenic approaches require transformation at a high frequency. Since it can be both technically demanding and time-consuming to construct vectors containing both transgenes, and the genetic linkage of these transgenes poses important advantages, including possible decreased aberrant phenotypes resulting from position effects, the possible methods for the delivery of both transgenes in a fashion similar to single-vector transformation are discussed with the above considerations in mind.

Simultaneous transformation: The possibility to co-transform plant cells with *Agrobacterium* infections was confirmed by the presence of two distinct T-DNAs in single cells of tobacco (McKnight et al., 1987) and by tomato that was transformed with two distinct T-DNAs carried in separate *A. tumefaciens* and *A. rhizogenes* strains respectively (McKnight et al., 1987). Eleven per cent of all regenerated tomato roots expressed markers from both binary vectors, whereas, in 16 tobacco regenerants, three expressed markers from both T-DNAs. In both cases, it could be demonstrated that the individual plant cells received DNA from both strains of infecting bacteria. Additionally, Mendelian segregation patterns indicated that the co-transformed T-DNAs were genetically unlinked. However, the transformation of *Brassica napus* with distinguishable T-DNAs containing different selectable markers (De Block and Debrouwer, 1991) indicated that these markers were frequently linked (78% of co-transformed cells). Importantly, high co-transformation frequencies were still obtained with the co-transformations (39-85% of transformed cells).

Using kanamycin and hygromycin markers, De Neve et al. (1997) were able to show that different T-DNAs that were co-transformed into either *Nicotiana* or *Arabidopsis* by different bacteria similarly were frequently linked. This was observed regardless of the plant species or the transformation method used. Krizkova and Hrouda (1998) presented evidence for frequent genetic linkage by using an expression cassette split onto two T-DNA segments. A promotorless neomycin phosphotransferase gene was contained on one T-DNA and the promotor to drive its expression was contained on another. Selection was based on expression of the *npt* gene. Transgenic plants that were obtained thus contained at least one linked direct repeat of T-DNA.

Since no multimeric T-DNA forms have been observed in *vir*-induced agrobacteria as part of the transfer process, it is generally thought that these linked T-DNAs arise in the plant cell (Zambryski, 1988). Two mechanisms are proposed to be responsible for the formation of these multimeric T-DNA forms (De Neve et al., 1997), namely the ligation model and the replication model. In the former model (Fig. 10), repeat structures result from the ligation of separate T-DNAs prior to or during integration in the plant genome. Alternatively, it is thought that these repeats could result from the replication and repair of single T-DNAs before or during insertion in the genome in the aptly termed replication model. In the former case, ligation of the T-DNAs could be mediated by plant enzymes or in co-operation with the Vir D2 protein, which is attached to the 5' end of the T-complex. This protein has been shown to be implicated in many processes in T-DNA transfer, including T-strand synthesis, T-DNA targeting to the nucleus, and precise integration into the genome (Tinland et al., 1995). In addition, Pansegrau et al. (1993) were able to show ligase activity for Vir D2 *in vitro*. De Neve et al. (1997) therefore speculated that it would be possible that Vir D2 could be involved in the *in vivo* ligation of separate T-DNAs. This would favour the formation of T-DNA repeats with an inverted orientation at the right border at a higher frequency than two other possible configurations (Fig. 10), as has been reported by several authors (De Block and Debrouwer, 1991; Grevelding et al., 1993; De Neve et al., 1997).

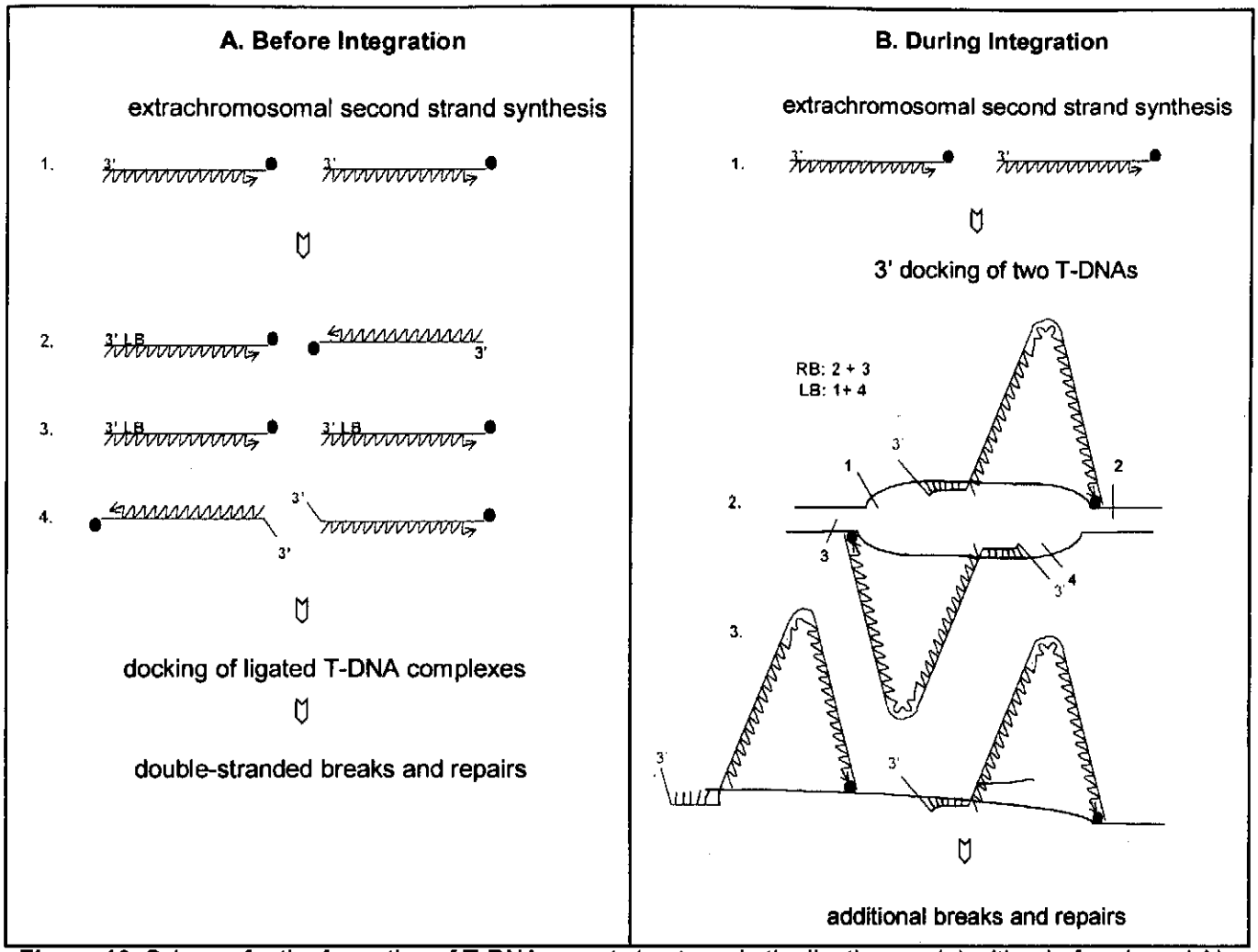

Figure 10. Scheme for the formation of T-DNA repeat structures in the ligation model, either before (panel A) or during (panel B) integration. In each, ligation can be accompanied by repair, giving rise to filler DNA or deletions at T-DNA junctions. A, three configurations for extrachromosomal ligation of double-stranded T-DNA. B, separate T-DNA integration at one target site. T-DNA 1 and T-DNA 2 would generate IC-RB after invasion of the upper and bottom strand respectively, followed by the ligation of repaired breaks at positions 2 and 3 (B, 2). IC-LB would result from T-DNAs in opposite plant DNA strands joining following ligation after repaired breaks at positions 1 and 4 (B, 2). TC would result from the invasion of both T-DNAs on a single strand with repair and ligation (B, 3). Red dots indicate Vir D2 bound to the 5' region of T-DNA; IC-LB are repeats about the left border, IC-RB repeats about the right border, and TC is tandem repeats (Adapted from De Neve et al., 1997).

High frequency co-transformation based on simultaneous transformation were already described in 1985 by Depicker et al. They found that the co-transformation frequency was the product of the frequencies of each single transformation when two strains of agrobacteria were used, whereas it was much higher when the T-DNAs were contained in a single bacterium. Thus, in the former case, the resulting co-transformation efficiency would be the result of two independent transformation events. This is in contrast with the results obtained by De Block and Debrouwer (1991), who found high co-transformation frequencies but only limited single transformation events. The assumption was made that only some of the cells contained in tissue explants were competent for transformation. This assumption was confirmed by De Buck et al. (1998), after co-transformation of *Arabidopsis* root explants and *Nicotiana* protoplasts. The authors concluded that cells that possess the

capacity for stabilisation of one T-DNA have higher chances of stabilisation of a second incoming T-DNA than cells that do not, based on the particular competence and capacity of such cells to integrate transiently expressed DNA fragments. The resultant co-transfer of independently transferred T-DNA copies is thus compatible with the ligation model of co-integration (De Neve et al., 1997).

In an attempt to obtain grapevine rootstocks resistant to nepoviruses, Torregrosa and Bouquet (1997) co-inoculated several *Vitis* rootstocks and *Vitis* x *Muscadinia* hybrids. Two strains were used: a virulent *A. rhizogenes* strain and an *A. tumefaciens* strain containing two binary plasmids. The plasmids harboured genes encoding the coat protein of grape chrome mosaic virus, kanamycin and hygromycin resistance and β-glucoronidase (GUS). Co-transformation frequencies ranged from four to 16% for *Vitis* cultivars and even lower for hybrids. Unfortunately, the authors were unable to establish a relationship between the expression of the transgenes. However, Perl et al. (2000) were able to regenerate stable transgenic grape plants (*Vitis vinifera* cvs. Red Globe and Velika) that are resistant to both paromomycin and hygromycin. Analysis of the transgenic lines is currently underway.

Sequential transformation: Co-transformation is also obtainable by sequential transformation of differing T-DNA copies. Both Delauney et al. (1988) and Sandler et al. (1988) produced transgenic plants containing sense and antisense constructs of the nopaline synthase or chloramphenicol acetyltransferase genes respectively. Again, kanamycin and hygromycin were utilised as selection markers.

Using distinct T-DNAs, both harbouring screening and selection markers, Matzke et al. (1989) attempted sequential co-transformation of SR1 tobacco plants. Notwithstanding the successful regeneration of double transformants, it was found that in half of the co-integrated cells, the initially transformed T-DNA could not be expressed as a direct result of sequential introduction of the second. The former was either inactivated by methylation, or expressed at a lower level. However, this phenomenon was reversible, as shown by the reactivation of gene expression when these plants were self- or backcrossed with untransformed tobacco plants. Promoter methylation was either reduced or abolished as a consequence of either event. Practically, the use of sequential co-transformation should be accompanied by selection for both antibiotics, thereby negating plants that do not express T-DNA that is able to confer resistance.

Particle bombardment: Co-integration of multiple genes has recently been reported by Hadi et al. (1996) and Chen et al. (1998), who were able to integrate 13 and 12 unlinked genes into soybean callus and rice plants through biolistic bombardment, respectively. In 1999, Tang et al. bombarded rice with three unlinked plasmids harbouring genes effective against rice pathogens. After optimisation of the molar ratios of the three plasmids, Tang et al. (1999) regenerated 160 rice plants. Of these, 70% of the analysed plants integrated all four genes contained in the three plasmids, while 70% of those expressed all four

transgenes, as confirmed by histochemical assays, Western blots, RT-PCR and growth on selective media, again indicating a high frequency of co-transformation. However, up to 30% of lines integrating the *gusA* gene did not express GUS at detectable levels (Tang et al., 1999). This might be due to the phenomenon of gene silencing or a loss of structural integrity of the cassette resulting from the transformation method. McKnight et al. (1987) postulated that large amounts of transferred DNA were prone to rearrangement and ligation when direct co-transformation methods, such as particle bombardment, are used. Conversely, the increased exposure of *Agrobacterium* cells to plant tissue would dictate that fewer copies are transferred, thus lowering the probability of the ligation of several DNA strands present in the extrachromosomal state, as happens in direct transformation methods.

It was previously shown that repeated T-DNA structures are correlated with gene silencing and the variability of transgene expression (Finnegan et al., 1994). It therefore would be highly advisable to evaluate the transformation method used for a specific application. Since most dicotyledonous plants are readily transformed by *Agrobacterium,* it is recommended that co-transformation through particle-bombardment be utilised for those species recalcitrant to *Agrobacterium.*

2.6 SUMMARY

The consideration of plant-pathogen interactions and recent information regarding plant defence responses has made it possible for geneticists to target certain aspects of that response when concerned with increased resistance. Considerable effort has been invested in the upregulation of single aspects of a plant's defence response, and has been met with a degree of success. Recent advances in plant transformation technology have made it possible to target multiple facets of the aforementioned response, thereby theoretically increasing the amount of resistance attainable in a transgenic line. This study aimed to focus on two aspects of the innate defence, namely PGIPs and phytoalexins. The gene encoding resveratrol synthase, which catalyses synthesis of the phytoalexin resveratrol, is available. In the former case, these proteins inhibit the action of fungal macerating enzymes, and thereby activate the host defence response. In the latter, direct actions on fungi are observed due to the presence of stilbene. Increased levels of these proteins and compounds will undoubtedly enhance resistance to fungal pathogens, and this has subsequently become the target for the upregulation of defence.

2.7 LITERATURE CITED

Abu-Goukh, A.A., Greve, L.C. and Labavitch J.M. 1983. Purification and partial characterization of 'Bartlett' pear polygalacturonase inhibitors. Physiol. Plant Pathol. 23: 111-122.

Agrios, G.N. 1997. Plant Pathology, 4th edition. Academic Press, San Diego.

Adrian, M., Rajaei, H., Jeandet, P., Veneau, J. and Bessis, R. 1998. Resveratrol oxidation in *Botrytis cinerea* conidia. Phytopathology 88: 472-476.

Bavaresco, L., Petegolli, D., Cantu, E., Fregoni, M., Chiusa, G. and Trevisan, M. 1997. Elicitation and accumulation of stilbene phytoalexins in grapevine berries infected by *Botrytis cinerea.* Vitis 36: 77-83.

Berger, D.K., Oelofse, D., Arendse, M.S., Du Plessis, E. and Dubery, I.A. 2000. Bean polygalacturonase inhibitor protein-1 inhibits polygalacturonases from *Stenocarpella maydis.* Physiol. Mol. Plant Path. 57: 5-14.

Bergmann, C.W., Ito, Y., Singer, D., Albersheim, P., Darvill, A.G., Benhamou, N., Nuss, L., Salvi, G., Cervone, F. and De Lorenzo, G. 1994. Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. Plant J. 5: 625-634.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical apllication. Annu. Rev. Plant Physiol. Mol. Biol. 48: 297-326.

Cervone, F., De Lorenzo, G., Degrà, L., Salvi, G. and Bergami, M. 1987. Purification and characterization of a polygalacturonase inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol. 85: 631-637.

Cervone, F., De Lorenzo, G., Pressey, R., Darvill, A.G. and Albersheim, P. 1990. Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? Phytochem. 29: 447-449.

Cervone, F., De Lorenzo, G., Caprari, C., Clark, A.J., Desiderio, A., Devoto, A., Leckie, F., Nuss, L., Salvi, G. and Toubart, P. 1993. The interaction between fungal endopolygalacturonase and plant cell wall PGIP (Polygalacturonase-inhibiting protein). In Mechanisms of Plant Defense Response ed by B. Fritig and M. Legrand, 64-67. Kluwer Academic Publishers.

Chapman, S., Kavanagh, T. and Baulcombe, D. 1992. Potato virus X as a vector for gene expression in plants. Plant J. 2: 549-557.

Chen, L., Marmey, P., Taylor, N.J., Brizard, J.P., Espinoza, C., D'Cruz, P., Huet, H., Zhang, S., De Kocho, A., Beachy, R.N. and Fauquet, C.M. 1998. Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnol. 16: 1060-1064.

De Ascensoa, A.R., Pretorius, I.S., Bellstedt, D.U., Burger, J.T. and Vivier, M.A. 2002. The isolation and characterisation of a gene encoding a polygalacturonase-inhibiting protein (PGIP) from *Vitis vinifera* L. Planta (in press).

De Block, M. and Debrouwer, D. 1991. Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Gen. 82: 257-263.

De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. 1998. *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Mol. Plant-Micr. Interact. 11: 449-457.

Delauney, A.J., Tabaeizadeh, Z. and Verma, D.P.S. 1988. A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants. Proc. Natl. Acad. Sci. USA 85: 4300-4304.

De Lorenzo, G., Cervone, F., Bellincampi, D., Caprari, C., Clark, A.J., Desiderio, A., Devoto, A., Forrest, R., Leckie, F., Nuss, L. and Salvi, G. 1994. Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. Bioch. Soc. Transac. 22: 394-397.

Dempsey, D.A., Silva, H. and Klessig, D.F. 1998. Engineering disease and pest resistance in plants. Trends Microb. 6: 54-61.

De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. 1997. T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J. 11: 15-29.

Depicker, A., Herman, L., Jacobs, A., Schell, J. and Van Montagu, M. 1985. Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol. Gen. Genet. 201: 477-484.

Desiderio, A., Aracri, B., Leckie, F., Mattei, B., Salvi, G., Tigelaar, H., Van Roekel, J.S.C., Baulcombe, D.C., Melchers, L.S., De Lorenzo, G. and Cervone, F. 1997. Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol. Plant-Micr. Interact. 10: 852-860.

Devoto, A., Clark, A.J., Nuss, L., Cervone, F. and De Lorenzo, G. 1997. Development and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. Planta 202: 284-292.

Ebel, J. 1998. Oligoglucoside elicitor-mediated activation of plant defense. BioEssays 20: 569-576.

Favaron, F., D'Ovidio, R., Porceddu, E. and Alghisi, P. 1994. Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 195: 80-87.

Favaron, F., Castiglioni, C., D'Ovidio, R. and Alghisi, P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Phys. Mol. Plant Path. 50: 403-417.

Finnegan, J. and McElroy, D. 1994. Transgene inactivation: plants fight back! Bio/technology 12: 883-888.

