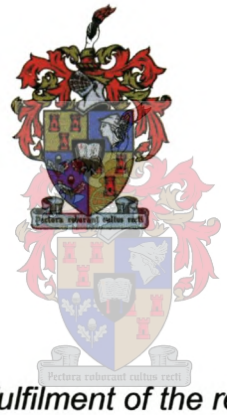
THE EXPRESSION OF YEAST ANTIFUNGAL GENES IN TOBACCO AS POSSIBLE PATHOGENESIS-RELATED PROTEINS

by

Esmé Maree Basson



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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.

SUMMARY

The resistance of plants to infection by phytopathogenic microorganisms is the result of multiple defence reactions comprising both constitutive and inducible barriers. While disease is the exception, such exceptions can be costly and even devastating. In particular, fungal diseases remain one of the major factors limiting crop productivity worldwide, with huge losses that need to be weighed up against massive cash inputs for pesticide treatments.

Part of the defence reactions of plants is the synthesis of pathogenesis-related proteins, such as the plant hydrolases, glucanases and chitinases. In recent years, attention has been paid to the implementation of these proteins in plant transformation schemes. The rationale for this approach was that these antimicrobial agents not only degrade the main cell wall components of fungi, but also produce glucosidic fragments that act as elicitors of the biosynthesis of defence metabolites by the host. Furthermore, since these active antimicrobial agents are individually encoded by single genes, these defence systems should and have been shown to be highly amenable to manipulation by gene transfer.

In this study, yeast glucanases from *Saccharomyces cerevisiae* were evaluated for their potential as antifungal proteins. The glucanases tested for their antifungal activity against *Botrytis cinerea* were the yeast *EXG1* and *BGL2* genes, encoding an exoglucanase and an endoglucanase respectively. An *in vitro* assay performed on these glucanases indicated that exoglucanase had a more detrimental effect on *B. cinerea* hyphal development and growth than the endoglucanase; the former caused typical disruption of the cells and leakage of cell material. The yeast exoglucanase was subsequently subcloned into a plant expression cassette containing the strong constitutive 35S promoter, yielding plasmids pEXG1 and pMJ-EXG1. The pMJ-EXG1 construct targeted the exoglucanase to the apoplastic region with a signal peptide from an antimicrobial peptide from *Mirabilis jalapa*, Mj-AMP2. The pEXG1 and pMJ-EXG1 constructs were mobilised into *Agrobacterium tumefaciens* to facilitate the subsequent tobacco transformation, which yielded transgenic

tobacco lines designated E and MJE respectively. Transgene integration was confirmed with southern blot and PCR analyses for both the E and MJE lines. The expression and heterologous production of the *EXG1*-encoded exoglucanase in the E-transgenic lines was shown with northern blots and activity assays respectively. Moreover, the high level of expression of the yeast exoglucanase led to a decrease in susceptibility of the E lines to *B. cinerea* infection in comparison to the untransformed tobacco controls. An average decrease in disease susceptibility of 40% was observed in an *in planta* detached leaf assay. Crude protein extracts from the E lines were also analysed in an *in vitro* quantitive fungal growth assay, inhibiting *in vitro* fungal growth by average 20%, thus further confirming the antifungal nature of the yeast exoglucanase.

Although integration of the MJ-EXG1 expression cassette was confirmed, no mRNA levels could be detected with northern blot or RT-PCR analysis of the MJE lines. These lines also did not show any *in vitro* antifungal activities or a decrease in susceptibility to *B. cinerea* infection in the detached leaf assay. It is suspected that this result is possibly linked to gene silencing, a phenomenon quite frequently associated with heterologous and/or overexpression of glucanases in plant hosts. It appears as if the targeted overexpression to the apoplastic space triggered the gene silencing response, since the intracellularly overexpressed product was produced and shown to display activity. The yeast exoglucanase thus joins the list of silenced glucanases in overexpression studies in plants.

Overall, this study confirmed the antifungal characteristics of the *Saccharomyces* exoglucanase and provides valuable information of the possibility of utilising yeast glucanases in a transgenic environment. A decrease in the susceptibility of tobacco to *B. cinerea* infection, as shown by the overexpressed *EXG1*-encoded exoglucanases, merits further investigation into the use of this gene in the engineering of disease-resistant crops.

OPSOMMING

Die weerstand van plante teen infeksie deur fitopatogeniese mikroörganismes is die resultaat van verskeie meervoudige verdedigingsreaksies wat beide konstitutiewe en induseerbare versperrings behels. Terwyl siekte die uitsondering eerder as die reël is, kan sulke uitsonderinge duur en selfs verwoestend wees. In die besonder is swamsiektes een van die vernaamste faktore wat gewasproduksie wêreldwyd beperk, met enorme verliese wat teen kontantinsette vir plaagdoders opgeweeg moet word.

Deel van die verdedigingsreaksie van plante is die sintese van patogeen-verwante proteïene, soos die planthidrolases, -glukanases en -chitinases. In die onlangse tyd is aandag geskenk aan die implementering van hierdie proteïene in plant transformasieskemas. Die grondrede hiervoor was dat hierdie antimikrobiese agente nie net die hoof selwandkomponente van swamme kan afbreek nie, maar ook glukosidiese fragmente produseer wat as ontlokkers van metabolietbiosintese vir die verdediging van die gasheer kan optree. Aangesien hierdie aktiewe antimikrobiese agente individueel deur enkele gene enkodeer word, blyk hierdie verdedigingsisteme om hoogs ontvanklik vir manipulasie deur geenoordrag te wees.

In hierdie studie is die gisglukanase van *Saccharomyces cerevisiae* vir hul potensiaal as antifungiese proteïene geëvalueer. Die glukanases wat vir hul antifungiese aktiwiteit teen *Botrytis cinerea* getoets is, was die gis *EXG1*- en -*BGL2*-gene, wat onderskeidelik vir 'n eksoglukanase en 'n endoglukanase enkodeer. 'n *In vitro* toets wat op hierdie glukanases uitgevoer is, het aangedui dat die eksoglukanase 'n meer skadelike effek op die hife-groei en -ontwikkeling van *B. cinerea* as die endoglukanase gehad het; eersgenoemde het die tipiese ontwrigting van die selle en die uitlek van selmateriaal tot gevolg gehad. Die gis-eksoglukanase is gevolglik in 'n plant uitdrukkingskasset wat die sterk konstitutiewe 35S promotor bevat, gesubkloneer, wat plamiede pEXG1 en pMJ-EXG1 opgelewer het. Die pMJ-EXG1-konstruk het die eksoglukanase na die apoplastiese gebied geteiken deur 'n seinpeptied vanaf 'n antimikrobiese peptied van *Mirabilis jalaba*, Mj-AMP2. Die pEXG1- en pMJ-EXG1-konstrukte is

in *Agrobacterium tumefaciens* gemobiliseer, wat die gevolglike tabaktransformasies gefasiliteer het wat die E en MJE transgeniese tabaklyne onderskeikelik gelewer het. Transgeen-integrasie is deur suidelike klad- en PKR-analises vir beide die E en MJE lyne bevestig. Die uitdrukking en heteroloë produksie van die *EXG1*-enkodeerde eksoglukanase is in die transgeniese E lyne deur noordelike klad en aktiwiteitstoetse onderskeidelik aangetoon. Verder het die hoë uitdrukkingsvlak van die gis-eksoglukanase tot 'n vermindering in die vatbaarheid van die E lyne vir *B. cinerea*-infeksie relatief tot die ongetransformeerde tabakkontroles gelei. 'n Gemiddelde vermindering in siektevatbaarheid van 40% is in 'n *in planta* verwyderde-blaartoets waargeneem. Ru proteïen-ekstrakte van die E lyne is ook in 'n *in vitro* kwantitatiewe swamgroeitoets geanaliseer en het *in vitro* swamgroei met tot gemiddeld 20% geïnhibeer, wat dus verder die antifungiese aard van die gis-eksoglukanase bevestig het.

Alhoewel die integrasie van die pMJ-EXG1 uitdrukkingskasset bevestig is, kon geen mRNA-vlakke met die noordelike klad- of RT-PCR-analises van die MJE-lyne waargeneem word nie. Hierdie lyne het ook geen *in vitro* antifungiese aktiwiteite of 'n vermindering in die vatbaarheid vir *B. cinerea*-infeksie getoon nie, soos in die verwyderde-blaartoets uitgevoer is nie. Dit word vermoed dat hierdie resultaat moontlik aan geenstilmaking gekoppel is, 'n verskynsel wat gereeld met heteroloë- en/of ooruitdrukking van glukanases in plantgashere gekoppel word. Dit blyk dat die ooruitdrukking wat tot die apoplastiese ruimte geteiken is, tot die geenstilmaking-respons aanleiding gegee het, aangesien die intrasellulêre ooruitgedrukte produk gemaak is en aktiwiteit getoon het. Die gis-eksoglukanase word dus deel van die lys van stilgemaakte glukanases in die ooruitdrukkingstudies van plante.

In die algemeen het hierdie studie dus die antifungiese kenmerke van die *Saccharomyces* eksoglukanase bevestig en waardevolle inligting oor die moontlike gebruik van gis-glukanases in 'n transgeniese omgewing verskaf. 'n Afname in die vatbaarheid van tabak vir infeksie deur *B. cinerea*, soos deur die ooruitdrukking van EXG1-enkodeerde eksoglukanase getoon is, verdien dus verdere ondersoek van die gebruik van hierdie geen in die skepping van siekteweerstandbiedende gewasse.

This thesis is dedicated to my parents

Hierdie tesis word aan my ouers opgedra

BIOGRAPHICAL SKETCH

Esmé Maree Basson was born in Paarl, South Africa on 7 of September 1976. She matriculated from Hugenot High School, Wellington in 1994. Esmé enrolled at the University of Stellenbosch in 1995 and obtained a B. Sc. (Agric) degree, majoring in Biochemistry, Genetics and Microbiology, in 1998. In 1999, she was accepted for a Master's degree in Wine Biotechnology at the Institute for Wine Biotechnology in Stellenbosch. She is currently employed at Juvenal SA (Pty) Ltd, an international supplier of cork stoppers.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and data from Chapter 3 will form part of an article to be submitted to *Transgenic Research* for publication.

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Chapter 2 **LITERATURE REVIEW**

β-Glucanases - with specific reference to pathogenesis-related proteins

Chapter 3 **RESEARCH RESULTS**

The yeast *EXG1* gene product confers antifungal activity against *Botrytis cinerea*, but is silenced in transgenic tobacco lines when the exoglucanase is targeted to the apoplastic space

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Fungi constitute a highly versatile group of eukaryotic carbonheterotrophic organisms that have successfully occupied most natural habitats. The vast majority of fungi are strict saprophytes; less than 10% of the approximately 100 000 known species are able to colonize plants and an even smaller fraction of these are capable of causing disease. Among the causal agents of infectious diseases of crop plants are phytopathogenic fungi, which play the dominant role not only by causing devastating epidemics, but also through less spectacular, although persistent and significant, annual crop yield losses. This has made the fungal pathogens of plants a serious economic factor that has attracted the attention of farmers, plant breeders and scientists alike (Knogge, 1996).

Since pathogen infection and insect infestation of all staple crops have led to catastrophic food shortages, plant breeders have introduced disease-resistance genes and insect genes into plants through extensive breeding programmes in an attempt to combat such crop failures. However, through the rapid evolution of microbial pathogens and insects, such resistance is often broken relatively quickly. Many chemicals have also been developed to help prevent disease and control herbivorous pests, but these are costly and can be toxic to the environment. Despite all the measures taken, global crop losses remain high; typically at 12-13% of potential production (Dempsey *et al.*, 1998).

The development of plant transformation techniques has opened new ways for creating disease-resistant and insect-resistant crops. Plants can now be engineered to synthesise a variety of antimicrobial and insecticidal products; alternatively, they can express pathogen-derived components that disrupt the infection process. Both strategies confer effective resistance and have yielded commercially improved crops (Dempsey *et al.*, 1998).

In 1971, Abeles *et al.* suggested that the glucanohydrolases, β-1,3-glucanase and chitinase, might function in defence against fungal

pathogens. At about the same time, the pathogenesis-related (PR) proteins were first described as a novel set of abundant proteins accumulating in leaves of resistant tobacco cultivars that react with hypersensitive response (HR) to infection with tobacco mosaic virus (TMV) (Antoniw *et al.*, 1980). Later, it was shown that these PR proteins include β-1,3-glucanases (the PR-2 family) and chitinases (the PR-3 family) (Linthorst, 1991). There now is compelling evidence that β-1,3-glucanase and chitinase, acting alone and in combination, can help defend plants against fungal infection (Bowles, 1990). It has been proposed that these glucanohydrolases act in at least two different ways: either directly, by degrading the cell walls of the pathogen, and/or indirectly, by promoting the release of cell wall-derived materials that can act as elicitors of defence reactions (Boller, 1993).

The direct actions of β-1,3-glucanase and chitinase proteins cause the hydrolysis of β-1,3-glucans and chitin respectively. These are major components of the cell walls of many pathogenic and potentially pathogenic fungi (Wessels and Sietsma, 1981). Treatment with either β-1,3-glucanases or chitinases can inhibit fungal growth *in vitro*, but combinations of the two enzymes have been shown to exhibit strong antifungal activity (Melchers *et al.*, 1993).

Biotechnology is starting to play an increasingly important role in modern agriculture as more tissue culture systems and transformation protocols are developed for crop species. According to Van der Biezen (2001), biotechnology is seen as the solution to environmentally friendly agriculture. This makes the study of antifungal proteins, such as glucanases, and their functioning in heterologous plant systems very important. The expression, antifungal activity and spectrum, as well as the stability of these proteins in heterologous systems, is optimally assessed first in model systems, such as tobacco. Without the knowledge gained from model systems, the genetic engineering of crop species will be a costly affair of trial and error.