Frediani, M., Cremonini, R., Salvi, G., Caprari, C., Desiderio, A., D'Ovidio, R., Cervone, F. and De Lorenzo, G. 1993. Cytological localization of the *PGIP* genes in the embryo suspensor cells of *Phaseolus vulgaris* L. Theor. Appl. Genet. 87: 369-373.

Fritig, B., Heitz, T. and Legrand, M. 1998. Antimicrobial proteins in induced plant defense. Curr. Opin. Immunol. 10: 16-22.

Gehlert, R., Schoppner, A. and Kindl, H., 1990. Stilbene synthase from seedlings of *Pinus sylvestris*: purification and induction in response to fungal infection. Mol. Plant-Micr. Interact. 3: 444-449.

Gelvin, S.B. 1998. The introduction and expression of transgenes in plants. Curr. Opin. Biotech. 9: 227-232.

Grevelding, C., Fantes, V., Kemper, E., Schell, J. and Masterson, R. 1993. Single-copy T-DNA insertions are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. Plant Mol. Biol. 23: 847-860.

Hadi, M.Z., McCullen, M.D. and Finer, J.J. 1996. Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep. 15: 500-505.

Hain, R., Bieseler, B., Kindl, H., Shröder, G. and Stöcker, R. 1990. Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol. Plant Mol. Biol. 15: 325-335.

Hain, R., Reif, H., Krause, E., Langebartels, R., Kindl, H., Vorman, B., Wiese, W., Schmelzer, E., Shreier, P.H., Stöcker, R. H. and Stenzl, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153-156.

Hipskind, J.D. and Paiva, N.L. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis.* Mol. Plant-Micr. Interact. 13: 551-562.

Hodson, M.J., Bar-Nun, N. and Mayer, A.M. 1987. Cytochemical localization of laccase in *Botrytis cinerea.* Transact. Brit. Mycol. Soc. 89: 572-575.

Hooykaas, P.J.J. and Schilperoort, R. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19: 15-38.

Hutcheson, S.W. 1998. Current concepts of active defense in plants. Annu Rev. Phytopath. 36: 59-90.

Jeandet, P., Sbaghi, M. and Bessis, R. 1993. The significance of stilbene-type phytoalexin degradation by culture filtrates of *Botrytis cinerea* in the vine-*Botrytis* interaction. In Mechanisms of Plant Defense Response ed by B. Fritig and M. Legrand, 84. Kluwer Academic Publishers.

Jeandet,P., Bessis, R., Sbaghi, M. and Meunier, P. 1995. Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. J. Phytopath. 143: 135-139.

Johnston, D.J., Ramanathan, V. and Williamson, B. 1993. A protein from immature raspberry fruits which inhibits endopolygalacturonase from *Botrytis cinerea* and other micro-organisms. J. Exp. Bot. 44: 971-976.

Jones, T.M., Anderson, A.J. and Albersheim, P. 1972. Host-pathogen interaction. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici.* Physiol. Plant Path. 2: 153-166.

Klee, H., Horsch, R. and Rogers, S. 1987. *Agrobacterium*-mediated plant transformation and its further application to plant biology. Annu. Rev. Plant Physiol. Mol. Biol. 38: 467-486.

Kobe, B. and Deisenhofer, J. 1995. Proteins with leucine-rich repeats. Curr. Opin. Struct. Biol. 5: 409-416.

Krizkova, L. and Hrouda, M. 1998. Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. Plant J. 16: 673-680.

Langcake, P. and Pryce, R.J. 1976. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Phys. Plant Path. 9: 77-86.

Leckband, G. and Lörz, H. 1998. Transformation and expression of a stilbene synthase gene of *Vitis vinifera*. L. in barley and wheat for increased fungal resistance. Theor. Appl. Genet. 96: 1004-1012.

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A., Nuss, L., Aracri, B., De Lorenzo, G. and Cervone, F. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO J. 18: 2352-2363.

Liswidowati, Melchior, F., Hohmann, F., Schwer, B. and Kindl, H. 1990. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta 183: 307-314.

Maleck, K. and Lawton, K. 1998. Plant strategies for resistance to pathogens. Curr. Opin. Biotechnol. 9: 208-213.

Marbach, I., Harel, E. and Mayer, A. 1985. Pectin, a second inducer for laccase production by *Botrytis cinerea*. Phytochem. 24: 2559-2561.

Mattei, B., Bernalda, M.S., Federici, L., Roepstorff, P., Cervone, F. and Boffi, A. 2001. The secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. Biochem. 40: 569-576.

Matzke, M.A., Primig, M., Trnovsky, J. and Matzke, A.J.M. 1989. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. 3: 643-649.

McKnight, T.D., Lillis, M.T. and Simpson, R.B. 1987. Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. Plant Mol. Biol. 8: 439-445.

Melchior, F. and Kindl, H. 1991. Coordinate and elicitor-dependent expression of stilbene synthase and phenylalanine ammonia-lyase genes in *Vitis* cv. Optima. Arch. Bioch. Biophys. 288: 552-557.

Nuss, L., Mahé, A., Clark, A.J., Grisvard, J., Dron, M., Cervone, F. and De Lorenzo, G. 1996. Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum.* Phys. Mol. Plant Path. 48: 83-89.

Pansegrau, W., Schoumacher, F., Hohn, B. and Lanka, E. 1993. Site-specific cleavage and joining of single-stranded DNA by virD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. Proc. Natl. Acad. Sci. USA 90: 11538-11542.

Perl, A., Lotan, O., Abu-Abied, M. and Holland, D. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nature Biotech. 14: 624-628.

Perl, A., Sahar, N., Farchi, S., Colova-Tsolova, V., Holland, D. and Gollop, R. 2000. Conventional and biotechnological breeding of seedless table grapes in Israel. *6th International Symposium on Grapevine Physiology and Biotechnology, Heraklion, Greece.*

Pezet, R., Pont, V. and Hoang-Van, K. 1991. Evidence for oxidative detoxication of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. Phys. Mol. Plant Path. 39: 441-450.

Pont, V. and Pezet, R. 1990. Relation between the chemical structure and biological activity of hydroxystilbenes against *Botrytis cinerea*. J. Phytopath. 130: 1-8.

Powell, A.L.T., Van Kan, J., Ten Have, A., Visser, J., Greve, L.C., Bennett, A.B. and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Micr. Interact. 13: 942-950.

Sandler, S.J., Stayton, M., Townsend, J.A., Ralston, M.L., Bedbrook, J.R. and Dunsmuir, P. 1988. Plant Mol. Biol. 11: 301-310.

Sarig, P., Zutkhi, Y., Monjauze, A., Lisker, N. and Ben-Arie, R. 1997. Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. Phys. Mol. Plant Path. 50: 337-347.

Sbaghi, M., Jeandet, P., Bessis, R. and Leroux, P. 1996. Degradation of stilbene-type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grapevines. Plant Path. 45: 139-144.

Sharrock, K.R. and Labavitch, J.M. 1994. Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. Physiol. Mol. Plant Path. 45: 305-319.

Schröder, J., Lanz, T. and Schröder, G. 1990. Genes for biosynthesis of stilbene-type phytoalexins. Plant Gene Transfer 311-318.

Somssich, I.E. and Hahlbrock, K. 1998. Pathogen defence in plants - a paradigm of biological complexity. Trends Plant Sci. 3: 86-90.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G. and Tonelli, C. 1994. Cloning and molecular analysis of genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. 24: 743-755.

Stark-Lorenzen, P., Nelke, B., Hänßler, G., Mühlbach, H.P. and Thomzik, J.E. 1997. Transfer of a grapevine stilbene synthase gene to rice (*Oryza sativa* L.). Plant Cell Rep. 16: 668-673.

Stotz, H.U., Powell, A.L.T., Damon, S.E., Greve, L.C., Bennet, A.B. and Labavitch, J.M. 1993. Molecular characterization of a polygalacturonase inhibitor from *Pyrus communis* L. cv Bartlett. Plant Physiol. 102: 133-138.

Stotz, H.U., Contos, J.J.A., Powell, A.L.T., Bennet, A.B. and Labavitch, J.M. 1994. Structure and expression of an inhibitor of fungal polygalacturonases from tomato. Plant Mol. Biol. 25: 607-617.

Tang, K., Tinjuangjun, P., Xu, Y., Sun, X., Gatehouse, J.A., Ronald, P.C., Qi, H., Lu, X., Christou, P. and Kohli, A. 1999. Particle-bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests. Planta 208: 552-563.

Tinland, B., Schoumacher, F., Gloecker, V., Bravo-Angel, A.M. and Hohn, B. 1995. The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. EMBO J. 14: 3585-3595.

Torregrosa, L. and Bouquet, A. 1997. *Agrobacterium rhizogenes* and *A. tumefaciens* co-transformation to obtain grapevine hairy roots producing the coat protein of grapevine chrome mosaic nepovirus. Plant Cell Tiss. Org. Cult. 49: 53-62.

Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L. and De Lorenzo, G. 1992. Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. Plant J., 2: 367-373.

Tropf, S., Kärcher, B., Schröder, G. and Schröder, J. 1995. Reaction mechanisms of homodimeric plant polyketide synthases (stilbene and chalcone synthases). J. Biol. Chem. 270: 7922-7928.

Vornam, B., Schon, H. and Kindl, H. 1988. Control of gene expression during induction of cultured peanut cells: mRNA levels, protein synthesis and enzyme activity of stilbene synthase. Plant Mol. Biol. 10: 235-243.

Wiese, W., Vorman, B., Krause, E. and Kindl, H. 1994. Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine fragment. Plant Mol. Biol. 26: 667-677.

Yao, C., Conway, W.S., Ren, R., Smith, D., Ross, G.S. and Sams, C.E. 1999. Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. Plant Mol. Biol. 39: 1231-1241.

Zambryski, P. 1988. Basic process underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. 22: 1-30.

Zambryski, P. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Mol. Biol. 43: 465-490.

CHAPTER 3

RESEARCH RESULTS

Evaluation of bean polygalacturonase-inhibiting proteins (PGIPs), alone and in combination with resveratrol synthase from *Vitis*, in transgenic tobacco as an antifungal strategy

A modified version of this manuscript will be submitted to
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Evaluation of bean polygalacturonase-inhibiting proteins (PGIPs), alone and in combination with resveratrol synthase from *Vitis*, in transgenic tobacco as an antifungal strategy.

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Abstract

Several classes of proteins have been confirmed to be crucial to the multi-layered defence mechanisms characteristic of plants. One of the best-known classes of defence compounds is the phytoalexins. One of the genes responsible for the production of the potent antifungal agent, resveratrol, is the resveratrol synthase gene from *Vitis vinifera* (*vst1*) that functions in the phenylpropanoid pathway. Another group of proteins, linked to the direct interaction and inhibition of polygalacturonases (PGs) and that is secreted by invading fungi, is the polygalacturonase-inhibiting proteins (PGIPs), which have been isolated from a variety of plant species. Apart from their inhibition effect on fungal PGs and their actions, the PGIPs have also been linked to the recognition and transduction of defence signals in plants as a result of their characteristic features. In this study, the *Phaseolus vulgaris pgip1* and *pgip2* genes, as well as the *vst1* gene, were introduced into tobacco to evaluate their respective and their combined effects on fungal infection when overexpressed in a transgenic environment. The set of expression cassettes that was prepared contained the various genes singly, under the control of a strong constitutive promoter, or contained the *vst1* gene in combination with the *pgip1* or *pgip2* genes, all under their own constitutive promoter-terminator regulatory sequences. Transgenic tobacco lines resulted where the *pgip1* and the *vst1* genes were integrated successfully and expressed in the tobacco genome. Co-transformation experiments resulted in transgenic lines, in which the *vst1* gene was integrated and expressed with both the respective *pgip* genes. The constructs harbouring the combinations of *vst1* and *pgip* genes on a single plasmid also yielded transgenic lines showing integration and expression of the transgenes. The various transgenic lines were evaluated for PGIP activity by an agarose diffusion assay and/or the presence of *trans*-resveratrol levels, as determined by dual mass spectrometrical analysis. Several lines with PGIP activities of up to 40% were identified, whereas the highest resveratrol level reached was 982 ng resveratrol per gram of dry weight, with the majority of the actively-producing lines yielding 200-400 ng resveratrol per gram of dry weight. An *in planta* infection study of the transgenic lines with *Botrytis cinerea* showed very good percentages of disease resistance, as well as good correlations with the levels of resveratrol and PGIP activity detected in these lines.

3.1 INTRODUCTION

Pathogenic fungi secrete a host of enzymes that are responsible for the hydrolytic breakdown of the plant cell wall (Jones et al., 1972). Pectin in the cell wall can be degraded by endo- and exopolygalacturonases (PGs), pectate lyase, pectin methylesterase and glucanase. The necrotrophic fungus, *Botrytis cinerea,* for example, releases nutrients from plant cell walls in the form of galacturonides, mainly brought about by the extensive action of endo-PGs. However, host plants attacked by these and other fungi have evolved several defence strategies. Included in these are inhibitors of fungal macerating enzymes, possessing the ability to limit tissue damage by the specific inhibition of these cell wall-degrading enzymes (Cervone et al., 1987). Polygalacturonase-inhibiting proteins (PGIPs) are glycoproteins with such an inhibitory action. These cell wall-bound proteins specifically inhibit the action of fungal endo-PGs. These PGs constitute the first class of cell wall-degrading enzymes produced in early host-pathogen interactions between fungi and their hosts (Jones et al., 1972). PG action results in the breakdown of pectin oligogalacturonides to smaller units, some of which possess host defence elicitor activity. However, these molecules characteristically exhibit degrees of polymerisation of between 10 and 15, and fungal PGs have the ability to cause the rapid breakdown of these active molecules to smaller, inactive units to circumvent the defence response. PGIPs specifically inhibit the activity of PG on these oligogalacturonides, thus leading to the continued existence of molecules with the ability to activate plant defence responses (Cervone et al., 1993). Furthermore, a structural feature of PGIPs, which is also present in plant resistance gene products, is required for specific pathogen resistance. This structural motif is largely responsible for PGIP-PG affinity and has been termed the leucine-rich repeat (LRR). A further role for the importance of PGIP in defence has been substantiated by the mutational analysis of *Botrytis* PGs. By mutating one of the encoding PG genes, *Bcpg1*, the pathogenicity of the fungus was reduced significantly (Ten Have et al., 1998), confirming the importance of fungal endo-PG in virulence. The hypothesis that appreciable levels of PGIP lead to increased resistance to fungal pathogens has been demonstrated elegantly in transgenic tomato plants (Powell et al., 2000; Berger et al., 2000).

Phytoalexins are secondary plant products that possess antifungal activity and are considered to be part of the general defence mechanism of plants. The synthesis of phytoalexins is induced by environmental conditions, such as ultraviolet irradiation and stress, as well as biotic factors such as pathogen ingress. In grapevine, a correlation has been shown between phytoalexin (resveratrol) content and resistance to the pathogen *B. cinerea* (Hain et al., 1993). This resistance is highly dependent on the rapid synthesis of appreciable levels of the fungitoxic substance. The presence of large amounts of resveratrol thus favours the host in plant-pathogen interactions. Overexpression of the resveratrol synthase gene in a number of plant species has been highly successful in increasing the host's innate resistance (Hain et al., 1993; Leckband and Lörz, 1998; Hipskind and Paiva, 2000).

Here, two PGIP-encoding genes from *Phaseolus vulgaris* (*pgip1* and *pgip2*) were individually introduced into tobacco, as well as individually co-transformed with a phytoalexin-producing gene (*vst1*) from *Vitis vinifera,* and compared with untransformed tobacco lines. The individual, as well as the possible synergistic effects of the transgenic proteins, were evaluated in an *in planta* infection study with *B. cinerea*. These results were interpreted together with the determined levels of activity for the transgenic PGIPs and the quantified resveratrol content of the relevant tobacco lines, leading to the conclusion that the overproduction of the various genes led to an improved resistance of the plants towards the fungal pathogen under the conditions tested.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial and fungal strains and culture conditions

All the bacterial strains and plasmids used in the study are listed in Table 1. All the media components listed as percentages refer to weight per volume (w/v), unless otherwise stated. *Agrobacterium* strains were routinely cultured in YEP medium (1% yeast extract, 1% peptone and 0.5% sodium chloride) at 28°C. *Escherichia coli* strains were grown in Luria Bertani (LB) medium (0.6% yeast extract, 1.2% tryptone and 1.2% sodium chloride) at 37°C, or LB-supplemented with the appropriate antibiotic for the selection of transformants or to retain selective pressure. *B. cinerea* cultures were obtained from the Department of Plant Pathology, Stellenbosch University. The spores were germinated and cultured on apricot halves, canned in natural juice. Before inoculation, the apricot halves were washed with sterile dH_2O, blotted dry and placed in a sterile tissue culture petri dish. Each apricot half was inoculated with a few spores of *B. cinerea* and incubated in the dark at 25°C for two weeks or until spore formation occurred. Spores were harvested and diluted to a density of 2.5×10^6 spores per ml in sterile water or grape juice, depending on the application.

3.2.2 DNA manipulations

All strains and plasmids used in this study are listed in Table 1. Standard techniques for DNA cloning were performed according to Sambrook et al. (1989). Restriction enzymes, Expand Polymerase and T4 DNA Ligase were purchased from Roche Molecular Biochemicals, whereas Klenow fragment (large fragment of DNA Polymerase I) was obtained from Amersham Pharmacia Biotech.

Table 1. Strains and plasmids used in this study.