In this study, a similar approach has been taken to study the activities of yeast glucanase genes from *Saccharomyces cerevisiae*. This forms part of an

initiative to upregulate and/or to improve the defence mechanisms of the plant against fungal pathogens.

1.2 SPECIFIC PROJECT AIMS

The overall aim of this project was to study the introduction of a yeast antifungal gene into a plant and to analyse the effect of this upregulated defence response on a fungal pathogen.

Monocotyledonous and dicotyledonous plants naturally respond to fungal attack with a complex network of defence mechanisms (Dixon and Harrison, 1990). These include the synthesis of PR proteins, such as the hydrolytic enzymes, β-1,3-glucanases and chitinases. These enzymes are known to degrade structural polysaccharides in the fungal cell wall (Kombrink *et al.*, 1988) and for inhibiting fungal growth *in vitro*. These respective genes therefore are candidates for an antifungal strategy (Sela-Buurlage *et al.*, 1993).

Several plant glucanase genes have been overexpressed in plants, rendering the host more resistant to fungal pathogens (Mauch *et al.*, 1988; Zhu *et al.*, 1994; Jongedijk *et al.*, 1995). Yeast glucanases have also been shown to be active against fungal pathogens. In 1998, Jijakli and Lepoivre characterised an exo-β-1,3-glucanase produced by the yeast, *Pichia anomala*, that showed antifungal activity against *Botrytis cinerea* on apples. These results strengthened the hypothesis that exo-β-1,3-glucanase activity is one of the mechanisms of action involved in the suppression of *B. cinerea* by antagonistic yeasts.

In this study, the glucanases from the yeast *Saccharomyces cerevisiae* were screened for effective antifungal activity against *B. cinerea*. The selected glucanase-encoded gene was then introduced into tobacco, *Nicotiana tabacum*, as a model system to test the antifungal functioning of the yeast protein in a plant environment.

This study forms part of a multidisciplinary research objective at the Institute for Wine Biotechnology that focuses on the genetic improvement of grapevine through recombinant DNA technology.

Specific aims of this study included the following:

i) The evaluation of supernatants from *S. cerevisiae* recombinant yeasts containing overexpression/secretion cassettes encoding the *Saccharomyces* EXG1p (exoglucanase) and BGL2p (endoglucanase) for their antifungal activity against *B. cinerea* spore cultures by using a time-scale microscopic analysis;

ii) The construction of plant expression cassettes that overexpress the EXG1 protein intracellularly or in the apoplastic space due to a fusion with the signal peptide from Mj-AMP2, an antifungal peptide from *Mirabilis jalapa*;

iii) The mobilisation of the expression cassettes into tobacco via *Agrobacterium tumefaciens* and the regeneration of transgenic tobacco lines that constitutively overexpress the yeast *EXG1* gene, accumulating the exoglucanase in the cytoplasm or apoplastic space;

iv) The analysis of the primary transformants for gene integration and copy number, for gene expression and glucanase activity;

v) The evaluation of the antifungal activities of the overexpressed heterologous glucanases in transgenic lines upon fungal infection by *B. cinerea*. The effect of the overexpressed glucanases was determined both *in vitro* and *in planta*.

The experimental details and results of this study are presented in Chapter 3 of this thesis.

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CHAPTER 2

LITERATURE REVIEW

β-Glucanases - with specific reference to plant pathogenesis-related proteins

LITERATURE REVIEW
β-Glucanases – with specific reference to plant pathogenesis-related proteins

2.1 INTRODUCTION

Plants are sedentary organisms. Their need to obtain a full spectrum of nutrients from the environment has led to the maximisation of their surface area to absorb the raw materials needed. These extensive aerial and subterranean boundaries with the environment cause them to be vulnerable to pathogens and to be readily affected by adverse conditions. Survival in a changing environment necessitates rapid responses to external stimuli, whether these signals arise from other organisms and predators, or from conditions such as waterlogging, high temperatures or drought. Plants use these environmental signals as developmental signals. Given the requirement to protect large surface areas, a strategy has evolved in which the ability to distinguish foreign from self resides in cells throughout the organism. One consequence of these recognition events can be the elicitation of a defence response (Bowles, 1990).

A defence response has to be a rapid and coordinated process in the area of injury, since wound sites are known to be important entry points for many pathogens. Research on defence responses, however, has shown that local events in the immediate zone of injury or pathogen invasion also trigger systemic events (Maleck and Lawton, 1998). These systemic events can be extremely rapid and generally involve a massive amplification of the response, since local changes occurring within one cell or a small group of cells produce systemic changes throughout the entire organ system(s).

In general, plants defend themselves against pathogens by a combination of two strategies: (i) structural characteristics that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant and (ii) biochemical reactions that take place in the cells and tissues of the plant and produce substances that either are toxic to the pathogen, or inhibit the growth of the pathogen in the plant (Agrios, 1997). Preformed or passive

structural characteristics include the amount and quality of wax and cuticle that cover the epidermal cells, the structure of the epidermal cell walls, the size, location, and shape of stomata and lenticels, and the presence of thick-walled cells that hinder the advance of the pathogen (Prusky and Keen, 1993).

It was, however, found that many pathogens fail to cause infection even though no apparent host structures inhibit them from doing so. This suggests that defence mechanisms of a chemical rather than a structural nature are responsible for the resistance to infection exhibited by plants against certain pathogens. These chemical defences include compounds released by the plant into its environment and inhibitors present in the plant cells before infection. Plant surface cells also contain variable amounts of hydrolytic enzymes, such as glucanases and chitinases, which typically cause the breakdown of pathogen cell wall components, thereby contributing to resistance. These proteins, together with others such as thaumatins, are known as the pathogenesis-related (PR) proteins. The induced defences are described as active defence mechanisms, because they occur in response to an invading pathogen and require host metabolism to function (Hutcheson, 1998).

At least three separate classes of active defence responses can be identified in the responding tissue of resistant plants that differ in eliciting signals: primary, secondary and systemically acquired responses.

Primary responses are localised in the cells that are in contact with the pathogen, in very close proximity to a pathogen structure, or, in the case of viruses, cells infected by the pathogen. In the latter case; they involve the recognition of specific signal molecules whose presentation and display by the pathogen are critical to their activity. The outcome of this primary response is usually programmed cell death (PCD) in the form of a hypersensitive reaction (HR).

Secondary responses occur in cells adjacent to the initial infection site in response to elicitors that are a result of the HR in the primary interaction. The third category is associated with systemically acquired resistance (SAR), which is hormonally induced throughout the plant (Hutcheson, 1998). If part of a plant has already reacted hypersensitively to a pathogen, the uninoculated parts of this plant develop an increased state of resistance, which is evidenced by

smaller lesions and a greater restriction of the infection upon challenge or inoculation by the same or any other unrelated, but necrotising, pathogen (Ye *et al.*, 1989).

In this review, β-glucanases as part of the PR proteins will be discussed in terms of their role in defence against pathogens and their possible application in the upregulation of the plant's innate defence response by means of genetic transformation technology.

2.2 β-GLUCANASES - WITH SPECIFIC REFERENCE TO PATHOGENESIS-RELATED PROTEINS

2.2.1 Pathogenesis-related proteins in general

As mentioned previously, the production of PR proteins is one of the most important defence mechanisms launched by the plant against pathogens. The HR to pathogen attack includes the production of PR proteins and is considered a very dramatic defence response induced in plants. Necrotic lesions are formed around the initial sites of pathogenic attack. The pathogen will be restricted to a zone of cells surrounding the necrotic lesions, due to a very intense defence response that is induced in this ring of cells (Lamb *et al.*, 1989). As was mentioned in the introduction, some of the changes are also induced distal to the infection site and even in uninoculated and uninfected parts of partially infected plants, though at a much lower rate than at the infection site. This scenario is representative of some of the PR proteins, specifically those whose production in uninoculated tobacco leaves represents 5-10% of their production in inoculated hypersensitively-reacting leaves. Since the PR proteins are induced parallel to the systemic acquired resistance (SAR) response, the former are believed to participate in the efficiency of the latter, but a causal relationship has not yet been proven. PR proteins, moreover, also represent very interesting and sensitive markers for the search for the signals involved in relaying induction stimuli from the primary site of necrosis to distant sites, where both SAR and PR proteins are induced (Lawton *et al.*, 1996).

Tobacco is still considered as a prototype system for the study of PR proteins, since these proteins were initially identified in tobacco plants that are resistant to infection by the tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). Initially, five families of PR proteins (PR 1-5) were identified; members from each of these families have subsequently been confirmed to have antifungal activity *in vitro* or in transgenic plants. Later, five additional families of PR proteins were classified by Dempsey *et al.* (1998) and a further three families were classified a year later (Kombrink, 1999) (Table 1).

TABLE 1. The thirteen families of pathogenesis-related (PR) proteins identified in tobacco (Kombrink, 1999).

Families	Description
PR-1	Novel 3-D structure; some antifungal activity
PR-2	β-1,3-glucanases
PR-3	Type I and II chitinases
PR-4	Homology to wound-induced *win1* and *win2* gene products; may have chitin-binding activity
PR-5	Related to the salt-induced osmotins of tomato and tobacco and the antimicrobial seed proteins of several cereals; show homology with the super sweet protein, thaumatin
PR-6	Proteinase inhibitors
PR-7	Proteinases
PR-8	Type III chitinases; lysozymal activity
PR-9	Peroxidases
PR-10	Intracellularly localised proteins; low molecular weight (16-18 kDa); novel sequences; 'ribonuclease-like' proteins
PR-11	Type of chitinases
PR-12	Defensins
PR-13	Thionins

PR proteins display very characteristic physiochemical properties that aid in their detection and isolation: i) they are very stable at low pH and remain soluble at low pH, in contrast with most other plant proteins, which are denatured under similar conditions; ii) they are relatively resistant to the action of proteolytic enzymes; iii) they exist predominantly as monomers of rather low molecular mass (8-50 kDa), and iv) they are localised in compartments such as the vacuole, the cell wall and/or the apoplast. Properties i), ii) and iv) are

interdependent, i.e. the high level of resistance of PR proteins to acidic pH and to protease action appear to be adaptations to survive the harsh environments in which they typically occur.

PR proteins from the grape berry, specifically thaumatin-like proteins and chitinases, have also been linked to haze formation in wine (Pocock *et al.*, 2000). These proteins are expressed in grapes even in the absence of stress, pathogenic attack or wounding and are probably involved in fruit development. It has subsequently been shown that a thaumatin-like protein accumulates in healthy grape berries at the onset of ripening (Tattersall *et al.*, 1997), whereas chitinase activity increases steadily during ripening (Robinson *et al.*, 1997). The effect of mechanical harvesting (a commercial form of wounding) on the PR protein levels in grapes and the subsequent wine (Pocock *et al.*, 2000) has also been examined. Mechanical harvesting; coupled with prolonged transport of the wounded fruit; resulted in higher PR protein levels in the juice and wine. This appeared to be due to wounding by the mechanical harvester, that elicited an increase in protein synthesis in the berries.

Most of the PR proteins are considered to be direct antimicrobial defence proteins or enzymes. The hydrolysis of structural components of the fungal cell walls through the actions of glucanases and chitinases in synergism is one of the best studied modes of action of PR proteins. Some PR proteins might also act antimicrobially in an indirect manner by releasing elicitor-active oligosaccharides, by catalysing cross-linking of macromolecules in the cell wall or even via membrane permeabilisation (Stintzi *et al.*, 1993).

β-Glucanases have both direct and indirect antimicrobial activities. The encoding genes are constitutively expressed at low levels, but are also induced upon pathogen attack. These properties contribute to the importance of this glucanases in the plant, specifically during the amplification of a defence response to invading pathogens. In the following sections, β-glucanases as PR-2 class proteins will be discussed in terms of their function and application in pathogenesis.

2.2.2 Substrates for β-glucanases

The occurrence of β-glucans is widespread throughout much of the biosphere, usually in the form of cellulose, which forms a major component of plant cell walls (Simmons, 1994). As β-glucanases hydrolyse β-glucans, it is obvious that information about β-glucans is highly relevant when reviewing β-glucanases, for β-glucanases undoubtedly moderate the amount, structure and function of β-glucans.

The plant β-glucan family consists of homopolymers of *D*-glucose linked in a β-configuration. Some are relatively simple molecules, consisting of linear chains of glucosyl residues joined by a single linkage type, whereas others are more complex and can comprise a variety of linkages in either linear or branched chains (Pitson *et al.*, 1993). Cellulose, for example, is a 1,4-β-glucan, whereas 1,3-β-glucan (referred to as callose) has only 1,3-β-linkages and is found at relatively low concentrations in various tissues in both monocotyledonous and dicotyledonous plants. 1,3-β-Glucan accumulation is developmentally, spatially and environmentally regulated and this component functions in a variety of physiological processes (Simmons, 1994).

Apart from their widespread occurrence in the plant kingdom, β-glucans are also synthesised by many fungi. Fungal glucans can either occur extracellularly or cytoplasmically, but their location in the fungal cell wall is most prominent, contributing to the overall structure of the cell wall (Buck *et al.*, 1986).

A wide range of glucans is found in filamentous fungi, such as *Botrytis cinerea*, ranging from branched β-1,3-, β-1,6-linked polymers, branched α-1,3-, α-1,4-linked polymers and linear α-1,3-linked polymers (Hunsley and Burnett, 1970; De Vries, 1974; Wessels and Sietsma, 1981). The role of fungal β-glucans is diverse, depending on their size, structure, physical and chemical properties and, perhaps most importantly, their location. The primary role of fungal cell wall β-glucans is as structural polymers, assisting in maintaining the rigidity of the cell wall. This is generally achieved in combination with other wall

components, most commonly chitin, but also α-glucans and assorted homo- and heteropolysaccharides (Farkas *et al.*, 1973).

Extracellular fungal-derived β-glucans may have many functions, including being implicated as the cause of induced wilting symptoms and related problems in certain plant species upon fungal infection (Ruel and Joseleau, 1990). Glucans in the extracellular fungal environment, like their cytoplasmic counterparts, are thought to act as reserve materials in some fungi, being metabolised once the readily utilisable carbon sources are depleted (Dickerson *et al.*, 1970). They may also play a role in protecting hyphal cells against dehydration. Encapsulation of the hyphae by extracellular mucilage, largely composed of polysaccharides such as glucans, may prevent the movement of water out of the cell upon desiccation, resulting in a limited loss of cell viability (Pitson *et al.*, 1993).

It is the previously mentioned protective role of glucans that makes them the perfect target for antimicrobial enzymes when protecting the plant against pathogen invasion. β-Glucanases, often together with chitinases, are thought to kill fungal pathogens by thinning the cell wall at the hyphal tip by degrading the chitin and β-glucan. This thinning causes swelling and, ultimately, the bursting and death of the hyphal tip (Arlorio *et al.*, 1992). The fungal hyphal tip is thought to be particularly susceptible to lysis, because its cell wall synthesis involves a delicate balance between β-glucan hydrolysis and synthesis and this balance could be disrupted by the plant β-glucanase activity (Arlorio *et al.*, 1992).

Fungal as well as plant β-glucans also appear to be effective elicitors of defence gene expression and therefore are good candidates for being primary signalling agents. The cell walls of the pathogen and host are in contact with each other during the initial stages of infection and the components derived from these structures thus would be appropriate primary communicators of pathogen attack.

2.2.3 Occurrence of β-glucanases

The β-1,3-glucanases are abundant, highly regulated enzymes that are widely distributed in most plants, fungi, yeasts and bacteria. Although the major interest in β-1,3-glucanases stems from their possible role in the response of plants to microbial pathogens, there is strong evidence that these enzymes are also involved in diverse physiological and developmental processes in uninfected plants. These processes include cell division, microsporogenesis, pollen germination and tube growth, fertilisation, embryogenesis, fruit ripening, seed germination, mobilisation of storage reserves in the endosperm of cereal grains, bud dormancy, as well as responses to wounding, cold, ozone and UVB radiation (Leubner-Metzger and Meins, 1999).

For the purpose of defence against pathogens and particularly fungal pathogens, β-glucanase is expressed constitutively at low levels and secreted to the apoplastic and intercellular spaces of the plant (Kombrink *et al.*, 1988), where it is positioned appropriately for encountering the pathogen. It has been shown that tomato and eggplant tissues infected with a vascular wilt pathogen contain an acidic 1,3-β-glucanase, primarily in the cell walls and in xylem vessels (Benhamou *et al.*, 1989). These acidic β-glucanases were found extracellularly and are thought to have an indirect role against pathogens, being involved specifically in the production of elicitors. Basic β-glucanases, in contrast, are found in the vacuole of the plant cell, where they have a direct inhibitory effect on pathogens (Stintzi *et al.*, 1993).

Yeast glucanases, just like plant glucanases, have also been indicated to have activity against pathogens. In 1998, Jijakli and Lepoivre reported on an exo-β-1,3-glucanase produced by the yeast *Pichia anomala*, an antagonistic yeast of *Botrytis cinerea*, which is the causal organism of grey mould disease of grapevine. It was found that the activity of exo-β-1,3-glucanases is one of the mechanisms of action involved in the suppression of *B. cinerea* by *P. anomala*. A similar discovery was made recently. Masih and Paul (2002) found *Pichia membranifaciens* strain FY-101 isolated from grape skin to be antagonistic to *B. cinerea*. Both these *Pichia* species can potentially be used as biological

control organisms against *B. cinerea*. Segal *et al.* (2002) also recently reported on the cloning and analysis of a secreted 1,3-β-glucanase of the yeast biocontrol agent *Candida oleophila*, supporting the hypothesis that β-glucanases play a very important role in defence against fungal pathogens.

S. cerevisiae has a complex system of glucanases, with several forms differing not only in physiochemical properties and substrate specificities, but also in their fate and appearance during the cell cycle (San Segundo *et al.*, 1993). Processes such as apical growth, branching and budding as well as mating and formation and release of ascospores, require the action of glucanases. Exoglucanases are all extracellular yeast products. Following secretion, they are first located somewhere between the plasma membrane and the surface of the cell wall (Hernández *et al.*, 1986).

Both endo- and exoglucanases are produced during vegetative growth. This is based on the fact that their content doubles in the cell upon transition from the S to the G2 phase. The exo-1,3-β-glucanases represent much more than 50% of the total glucanase activity in vegetative cells (Cenamor *et al.*, 1986). Exoglucanase production is growth-associated, unlike that of the metabolic hydrolases, which usually are subject to catabolite repression (Cenamor *et al.*, 1986). The major form of exoglucanases synthesised constitutively by *S. cerevisiae*, is Exg1p of about 56kD. It is not substrate specific, hydrolysing both 1,3-β- and 1,6-β-linkages. It also has β-glucosidase activity, since it acts on synthetic β-glucosides, such as ρ-nitro-phenol-β-D-glucopyranoside (PNPG) and 4-methylumbelliferyl-β-D-glucosides (MUG) (Farkas *et al.*, 1973).

S. cerevisiae glucanases are also thought to be autolysins, since β-glucan is the most abundant component of the cell wall of this yeast. The finding that both protoplasts and intact cells secrete enzymes possessing endo-β-1,3-glucanase specificities into the growth medium, seems to be of basic importance for cell wall extension and degradation (Farkas *et al.*, 1973). With regard to the chemical structure of the cell wall glucan in *S. cerevisiae*, endo-β-1,3-glucanase seems to play the crucial role in the degrading processes involved in cell wall growth (Larriba *et al.*, 1995). It appears that

endo-β-1,3-glucanases are involved in the slow, but significant, autohydrolysis that proceeds in isolated cell walls of *S. cerevisiae*, since oligosaccharides of laminaribiose series are liberated from the cell walls during autohydrolysis. One of the major proteins of *S. cerevisiae* is an endo-β-glucanase (*BGL2* gene product). It is incorporated into the cell wall and the protein is about 29kD in size (Klebl and Tanner, 1989).

2.2.4 Classification of β-glucanases

The production of β-glucan-degrading enzymes is a characteristic attributed to a wide variety of organisms. Several types of β-glucan-degrading enzymes exist, and they are classified according to the type of β-glucosidic linkage(s) they cleave and their mechanism of substrate attack (Pitson *et al.*, 1993). Stintzi *et al.* (1993) have isolated five distinct members from tobacco (Table 2), four acidic enzymes and a basic enzyme, whereas Meins *et al.* (1992) grouped tobacco 1,3-β-glucanases into three structural classes based on a comparison of their deduced amino acid sequences (Table 3). The acidic enzymes are extracellular (Kaufmann *et al.*, 1987) and are thought to play an indirect role in plant defence by releasing elicitors or by their involvement in SAR in plants (Boller, 1993). The basic counterpart, however, occurs in the vacuoles (Van den Bulcke *et al.*, 1989) and has a direct effect on pathogens, often exhibiting *in vitro* antifungal activity. Similar structural classes have been reported for tomato, potato and other plant species (Oh and Yang, 1995).

The ca. 33 kDa class I enzymes (β-glucanase I) of *Nicotiana tabacum*, which constitute the PR-2e subgroup of tobacco PR proteins, are basic proteins localised in the cell vacuole (Sticher *et al.*, 1992). β-Glucanase I is produced as a preproprotein with an N-terminal hydrophobic signal peptide, which is co-translationally removed, and a C-terminal extension that is N-glycosylated at a single site. The proprotein is transported from the endoplasmic reticulum via the Golgi apparatus to the vacuole, where the C-terminal extension is removed to render the mature, non-glycosylated enzyme (Sticher *et al.*, 1992). Considerable indirect evidence exists that, by analogy with tobacco class I

chitinases (Neuhaus *et al.*, 1991) and barley lectin (Bednarek and Raikhel, 1991), the C-terminal extension contains a signal for targeting to the vacuole (Melchers *et al.*, 1993). Recent results obtained with cultured tobacco cells provide strong evidence that vacuolar class I β-glucanases and chitinases can be secreted into the medium via a novel pathway (Kunze *et al.*, 1998).

TABLE 2. PR-2 family members (β-1,3-glucanases) of tobacco and other *Nicotiana* species (Stintzi *et al.*, 1993).

Class [a]	Member name	Trivial name	Origin [b]	MW (kDa) [c]	Pi	Localisation
I	PR-2e	Glb	Nt (T)	33	Basic	Vacuole
I	PR-2e	Gla	Nt (S)	33	Basic	Vacuole
I	PR-2e	Gglb50	Nt (S)		Basic	Vacuole
I	PR-2e	Gln2	Nt (S)		Basic	Vacuole
I		Gn2 [d]	Np		Basic	Vacuole
I		Gn1 [d]	Np	34	Basic	Vacuole
II	PR-2a	PR-2	Nt	35	Acidic	Secreted
II	PR-2b	PR-N	Nt	35	Acidic	Secreted
II	PR-2c	PR-O	Nt	35	Acidic	Secreted
II		PR-2d	Nt		Acidic	Secreted
II	Stylar β GLU [e]	Sp41a	Nt	41	Acidic	Secreted
II	Stylar β GLU [e]	Sp41b	Nt	41	Acidic	Secreted
III	PR-2d	PR-Q'	Nt	35	Acidic	Secreted
IV	Anther β GLU [e] [f]	Tag1	Nt	35	Acidic	Secreted

(a) Classification according to amino acid sequence identity of the mature proteins.

(b) *Nicotiana tabacum* (Nt); *N. plumbaginifolia* (Np); T and S refer to the *N. tometosiformis* and *N. sylvestris* progenitors.

(c) Approximate molecular weight of mature protein.

(d) Amino acid sequence identity to tobacco βGlu I enzymes of the *N. plumbaginifolia* enzymes is ca. 98% for Gn2, but only ca. 76% for Gn1.

(e) Not induced by pathogens, i.e. a "PR-like protein".

(f) Tag1 is assigned to a new class, since it shares only 37-38% amino sequence identity to Gla, PR-2 and PR-Q' (Leubner-Metzger and Meins, 1999).

The known mature β-glucanase I protein of tobacco and β-glucanase II of *N. plumbaginifolia* share ca. 98% amino acid identity (Gheysen *et al.*, 1990). The β-glucanase I of *N. plumbaginifolia* is structurally more distinct and shares

only 76% identity with the mature β-glucanase I protein. The tobacco β-glucanase I multigene family consists of very similar homologues derived from the *N. sylvestris* and *N. tomentosiformis* progenitors of tobacco, as well as of recombinants of the two progenitor types (Sperisen *et al.*, 1991).

In contrast to β-glucanase I, the class II and III members of the PR-2 family are secreted into the extracellular space (Meins *et al.*, 1992). The tobacco class II β-glucanases PR-2a, PR-2b and PR-2C and the class III β-glucanase PR2-d, which are also known as PR-2, PR-N, PR-O and PR-Q respectively, are acidic proteins without the C-terminal extension that is present in the class I enzymes ranging from ca. 34 to 36 kDa (Beffa *et al.*, 1993). The class II tobacco isoforms are at least 82% identical in amino acid sequence and differ from the class I enzymes at a minimum of 48.8% of the amino acid positions (Meins *et al.*, 1992). Class II also included the two acidic 41 kDa stylar β-glucanase isoforms, Sp41 and Sp41b, which are exclusively expressed in the styles of tobacco flowers. They do not appear to be induced by pathogen infection and, hence, are referred to as "PR-like proteins" (Meins *et al.*, 1992).

The acidic 35 kDa PR-2d (PR-Q) protein is the sole representative of tobacco class III β-glucanases and differs in sequence by at least 43% from the class I and class II enzymes (Domingo *et al.*, 1994). Two highly homologous cDNA clones for class III β-glucanase have been isolated from tomato plants infected with a viroid. Based on deduced amino acid sequences, TomPR-Q'a is an acidic isoform with 86.7% similarity to tobacco PR-Q', whereas TomPR-Q'b is a basic isoform with 78.7% similarity to tobacco PR-Q'.

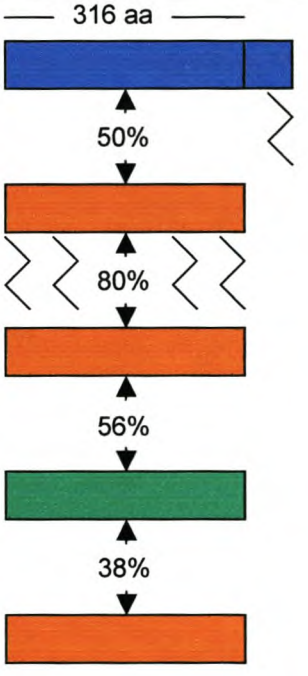
The Tag1 protein appears to represent a novel class of tobacco β-glucanases. It is a "PR-like" protein that is expressed specifically in tobacco anthers (Bucciaglia and Smith, 1994). Tag1 is similar to the tobacco class I β-glucanases and is encoded by a small gene family with at least two members derived from the *N. sylvestris* and *N. tomentosiformis* progenitors of tobacco. On the basis of the deduced amino acid sequence, it is proposed that Tag1 encodes a polypeptide with an N-terminal signal peptide, but no C-terminal extension, suggesting that the protein may be secreted. The mature form of Tag1 is an acidic 35 kDa protein, which shares consensus sequences that are

found in all classes of tobacco β-glucanases. It is 37-38% identical to the sequence of the mature protein of tobacco class I Gla, class II PR-2 and class III PR-Q'. Based on the criteria used earlier to classify tobacco β-glucanases (Meins *et al.*, 1992), Leubner-Metzger and Meins (1999) have assigned Tag1 to a new class, namely class IV of the PR-2 family (Table 2).