Strain or plasmid(s)	Description	Source or reference
***E. coli* strains**		
DH5α	supE44 *lac*U169[Φ80*lacZ*M15 *hsd*R17*rec*A1*gyr*A96*thi*-1*rel* A1	Life Technologies (GIBCO/BRL)
***A. tumefaciens* strains**		
EHA 105	Disarmed, succinomopine-type strain	Hood et al., 1993
Plasmids		
PHVXII	Yeast cloning vector, PGK promoter and terminator	Volschenk et al., 1997
pART 7	Cloning vector, CaMV 35S promoter, ocs 3' terminator	Gleave, 1992
pART 7-vst1	*vst1* ORF in pART 7 backbone	This study
pBlue-vst1	*vst 1* cassette in pBluescript backbone	This study
pGEM T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega
pGEM-pgip2	pGEM backbone containing *pgip2* plant expression cassette	This study
pBluescript (SK+)	Cloning vector	Stratagene
pCAMBIA 1300	Plant expression vector Hygromycin resistance marker	CAMBIA
pCAMBIA 2300	Plant expression vector Kanamycin resistance marker	CAMBIA
pFAJ3068	Plant expression vector	Prof. Broekaert Katholieke Universiteit, Leuven
pPVX201-pgip2	Plant expression vector	Leckie et al., 1999
pGA482	Plant expression vector	Dr. D. Berger, ARC Roodeplaat Research Laboratory, Pretoria
pC1300-pgip	pCAMBIA 1300 containing 2 kb *pgip1* cassette	This study
pC2300-pgip	pCAMBIA 2300 containing 2 kb *pgip1* cassette	This study
pC1300-vst	pCAMBIA 1300 containing 3.4 kb *vst1* cassette	This study
pC2300-vst	pCAMBIA 2300 containing 3.4 kb *vst1* cassette	This study
pC1300-pgip-vst	pC1300 pgip backbone containing a 3.4 kb *vst1* cassette	This study
pC2300-pgip-vst	2300 pgip backbone containing a 3.4 kb *vst1* cassette	This study
pFAJ-pgip2	pFAJ3068 containing a 1 kb *pgip2* fragment	This study
pC2300-vst-pgip2	2300 vst backbone containing a 1.9 kb *pgip2* cassette from FAJ3068	This study

PCR primers, their templates and their applications are listed in Table 2. PCR amplifications were performed with Expand Polymerase (Roche Molecular Biochemicals) in 50 µl reaction mixtures, typically consisting of 1x Expand Polymerase PCR buffer (without $MgCl_2$), 200 µM dNTPs, 200 nM of each primer, 5 ng template DNA and $MgCl_2$, added to the 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 1 minute per 1 kb amplified. Reactions proceeded for 30 cycles in a Biometra trio-thermoblock cycler.

3.2.3 Construction of expression cassettes

The bean *pgip1* open reading frame under the control of the dual 35S promoter (Kay et al., 1987) and the tobacco etch viral leader fragment were excised as a 2 kb *Pst*I fragment from plasmid pGA482 (kindly provided by D. Berger). This fragment was cloned into the corresponding sites of pCAMBIA 1300 and 2300, rendering pC1300-pgip and pC2300-pgip, respectively. Oligonucleotides Vst F' and Vst R' (Table 2) were synthesised and used for PCR amplification of the *V. vinifera vst1* gene (kindly provided by P. Schreier). The amplification product was digested with *Hin*dIII and cloned into the *Hin*dIII site of pART7, rendering pART7-vst1, a vector with the *vst1* cassette, under the control of the CaMV 35S promoter and octopine synthase 3' terminator. The *vst1* cassette was released from pART7-*vst1* on a 3.4 kb *Not*I fragment and subcloned into the *Not*I site of pBluescript(SK+) (Stratagene, La Jolla, CA), rendering pBlue-vst1. This cassette was released from pBlue-*vst1* by *Bst*X1 and *Sal*I digestions and mobilised into the corresponding sites of pCAMBIA 1300 and pCAMBIA 2300, yielding pC1300-vst and pC2300-vst, respectively. The same cassette was cloned into plasmids pC1300 pgip and pC2300 pgip, yielding clones pC1300-pgip-vst and pC2300-pgip-vst, respectively. Oligonucleotides PGIP2-5' and PGIP2-3' (Table 2) were synthesised and used for the amplification of bean *pgip2* from plasmid pPVX201-pgip2 (kindly provided by F. Cervone). The fragment was digested with *Xho*I and *Sac*I and cloned into the corresponding sites of pFAJ3068, rendering pFAJ-pgip2, containing a *pgip2* cassette under control of the CaMV 35S promoter and nopaline synthase terminator. This cassette was amplified from this plasmid using oligonucleotides CaMV 35S and Nos T (Table 2), and subcloned into pGEM T-Easy, rendering pGEM-*pgip2* (Promega, Madison, WI). Digestion with *Sal*I excised an 1.9 kb fragment, which was cloned into the corresponding sites of pC2300-vst, yielding 2300-vst-pgip2. The plant expression constructs were verified by sequencing for integrity and are depicted in Fig. 1.

Table 2. Primer pairs, templates and products yielded by PCR amplification in this study.

Primer	Sequence	Paired with	Template	Product
Vst F' (*Hin*dIII)	5'-GATCAAGCTTCAATGGCTTCAGTCGAGGAA-3'	Vst R'	pHVXII	1181 bp *vst1* gene
Vst R' (*Hin*dIII)	5'-GATCAAGCTTTTAATTTGTCACCATAGGAA-3'	Vst F'	pHVXII	1181 bp *vst1* gene
PGIP2-5' (*Xho*I)	5'-GCCTCGAGATGACTCAATTCAATATCCCAG-3'	PGIP2-3'	pPVX201-pgip2	1029 bp *pgip2* gene
PGIP2-3' (*Sac*I)	5'-GCACGAGCTCTTAAGTGCAGGCAGGAAG-3'	PGIP2-5'	pPVX201-pgip2	1029 bp *pgip2* gene
CaMV 35S	5'-GCTGACTGAGACTTTTCAACAAAGGG-3'	Nos T	pFAJ-pgip2	1.9 kb *pgip2* cassette
Nos T	5'-CGATCTAGTAACATAGATGACA-3'	CaMV 35	pFAJ-pgip2	1.9 kb *pgip2* cassette

Restriction sites included in the primers are underlined and indicated in brackets under "Primer".

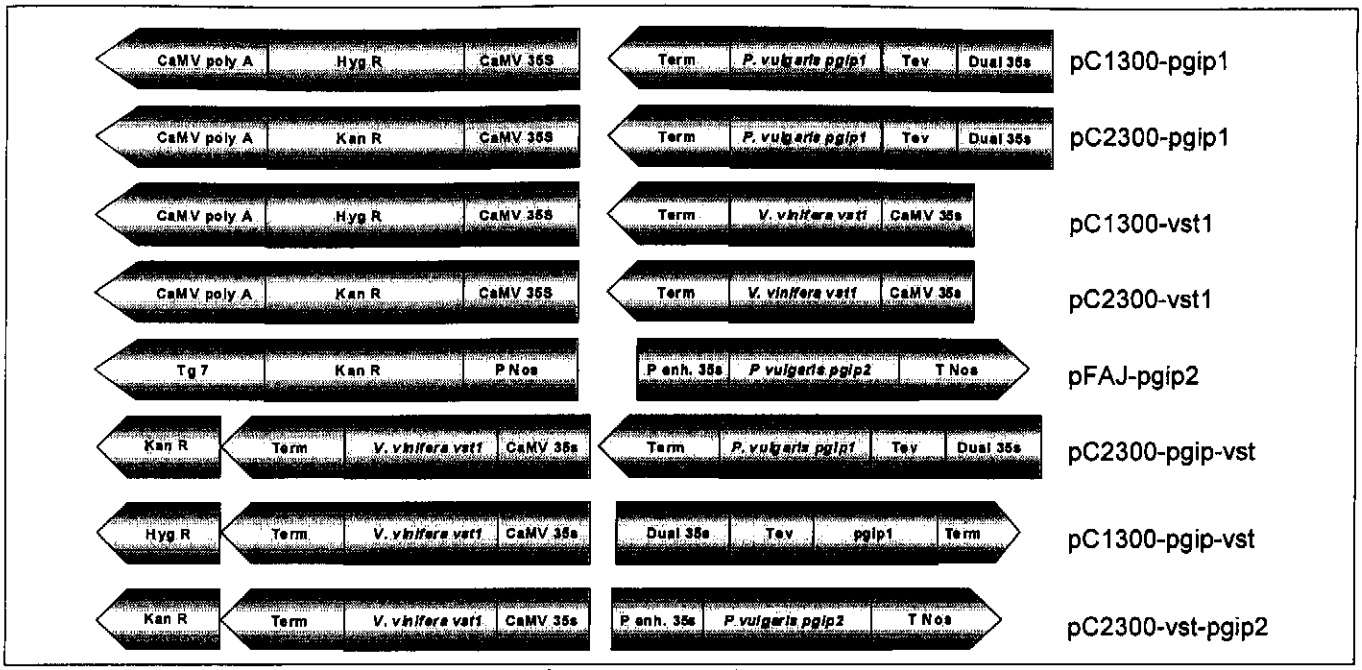


Figure 1. Diagrammatic representation of plant expression cassettes harbouring the polygalacturonase-inhibiting protein (PGIP) encoding genes from *Phaseolus vulgaris*, *pgip1* and *pgip2*, the resveratrol synthase gene from *Vitis vinifera* (*vst1*), and various combinations of the T-DNA structures utilised for the transformation of tobacco plants. CaMV poly A, cauliflower mosaic virus polyadenylation signal; Hyg R, hygromycin phosphotransferase gene; CaMV 35S, cauliflower mosaic virus 35S promoter; Term, cauliflower mosaic virus 35S polyadenylation signal; Tev, tobacco etch viral 5' nontranslated region; Kan R, neomycin phosphotransferase II gene; P enh. 35S and Dual 35S, cauliflower mosaic virus promoter with duplicated enhancer region; Tg7, terminator of T-DNA gene 7; P Nos, promoter of nopaline synthase gene; T Nos, terminator of nopaline synthase gene.

3.2.4 Plant transformation

The plant expression cassettes were mobilised into *A. tumefaciens* strain EHA105 by triparental mating. *Agrobacterium* strains harbouring pC1300-pgip, pC2300-pgip, pC1300-vst, pC2300-vst and pFAJ-pgip2 were used to infect leaf discs of *Nicotiana tabacum* cv. SR-1, essentially as described by Horsch et al. (1985). The leaf discs from tobacco plants were cocultivated with the bacterial suspension for 20 min, blotted on sterile Whatman filter paper (Whatman International Ltd), transferred to plant regeneration medium without antibiotics (Table 3) and incubated at 22°C in darkness for 2 days. The leaf discs were then transferred to selective medium (regeneration medium, Table 3) and incubated at 28°C. After three weeks, hygromycin or kanamycin resistant tobacco shoots were regenerated. The shoots were then placed on rooting media with selective pressure (Table 3) and allowed sufficient time for root formation. Once this occurred, the plantlets were subcultured and maintained on maintenance medium (Table 3) at 28°C under a 16 h photoperiod without any selection.

Co-transformations of pC1300-pgip1, pC2300-vst1, and pC1300-vst1 and pFAJ-pgip2 were also performed according to Horsch et al. (1985), with an equal mixture of the relevant *Agrobacterium* suspensions. Shoots were regenerated on a medium containing both hygromycin and kanamycin at 5 mg/L^{-1} and 60 mg/L^{-1} respectively. Thereafter,

resistant shoots were placed on full strength antibiotic medium (Table 3). Transgenic lines were subcultured clonally, some of the clonal copies of these primary transformants were maintained *in vitro*, while others were hardened off and maintained under greenhouse conditions for further evaluations.

Table 3. Media used for the cultivation of plant material.

Organic constituent	Regeneration	Rooting	Maintenance
Difco-agar ($g.L^{-1}$)	8	8	8
myo-inositol ($mg.L^{-1}$)	100	100	100
Nicotinic acid ($mg.L^{-1}$)	1	1	1
Pyridoxin HCl ($mg.L^{-1}$)	1	1	1
Thiamin HCl ($mg.L^{-1}$)	10	10	10
6-Benzylaminopurine (µM)	10	0	0
β-Naphtoxyacetic acid (µM)	0	0.5	0
Sucrose ($g.L^{-1}$)	30	15	15
Cefotaxime ($mg.L^{-1}$)	400	400	0
Kanamycin ($mg.L^{-1}$)	120	120	0
Hygromycin ($mg.L^{-1}$)	10	10	0
pH (1M KOH)	5.8	5.8	5.8

Mineral salts utilised in media were as described in Murashige and Skoog (1962).

3.2.5 Southern blot analysis

Transgene integration into the regenerated plant lines was confirmed by Southern blot analysis. The total DNA from 40-50 mg of *in vitro* leaf tissue was isolated according to McGarvey and Kaper (1991). An extraction buffer consisting of 3% (w/v) cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylene-diamine tetra-acetic acid (EDTA) and 1 M Tris-HCl (pH 8) was used for the extraction. Samples were digested with 10 units of *Sful*, subjected to 0.8% (w/v) agarose gel electrophoresis and blotted onto Hybond N nylon membranes (Amersham Pharmacia Biotech), as described by Sambrook et al. (1989). The full-length genes were PCR-labelled as probes using the 10x DIG dNTP labelling mixture (Roche Diagnostics), according to the manufacturer's specifications. Lambda DNA digested with *Bst*EII was used as a molecular size marker. Hybridisations were performed at 42°C for 16 h using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications.

3.2.6 RNA isolations and Northern blot analysis

Total RNA was isolated from 40-50 mg of *in vitro* leaf tissue using the TRIzol® Reagent (Life Technologies (GIBCO/BRL)). The total RNA was subjected to 1.2% (w/v)

formaldehyde gel electrophoresis and blotted onto Hybond N nylon membranes (Amersham Pharmacia Biotech), according to standard procedure (Sambrook et al., 1989). The probes utilised for Southern blot analysis were used for hybridisation at 50°C for 16 h, using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications. Transcript sizes were confirmed by comparing co-migration with a Gibco 0.24-9.5 kb RNA ladder.

3.2.7 Liquid chromatography-dual mass spectrometry analysis for detection of resveratrol

Independent transgenic tobacco lines expressing stilbene synthase were analysed for the presence of resveratrol. Metabolites were extracted in acetone, from three to four grams of young leaf material, ground in liquid nitrogen and freeze-dried. Extraction proceeded for 30 minutes at 60°C. The supernatant was dried under nitrogen for several minutes. The dried material was redissolved in 700 µl of methanol, after which 300 µl of water was added. The sample was vortexed vigorously, then centrifuged to remove the insoluble debris. The supernatant was again dried under nitrogen and dissolved in 500 µl of 25 mM citrate-phosphate buffer (pH 5.2), containing 0.5 mg/ml β-glucosidase from almonds (Sigma), to liberate sugar moieties bound to the resveratrol. Samples were incubated for one hour at 37°C, after which free resveratrol was extracted with ethyl acetate. These extracts were dried under nitrogen and redissolved in 50% (v/v) acetonitrile. Mass spectrometric analysis was developed in conjunction with the Central Analytical Facility (Stellenbosch University) and performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Samples were injected by a Waters 717 Plus autosampler and transported to the ionisation source in a carrier stream of solvent A (acetonitrile/water: 1/1 (v/v)), pumped by a Pharmacia LKB 2249 gradient pump.

Ions were detected in the negative mode and the ionisation was optimal at a capillary voltage of 3.5 kV, a cone voltage of 45 V and a source temperature of 120°C. The nebuliser gas used was nitrogen, at a gas flow of 40 L/h. For detection of the molecular ion of resveratrol, the first analyser was scanned through a range of m/z = 100-300 at a scan rate of 150 amu/sec. A representative scan was produced by the addition of scans across the elution peak and subtraction of the background. For fragmentation analysis, the molecular ion was selected by the first analyser and passed into the fragmentation cell, where collisionally-induced dissociation was accomplished by the addition of argon at a pressure of $2x10^{-3}$ mbar and the application of a collision energy of 30 eV. The fragmentation pattern was generated by scanning the second analyser from m/z = 10-240 at 150 amu/sec.

For the quantitative analysis, the samples were subjected to separation by HPLC prior to mass spectrometry. A Phenomenex Luna 3µ C18, 2 x 150 mm column was used and

the mobile phase was 50/50: acetonitrile/water (v/v), at a flow rate of 100 µl/min. Resveratrol was quantified in leaf extracts by multiple reaction monitoring, using the molecular anion at m/z = 227 as the precursor and the fragment at m/z = 142 as the product ion. The standards were analysed to construct a calibration curve from which the concentration of resveratrol in the extract samples was calculated. Light-induced isomerisation of *trans*-resveratrol was also followed over a period of time after the application of 312 UV nm light.

3.2.8 Isolation of crude protein extracts from transgenic lines for PGIP activity assay

Total protein extracts were prepared from two to three grams of young leaf tissue of the various transgenic lines. The extraction buffer consisted of 1 M NaCl, 0.1 M sodium acetate (NaAcetate) (pH 6.0), 1% (w/v) polyvinylpyrrolidone, and 10 mM β-mercapto-ethanol. Proteins were extracted overnight at 4°C with regular agitation. Insoluble debris was removed by centrifugation, and the supernatant was dialysed overnight against 20 mM NaAcetate (pH 5.2), utilising Spectra/Por membrane 4 (Spectrum). The crude extracts were subjected to freeze-drying and subsequently dissolved in 50 mM NaAcetate (pH 5.2).

The inhibition of PG activity by the PGIPs was determined by radial diffusion in agarose in an agarose diffusion plate assay (Taylor and Secor, 1988). *Aspergillus niger* PG (commercial preparation) was incubated with crude protein extracts for 20 h at 37°C. Clearing zones are indicative of PG activity, and are quantified by measuring the diameter of the zones. These were compared with clearing zones that developed when the inhibitor (protein extracts containing PGIP) was added to the PGs, causing a reduction in the diameter of the clearing zones. Zones of inhibition were visualised by staining the agarose plates with 0.05% (w/v) Ruthenium red (Sigma).

3.2.9 Fungal inhibition analysis on detached leaves from transgenic lines

Leaves from one-month-old hardened-off greenhouse plants were removed and the petioles inserted into 0.8% (w/v) water agar in a Magenta vessel (Sigma) for a detached leaf infection assay against *B. cinerea*. Three leaves per plant from each plant line were inoculated. Two aliquots of 2 µl (harbouring 2500 spores/µl) of the spore suspension were dropped onto the adaxial surface of the leaf without wounding, after which the vessels were closed to maintain a high humidity. The spore viability was tested by plating 5×10^3 spores onto water agar plates. The leaves were maintained at 22°C for seven days. The disease lesions were scored after 96 hours and again after seven days, according to a 10-point lesion index scale that was developed. The lesions were also measured after 96 h and seven days and compared to the lesions on the inoculated untransformed tobacco leaves. These lesion sizes were used to calculate the percentage disease resistance of

the transgenic lines. The lesions were photographed after 96 hours, and the whole leaves were photographed after three weeks.