The specific enzymatic activities and substrate specificities of different β-glucanases vary considerably. The β-glucanase I and class II PR-2c proteins appear to be 50 to 250 times more active in degrading the β-1,3-glucan substrate laminarin than the class II PR-2a and PR-2b and the class III PR-2d enzymes (Linthorst *et al.*, 1991).

TABLE 3. The tobacco β-1,3-glucanases (PR-2 family), showing the extent of homology between the different members when comparing mature polypeptides (Leubner-Metzger and Meins, 1999).

Class	Member name	Trivial name	MW (kDa)	pI	Homology
I	PR-2e	Gla	33	9.5	316 aa
					50%
II	Stylar βGLU	Sp41a	41	5.7	
					80%
II	PR-2a	PR-2	35	5.2	
					56%
III	PR-2d	PR-Q'	35	5.1	
					38%
IV	Anther βGLU	Tag1	35	4.9	



2.2.5 Mode of action of β-glucanases

β-Glucanases are enzymes that hydrolyse the β-*O*-glucosidic linkages of β-glucan chains, leading to the release of glucose and oligosaccharides. They may be classified as exo- or endo-β-glucanases (Nebreda *et al.*, 1986). Exo-1,3-β-glucanases hydrolyse the β-*O*-glucosidic linkages at the nonreducing end of the polymer chain, leading to the release of glucose. These glucanases are distinguishable from endo-1,3-β-glucanases that attack the linkages at intermediate points of the polymer chain, releasing oligosaccharides. Endoglucanases produce controlled nicks in the glucan structure and exoglucanases processes the tail generated by the endoglucanases (Larriba *et al.*, 1995).

Plant 1,3-1,4-β-glucanases specifically hydrolyse plant cell wall 1,3-1,4-β-glucans in the graminaceous monocotyledons during normal wall metabolism. Plant β-1,3-glucanases specifically hydrolyse the β-1,3-glucans of fungal cell walls. This is because plant 1,3-β-glucanases catalyse the hydrolysis of 1,3-β-glucosyl linkages when several continuous 1,3-β-glucosyl residues are present and not when 1,3-β-glucosyl linkages are interspersed with 1,4-glucosyl residues, as typically occurs in plant 1,3-1,4-glucans (Høj *et al.*, 1988, 1989). A defence strategy involving 1,3-β-glucanase thus seems capable of offering a fairly specific attack against a fungal pathogen without inflicting damage to the cell wall of the plant.

It has been noted that there is no correlation between the level of sequence similarity and the relative catalytic activity of the various enzymes. This raises the problem of differences in substrate specificity between glucanases of a given plant species and also between glucanases of different plant species (Ham *et al.*, 1991).

2.2.6 The pathogenesis-related regulation of β-glucanases

β-Glucanases show developmental regulation and regulation in response to treatment with hormones or infection with pathogens. In general, β-glucanases

are induced in plants infected by viral, bacterial and fungal pathogens. Similarly, elicitors, including fungal glucans, can induce the accumulation of these enzymes (Boller, 1993).

Class I β-glucanase proteins and their mRNAs are induced in TMV-infected leaves of tobacco as part of the local HR response (Meins *et al.*, 1992). Basic PR-2 proteins have also been found to accumulate in and around lesions, as described by Heitz *et al.* (1994). The PR-related class II and class III β-glucanases are induced locally, in TMV-infected leaves, and systemically, in non-infected leaves of the same plant (Côte *et al.*, 1991). The close correlation between systemic induction of class II and class III β-glucanases has led to the use of these genes as markers for SAR (Delaney, 1997). Systemic accumulation of salicylic acid (SA) is associated with the HR of tobacco, *Arabidopsis thaliana*, and certain other plants (Delaney, 1997). Treatment of mature, wild-type tobacco plants with SA, strongly induced the accumulation of mRNAs of PR-related class II and III β-glucanases and certain other PR proteins (Ye *et al.*, 1992), whereas the promoter activity of the class II *PR-2b* and *PR-2d* genes is induced in response to SA. While SA is probably not the long-distance systemic signal for SAR activation, it is required for the transduction of this signal in leaves distal from the primary infection site (Delaney, 1997). Transgenic tobacco plants failing to accumulate significant amounts of SA, for example, are unable to develop an SAR and do not provide the plant with pathogen resistance distal from a primary infection site.

2.3 APPLICATION OF β-GLUCANASES IN THE UPREGULATION OF DISEASE RESISTANCE IN PLANTS

2.3.1 The principle of upregulated disease resistance in plants

Utilisation of a complex array of cues for defence gene activation may facilitate the flexible deployment of the battery of defence responses in several rather different biological circumstances. The rapid stimulation of defence gene transcription following the perception of an appropriate signal has been

observed in many systems. This defence gene transcription had markedly different patterns of gene activation in resistant plants compared to susceptible plants (Dixon and Harrison, 1990). This provides a conceptual basis for the initial development of strategies to engineer enhanced resistance to microbial attack by gene transfer.

One strategy is to express, in a constitutive manner, defence genes that are normally only induced as a result of pathogen attack. The most attractive initial candidates for this approach are genes encoding chitinases and β-glucanases that form part of the primary induced responses. Since single genes individually encode these active antimicrobial agents, these defence systems could be targeted for manipulation by gene transfer (Lamb *et al.*, 1992).

The suggestion therefore is to transfer the microbial genes responsible for disease control directly into the plant as a way to overcome the often troublesome and unpredictable application of the whole organism in biological control strategies. The real challenge of gene transfer or genetic engineering is, firstly, how to get the new DNA into the cell and integrate it stably and, secondly, designing constructs that will ensure good levels of expression of the foreign gene product. The latter is particularly relevant in the case of engineering resistance to pathogens, as most pathogens infect specific areas of host cells or tissues. It therefore is of great importance that any defence product is present in the optimum place to inhibit pathogen growth. For instance, it will be ineffective to accumulate an antimicrobial protein in the cell vacuole when the microbe concerned is multiplying in the intercellular spaces.

2.3.2 Application of β-glucanases in genetic transformation schemes

β-Glucanases and chitinases are the foundation of most biological control systems, although a full-scale application of fungal biocontrol agents in commercial agriculture has been delayed because of the inconsistent results obtained by introducing these sensitive microorganisms into the ever-changing environment. There is strong evidence that the expression of β-glucanase

transgenes, alone or in combination with chitinase-encoding genes controlled by strong constitutive promoters, can reduce the susceptibility of plants to infection by certain fungi (Lamb *et al.*, 1992).

Transgenic tobacco plants expressing a soybean β-1,3-glucan elicitor releasing β-glucanase or the tobacco class II β-glucanase PR-2b, show reduced symptoms when infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Peronospora tabacina* (Yoshikawa *et al.*, 1993). β-1,3-Glucans are the major components of the cell walls of oomycetes, a group of fungi that do not contain chitin (Wessels and Sietsma, 1981). In many cases, a pronounced synergistic effect is obtained when β-glucanase and chitinase transgenes are expressed in combination. Tomato plants expressing tobacco class I β-glucanase and chitinase transgenes show reduced susceptibility to infection by *Fusarium oxysporum* sp. *lycopersici*, whereas the expression of either gene alone is not effective (Jongedijk *et al.*, 1995). Sela-Buurlage *et al.* (1993) transformed tobacco plants with transgenes encoding modified class I tobacco β-glucanases and chitinases that are targeted for secretion. They found that the extracellular fluid from the leaves of plants expressing both β-glucanases and chitinases, showed strong antifungal activity against *F. solani*, whereas this effect was less for plants expressing either transgene alone. Alfalfa plants expressing both alfalfa *Aglu1* acidic β-glucanase and rice *Rch10* basic chitinase transgenes showed reduced disease symptoms when infected with the oomycetes pathogen *P. megasperma* sp. *medicaginis*, which does not contain chitin in its cell walls, whereas no reduction in symptoms was observed with several chitin-containing fungal pathogens (Masoud *et al.*, 1996). Therefore β-glucanases conferred resistance.

The induction of β-glucanases as part of the hypersensitive reaction is a stereotypic response, i.e. the pattern of induction is similar for viral, bacterial and fungal pathogens. Although antifungal β-glucanase I appears to be tailored for defence against fungi, recent studies of β-glucanase I-deficient mutants generated by antisense transformation suggest that these enzymes also play an important role in viral pathogenesis. These mutants showed greatly reduced levels of β-glucanase I. It was also found that a novel intracellular form of

β-glucanase, that is serologically distinct from any of the known tobacco β-glucanases, is induced by virus infection in β-glucanase I-deficient plants, but not in wild type plants. Thus, plants can compensate for a deficiency in enzyme activity by producing a functionally equivalent replacement. The fact that compensation occurred specifically in response to virus infection suggests that β-glucanases play an important role in pathogenesis (Beffa *et al.*, 1993; Ham *et al.*, 1997).

Unexpectedly, the β-glucanase I-deficient mutants showed markedly reduced lesion size, lesion number and virus yield. It was found that callose deposition in and around virus-induced lesions is increased in β-glucanase I-deficient tobacco, suggesting that decreased susceptibility to the virus resulted from increased callose deposition in response to infection. Callose deposition is known to act as a physical barrier to the spread of a virus. These findings are of particular interest, because they suggest a novel means, based on antisense transformation with host genes, for protecting plants against viral infection (Beffa *et al.*, 1996). The intriguing possibility is also raised that viruses can use a defence response of the host against fungal infection (production of β-glucanase I) to promote their own replication and spread.

2.3.3 Gene silencing

Since β-glucanase genes are so tightly regulated, they could be susceptible to a phenomenon called post-transcriptional gene silencing. This phenomenon has been observed in transgenic plants overexpressing a foreign β-glucanase gene. The interaction between transgenes and host genes with similar sequences frequently leads to trans-inactivation of expression at the mRNA level. Once established, the inactive (silent) state is stable: it can persist in vegetatively growing plants and, in some cases, is meiotically transmitted to the progeny. Nevertheless, silenced genes can return to an expressed state, indicating that the stable changes are potentially reversible and, hence, a form of epigenetic modification (Meins, 1996). It is now recognised that this phenomenon, called homology-dependent gene silencing (HDGS) (Meyer and Saedler, 1996),

occurs generally in plants. Little is known about the mechanism for HDGS or its functional significance. Evidence suggests that HDGS can also help protect the plant against virus infection (Covey *et al.*, 1997).

There is no doubt that the gene silencing phenomenon hampers the general exploitation of economically important crops that are resistant to the major pathogens causing crop losses (De Neve *et al.*, 1999). The different aspects of the generation of transgenic plants that influence the likelihood of activating transgene silencing include the integration site of the gene, the plant species and transformation method, the growth conditions, the copy number of the transgene and the construct design (Meins, 1996).

Although it is not yet possible to completely eliminate transgene silencing, the careful design of transformation vectors, the choice of transformation technique and the selection of transformants based on their characteristics at the molecular level should drastically reduce the number of transgenic plants that turn out to be sensitive to transgene silencing (De Wilde *et al.*, 2000). Silencing has not evolved as a mechanism to regulate or inactivate transgene expression, but probably is the reflection of natural plant processes, such as the regulation of gene expression of multigene families or the interaction with parasitic sequences, including viruses, transposable elements and viroids. Hence, it is possible that the study of these processes will reveal how to overcome silencing. It therefore has not only been discovered that plants can use gene silencing to respond to the foreign nucleic acids of viruses (Kooter *et al.*, 1999), but also that viruses, in turn, have evolved their own counter-defence, namely the suppression of gene silencing (Smyth, 1999).

In conclusion, the application of β-glucanases in genetic transformation schemes seems to be a viable and practical option. Although glucanases are notorious for gene silencing in a transgenic environment, careful planning and further investigation might resolve this problem.

The fact that they are directly involved in the degradation of fungal cell walls, together with the fact that they already are part of the plant's innate defence system, renders this enzyme of great importance in future transgenic studies.