3.3 RESULTS

3.3.1 Transformation of tobacco with *pgip* genes and the *vst1* gene

Plant expression cassettes containing various combinations of *pgip* genes from bean and the *vst1* gene from *V. vinifera* (Fig. 1) were transformed into tobacco via *Agrobacterium* transformations to yield primary transformants resistant to either kanamycin or hygromycin, or both. All the constructs prepared and transformed yielded several transgenic plantlets (typically 10-20 per construct), except the pFAJ-pgip2, from which no viable transgenics could be obtained, even after several transformation events.

3.3.2 Integration and expression of the transgenes in tobacco lines

Southern blot analysis of genomic DNA from an untransformed control and the various transgenic lines were used to confirm the integration of the various genes into the F_0 progeny. The Southern blot analysis indicated that the copy number of the lines transformed with either the *pgip* genes or the *vst1* genes varied between one and two, with the majority of transgenic lines having one copy (results not shown). Plant lines containing *pgip* genes in combination with the *vst1* gene showed transgene incorporation of between one and three copies of each gene (two signals equal one copy) (Fig. 2).

Northern blot analysis of the total RNA isolated from the transgenic lines confirmed the expression of the various genes in some of the F_0 progeny (Fig. 3). Table 4 summarises the total number of transgenic plant lines for each construct that showed gene expression.

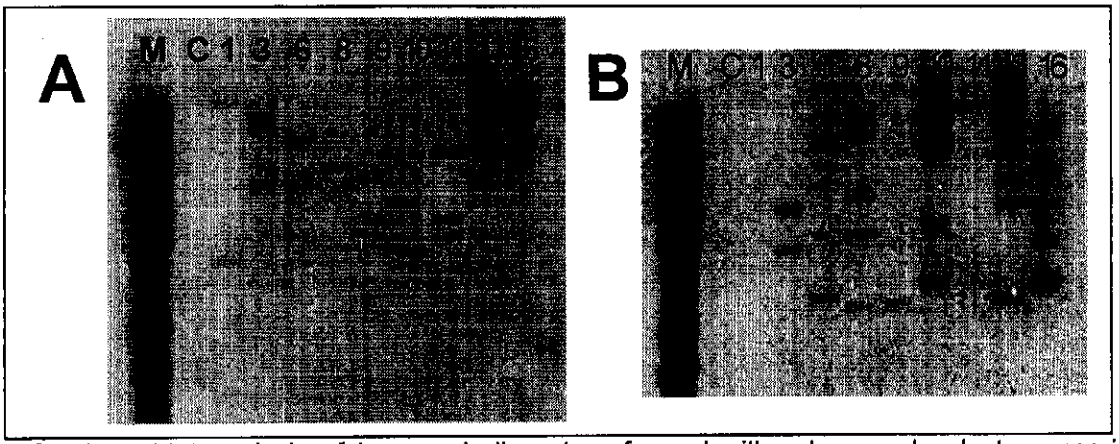


Figure 2. Southern blot analysis of transgenic lines transformed with a bean polygalacturonase-inhibiting protein (PGIP) encoding gene, *pgip1*, in combination with the resveratrol synthase-encoding gene from *Vitis vinifera* (*vst1*), yielding lines TP1V. Genomic DNA of TP1V lines hybridised with a DIG-labelled *vst1* (A), or *pgip1* (B) probe. Lane M represents the marker DNA, lane C represents genomic DNA from an untransformed tobacco line, whereas the rest of the numbers in the lanes represent the various TP1V lines.

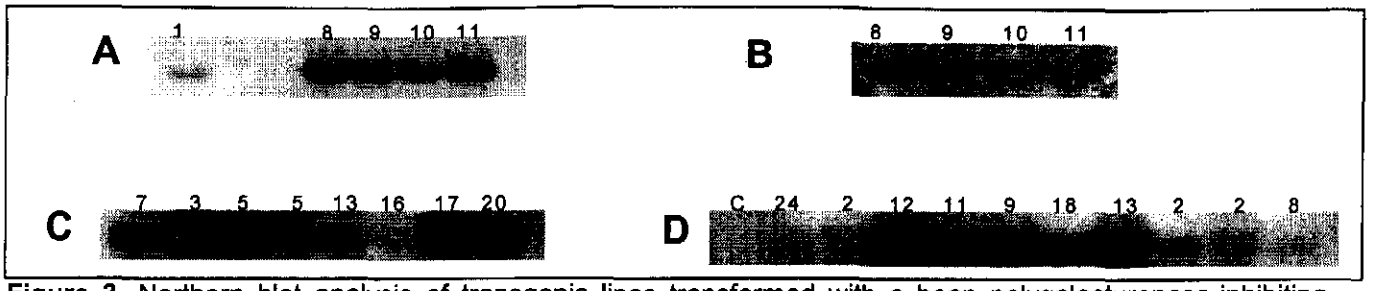


Figure 3. Northern blot analysis of transgenic lines transformed with a bean polygalacturonase-inhibiting protein (PGIP) encoding gene, *pgip1*, in combination with the resveratrol synthase encoding gene from *Vitis vinifera* (*vst1*), yielding lines termed TP1V. Total RNA of TP1V lines hybridised with a DIG-labelled *vst1* (A), or *pgip1* (B) probe. The numbers in the lanes from (A) and (B) represent the various TP1V lines showing expression of the respective genes. Lines individually transformed with *pgip1* (C) or *vst1* (D), showing individual lines with transcripts of the respective genes. Lane C represents total RNA from an untransformed tobacco line, showing no hybridisation signal.

Table 4. Total Northern positive transgenic plants generated in this study.

Constructs transformed into tobacco	Transgene harbouring line	Plant lines expressing transgene(s)
pC1300-pgip1, pC2300-pgip1	TP1	9
pC1300-vst1, pC2300-vst1	TV	16
pC1300-pgip-vst, pC1300-pgip-vst, pC1300-pgip1 + pC2300-vst1	TP1V	8
PC2300-vst-pgip2, pFAJ-pgip2 + pC1300-vst1	TP2V	7

3.3.3 Determination of PGIP activity in transgenic lines

Inhibition of PG activity was determined by measuring the inhibition of PGs from *A. niger* in an agarose diffusion plate assay, which was performed in triplicate for each leaf extract from plant lines TP1, TP1V and TP2V (Fig. 4). Extracts from untransformed control lines were used as a control and it was evident that these lines also showed some inhibition (approximately 23%) against the PGs from *A. niger* PG. The transgenic lines tested exhibited varying degrees of PG inhibition, ranging from 12-38% inhibition (Table 5). Several of the lines had lower PG inhibition than the untransformed control, but a few (TP1 16, TP1 20, TP1V 10, TP1V 11 and TP1V 19) gave inhibition percentages well in excess of that of the control.

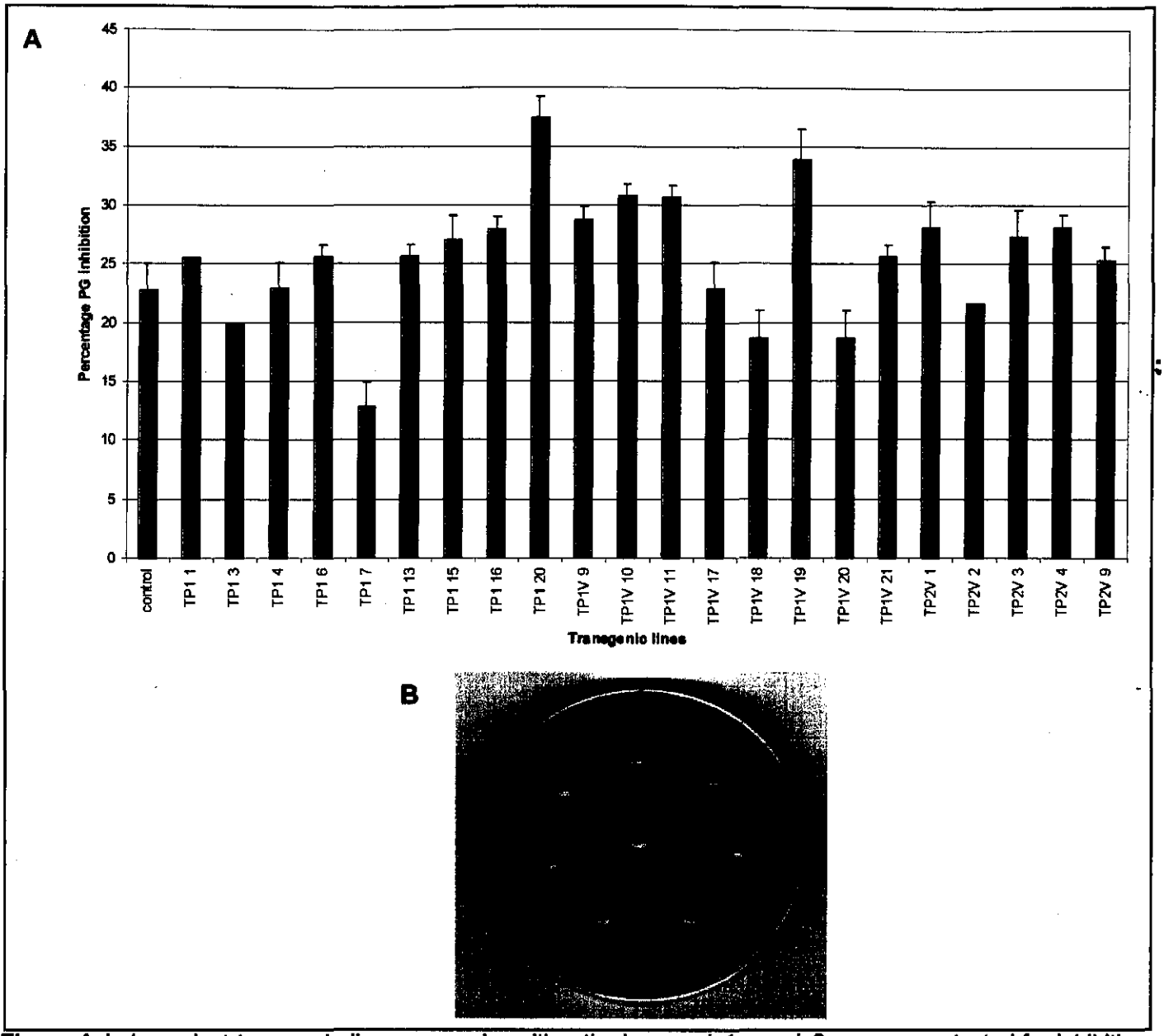


Figure 4. Independent transgenic lines expressing either the bean *pgip1* or *pgip2* genes were tested for inhibition against *A. niger* polygalacturonase (PGs), as compared to an untransformed control plant in an agarose diffusion plate assay (A). A clear zone is indicative of PGs degrading the pectic substrate in the medium, whereas a reduction in zone size is indicative of PG inhibition by transgenic PGIPs present in the leaf extracts from the transgenic lines. Well C, untransformed control plant, showing inhibition of PGs as compared to uninhibited PG (middle); 1, TP1 1; 2, TP1 4; 3, TP1 6; 4, TP1 13; 5, TP1 16; 6, TP1 20 (B).

Table 5. Percentage inhibition of *A. niger* PG by crude protein extracts of transgenic lines harbouring *pgip* genes.

Percentage *A. niger* PG inhibition	*pgip* harbouring lines
10-15	TP1 7
15-20	TP1V 8, 17
20-25	TP1 4; TP1V 17; TP2V 2
25-30	TP1 1, 6, 13, 15, 16; TP1V 9, 21 TP2V 1, 3, 4, 9
30-35	TP1V 10,11;TP1V 19
35-40	TP1 20

3.3.4 Determination of resveratrol levels in transgenic plant lines

The extraction and detection methodologies employed were successful in detecting and quantifying resveratrol in extracts from plant lines TV, TP1V and TP2V. It was also found that light-induced isomerisation occurred within 30 s of 312 nm UV light being applied to the standards of *trans*-resveratrol (Fig. 5). This was enough time to convert all the *trans*-resveratrol to *cis*-resveratrol, after which formation of the former was initiated. Degradation of the compound occurred after 5 min.

With the analysis method calibrated against the commercially available pure resveratrol, it was possible to quantify the resveratrol content in the various plant lines tested (Fig. 6). The transgenic lines had a varied ability to produce this compound. The bulk of the producing lines rendered 200-400 ng/g resveratrol dry weight, with the highest producing line, TV 12, showing almost 1 μg/g resveratrol dry weight after the leaf extracts were incubated with the β-glucosidase to remove the glucose moieties typically associated with resveratrol in plant extracts. It was found that no resveratrol could be detected in samples that had not been treated with β-glucosidase (data not shown). From the analyses, it was evident that the plant lines also produced the *cis*-isomer of resveratrol in considerable quantities (Fig. 7). Since the antifungal effect imparted by this isomer is unknown, quantification only proceeded with the *trans*-isomer.

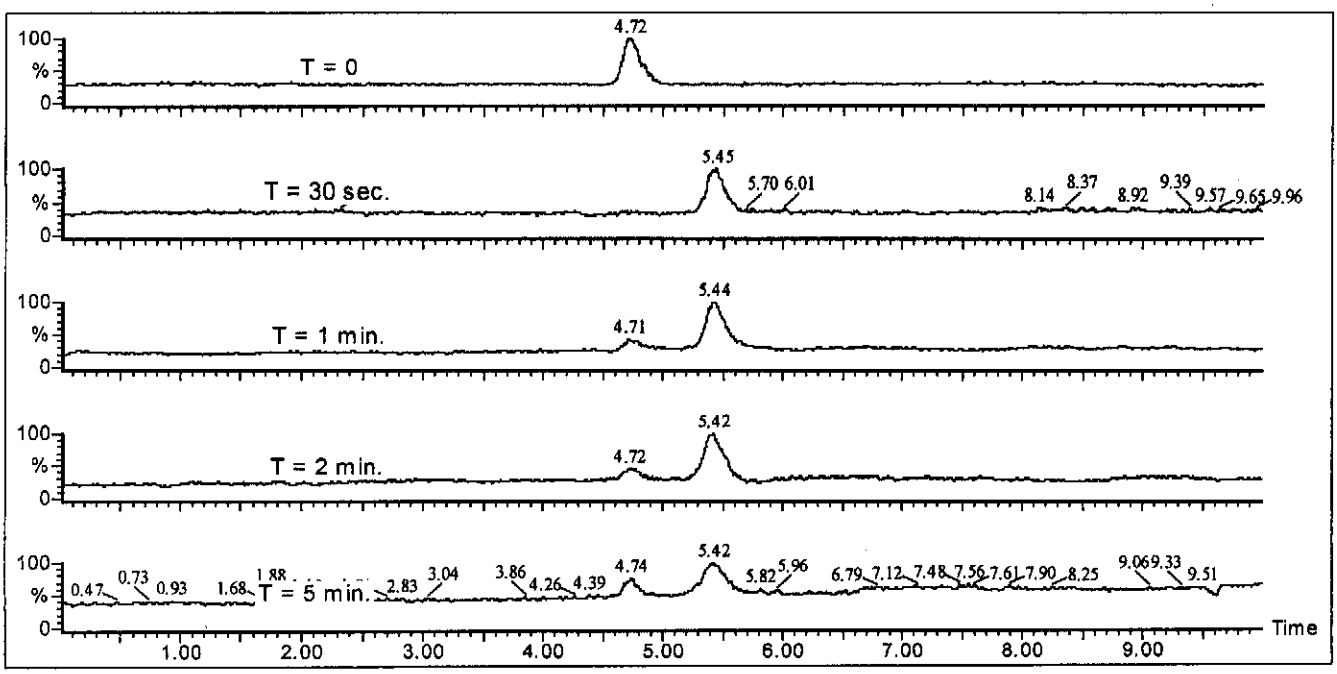


Figure 5. Chromatograms of ultraviolet irradiated *trans*-resveratrol standards as detected by dual mass spectrometrical analysis following separation on a C18 column by HPLC analysis. After 30 s exposure to 312 nm UV light, all *trans*-resveratrol was converted to *cis*-resveratrol, after which reversion to the *trans* isomer was initiated and degradation took place.

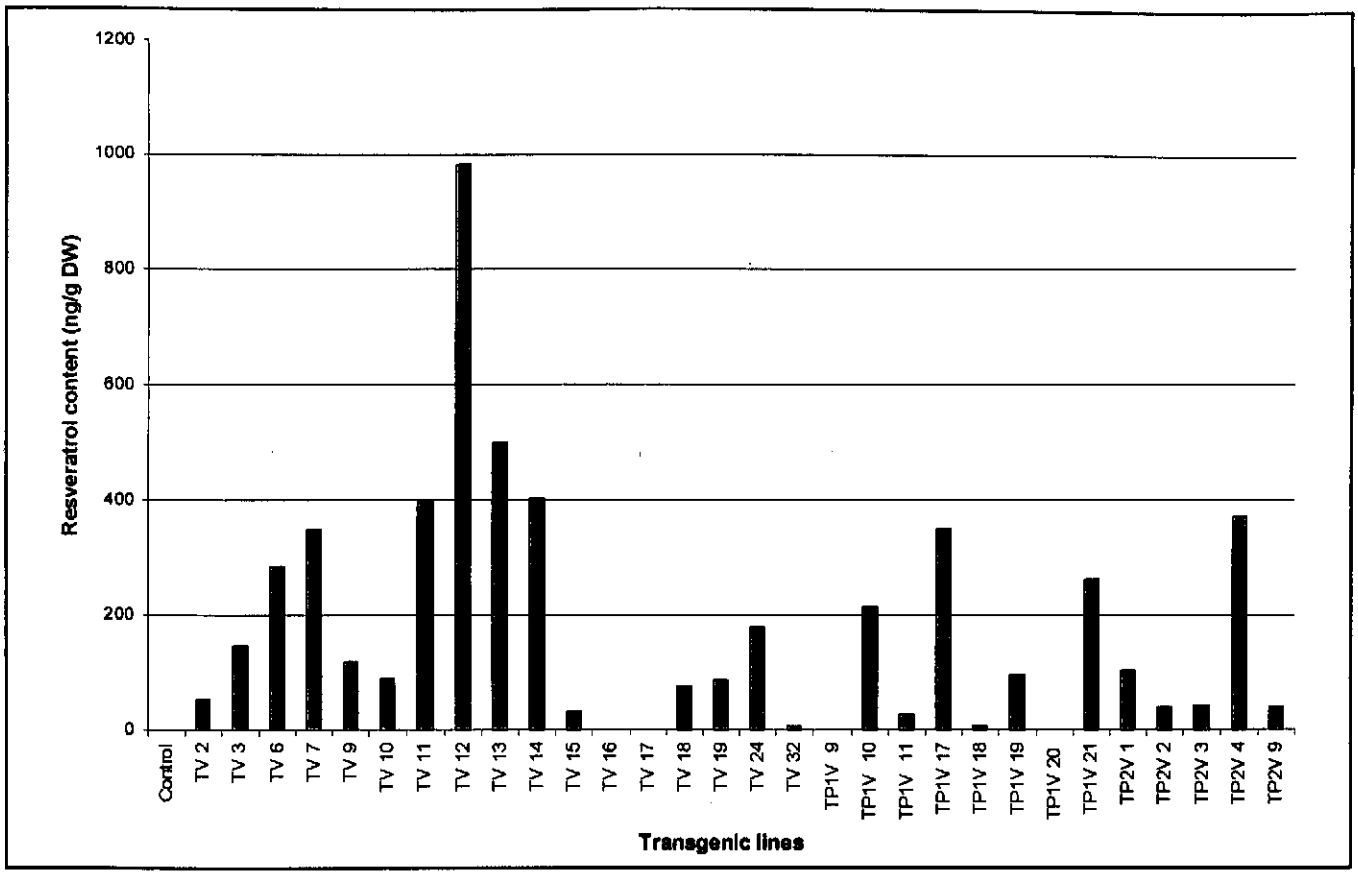


Figure 6. Resveratrol content of independent transgenic lines expressing the *Vitis vinifera* resveratrol synthase gene (*vst1*). Standards were analysed to construct a calibration curve from which the concentration of resveratrol in the extract samples was calculated following detection by dual mass spectrometrical analysis after separation on a C18 column by HPLC analysis.