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CHAPTER 3

RESEARCH RESULTS

The yeast *EXG1* gene product confers antifungal activity against *Botrytis cinerea*, but is silenced in transgenic tobacco lines when the exoglucanase is targeted to the apoplastic space

Data from Chapter 3 will form part of an article
to be submitted for publication in
Transgenic Research

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The yeast *EXG1* gene product confers antifungal activity against *Botrytis cinerea*, but is silenced in transgenic tobacco lines when the exoglucanase is targeted to the apoplastic space

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ABSTRACT

An exoglucanase from *Saccharomyces cerevisiae*, encoded by *EXG1*, inhibits the fungal pathogen, *Botrytis cinerea*, *in vitro*. To evaluate the possible application of *EXG1* in the genetic transformation of grapevine and other economically important crops, the exoglucanase was studied in tobacco. The *EXG1* gene construct (pEXG1), containing the phosphoglycerate kinase I (*PGK1*) promoter and terminator, was introduced into tobacco via *Agrobacterium* transformation. A fusion between *EXG1* and the signal peptide from *Mirabilis jalapa*, *MJ*, which directs the protein to the apoplastic region of the plant cell, was also constructed and introduced into tobacco (pMJ-EXG1). Southern blot and PCR analysis of the resulting transgenic tobacco lines confirmed the integration of both constructs. Northern blot analysis confirmed the transcription of the *EXG1* gene construct, whereas no transgene expression was observed for the MJ-EXG1 gene construct. Crude proteins of both transgenic lines were extracted and analysed for exoglucanase activity in a β-glucosidase assay. A microtiter bioassay was conducted for both transgenic lines to measure inhibition against *Botrytis cinerea*. EXG1 and MJ-EXG1 *in vitro* transgenic tobacco plant leaves were also challenged with *Botrytis cinerea* spores in an infection study in which lesion size and type were compared to the control tobacco plants. These assays revealed an average inhibition of 20% and a reduction in disease susceptibility as high as 65% in the EXG1 transgenic lines to *Botrytis cinerea*. However, no significant β-glucosidase activity or inhibition against *B. cinerea* spores could be detected for the MJ-EXG1 transgenic plant lines. We propose a gene silencing effect in the above-mentioned case.

Keywords: *Saccharomyces cerevisiae* – exoglucanase – transgenic – inhibition - gene silencing

3.1 INTRODUCTION

The possibility to genetically transform plant species has been linked to a host of possible outcomes, but one of the main focuses has been manipulated disease resistance. Studies on defence responses in plants have focused mainly on the endohydrolases that exhibit β-1,3-glucanase and chitinase activities. β-Glucans and chitin, the substrates for these enzymes, are common components of the surface structures of pathogens and pests. β-Glucans are major cell wall constituents of common fungal pathogens (Bowles, 1990), whereas chitin is an abundant product of the microbial walls and the exoskeleton of insects. There is evidence that the action of these two endohydrolases has detrimental effects, such as the inhibition of hyphal growth (Mauch *et al.*, 1988) and the probable release of signalling molecules (β-glucans) that activate defence genes (Okinaka *et al.*, 1995). Extended studies have also confirmed that plant and fungal glucanases and chitinases are excellent candidates for manipulated disease resistance and/or biological control (Mauch *et al.*, 1988; Zhu *et al.*, 1994; Jongedijk *et al.*, 1995; Masih and Paul, 2002).

The demonstration that activation of defence gene transcription underlies the expression of many inducible protective mechanisms suggests a direct strategy for engineering enhanced natural resistance through the constitutive expression of normally inducible defences (Lamb *et al.*, 1994). Since the endohydrolases are individually encoded by single genes, these defence systems should be highly amenable to manipulation by gene transfer.

The yeast *Saccharomyces cerevisiae* produces a variety of enzymes that exhibit antifungal activity, including glucanases and chitinases (San Segundo *et al.*, 1993). To evaluate the potential and limitations of yeast glucanases as possible antifungal agents, an exo- and endoglucanase from *S. cerevisiae* were evaluated *in vitro* for their activity against the fungal pathogen, *Botrytis cinerea.* This led to the selection of the exoglucanase for strong expression in the cytoplasm and in the apoplastic space of transgenic tobacco plants as a model

system. Transgenic lines harbouring the relevant constructs were evaluated for their ability to combat infection by *B. cinerea*.

In this article, we demonstrate the *in vitro* and *in planta* antifungal activity of yeast exoglucanases and the possible phenomenon of gene silencing when the exoglucanase is targeted to the apoplastic area of the plant cells.

3.2 MATERIALS AND METHODS

3.2.1 Strains and culture conditions

All microbial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) medium (0.6% yeast extract, 1.2% tryptone and 1.2% sodium chloride) or in LB supplemented with the appropriate antibiotic for the selection of transformants or to retain selective pressure. All media components listed refer to weight per volume (w/v) of the medium, unless indicated otherwise. *Saccharomyces cerevisiae* strains were grown at 30°C in SCD medium (2% glucose, 0.67% yeast nitrogen base) supplemented with the amino acids histidine, lysine, tryptophan and uracil. The *Agrobacterium tumefaciens* strain was routinely cultured in YEP medium (1% yeast extract, 1% peptone and 0.5% sodium chloride) at 28°C.

A sporulating culture of *Botrytis cinerea* was obtained from the Department of Plant Pathology, Stellenbosch University. The spores were germinated and inoculated on apricot halves canned in natural syrup to sustain a high level of virulence. Prior to inoculation, the apricot halves were washed with sterile water, dried on sterile paper towels and placed inside sterile tissue culture petri dishes (100 mm x 20 mm). Each apricot half was inoculated with a few spores of *B. cinerea* and incubated in the dark at 25°C until sporulation. Spores were harvested with a bent glass rod and 3 ml of sterile water containing Tween 20 (90 µl/l). The spore concentration was determined with a haemocytometer for subsequent applications (Coertze and Holz, 2002).

TABLE 1. Strains and plasmids used in this study

Strains or plasmid(s)	Genotype or description	Source or reference
***E.coli* strains**		
DH5 α	supE44 lacU169 [*Φ80 lac*ZM15 *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
***B. cinerea* strain**	A highly virulent strain isolated in the Stellenbosch area from grapevine	Department of Plant Pathology, Stellenbosch University
Yeast strains		
FY 834	*MAT*α *his3leu2lys2trp1ura3*	Winston *et al.*, 1995
Plasmids		
pSP73	pGEM based multiple cloning vector	Promega
pGEM-T Easy	pGEM5Zf (+) based PCR cloning vector	Promega
pHVX2	YEplac-based plasmid; Ap^R; containing PGK1 promoter and terminator	Volschenk *et al.*, 1997
pART7	pGEM9Zf (+) based primary cloning vector	Gleave, 1992
pART27	pGEM9Zf (+) based binary vector	Gleave, 1992
pEX192	pUC19-based plasmid; Ap^R; 3.95 kb *S. cerevisiae* genomic insert, comprising the exoglucanase (*EXG1*) gene	Van Rensburg *et al.*, 1997
pSCB219	pUC19-based plasmid; Ap^R; 2.7kb *S. cerevisiae* genomic insert, comprising the endoglucanase (*BGL2)* gene	Van Rensburg *et al.*, 1997
pFAJ3068	A plant expression vector; Kan^R, driven by Nos promoter; *Hs-AFP1*, driven by the enhanced 35S promoter and fused to *M. jalapa* signal peptide encoding sequence	Broekaert (F.A Jansens Laboratory of Genetics, Catholic University of Leuven, Belgium)
pSP/*EXG1*	1.3 kb pEX192 PCR fragment in pSP73	This study
pART7/*EXG1*	1.3 kb pSP/EXG1 fragment in pART7	This study
pART27/*EXG1*	3.5 kb pART7/EXG1 *Not*I fragment in pART27	This study
pGEM / *EXG1-MJ*	0.94 kb pEX192 PCR fragment in pGEM-T easy	This study
pFAJ/*EXG1-MJ*	0.94 kb pGEM/EXG1-MJ *Sac*I fragment in pFAJ/MJ	This study
pGEM/*MJ*	0.16kb pFAJ3068 PCR fragment in pGEM-T easy	This study
pHVX/*EXG1*	1.3 kb pEX192 PCR fragment in pHVX2	This study
pHVX/*BGL2*	0.939 kb pSCB219 PCR fragment in pHVX2	This study

3.22 Construction and transformation of yeast and plant cassettes

Standard techniques for DNA cloning were performed according to Sambrook et al., (1989). Restriction enzymes and PCR amplification reagents were obtained from Roche Diagnostics and used according to the supplier's recommendations. T4 DNA ligase and the pGEM-T easy™ kit were obtained from Promega. Sequencing was done by the DNA Sequencing Facility, Department of Genetics, Stellenbosch University, using an ABI Prism 377 automated DNA sequencer from PE Biosystems. All PCR primers used and their application are listed in Table 2.

TABLE 2. Primer pairs and sequences used in this study

Primer	Sequence	Paired with	Template	Product
exg1-F (*Bam*HI)*	5'-TAG CGG ATC CAC CAA CTA AAA TGC TTT C-3'	exg1-R	pEX192	1.3 kb of *EXG1*
exg1-R (*Hin*dIII)*	5'-TAG CAA GCT TTT GAG GGC GACT TAG TTA G-3'	exg1-F		
exg1-mj (*Sac*I)*	5'-GGC CGA GCT CCT TTC GCT TAA AAC-3'	exg1-R	pEX192	1.3 kb of *EXG1* without ATG
bgl2-L (*Eco*RI)*	5'-GAT CGA ATT CAG ATG CGT TTC TCT ACT ACA-3'	bgl-R	pSCB219	940 bp of *BGL2*
bgl2-R (*Xho*I)*	5'-GAT CTC TAG AGT TCA TGA AAA GTC ACA GTC-3'	bgl-L		
mj-F (*Xho*I)*	5'-GGC GCT CGA GTA TTT TTA CAA CAA TTA CCA AC-3'	mj-R	pFAJ3068	160 bp of *MJ*
mj-R (*Sac*I)*	5'-GGC CGA GTC TTG CTT CTA GCA TGC CGG ACA TGG-3'	mj-F		

* Restriction sites included in primer sequences are underlined

PCR reactions were performed in 50 μl reaction mixtures, typically consisting of 1x Expand High Fidelity PCR Buffer without $MgCl_2$, 10 mM dNTPs, 12.5 μM of each primer, 5 ng template DNA and $MgCl_2$ (added to the optimal concentration). Reactions proceeded in a Biometra Trio-thermoblock cycler.

The yeast exo- and endoglucanase genes were PCR amplified from pEX192 and pSCB219 respectively. The primer pairs used are indicated in

Table 2; the amplification programme included 34 cycles of denaturation at 94°C for 2 min, annealing at 60°C for 1 min and elongation at 72°C for 2 min. The resulting PCR products were digested and cloned, under control of the *PGK1* promoter and terminator, into the *Eco*RI/*Xho*I sites of the yeast episomal plasmid, pHVX2 (Fig.1). The resulting recombinant plasmids, designated pHVX/*EXG1* and pHVX/*BGL2* respectively, were verified by sequencing and transformed into *S. cerevisiae* strain FY834 (leucine marker) with the LiAc-TE method according to Sambrook *et al.* (1989). The transformed yeast strains were subsequently used for *in vitro* antifungal activity assays against *B. cinerea*, utilising a time-course microscopic antifungal assay.

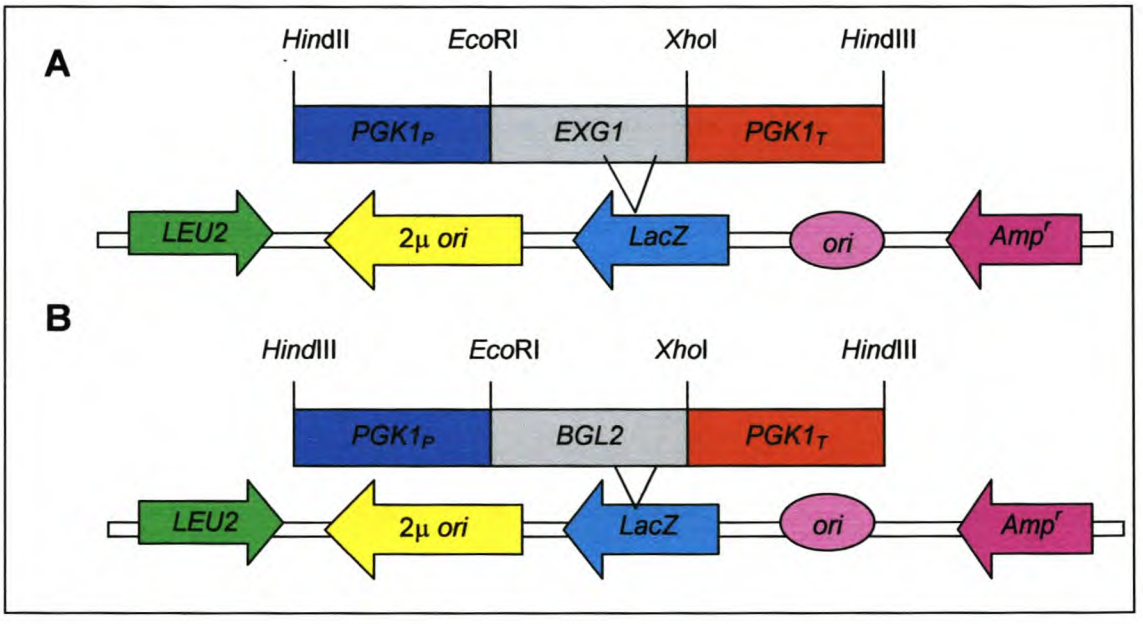


FIGURE 1: The yeast expression cassettes containing the exoglucanase (**A**) and the endoglucanase (**B**) from *Saccharomyces cerevisiae*. The abbreviations represent the following: ***EXG1***, Mature protein-encoding domain of *EXG1*; ***BGL2***, Mature protein-encoding domain of *BGL2*; ***PGK1*$_P$**, Phosphoglicerate kinase I promoter; ***PGK1*$_T$**, Phosphoglicerate kinase I terminator; **2µ *ori***, 2µ plasmid origin of replication; ***ori***, origin of replication; ***LEU2***, leucine marker; ***LacZ***, β-galactosidase encoding gene and ***Amp*^{r}**, ampicillin resistance marker.

Based on the results from the antifungal activity assays with the recombinant yeasts, the *EXG1* gene was mobilised into plant expression vectors. All the plant expression vectors that were constructed relied on kanamycin selection in *E. coli*, *Agrobacterium tumefaciens*, as well as in tobacco. The yeast *EXG1* gene was PCR amplified (1.3 kb) from pEX192 with primer pairs exg1-F and

exg1-R and subcloned into the *Bam*HI/*Hin*dIII sites of pSP73, yielding pSP/*EXG1*. The *EXG1* gene was liberated from this construct by digestion with *Cla*I and *Hin*dIII and subcloned into the corresponding sites of pART7, rendering pART7/*EXG1*. The latter was subsequently digested with *Not*I to release an *EXG1* cassette for subcloning into the binary vector, pART27, to yield the plant transformation vector, pART27/*EXG1* (Fig. 2).

The *EXG1* gene was also subcloned as a fusion product with a signal peptide from *Mirabilis jalapa* (Mj-Amp2) to yield a second plant transformation vector, pFAJ/MJ-EXG1 (Fig. 2). The signal peptide was obtained from pFAJ3068 and PCR amplified with primers mj-F and mj-R. The *Xho*I/*Sac*I-digested PCR product (0.16 kb) was cloned into the corresponding sites of pGEM-T easy and was designated pGEM/MJ. *EXG1*, lacking an ATG, was obtained from pEX192 by the same PCR procedures used to obtain *EXG1*, except for the use of a modified 5' primer with an additional *Sac*I site. The PCR product (1.3 kb) was cloned into the *Sac*I site of pGEM/*MJ* and the vector was designated pGEM/MJ-EXG1. The latter was digested with *Xho*I and *Hin*dIII and cloned back into pFAJ3068 to yield pFAJ/MJ-EXG1. Both plant transformation vectors were verified by sequencing.

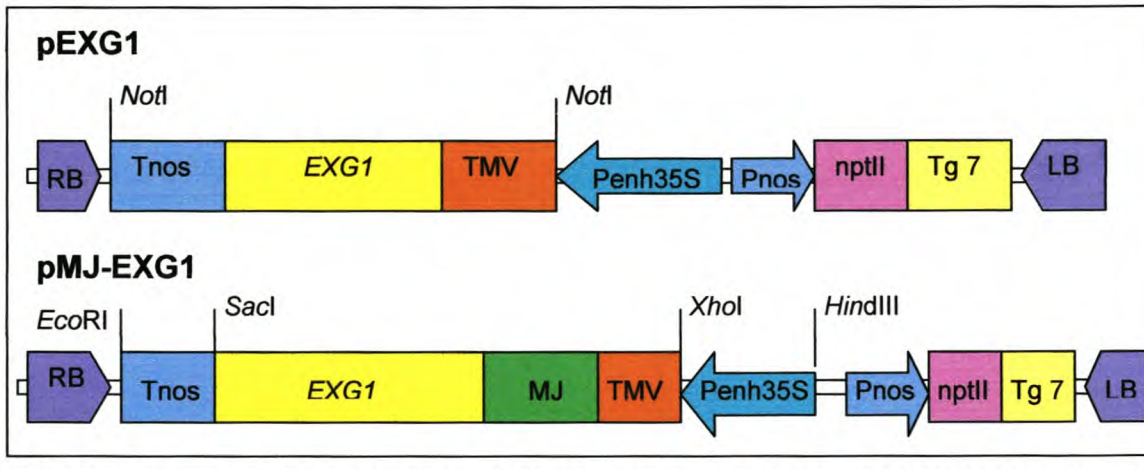


FIGURE 2: The plant expression cassettes used in tobacco transformation with an exoglucanase from *Saccharomyces cerevisiae*. The abbreviations represent: **RB**, T-DNA right border; **LB**, T-DNA left border; **TMV**, Tobacco mosaic virus 5' leader sequence; **Penh35S**, Promoter of 35S RNA of cauliflower mosaic virus with duplicated enhancer region; **Pnos**, Promoter of T-DNA nopaline synthase gene 7; **Tnos**, Terminator of T-DNA nopaline synthase gene; ***EXG1***, Mature protein- encoding domain of ***EXG1***; **MJ**, Signal peptide-encoding domain of Mj-AMP2 cDNA.

3.2.3 Analysis of recombinant *S. cerevisiae* for activity against *B. cinerea*

To assess the antifungal activity of the *S. cerevisiae* endo- and exoglucanase against *B. cinerea*, the respective recombinant yeasts were grown overnight in 50 ml of selective media. Cells were centrifuged and 15 ml of the supernatant were collected. Approximately 2000 *B. cinerea* spores in 100 µl half strength potato dextrose broth (PDB) (Difco) were incubated together with 50 µl of supernatant. The control consisted of 2000 spores in 100 µl half strength PDB, together with 50 µl of supernatant of yeast transformed with the backbone vector (pHVX2). The samples were incubated in the dark at 25°C for three days. Microscopical photographs were taken every 24 hours for each sample, under 100X or 400X magnification, to assess the effect of the glucanases on the morphology of *B. cinerea*.

3.2.4 Triparental mating and preparation of *Agrobacterium tumefaciens* for tobacco transformation

Tri-parental mating was done according to the methods described by Horsch *et al.* (1988). The subsequent *Agrobacterium tumefaciens* EHA105 transformants, EHA105/*EXG1* and EHA105/*MJ-EXG1*, were verified by PCR analysis. A single *A. tumefaciens* colony that had been confirmed to contain the specific plant expression vector, was inoculated into 5 ml of LB containing 0.1% glucose, 30 µg/ml rifampicin, 50 µg/ml streptomycin and 50 µg/ml kanamycin and grown overnight at 28°C. One millilitre of this culture were inoculated into 50 ml of LB (0.1% glucose and no antibiotics) and incubated at 28°C until OD_{600} = 1. The culture was centrifuged at 5000x g for 10 min and the pellet was resuspended in 50 ml of LB containing 0.1% glucose and used for the subsequent transformation procedures.

3.2.5 Tobacco transformation and plantlet regeneration

Tobacco (*Nicotiana tabacum*, petit Havana, SR1) leaf discs were transformed with the above-mentioned *A. tumefaciens* transformants according to Horsch *et al.* (1988). Shoots were selected on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1 µg/ml N^6-BA, 0.1 µg/ml α-naphthalene acetic acid, 400 µg/ml Claforan and 120 µg/ml kanamycin. Resistant shoots were transferred to MS medium with 400 µg/ml Claforan and 120 µg/ml kanamycin for rooting. The resulting transgenic tobacco plantlets were sub-cultured to maintenance medium comprising 15 mg/ml sucrose, 10 µg/ml thiamin HCl, 100 µg/ml myo-inositol, 1 µg/ml nicotinic acid and pyridoxin HCl and maintained at 25°C under a 16 hour light cycle. All media prepared had a pH of 5.8 adjusted with 1 M KOH. These primary *in vitro* cultures were used for all subsequent analyses of the *EXG1* and *MJ/EXG1*-containing transgenic tobacco lines; these lines were designated E and MJE respectively.

3.2.6 PCR and southern blot analyses of transgenic tobacco lines

To confirm integration of the transgenes, southern blot analyses were performed on the E lines, whereas gene integration was confirmed on the MJE lines with PCR.

For the southern blot analyses, genomic DNA was extracted from 40-50 mg of tobacco leaf tissue using a protocol by McGarvey and Kaper (1991) that was modified slightly. The extraction buffer consisted of 3% CTAB (cetyltrimethylammoniumbromide), 1.4 M NaCl, 0.02 M EDTA (pH 8.0) and 1M Tris-HCl (pH 8.0). DNA (20 µg) was digested with *Hin*dIII, separated on a 0.8% agarose gel, and transferred by capillary transfer to a Hybond-N nylon membrane following the manufacturer's instructions (Amersham Pharmacia Biotech). Probe DNA (*EXG1* PCR product) was PCR-labelled using the 10× DIG dNTP labelling mixture (Roche Diagnostics) according to the manufacturer's specifications. Hybridisations were performed at 42°C for 19 hours using the standard DIG hybridisation buffer supplemented with 50% (w/v)

formamide (Roche Diagnostics). Signal detection proceeded according to the manufacturer's specifications. The *Hin*dIII enzyme digests once in the *EXG1* coding sequence, therefore every two hybridisation signals detected represented a single gene copy.

Genomic DNA from the putative transformants was used, together with primers mj-F and exg1-R (Table 2), in a PCR analysis to confirm the positive integration of the pMJ-EXG1 cassette. Reactions proceeded in a Biometra Trio-thermoblock cycler.

3.2.7 RNA isolation and analyses from transgenic lines to confirm gene expression

Northern blot analyses were performed on all the transgenic lines to confirm the gene expression of the transgenes.

For the northern blot hybridisation, the total RNA was isolated from 50 mg of leaf tissue according to the TRIzol reagent protocol (GIBCO-BRL). RNA (30 μg) was separated on a formaldehyde gel (Davies *et al.*, 1986) and transferred by capillary transfer to a Hybond-N nylon membrane following the manufacturer's instructions (Amersham Pharmacia Biotech). Probe DNA (*EXG1* PCR product) was DIG-labelled as for the southern blot. Hybridisation proceeded at 45°C. Detection was performed according to the manufacturer's protocol (Roche).

RT-PCR analysis was performed on 4 of the MJE transgenic plant lines to verify the results obtained with the northern blot analysis done on these plants. RNA was isolated and verified for integrity on a formaldehyde gel according to the methods described above. The RT-PCR reactions were executed using the *EXG1*-specific primers, exg1-mj and exg1-R (Table 2). The *C. therm* one-step RT-PCR kit (Roche) was used according to the manufacturer's guidelines. cDNA synthesis was performed at 50°C for 30 min, followed by a PCR reaction; denaturation occurred at 94°C for 30 sec. The annealing temperature was 55°C for 30 min, with elongation at 72°C for 2 min. The programme was run for 30 cycles. Reactions proceeded in a Biometra

Trio-thermoblock cycler. The PCR products were separated on a 1% (w/v) agarose gel.

3.2.8 Protein isolation

Crude protein extracts were prepared from the different transgenic tobacco lines. Two or three grams of young leaf tissue were ground to a fine powder in the presence of liquid nitrogen. The tissue was homogenised (Polytron 450) in 1 ml of ice cold extraction buffer (50 mM phosphate buffer pH 7.0, 5 mM β-mercaptoethanol, 5 mM EDTA). The samples were centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was transferred to clean Falcon tubes and stored overnight at -20°C. After thawing on ice, the samples were centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was transferred to clean Sterilin tubes and stored at 4°C. The crude protein extracts were dialysed overnight against water and freeze-dried. The proteins were weighed and dissolved in dH_2O to a final concentration of 10 mg/ml.

3.2.9 β-Glucosidase activity assay

In an effort to determine if the *EXG1* gene product was still active, crude protein samples were isolated from the E and MJE transgenic tobacco lines (as previously described) and subjected to a β-glucosidase activity assay, which provides a direct indication of exoglucanase activity. The total crude proteins were concentrated with 30 kDa cut-off Micron™ columns (Millipore) and quantified using the Bradford method (Bio-Rad). Crude protein was added to the wells of microtiter plates (96-well plates from Falcon) in a 50 µl reaction to a final concentration of 5 µg/ml. The reaction contained 4 mM ρ-nitrophenyl-β-D-glucopiranoside as substrate and 0.1 M NaOAc (pH 5.5 buffer). The assay was prepared in six parallels. The samples were incubated at 37°C for 1 hour. The reaction was stopped by adding 100 µl of 1 M Na_2CO_3 to the reaction mixture. The OD_{405nm} of all the samples was determined with a microtiter plate reader (PowerwaveX, Bio-Tek Instruments Inc.). The reading at

time zero served to normalise the samples. The corrected absorption values were converted to µM ρ-nitrophenyl released per hour using a ρ-nitrophenyl standard curve (not shown). One unit of crude protein activity is defined as nM ρ-nitrophenyl released per hour by 1 µg of crude protein extract.

3.2.10 Microspectrophotometric assay of fungal inhibition in transgenic tobacco plants

The crude protein extracts from the E and MJE transgenic tobacco lines were analysed for their antifungal activities against *B. cinerea* using a quantitive spectrophotometric inhibition assay (Broekaert *et al.*, 1990). Spores of *B. cinerea* were harvested and counted in a hematocytometer. They were added to the wells of microtiter plates (96-well plates from Falcon) to yield a final concentration of approximately 5000 spores/µl in a final volume of 100 µl, containing 50 µl of growth medium as used for pre-germination, 20 µl of substrate (half-strength PDB) and crude protein extracts at a concentration of 5 mg/ml protein. Each assay was prepared in six parallels, permitting a maximum number of 36 repetitions per plate. The mixtures were equilibrated in the wells for 1 h at 25°C before the first reading (time=0) was taken at A_{595}. The plates were further incubated at 25°C and absorbance (A_{595}) was measured at intervals of 24 until 48 h after inoculation, using a programmed scanner and averaging six successive wells per sample. The microplate readings were performed on the PowerwaveX microplate reader (Bio-Tek Instruments Inc.). The activity of the different crude protein extracts was expressed in terms of percentage growth inhibition, which is defined as

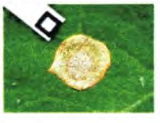
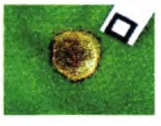
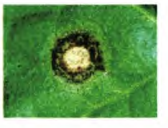
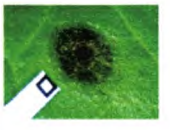
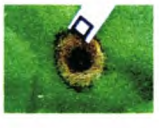
$$100 \times \frac{\text{Corrected A595 Control} - \text{Corrected A595 Sample}}{\text{Corrected A595 Control}}$$

The percentage inhibition of each of the different crude protein extracts was plotted against the others.