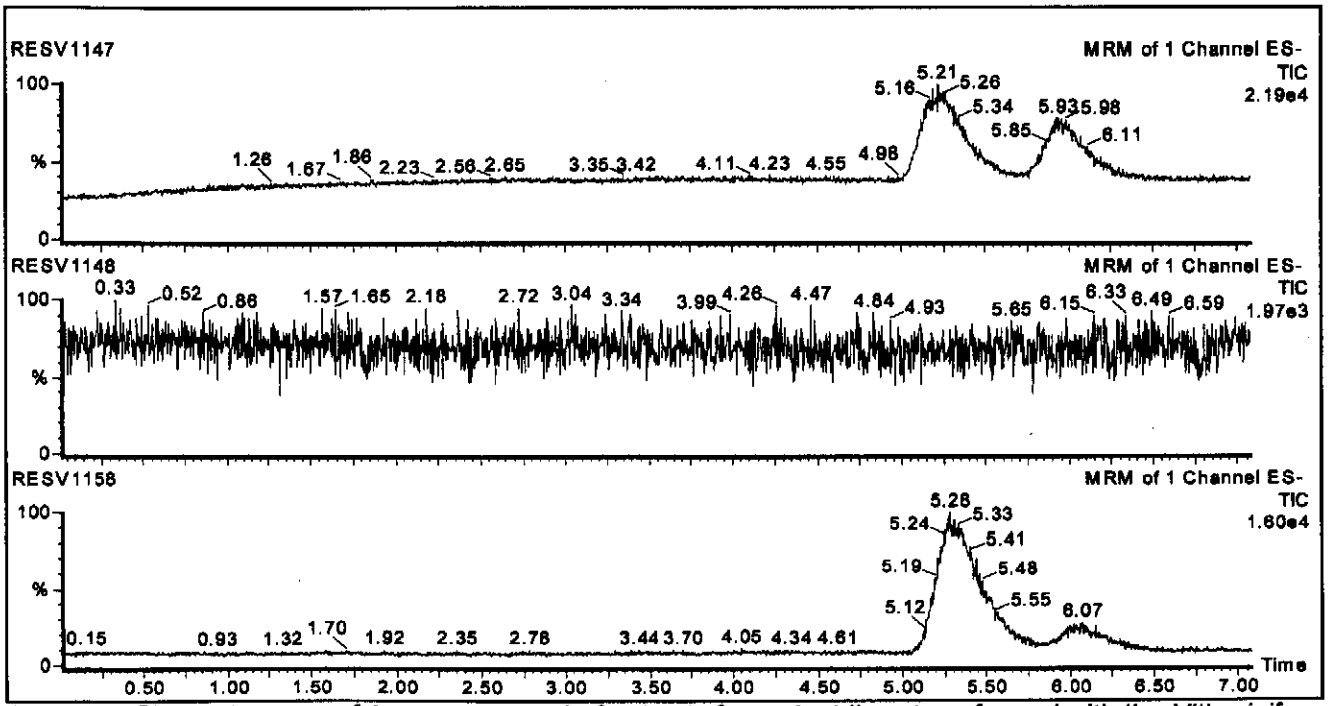


Figure 7. Chromatograms of *trans*-resveratrol of extracts from plant lines transformed with the *Vitis vinifera* resveratrol synthase gene (*vst1*), as detected by dual mass spectrometrical analysis following separation on a C18 column by HPLC analysis. Chromatograms RESV1147, RESV1148 and RESV1158 correspond to plant line TV 13; the untransformed control and plant line TV 12. In lines TV 12 and 13, the first and second observed peaks correspond to *trans* and *cis*-resveratrol, respectively. Quantification of resveratrol levels pertains only to the measured *trans* isomer.

3.3.5 Fungal inhibition studies on transgenic tobacco lines

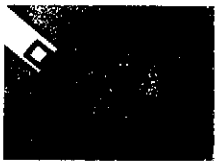
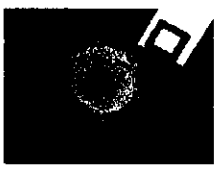
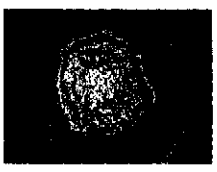
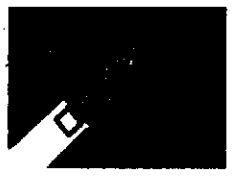
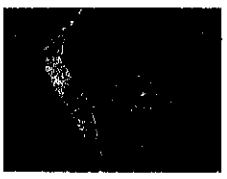
A lesion grading system (a 10-point lesion index scale) was devised in order to score the lesions that developed on the detached leaves from the various transgenic lines infected with *B. cinerea* (Table 6). The detached leaves were inoculated with high spore concentrations and incubated in conditions favourable for disease development. The spore viability of the *Botrytis* spore suspension used was in excess of 99%.

The transgenic lines showing active expression of the various transgenes were evaluated for their ability to inhibit fungal infection in comparison to the untransformed control. The tissue damage caused by *B. cinerea* on the untransformed control tobacco leaves was severe and actively-spreading lesions developed (type 10 lesions) (Table 6). The lesion sizes on the various plant lines were measured after four and seven days of incubation post-inoculation and used to calculate the percentage disease resistance in comparison to the untransformed control plants (Figs. 8-10). Transgenic TP1 lines showed a range of resistances, starting at 25% and approaching 70% in line TP1 16 (Fig. 8). The lesion types of several of these lines (TP1 3, TP1 15 and TP1 16) exhibited the most resistant phenotype on the index scale (type 1 lesions, Table 6). The transgenic lines expressing *pgip* genes and the *vst1* gene, TP1V and TP2V, also showed very good percentages of disease resistance when compared to the untransformed control lines. Some of the lines showed lower levels of resistance, from five to 20%, but the majority of the lines exhibited disease resistance from 40-80% (Fig. 9). The lines with lower resistance levels exhibited more susceptible lesion types (TP1V 21, type 9), whereas lines such as TP1V 9 were characterised by highly resistant lesions (type 1) (Fig. 11).

It was also clear that the levels of resistance of several of the lines increased from day four to day seven post-inoculation. This was very prominent in the lines containing *pgip* and *vst1* genes (Fig. 9), with most of the lines showing an average increase in resistance of between five and 10% at the measurement on day seven. The same tendency was observed in some of the lines expressing only the *pgip* or *vst1* genes, although to a lesser extent (Figs. 8 and 9 respectively).

The lesions were photographed four days (Fig. 11) and three weeks (Fig. 12) post-inoculation. The appearance of the leaves, as well as the presence of fungal survival structures, was evaluated on all the infected transgenic lines and the control after three weeks of incubation. The very high inoculum and extremely favourable conditions caused high levels of fungal infection in the untransformed control, leading to excessive physical damage on the leaves (Fig. 12). The pathogen was also able to form a large number of reproductive organs and survival structures. Correlating with the excellent disease resistances observed in several of the transgenic lines was the complete lack of fungal reproductive organs or survival structures on the infected leaves of these lines after three weeks. Moreover, the leaves were still green and healthy and showed no signs of further disease development (Fig. 12).

Table 6. The lesion-type index used in a *Botrytis* infection study. Transgenic and control tobacco leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16 h light/8 h dark cycle. To score the lesion types, photographs were taken after four days and a lesion-type index was drawn up. The scale proceeds from the most resistant phenotype (type 1) to the most susceptible (type 10).

Lesion appearance*	Lesion description	Lesion appearance*	Lesion description
	**Type 1** Dry; limited expansion; no hyphal growth; dark or light brown		**Type 6** Moist; expanding; no or minor hyphal growth; dark or light brown
	**Type 2** Moist; limited expansion; no hyphal growth; dark or light brown		**Type 7** Dry; expanding; hyphal growth; translucent
	**Type 3** Dry, minor expansion, no hyphal growth, dark or light brown		**Type 8** Dry; expanding; hyphal growth; dark or light brown
	**Type 4** Dry; limited expansion; hyphal growth; dark or light brown		**Type 9** Moist; expanding; hyphal growth; dark or light brown; physical damage
	**Type 5** Moist; minor expansion; hyphal growth; dark or light brown		**Type 10** Moist; expanding; hyphal growth; translucent; physical damage

* The rectangular block on the white paper strip represents an area of 1mm^2 and was used to standardise lesion magnification

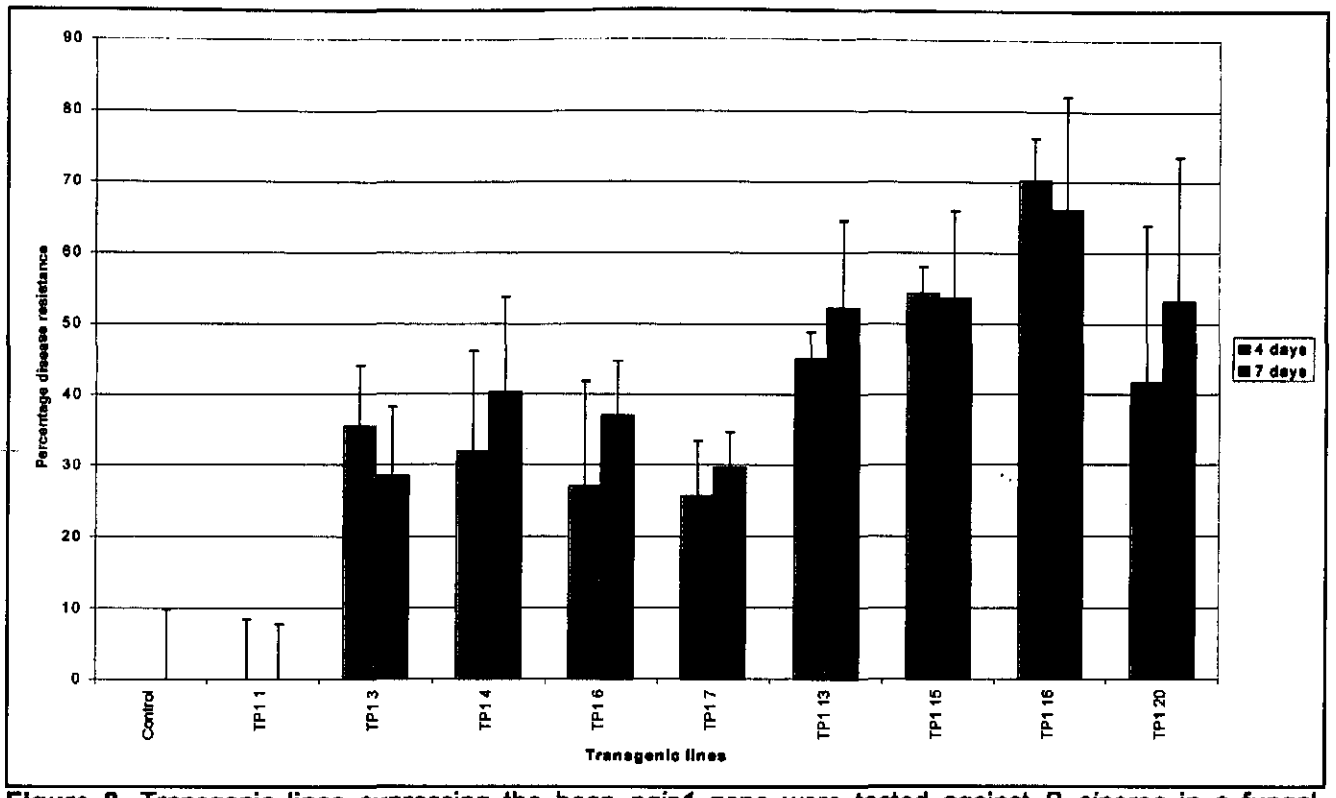


Figure 8. Transgenic lines expressing the bean *pgip1* gene were tested against *B. cinerea* in a fungal infection assay on detached leaves. The sizes of the lesions that developed were measured and processed to indicate percentage disease resistance in comparison to untransformed tobacco plants.

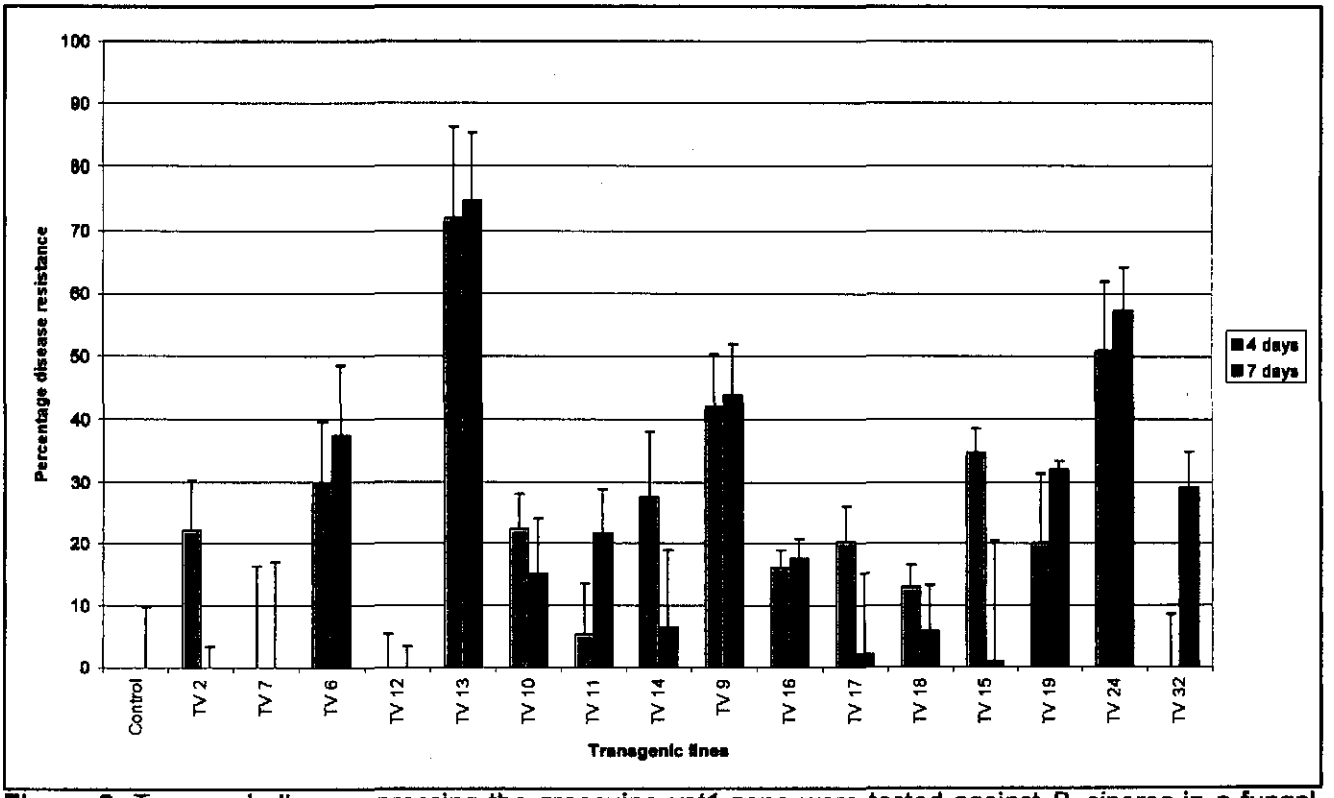


Figure 9. Transgenic lines expressing the grapevine *vst1* gene were tested against *B. cinerea* in a fungal infection assay on detached leaves. The sizes of the lesions that developed were measured and processed to indicate percentage disease resistance in comparison to untransformed tobacco plants.

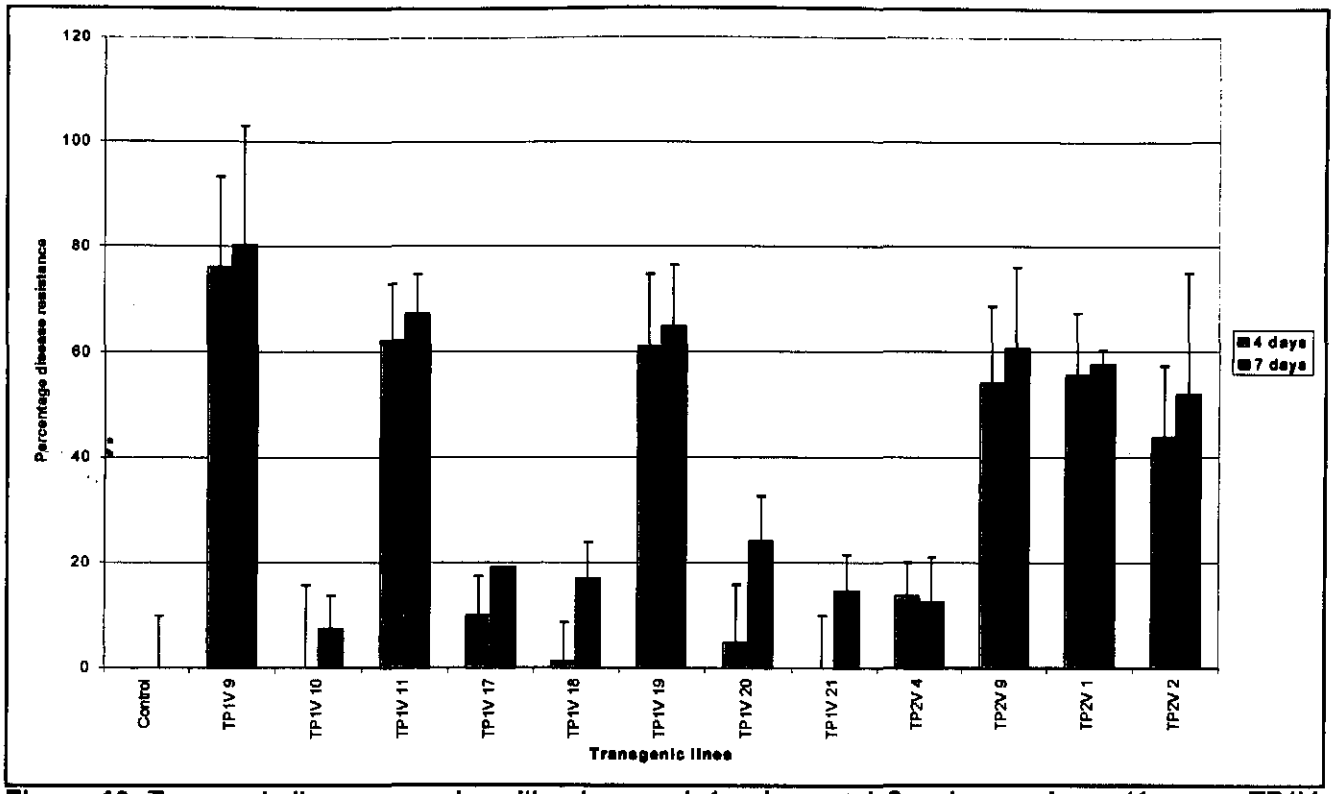


Figure 10. Transgenic lines expressing either bean *pgip1* or bean *pgip2* and grapevine *vst1* genes, TP1V and TP2V, respectively, were tested against *B. cinerea* in a fungal infection assay on detached leaves. The sizes of the lesions that developed were measured and processed to indicate percentage disease resistance in comparison to untransformed tobacco plants.

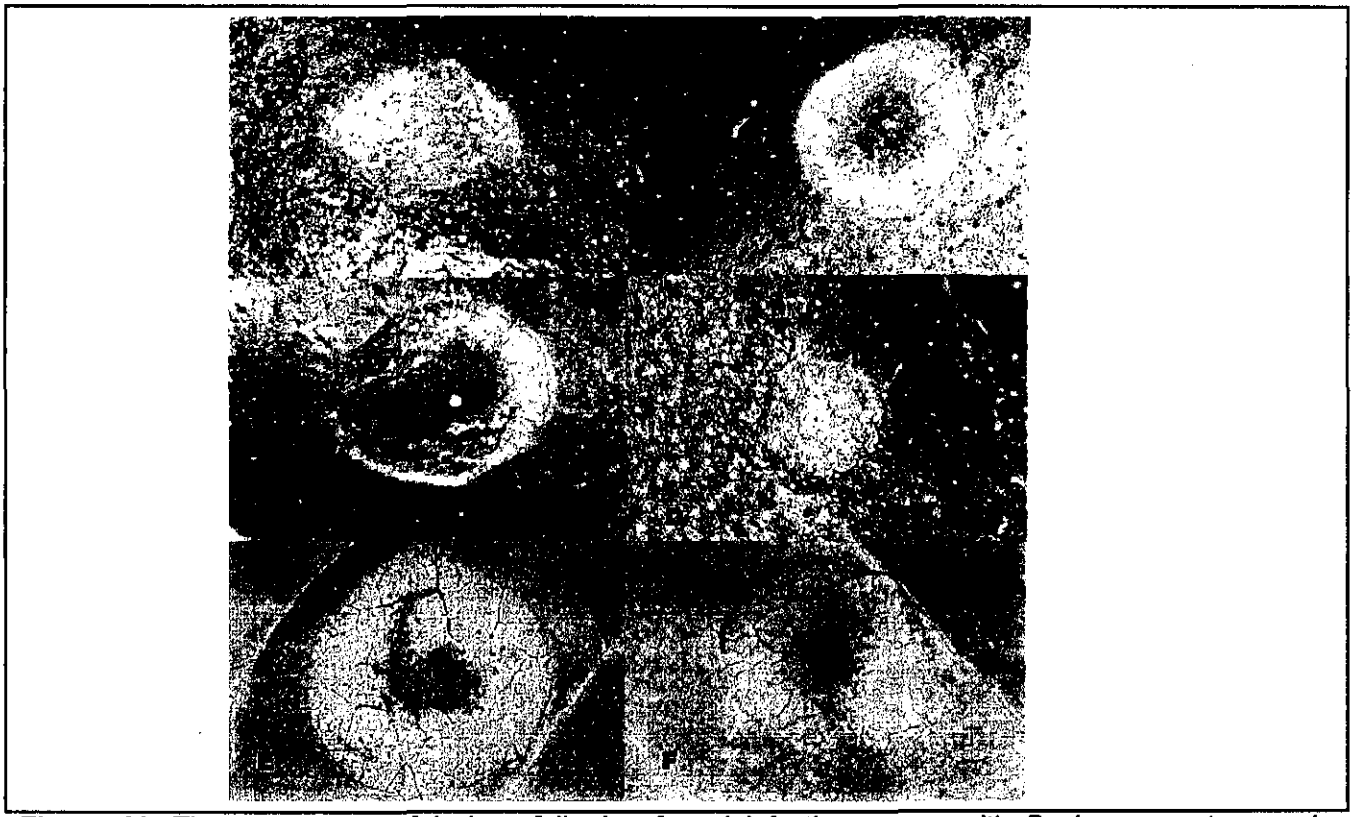


Figure 11. The appearance of lesions following fungal infection assays with *B. cinerea* on transgenic tobacco plants transformed with bean *pgip* genes, TP1, and/or grapevine *vst1* genes, TP1V and TP2V, TV respectively. Photomicrographs showing lesion appearance on transgenic lines after four days of incubation: A, TP1V 9 (lesion type 1); B, TV 13 (lesion type 1); C, TP1 13 (lesion type 5); D, TP1 16 (lesion type 1); E-F, Control (lesion type 10).

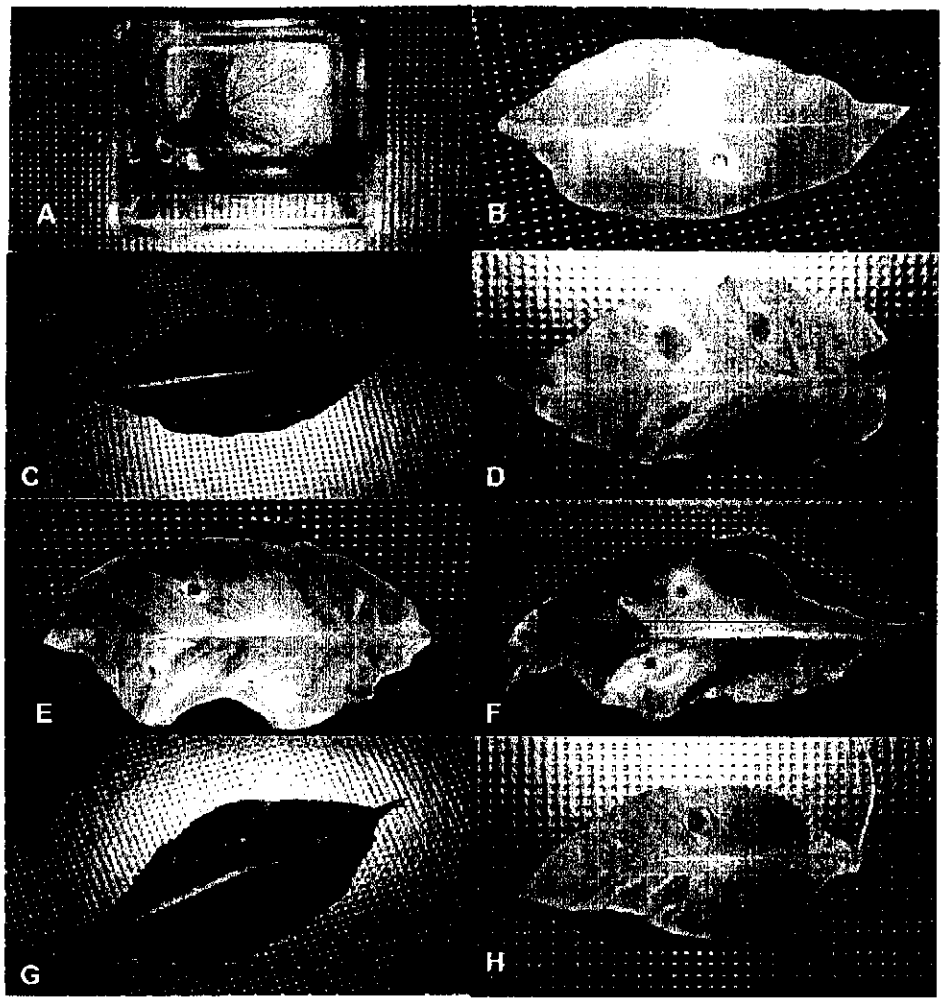


Figure 12. The appearance of lesions following fungal infection assays with *B. cinerea* on transgenic tobacco plants transformed with bean *pgip1* TP1, and/or grapevine *vst1* genes, TP1V and TV, respectively. Photomicrographs showing lesion appearance of transgenic lines after three weeks of incubation: A, Control; B, TP1V 17; C, TP1V 9; D, TV 12; E, TP1V 10; F, TP1 15; G, TP1 16; H, TP1V 21.

3.4 DISCUSSION

Transformation of tobacco with bean PGIPs and/or resveratrol synthase

The bean *pgip1* and *pgip2* and the *vst1* gene from *V. vinifera* were placed under the control of strong constitutive promoters, alone or in combination (Fig. 1), and were successfully integrated into the tobacco genome, yielding several primary transgenic lines. Northern blot analyses indicated that several of the lines, i.e TP1V 13 and 16, harbouring *pgip1* and *vst1*, did not express the transgenes. This might correlate with gene integration at a high frequency. The lines showing integration of both genes, but no expression levels, typically lacked expression of both genes. In co-transformation experiments by De Block and Debrouwer (1991), as well as by De Buck et al. (1998), it was found that two T-DNAs incorporating simultaneously during the transformation event influence the other's integration positively. The authors concluded that cells competent in the stabilisation of one T-DNA might have an increased chance to stabilise a second integrating T-DNA. It therefore seems that the gene silencing that occurred in these lines affected both

transgenes equally. On the other hand, most of the transgenic lines in which *pgip1* or *vst1* were integrated showed single copies in the transgenic genomes, as well as good expression from these genes.

Evaluation of transgenic tobacco for PGIP activity and/or resveratrol content

The presence of active PGIP in the lines harbouring either of the bean *pgip* genes was determined with an agarose diffusion assay against PGs from *A. niger*. This assay showed varying, but overall fairly low levels of inhibition for the different lines (Fig. 4), especially since the untransformed tobacco control also inhibited the PGs quite strongly. The fact that several different plant expression cassettes all yielded fairly low PG inhibition activities rules out the possibility that transformation or integration artifacts are to blame for these low PGIP activities. Although a satisfactory explanation for the low-level inhibition cannot be deduced from these results, they might suggest that *N. tabacum* possesses a native PGIP(s) with the ability to interact with *A. niger* PG.

Despite the low levels of PG inhibition detected for the transgenic lines with overexpressed PGIPs, these lines yielded excellent inhibition of *B. cinerea* infection in an *in planta* assay (Figs. 8 and 10). It has been shown that PGIPs not only inhibit PGs, but also activate other plant defence responses (Cervone et al., 1993). This might explain the low levels of active PGIP coinciding with high levels of resistance. Transgenic lines with higher PG activity (> 25% inhibition, Table 5) also showed better antifungal activity (Figs. 4, 8 and 10). Moreover, plant line TP1 7 had the lowest PG inhibition and also showed poor resistance to *B. cinerea* infection on detached leaves. Thus, a correlation exists between disease resistance and PGIP activity in these transgenic lines.

The transgenic lines expressing the *vst1* gene contained varying levels of resveratrol, as detected by dual mass spectrometrical analysis after the leaf extracts were exposed to β-glucosidase treatment (Fig. 7). A good correlation also existed between the transgenic lines producing high levels of resveratrol and resistance to *B. cinerea* in the plant infection assay. Transgenic lines TV 12 and 13 produced the highest levels of resveratrol and both proved to be extremely resistant after three weeks, with the latter displaying resistance as early as three days post-inoculation (Fig. 12). These lines revealed no hyphal growth or fungal reproductive organs on the lesions, even after three weeks of incubation under conditions favourable for disease development. This might suggest that the levels of resveratrol attained in these lines are sufficient to curb pathogen spread. However, the levels are lower than previously reported in tobacco (Hain et al., 1993). These authors, however, utilised two tandem *vst* genes, both under control of their own inducible promoters.

The quantification methods used in this study did not account for resveratrol polymerisation products that also have potent antifungal effects. The presence of these products in the transgenic tissues cannot be discounted. The plant extracts also showed the presence of *cis*-resveratrol and, although it has not been proven, it might also possesses antifungal activity, contributing to the observed disease resistances in the

relevant transgenic lines. Furthermore, chalcone synthase, an enzyme utilising the same precursors as resveratrol synthase, might compete for the subtrates necessary for the formation of the compound resveratrol.

The plant lines showing the best inhibitory activity after three weeks seldom allowed for the formation of fungal survival structures, thereby limiting the spread of the pathogen (Fig. 12). The combined set of results confirmed that overexpression of the genes encoding proteins involved in antifungal activities and/or the upregulation of the plant's defence mechanism can provide increased resistance to fungal infection. The results also showed that the observed resistances could be correlated with the levels of the various transgenic proteins introduced into the lines. This is a promising result that should be followed up with a larger set of transgenic lines, to further evaluate the possible synergistic action between the PGIPs and resveratrol in biotechnological applications to improve the plant's endogenous disease resistance mechanisms.

3.5 LITERATURE CITED

Berger, D.K., Oelofse, D., Arendse, M.S., Du Plessis, E. and Dubery, I.A. 2000. Bean polygalacturonase-inhibitor protein-1 inhibits polygalacturonases from *Stenocarpella maydis.* Physiol. Mol. Plant Path. 57: 5-14.

Cervone, F., De Lorenzo, G., Degrà, L., Salvi, G. and Bergami, M. 1987. Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol. 85: 631-637.

Cervone, F., De Lorenzo, G., Caprari, C., Clark, A.J., Desiderio, A., Devoto, A., Leckie, F., Nuss, L., Salvi, G. and Toubart, P. 1993. The interaction between fungal endopolygalacturonase and plant cell wall PGIP (Polygalacturonase-inhibiting protein). In Mechanisms of Plant Defense Response, ed by B. Fritig and M. Legrand, 64-67. Kluwer Academic Publishers.

De Block, M. and Debrouwer, D. 1991. Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Gen. 82: 257-263.

De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. 1998. *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Mol. Plant-Micr. Interact. 11: 449-457.

Gleave, A.P. 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. 20: 1203-1207.

Hain, R., Reif, H., Krause, E., Langebartels, R., Kindl, H., Vorman, B., Wiese, W., Schmelzer, E., Shreier, P.H., Stöcker, R.H. and Stenzl, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153-156.

Hipskind, J.D. and Paiva, N.L. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis.* Mol. Plant-Micr. Interact. 13: 551-562.

Hood, E.E., Gelvin, S.B., Melchers, S. and Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants (EHA 105). Trans. Res. 2: 208-218.

Horsch R.B., Fry J.E, Hoffmaun N.L, Eichholtz D., Rogers S.G. and Fraley R.T. 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Jones, T.M., Anderson, A.J. and Albersheim, P. 1972. Host-pathogen interaction. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Path. 2: 153-166.

Kay, R., Chan, A., Daly, M. and McPherson, J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299-1302.

Leckband, G. and Lörz, H. 1998. Transformation and expression of a stilbene synthase gene of *Vitis vinifera*. L. in barley and wheat for increased fungal resistance. Theor. Appl. Genet. 96: 1004-1012.

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A., Nuss, L., Aracri, B., De Lorenzo, G. and Cervone, F. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO J. 18: 2352-2363.

McGarvey, P. and Kaper, J.M. 1991. A simple and rapid method for screening transgenic plants. Biotechniques 11: 428-432.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Powell, A.L.T., Van Kan, J., Ten Have, A., Visser, J., Greve, L.C., Bennett, A.B. and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact.13: 942-950.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Taylor, R.J. and Secor, G.A. 1988. An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopath. 78: 1101-1103.

Ten Have, A., Mulder, W., Visser, J. and Van Kan, J.A.L. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. 11: 1009-1016.

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F.F., Subden, R., Young, R.A., Lonvaud, A., Denayrolles, M. and Van Vuuren, H.J.J. 1997. Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. *Nat. Biotechnol.* 15: 253-257.