3.2.11 *Botrytis cinerea* infection studies

The E and MJE *in vitro* plant lines were subjected to *B. cinerea* infection studies. The top three fully grown leaves from each plant were transferred to Magenta pots containing water agar (8 g l^{-1}). Each leaf was inoculated with two spots of 5000 *B. cinerea* spores, suspended in 100% pure grape juice. The leaves were incubated at 22°C under a 16 h light/8 h dark cycle for three weeks.

TABLE 3. The lesion-type index used in the *B. cinerea* 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 lesion types, photographs were taken after 48 hours and a lesion type index was drawn up. The scale proceeds from the most resistant phenotype (type 1) to the most susceptible (type 10).

Photo	Lesion description	Photo	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.

The disease symptoms were scored after 48 hours and 120 hours by measuring the lesion size. The lesion types were also observed for each transgenic and control tobacco line. The appearance of the lesions was used to establish a lesion-type index with a ten-point scale that is presented in Table 3. The lesion size data were used to draw up a disease resistance index of all the transgenic tobacco lines.

3.3 RESULTS

3.3.1 Microscopic analyses of *B. cinerea* inhibition by the exo- and endoglucanase from *S. cerevisiae*

The antifungal activity of the different glucanases was clearly visible from the microscopical analysis. The exoglucanase Exg1p shows effective inhibition of *B. cinerea* due to hyphal tip degradation and the leakage of the cell material. It also inhibits the elongation of the hyphae, leading to a restriction in the spread of the hyphae (Fig. 3A-C). The endoglucanase Bgl2p, in contrast, had no significant effect on fungal hyphae morphology (Fig. 3E) and the sample containing this glucanase showed very similar hyphae growth to that of the control (Fig. 3D).

3.3.2 Tobacco transformation and regeneration

The plant expression cassettes, pEXG1 and pMJ-EXG1, were successfully mobilised into *N. tabacum* SR1, yielding 20 putative transgenic lines of each, designated E and MJE respectively. The *EXG1* construct was developed to study the *in planta* activity of Exg1p under the native signal peptide. The MJ-EXG1 construct was developed to study the *in planta* activity of Exg1p when directed to the apoplastic region of the plant cell.

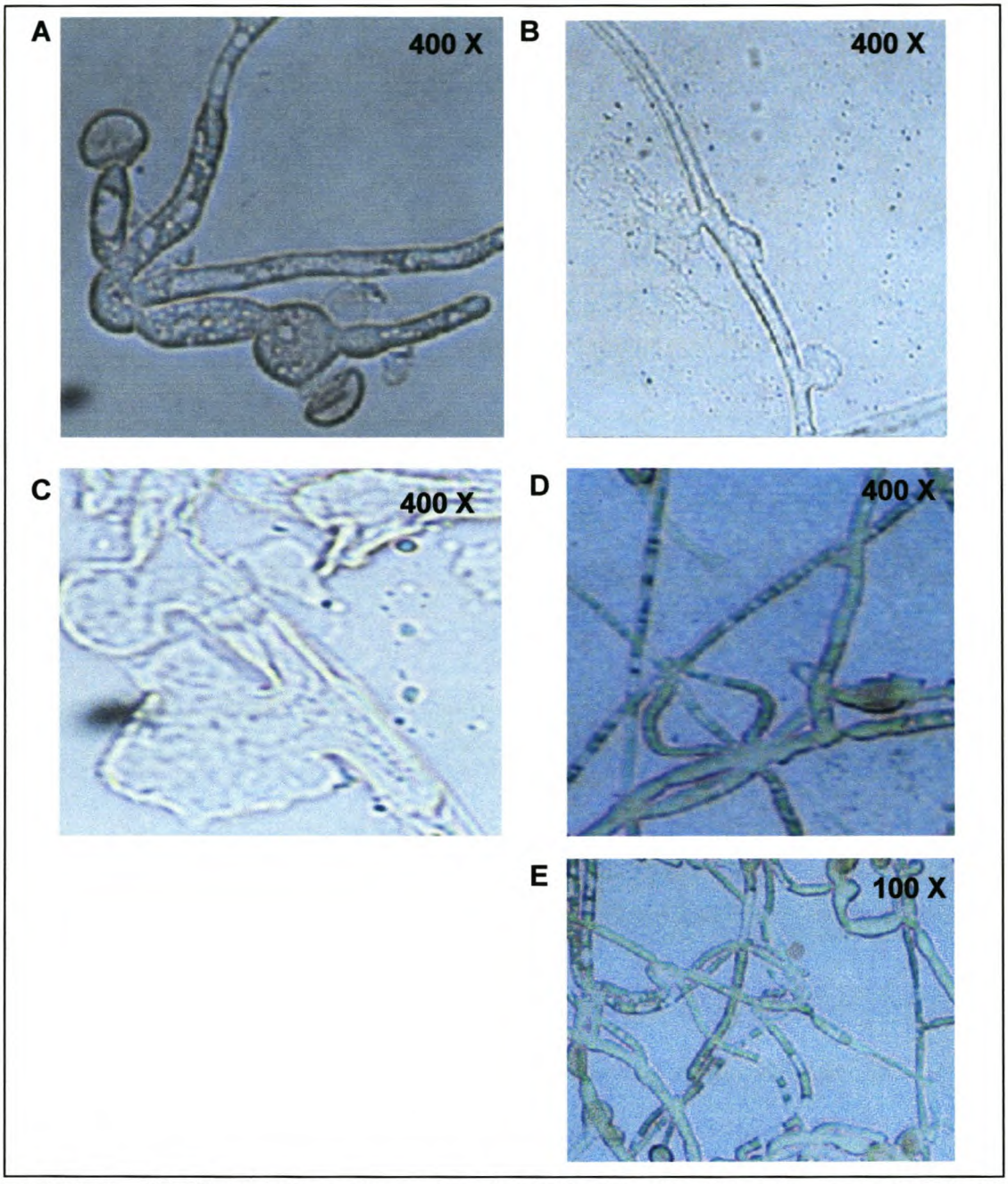


FIGURE 3. (**A**) Microscopic analyses of crude Exg1p from *Saccharomyces cerevisiae* incubated with *Botrytis cinerea* spores. The antifungal effect observed after 24 h and 48h (**B** and **C**) for *Botrytis cinerea* hyphae development during the incubation of 50 µl Exg1p-containing yeast supernatant in the presence of *B. cinerea* spores. The enlarged vacuoles and leaking out of cell material induced by the Exg1p restricts the development of the hyphae, resulting in the restricted growth of the fungal pathogen. (**D**) The natural growth pattern of *B. cinerea* observed in the absence of the antifungal agent. The rapid spreading hyphae are a sign of a healthy and unrestricted fungal pathogen. (**E**) The antifungal effect observed on *B. cinerea* hyphae development after 48 h during the incubation of 50 µl Bgl2p-containing yeast supernatant in the presence of *B. cinerea* spores.

3.3.3 Southern blot and PCR analyses of transgenic tobacco lines

The integration of the *EXG1* construct into the tobacco genome was confirmed by southern blot analysis for 13 of the 20 primary transformants (Fig. 4). The copy number of the gene in the plants varied between one and three copies. PCR analysis of genomic DNA from the 20 primary transformants with the pMJ-EXG1 cassette confirmed the successful integration of the cassette into the tobacco genome of 12 of the primary transformants (Fig. 5).

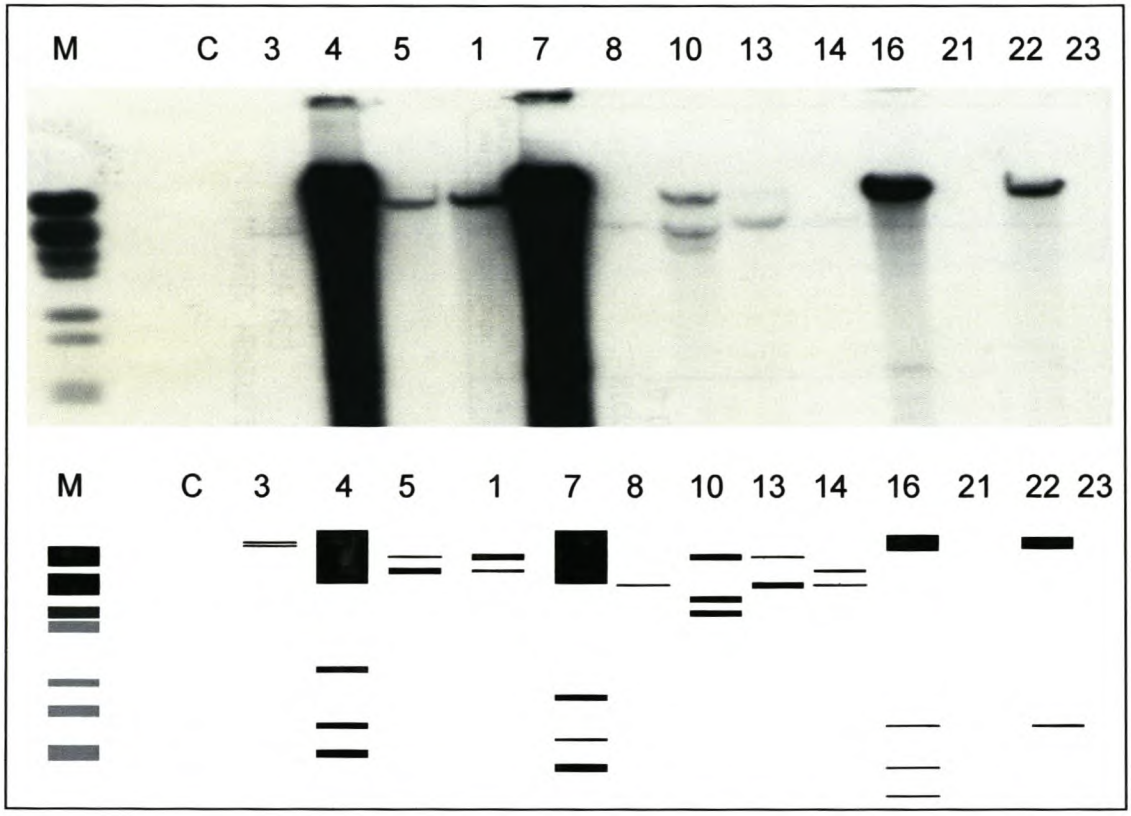


FIGURE 4. Southern blot analysis and line diagram of *Hind*III-digested genomic DNA from the parental tobacco lines transformed with the *EXG1* gene from *Saccharomyces cerevisiae* and probed with a DIG-labelled *EXG1* sequence. Lane **M** represents the DNA marker, consisting of *Bst*EII-digested Lambda DNA. Lane **C** represents a control tobacco line. The numbers in the subsequent lanes represent the different transgenic lines tested.

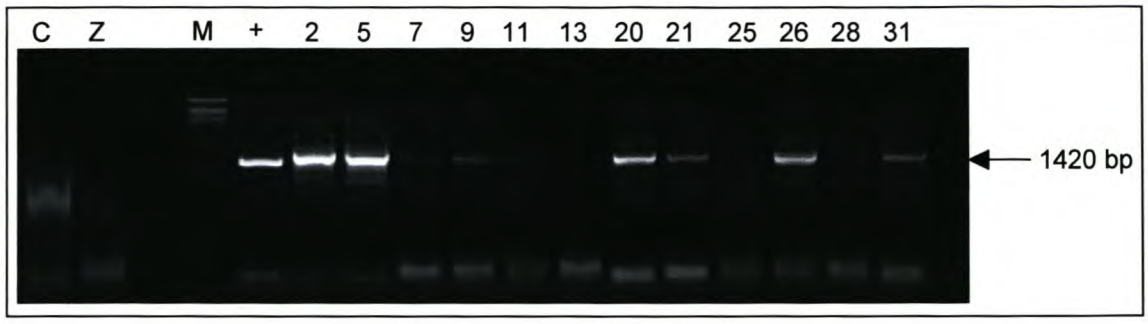


FIGURE 5. PCR analysis of genomic DNA from parental tobacco lines transformed with the pMJ-EXG1 cassette and PCR screened with *MJ/EXG1*-specific primers. Lanes **C** and **Z** represent a control tobacco plant line and zero-DNA control respectively. Lane **M** represents the DNA marker, *Bst*EII-digested Lambda DNA. Lane **+** represents a positive control, pMJ-EXG1 plasmid DNA. The numbers in the subsequent lanes represent the different transgenic lines tested.

3.3.4 Confirming gene expression in the transgenic tobacco lines

Northern blot analysis revealed the active transcription of the pEXG1 expression cassette (Fig. 6). A transcript of 1300 bp, which correlates with the *EXG1* sequence, was visualised as a dark band in seven of the E plant lines. There was also no clear correlation between copy number and expression. No mRNA signals could be detected for the MJE tobacco lines by northern blot analysis (results not shown). In a further attempt to confirm gene expression in the MJE tobacco lines, RT-PCR analysis was performed, but no product could be observed that indicated gene expression (Fig. 7).