CHAPTER 4

RESEARCH RESULTS

The production of resveratrol by *Saccharomyces cerevisiae*

A modified version of this manuscript will be submitted to *FEMS Yeast Research*

RESEARCH RESULTS

The production of resveratrol by *Saccharomyces cerevisiae.*

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Stilbenes are secondary plant products that are produced through the phenylalanine/polymalonate pathway. The stilbene resveratrol is a stress metabolite produced by *Vitis vinifera* during fungal infection, wounding or UV radiation. Resveratrol is synthesised particularly in the skins of grape berries and only trace amounts are present in the fruit flesh. Red wine contains a much higher resveratrol concentration than white wine, due to skin contact during fermentation. Apart from its antifungal characteristics, resveratrol has also been shown to have cancer chemopreventive activity and to reduce the risk of coronary heart disease. It acts as an antioxidant and anti-mutagen and has the ability to induce specific enzymes, which metabolise carcinogenic substances. The objective of this pilot study was to investigate the feasibility of developing wine yeasts with the ability to produce resveratrol during fermentation in both red and white wines. To achieve this goal, the phenylpropanoid pathway in yeast would have to be modified to produce *p*-coumaroyl-CoA, one of the substrates required for resveratrol synthesis. The other substrate, malonyl-CoA, is already found in yeast and is involved in *de novo* fatty acid biosynthesis. We hypothesised that the production of *p*-coumaroyl-CoA can be achieved by cloning and expressing the *4CL9* or *4CL216* (coenzyme A ligase) and resveratrol synthase (*vst1*) genes in laboratory strains of *Saccharomyces cerevisiae*. Yeast has the ability to metabolise *p*-coumaric acid, a substance already present in grape must. Transformants expressing both the introduced genes were obtained and tested for resveratrol production with the addition of the precursors necessary for resveratrol production. The results obtained have shown that yeast was able to produce the resveratrol analogue piceid. β-glucosidase treatment of organic extracts of yeast has resulted in the detection of the compound resveratrol. This is the first report of the reconstruction of a biochemical pathway in a heterologous host to produce the important antioxidant, resveratrol.

4.1 INTRODUCTION

The stress metabolite resveratrol has enjoyed considerable tenure as a research subject in diverse fields, ranging from disease resistance in plants to its contribution to human health. Both structural and biochemical mechanisms are involved in providing a plant with resistance to its pathogens (Jeandet et al., 1995). The active defence mechanism in

plants, in response to infection, involves the induced accumulation of antimicrobial, low molecular weight secondary metabolites known as phytoalexins (Hammerschmidt, 1999). Stilbene synthases synthesise the backbone of the stilbene phytoalexins that have antifungal properties and contribute to the pathogen defences of the plant (Tropf et al., 1995). The speed and intensity with which stilbene compounds are formed are indicators of the plant's ability to resist fungal infection. The rapid increase in resveratrol concentration in grapevine after infection by *Botrytis cinerea* gives a good indication of the crucial role it plays in disease resistance. The primary area of defence for the grape against fungal infection is the grape skin. As a result, resveratrol is synthesised particularly in the skin cells of the grape, whereas only small amounts can be detected in the fruit flesh (Jeandet et al., 1995). Indeed, as soon as the fungus has penetrated into the inner tissues through the grape surface, it is able to develop unhindered in the fruit (Considine, 1982).

During red wine vinification, the must is fermented with the grape skins. As the temperature and alcohol content increase, phenolic substances, including resveratrol, are extracted into the wine. Must used for the production of white wine is fermented in the absence of the grape skins and therefore contains very little of these compounds. Due to this difference in vinification practices, only the consumption of red wine that has been linked to the "French paradox". This dietary anomaly suggests that although the French seemingly follow a high-fat diet and low exercise lifestyle, they have a remarkably low incidence of coronary heart disease. The oxidative modification of low-density lipoproteins is recognised as an important factor in the development of atherosclerosis (Steinberg, 1991). Resveratrol has been implicated in this beneficial action of red wine, mainly due to its ability to act as an antioxidant and as an inhibitor of platelet aggregation (Kopp, 1998). Based on the structural similarity between resveratrol and the synthetic estrogen diethylstilbesterol, Gehm et al. (1997) have shown that resveratrol acts as a phytoestrogen. Given the known cardioprotective benefits of estrogens (Lobo, 1995), these findings are extremely appealing.

Dietary intake contributes to both the development and prevention of human cancer (Huang, 1997). Certain phytochemicals in fruit, vegetables, spices, beverages and foods have been identified as potential cancer chemopreventive agents. The antioxidant and anti-inflammatory activities of resveratrol enable it to inhibit biochemical changes involved in tumor initiation, promotion and progression (Huang, 1997). Given these health benefits, the aim of this study was to investigate the feasibility of developing wine yeasts for the production of resveratrol during fermentation in both red and white wines. This required the modification of the phenylpropanoid pathway in yeast. Malonyl-CoA, one of the substrates required for the production of resveratrol, is present in the yeast and is actively involved in fatty acid biosynthesis (Lampl et al., 1996). The other substrate, *p*-coumaroyl-CoA, can be produced from *p*-coumaric acid, which is found in small quantities in grape must (Goldberg et al., 1998) and has been shown to be accumulated by yeast. The amount present in the grape must as well as the amount accumulated by yeast could, however, be a limiting

factor in the production of resveratrol. The pathway of resveratrol production is shown in Fig. 1.

In this study, the genes encoding coenzyme A ligase, *4CL216* and resveratrol synthase (*vst1*) were introduced into a laboratory strain of *Saccharomyces cerevisiae* under strong constitutive promoters. These recombinant strains were tested for gene integration and the presence of resveratrol.

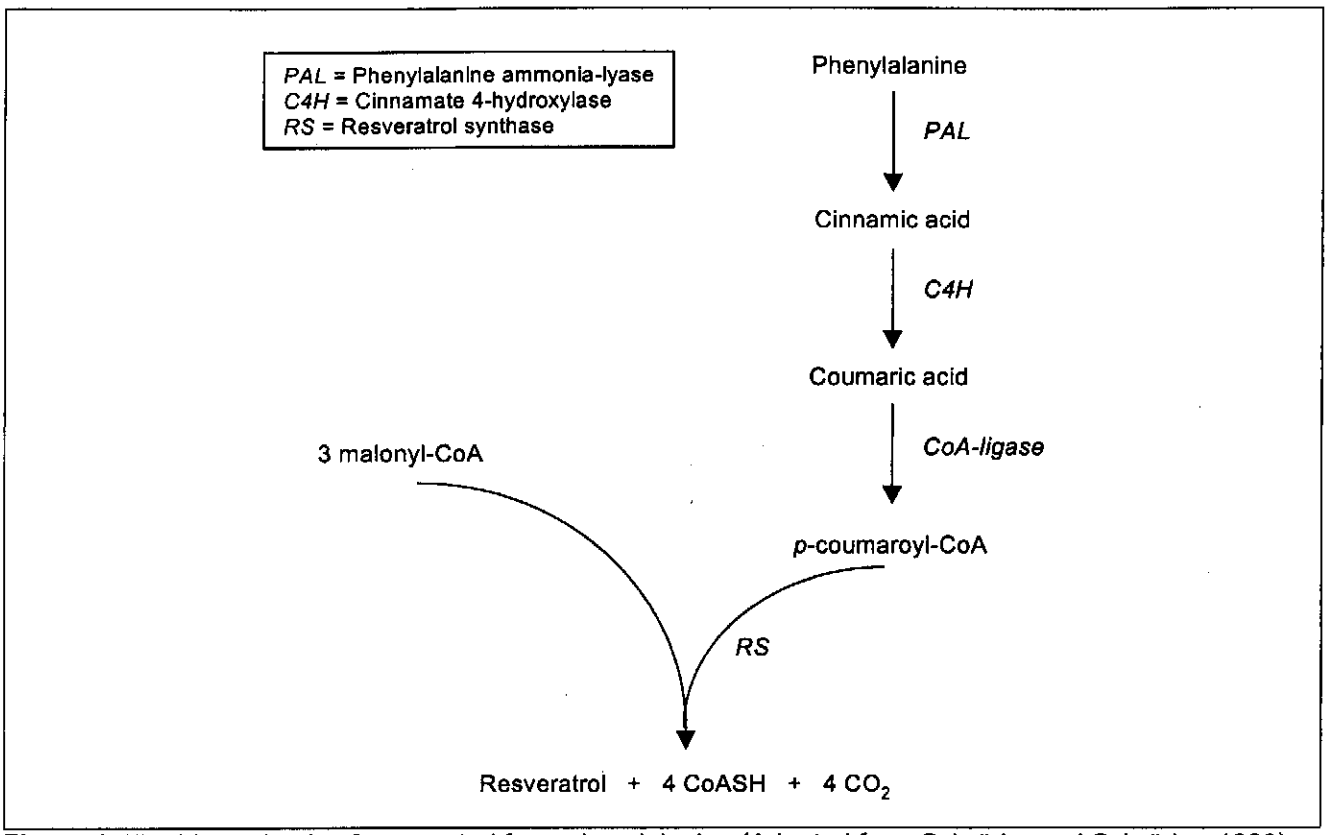

Figure 1. The biosynthesis of resveratrol from phenylalanine (Adapted from Schröder and Schröder, 1990).

4.2 MATERIALS AND METHODS

4.2.1 Microbial strains and culture conditions

All yeast and bacterial strains used in this study, their relevant genotypes and all the plasmids used are listed in Table 1. *Escherichia coli* DH5α cells were used for bacterial transformations and were grown at 37°C in Luria-Bertani (LB) broth, containing 1.2% tryptone, 1.2% sodium chloride and 0.6% yeast extract (Sambrook et al., 1989). All percentages are given as weight per volume (w/v) unless otherwise stated. All bacterial transformations and isolation of DNA were done according to Sambrook et al. (1989). Selection was done on solid media (LB agar) containing 100 µg/ml ampicillin (Roche).

S. cerevisiae cells were grown at 30°C in synthetic media SCD and SCDL [containing 0.67% yeast nitrogen base without amino acids (Difco), supplemented with the required amino acids and 2% glucose (for SCD) or 0.8% glucose (for SCDL)] as well as in rich

media, YPD (containing 1.2% yeast extract, 2.5% peptone and 1.2% glucose) or YPG (containing 1.2% yeast extract, 2.5% peptones and 1.2% galactose). Solid media contained 2% agar.

Table 1. Microbial strains and plasmids used in this study.

Microbial strains or plasmids	Genotype or characteristics	Source
Bacterial strain		
Escherichia coli DH5α	*F' endA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 recA1 gyrA (Nalr) relA1 Δ(laclZYA-argF)U169 deoR [F80dlac DE(lacz)M15]*	GIBCO-BRL/Life Technologies
Yeast strain		
FY23	MATa *leu2 trp1 ura3*	Winston et al., 1995
Plasmids		
pHVXII	*PGK1 P/T*	Volschenk et al., 1997
pPAL	Bluescript, *PAL*	Liew et al., 1996
p4CL9	Bluescript, *4CL9*	Allina et al., 1998
p4CL216	Bluescript, *4CL216*	Allina et al., 1998
pVST1	Bluescript, *vst1*	Hain et al., 1993
pDLG3	*ADH2 P/T; URA3*	La Grange et al., 1997
pDLG34CL9	*ADH2 P/T; URA3; 4CL9*	This study
pDLG34CL216	*ADH2 P/T; URA3; 4CL216*	This study
YEpeno2	*ENO2 P/T*	Unpublished
YEpeno2Vst1	*ENO2 P/T; URA3; vst1*	This study
YEplac181Vst1	*ENO2 P/T; LEU2; vst1*	This study

4.2.2 DNA manipulations and plasmid construction

Standard procedures for the manipulation of DNA were used throughout the study (Ausubel et al., 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity DNA polymerase were used in the enzymatic manipulation of DNA (Roche), according to the specifications of the supplier. The laboratory yeast strain FY23 was transformed according to standard protocols and the positive transformants were selected on SC media without the amino acids leucine and uracil (Ausubel et al., 1994).

The *vst1* gene (from *Vitis*) was digested from pVST1 and inserted at the *Eco*RI site of pJCP, generating pJCPVst1. The correct orientation was confirmed by digestion with *Kpn*I. The *vst1* gene was cut from pVST1 at the *Eco*RI sites and cloned into YEpeno2 under the control of the *ENO2* promoter generating Yepeno2Vst1. The correct orientation was confirmed by digestion with *Kpn*I. The entire expression cassette was cut out at the *Bam*HI-*Xba*I site and cloned into the corresponding sites of YEplac181, containing the *LEU2* auxotrophic marker, generating YEplac181Vst1. The coenzyme A ligase *4CL9* and

4CL216 genes were amplified using PCR (Table 2) from p4CL9 and p4CL216 and sub-cloned into the pGEM-T vector system. The genes were then subcloned into pDLG3 at the *Bgl*II-*Xho*I sites under control of the *ADH2* promoter, yielding pDLG34CL9 and pDLG34CL216 respectively. pDLG3 contains the *URA3* auxotrophic marker.

Table 2. Primers used in this study.

Template	Primer	RE sites
PGK $_P$ in pHVXII	5' GACTGATATCCCCGGGAGCTTTCTAACTGATCTACT 3'	*Eco*RV *Sma*I
PGK $_T$ in pHVXII	5' GACTACTAGTTAACGAACGCAGAATTTTGG 3'	*Spe*I
ENO2 (Forward)	5' GACTCCCGGGGTCGACGCTGCGGGTATAGA 3'	*Sma*I
ENO2 (Reverse)	5' TGCACTGCAGCATTATTATTGTATGTTATAGTA 3'	*Pst*I
4CL9 (Forward)	5' GATCAGATCTATGGAGGCCAATAAGGATCA 3'	*Bgl*II
4CL216 (Forward)	5'GATCAGATCTATGGAGGCGAAAAATGATCA 3'	*Bgl*II
4CL9, 4CL216 (Reverse)	5' GTACCGGGCCCCCTCGAG '3	*Xho*I
PAL (Forward)	5' GATCAGATCTATGGAAGTTTCGAAGGAGAA 3'	*Bgl*II
PAL (Reverse)	5' GATCTCTAGACTTCTTCCACAGTTGACATC 3'	*Xba*I

Restriction sites are indicated by the underlined nucleic acids.

4.2.3 RNA isolation and Northern blot analysis

FY23 recombinant yeast cells were grown in 10 ml of selective media for 48 h. Total RNA was isolated using the FastRNA kit-RED product (BIO 101). RNA (10 μg) from each culture was subjected to formaldehyde gel electrophoresis. The RNA was then transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) and hybridised to radioactively labelled probes according to standard Northern blot procedures (Ausubel et al., 1994). The 1646-bp *4CL9*, 1673-bp *4CL216* and 1178-bp *vst1* PCR products were used as probes. All probes were labelled with ^{32}P-dATP, using the Prime-It II random labelling kit (Stratagene).

4.2.4 Resveratrol assays

FY23 transformed with pDLG34CL216 and YEplac181Vst1 and an FY23 control strain were inoculated from an overnight pre-culture into 100 ml of SCDL containing 10 mg/L malonyl-CoA, coenzyme A and *p*-coumaric acid. Cultures were generated using individual transformants. The cells were grown at 30°C for 48 h. The cultures were then centrifuged at 5000 rpm for 5 min and the supernatant was removed. The remaining cells were then re-suspended in 100% ice cold methanol, after which the cells were broken with glass beads. The extraction of soluble compounds was allowed to progress for two days at

- 20°C. The cell debris was subsequently removed by centrifugation at 5000 rpm for 5 min. The supernatants were dried under nitrogen and dissolved in 500 µl of 25 mM citrate-phosphate buffer containing 0.5 mg/ml β-glucosidase from almonds (Sigma). After incubation at 37°C for one hour, the free resveratrol was extracted three times with ethyl acetate and dried under nitrogen. The pellet was dissolved in 50% acetonitrile. Mass spectrometric analysis was developed in conjunction with the Central Analytical Facility (Stellenbosch University) and performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Samples were injected by a Waters 717 Plus autosampler and transported to the ionisation source in a carrier stream of solvent A (acetonitrile/water: 1/1 (v/v)), pumped by a Pharmacia LKB 2249 gradient pump.

Ions were detected in the negative mode and the ionisation was optimal at a capillary voltage of 3.5 kV, a cone voltage of 45 V and a source temperature of 120°C. The nebuliser gas used was nitrogen at a gas flow of 40 L/h. For detection of the molecular ion of resveratrol, the first analyser was scanned through a range of m/z = 100-300 at a scan rate of 150 amu/sec. A representative scan was produced by the addition of scans across the elution peak and by subtracting the background. For the fragmentation analysis, the molecular ion was selected by the first analyser and passed into the fragmentation cell, where collisionally-induced dissociation was accomplished by the addition of argon at a pressure of $2x10^{-3}$ mbar and the application of a collision energy of 30 eV. The fragmentation pattern was generated by scanning the second analyser from m/z = 10-240 at 150 amu/sec.

For quantitative analysis, the samples were subjected to separation by HPLC prior to mass spectrometry. A Phenomenex Luna 3µ C18, 2 x 150 mm column was used and the mobile phase was 50/50: acetonitrile/water (v/v), at a flow rate of 100 µl/min. Resveratrol was quantified in leaf extracts by multiple reaction monitoring, using the molecular anion at m/z = 227 as the precursor and the fragment at m/z = 142 as the product ion. Standards were analysed to construct a calibration curve, from which the concentration of resveratrol in the extract samples was calculated.

4.3 RESULTS AND DISCUSSION

4.3.1 Construction of expression cassettes, yeast transformation and comfirmation of gene expression

The production of resveratrol by yeast was attempted by the introduction of certain key enzymes to produce the key substrates and by modifying the enzymes necessary, but not present in the yeast. The recombinant plasmids pDLG34CL216 and YEplac181Vst1, harbouring the coenzyme A ligase and resveratrol synthase genes, were transformed into

the laboratory yeast strain FY23. Northern blot analysis of the recombinant yeast strains showed active transcription of the *4CL216* and *vst1* genes (Fig. 2), whereas the control strain did not show any hybridisation signal. The detected signal in the lanes containing RNA from the transformed strains corresponded to the expected sizes of the various genes when compared to a Bio-Rad Laboratories RNA molecular size marker.

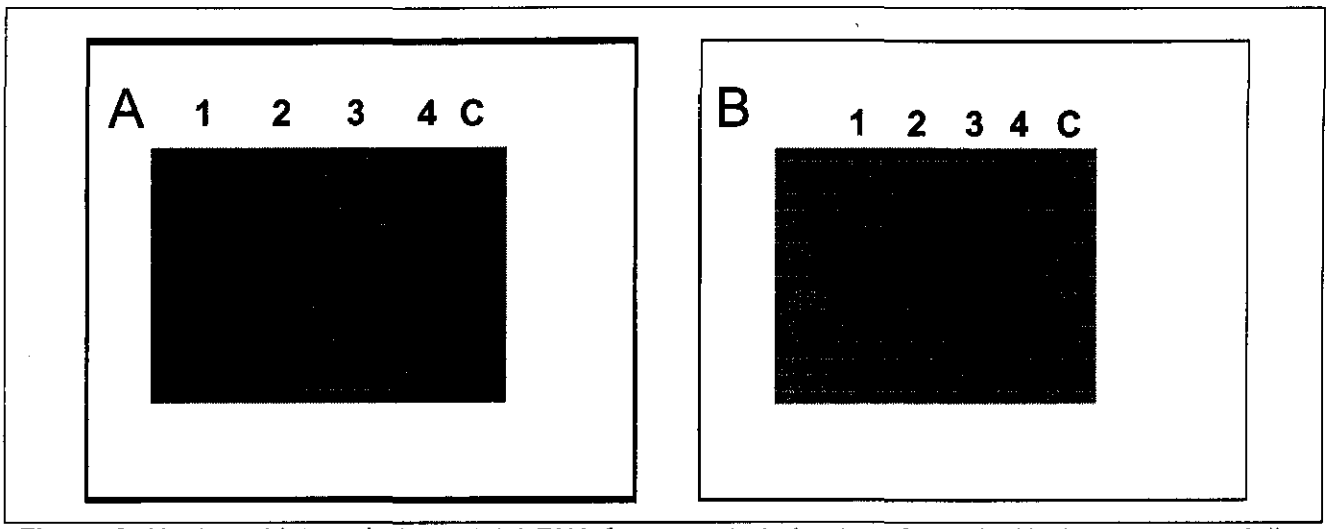

Figure 2. Northern blot analysis on total RNA from yeast strains transformed with the coenzyme A ligase (*CL216*) and resveratrol synthase (*vst1*) genes. FY23 transformed with *vst1* and *CL216*, probed with the radioactively-labelled *vst1* gene (A), or with the radioactively-labelled *CL216* gene (B). Hybridisation signals corresponded to transcripts of the correct size. FY23 was used as a control.

4.3.2 Resveratrol assays

Dual mass spectrometrical assays on recombinant yeast strains harbouring the coenzyme A ligase and resveratrol synthase genes confirmed the presence of *trans*-resveratrol in the yeast cells. Only after the samples were treated with β-glucosidase to release the glucose moieties that are typically bound to resveratrol, were detectable levels of this compound obtained (Fig. 3). Low levels of resveratrol (approximately 28 ng) were detected in the treated samples under the conditions tested. These results however, are very promising, since they confirmed that the introduced genes and their products were active in the heterologous yeast system. It is, moreover, the first report of the construction of a pathway to produce resveratrol in a heterologous system by introducing some of the biosynthetic genes into the producer organism. Although the levels of resveratrol that were obtained are rather low, several optimisations can still be implemented to increase these levels. More resveratrol could be extracted if optimal extraction procedures are elucidated. Also, optimal culture conditions, the time of harvest of the cells, as well as the stability of resveratrol in the heterologous yeast environment need to be evaluated. This is of particular interest if the long-term goal of developing wine yeast strains capable of resveratrol production is to be realised. Another important aspect that is still under investigation is the availability of the necessary precursors at adequate levels in the yeast

environment for optimal resveratrol production. Although the precursors do occur in the yeast, they are also utilised in the fatty acid biosynthetic pathway, which might cause competition for these precursors.

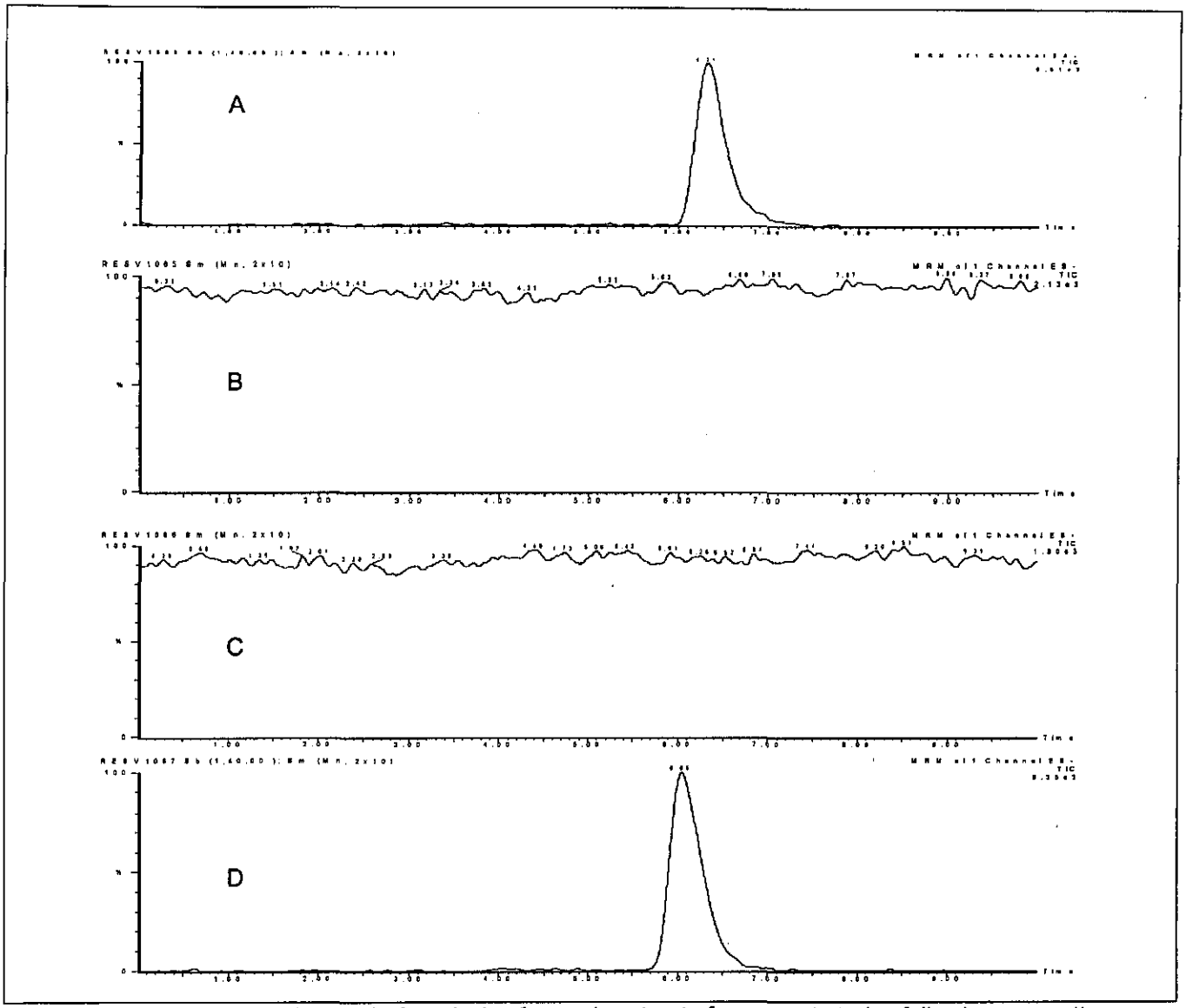

Figure 3. Dual mass spectrometrical analysis of organic extracts from yeast strains following separation on a C18 column by HPLC analysis for resveratrol content. Chromatograms of the resveratrol standard at 50 ng/ml (A), the FY23 yeast control strain after β-glucosidase treatment (B), resveratrol extraction of recombinant yeast without β-glucosidase treatment (C), resveratrol extraction of recombinant yeast, after β-glucosidase treatment (D).

Ultimately, wine yeasts will be transformed with the aforementioned genes and the wines will be evaluated for increased resveratrol content. The preliminary results obtained indicate the possibility of increasing the amounts of resveratrol in both white and red wine by producing this compound in the wine yeast during the fermentation. This pilot study has also paved the way for a more in-depth analysis of the optimised production of resveratrol from yeast and of the various restricting factors influencing this manipulated pathway.

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4.5 LITERATURE CITED

Allina, S.M., Pri-Hadash, A., Theilmann, D.A., Ellis, B.E. and Douglas, C.J. (1998). 4-Coumarate: Coenzyme A ligase in hybrid poplar. Plant Physiol. 116, 743-754.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994). Current protocols in molecular biology. John Wiley & Sons, New York.

Considine, J.A. (1982). Physical aspects of fruit growth: cuticular fracture and fracture patterns in relation to fruit structure in *Vitis vinifera*. J. Hortic. Sci. 57, 79-91.

Gehm, B.D., Mcandrews, J.M., Chien, P.U. and Jameson, J.L. (1997). Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc. Natl. Acad. Sci. USA 94, 14138-14143.

Goldberg, D.M., Tsang, E., Karumanchiri, A. and Soleas, G.J. (1998). Quercetin and *p*-coumaric acid concentrations in commercial wines. Am. J. Enol. Vitic. 49, 142-151.

Hain, R., Reif, H.J., Krause, E., Langebartels, R., Kindle, H., Vornom, B., Wiese, W., Schmelzer, E., Schreier, P.H., Stöcker, R.H. and Stenzel, K. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361, 153-156.

Hammerschmidt, R. (1999). Phytoalexins: What have we learned after 60 years? Annu. Rev. Phytopathol. 37, 285-306.

Huang, M-T. (1997). Diet for cancer prevention. Food Sci. 24, 713-727.

Jeandet, P., Bessis, R., Sbaghi, M. and Meunier, P. (1995). Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. J. Phytopath. 143, 135-139.

Kopp, P. (1998). Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur. J. Endocrinol. 138, 619-620.

La Grange D.C., Pretorius, I.S. and Van Zyl, W.H. (1997). Cloning of the *Bacillus pumulus* β-xylosidase gene (xynB) and its expression in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 47, 262-266.

Lampl, M., Ivessa, A., Spreitzer, B., Schneiter, R. and Kohlwein, S. (1996). Acetyl-CoA carboxylase function in yeast. Yeast Gen. and Mol. Biology, Madison, Wisconsin.

Liew, C-J., Goh, C-J., Loh, C-S. and Lim, S-H. (1996). Cloning and nucleotide sequence of cDNA encoding phenylalanine ammonia lyase from *Bromheadia finlaysoniana*. Plant Physiol. 112, 863.

Lobo, R.A. (1995). Benefits and risks of estrogen replacement therapy. Am. J. Obstet. Gynecol. 173, 982-989.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schröder, J. and Schröder, G. (1990). Stilbene and chalcone synthases: Related enzymes with key functions in plant-specific pathways. Z. Natuurforsch. 45, 1-8.

Steinberg, D. (1991). Antioxidants and atherosclerosis: a current assessment. Circulation 84, 1420-1425.

Tropf, S., Karcher, B., Schröder, G. and Schröder, J. (1995). Reaction mechanisms of homodimeric plant polyketide synthases. Am. Soc. Biochem. Mol. Biol. 270, 7922-7928.

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F.F., Subden, R., Young, R.A., Lonvaud, A., Denayrolles, M. and Van Vuuren, H.J.J. (1997). Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nat. Biotechnol. 15, 253-257.

Winston, F., Dollard, C. and Ricupero-Hovasse, S.L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast 11, 53-55.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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Factors associated with product quality are nonnegotiable in the agricultural community. Typically, these factors are controlled and strongly influenced by the producers. Due to the prevailing climatic influences and the unpredictable nature of infection, the fact remains that pathogens and pests are not controlled well. Disease and infection however, are major determinants of crop quality and also quantity. Not only in South African vineyards and other fruit crops, but also globally, the fungi responsible for grey mould (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*) have contributed significantly to the decay and yield and quality losses of these fruit crops. Current strategies to curb the spread and destruction of these pathogens focus on biological control, the extensive use of fungicides or, lastly, breeding for pathogen resistance. All these methods are hampered by drawbacks, be it climate dependency for biological control, consumer resistance towards chemical fungicides or the time and labour intensiveness of the breeding of cultivars resistant to these pests. This study has focused on the use of genetic transformation technology to enhance disease resistance in plants.

Due to the boom in technological developments and their application, also in the plant sciences, a wealth of knowledge has become available concerning the organisation of plant genomes, plant genetics, plant physiology (Agrios, 1997) and plant-pathogen interactions (Ebel, 1998; Fritig et al., 1998; Hutcheson, 1998; Somssich and Hahlbrock, 1998). The understanding of the plant's innate disease resistance response and the various genes and their encoded products in these processes have led to the current, intensive efforts to evaluate these genes in transgenic environments (Hain et al., 1993; Berger et al., 2000; Hipskind and Paiva, 2000; Powell et al., 2000). This study has taken a similar approach, choosing the bean polygalacturonase-inhibiting protein (PGIP) encoding genes (*pgip1* and *pgip2*) and the resveratrol synthase encoding gene from *Vitis vinifera* (*vst1*) to analyse for antifungal activity in a tobacco model system. PGIPs provide resistance by favouring the existence of oligomers that are capable of eliciting defence responses, and by inhibiting fungal macerating enzymes (Cervone et al., 1993). The resveratrol synthase gene has previously been shown to enhance resistance to fungal pathogens when overexpressed in plant systems (Hain et al., 1993; Hipskind and Paiva, 2000). These two genes have been transformed, alone and in combination, into *Nicotiana tabacum* SR1 plants.

Transgenic lines were obtained that show integration patterns as well as the expression of all transgenes. This study was initially aimed at evaluating the possible additive and synergistic effects of these genes, but although good correlations were obtained between transgene product activity and resistance to the fungal pathogen *Botrytis*, no clear correlations could be drawn to speculate on the synergism between the

genes. However, strong disease resistance levels were obtained in several of the transgenic lines, reaching levels of up to 80%. Even three weeks post-inoculation, these lines showed resistances to the effect that no fungal survival structures were formed. These results bode well for the transfer of this strategy to grapevine and other species, since the spread of the pathogen could be curbed under the conditions used for the test. From the results it was clear that initial infections occurred, but that the pathogens were limited to the infection sites.

When the transgenic lines harbouring the bean *pgip* genes were tested for PGIP activity, relatively low levels of PGIP activity against endopolygalacturonases (PGs) of *A. niger* were detected. These lines, however, yielded excellent resistances against the fungal pathogen under high inoculum pressure and infection conditions favouring the pathogen. This might be due to the fact that the PGIPs activated the plant's resistance mechanism very effectively, leading to the high levels of disease resistance observed. The low level activity detected could also be the result of the crude preparation of protein utilised. Purified protein, together with more sophisticated analyses, are needed for the precise indication of activity.

The transgenic lines expressing the resveratrol synthase gene were subjected to accurate and sensitive mass spectrometrical analysis, indicating varying amounts of resveratrol in the organic extracts. These levels could only be detected following glucosidase treatment of the extracts to remove the glucose residues from the *trans*-resveratrol that was measured in the assay. Several of the chromatograms also showed significant peaks for *cis*-resveratrol that might also contribute to the excellent antifungal activities observed in these lines. The lines showing high resveratrol content also showed the best disease resistance levels three weeks post-inoculation.

Another objective of this study, as a collaborative effort in our Institute, was to evaluate the possibility of producing resveratrol from yeast. This aim pertained to the several positive effects on human health linked to the presence of resveratrol. These effects range from antioxidant activity and the inhibition of platelet aggregation (Kopp, 1998) to the cardioprotective benefits it shares with estrogens because of their structural similarity (Lobo, 1995). To achieve the production of resveratrol from the yeast *Saccharomyces cerevisiae*, it was necessary to introduce a coenzyme A ligase-encoding gene and the *vst1* gene into the yeast; the other precursors in the pathway are present in the yeast and form part of the fatty acid biosynthetic pathway. Expression of the introduced genes could be shown, as well as the presence of resveratrol in organic yeast extracts treated with glucosidase. Although these levels were fairly low, several optimisations in both the culture conditions and the extraction method will probably lead to higher detectable levels.

However, this is an important breakthrough, since it is now possible to engineer novel wine yeast strains to produce this important compound effectively while fermenting the must to make wine. Since the resveratrol from the yeast would not be dependent on the vinification procedures, it would lead to enhanced levels of resveratrol in both red and white wines, thereby increasing the health aspects of the wine in general.

In conclusion, this study forms part of the large and rapidly expanding initiative to produce transgenic plants harbouring recombinant copies of certain genes, in this case antifungal genes. This type of initiative adds valuable information to the scientific body of knowledge regarding the various genes, their encoded products and, most importantly, their modes of action and their efficacy in the specific process studied. This study has confirmed the important roles of PGIPs and resveratrol in manipulated disease resistance in plants.

5.2 LITERATURE CITED

Agrios, G.N. 1997. Plant Pathology, 4th edition. Academic Press, San Diego.

Berger, D.K., Oelofse, D., Arendse, M.S., Du Plessis, E. and Dubery, I.A. 2000. Bean polygalacturonase inhibitor protein-1 inhibits polygalacturonases from *Stenocarpella maydis.* Physiol. Mol. Plant Path. 57: 5-14.

Cervone, F., De Lorenzo, G., Caprari, C., Clark, A.J., Desiderio, A., Devoto, A., Leckie, F., Nuss, L., Salvi, G. and Toubart, P. 1993. The interaction between fungal endopolygalacturonase and plant cell wall PGIP (Polygalacturonase-inhibiting protein). In Mechanisms of Plant Defense Response, ed by B. Fritig and M. Legrand, 64-67. Kluwer Academic Publishers.

Ebel, J. 1998. Oligoglucoside elicitor-mediated activation of plant defense. BioEssays 20: 569-576.

Fritig, B., Heitz, T. and Legrand, M. 1998. Antimicrobial proteins in induced plant defense. Curr. Opin. Immunol. 10: 16-22.

Hain, R., Reif, H., Krause, E., Langebartels, R., Kindl, H., Vorman, B., Wiese, W., Schmelzer, E., Shreier, P.H., Stöcker, R. H. and Stenzl, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153-156.

Hipskind, J.D. and Paiva, N.L. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis.* Mol. Plant Micr. Interact. 13: 551-562.

Hutcheson, S.W. 1998. Current concepts of active defense in plants. Annu Rev. Phytopath. 36: 59-90.

Kopp, P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur. J. Endocrinol. 138: 619-620.

Lobo, R.A., 1995. Benefits and risks of estrogen replacement therapy. Am. J. Obstet. Gynecol. 173: 982-989.

Powell, A.L.T., Van Kan, J., Ten Have, A., Visser, J., Greve, L.C., Bennett, A.B. and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant-Micr. Interact. 13: 942-950.

Somssich, I.E. and Hahlbrock, K. 1998. Pathogen defence in plants - a paradigm of biological complexity. Trends Plant Sci. 3: 86-90.