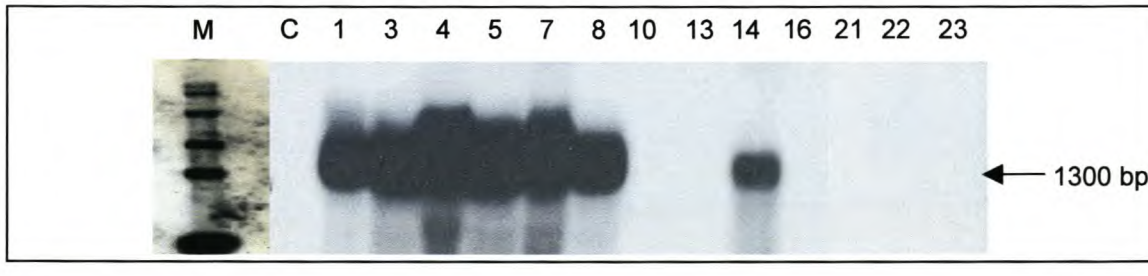


FIGURE 6. Northern blot analysis conducted on total RNA isolated from tobacco transformed with *EXG1* (rendering transgenic E-lines). Detection was achieved with a DIG-labelled *EXG1* sequence. Lane **C** represents an untransformed tobacco plant line. Lane **M** represents the RNA ladder from GIBCO. The numbers in the subsequent lanes represent the different transgenic tobacco lines.

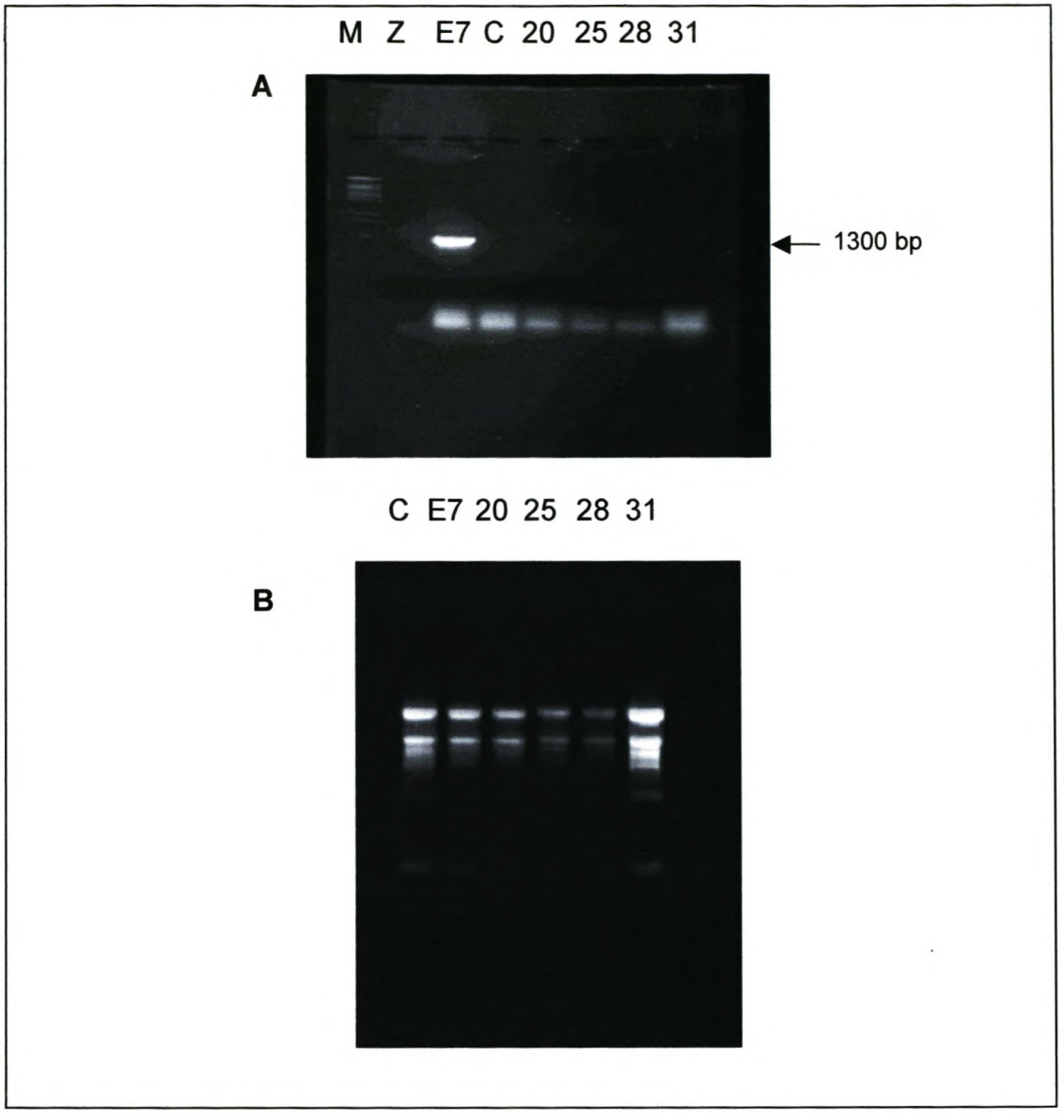


FIGURE 7. (**A**) RT-PCR was performed on the total RNA isolated from tobacco transformed with the MJ-EXG1 expression cassette. RNA from a northern positive transgenic plant line expressing the *EXG1* gene, E7, was used as a positive control. Lanes **Z** and **C** represent a zero-RNA control and RNA from an untransformed tobacco control respectively. Lane **M** represents the DNA marker, consisting of *Bst*EII-digested Lambda DNA. The numbers in the subsequent lanes represent the different transgenic lines. (**B**) The RNA samples used in the RT-PCR were separated on a 1% (w/v) agarose gel.

3.3.5 β-Glucosidase activity assays

The untransformed control tobacco lines showed endogenous β-glucosidase activity ranging from 4000 – 4200 units of activity. Crude protein extracts from the E-lines showing transgene expression (E1, E3, E4, E5, E7, E8 and E14) all showed higher levels of activity compared to that of the control tobacco lines. Only one line, E7, showed significantly higher β-glucosidase activity, with a three-fold increase in activity units compared to that of the untransformed control. The crude protein extracts from the E tobacco lines showing no transgene expression (E10, E13, E16, E21, E22 and E33), however, showed lower levels of activity compared to the untransformed control. The crude protein extracts from the MJE tobacco lines showed variable levels of activity, with the majority being lower than the untransformed control.

3.3.6 Microtiter antifungal assays

Microtiter antifungal assays were conducted on crude protein extracts from the E transgenic lines that showed transgene expression of *EXG1* and from all of the MJE transgenic lines (Fig. 8). This assay was conducted to quantitatively establish the fungal inhibition of E and MJE transgenic lines against *B. cinerea*. Six of the E transgenic lines had low percentages of growth inhibition (12-20%) against *B. cinerea*, whereas transgenic line E7 showed a significant level of growth inhibition (40%) (Fig. 8A). As expected, all of the MJE transgenic lines did not exhibit a significant percentage of growth inhibition (3-20%) compared to that of the E transgenic lines.

3.3.7 Botrytis cinerea infection studies

A detached leaf assay was conducted on the E (showing transgene expression) and MJE tobacco lines to study the extent of reduction in disease susceptibility to *B. cinerea* fungal infection. This assay showed very similar results to the microtiter plate assay.

TABLE 4. The β-glucosidase activity of crude proteins extracted form transgenic tobacco transformed with the *S. cerevisiae EXG1* gene (transgenic lines designated E) and the *S. cerevisiae EXG1* gene under the Mj-AMP2 signal peptide (transgenic lines designated MJE).

E Northern +		E Northern -		MJE Northern -	
Plant lines	Units of activity	Plant lines	Units of activity	Plant lines	Units of activity
C1*	4 002 ± 550	C1*	4 200 ± 600	C1*	4 200 ± 600
C2*	4 080 ± 350	C2*	4 128 ± 400	C2*	4 202 ± 350
1	4 428 ± 664	6	3 128 ± 310	2	3 280 ± 360
3	5 245 ± 629	10	3 369 ± 350	5	2 536 ± 250
4	4 389 ± 572	13	2 657 ± 208	7	3 260 ± 293
5	4 356 ± 499	16	2 892 ± 205	9	3 057 ± 244
7	11 506 ± 1725	22	2 160 ± 160	11	2 119 ± 318
8	4 828 ± 627			13	2 073 ± 248
14	5 069 ± 456			20	5 560 ± 834
				21	5 384±
				25	3 830±
				26	2 861±
				28	5 338±
				31	4 802±

*C1 and C2 represent two different untransformed control tobacco plants. One unit of activity is described as the amount of ρ-nitrophenol (in nM) released per hour by 1 µg of crude protein at 37°C.

The detached leaves were inoculated with high spore concentrations and incubated under conditions favourable for disease development. The lesions that developed were measured and were compared visually to a lesion-type index (Table 3). The tissue damage to the untransformed control tobacco leaves, caused by *B. cinerea*, was severe and spread actively (lesion type 9) (Table 5). The average lesion sizes on the untransformed controls were 8 mm after 48 h, increasing to 10 mm by 120 h post-inoculation. In contrast, all the E tobacco lines showed a smaller lesion size compared to the control after 48 and 120 h (Fig. 9), with the observed lesion types ranging from type 3 to type 7 (Table 3). Transgenic line E7, however, showed a significant three-fold reduction in lesion size, with an observed lesion of type 2 (Table 5). The MJE lines showed lesion types and sizes very similar to those of the untransformed control plants (Fig. 10 and Table 5).

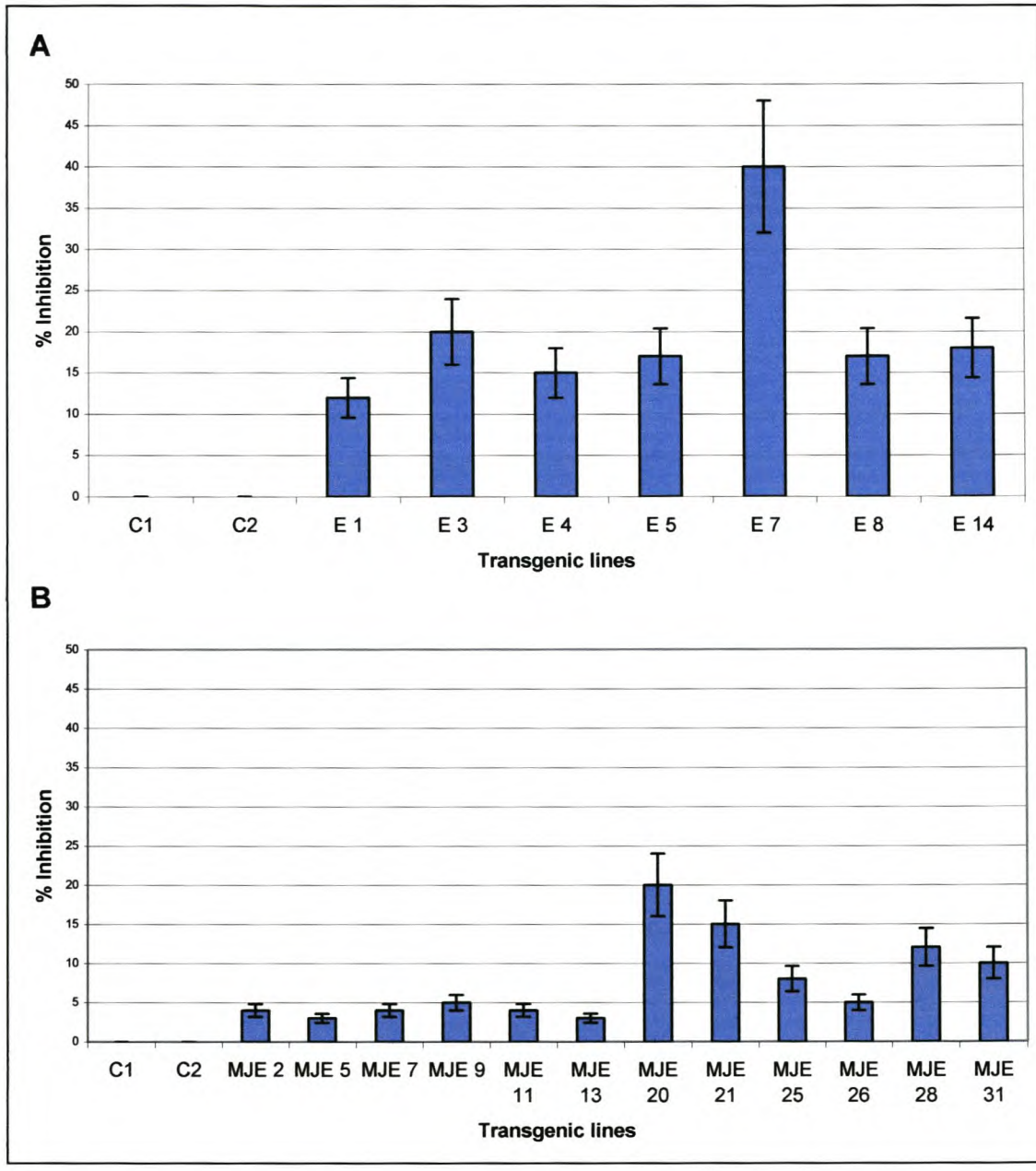


FIGURE 8: The percentage growth inhibition of crude protein extracts from tobacco lines transformed with (**A**) the *EXG1* gene from *Saccharomyces cerevisiae* (transgenic line designated E) and (**B**) the *EXG1* gene from *Saccharomyces cerevisiae* under the Mj-AMP2 signal peptide (transgenic lines designated MJE) against *Botrytis cinerea*. Crude protein extracts (5 mg/ml) were assayed against 2000 *B. cinerea* spores in half-strength potato dextrose broth and incubated at 25°C for 48 h. The A_{595} (after 48 h) was used to determine the percentage growth inhibition normalised against the negative control (untransformed tobacco protein). The values were corrected by subtracting the time zero measurements at A_{595}. Statistical analysis was performed with GraphPadPrism; the standard deviation never exceeded 20%. C1 and C2 represent two different untransformed control tobacco plants.

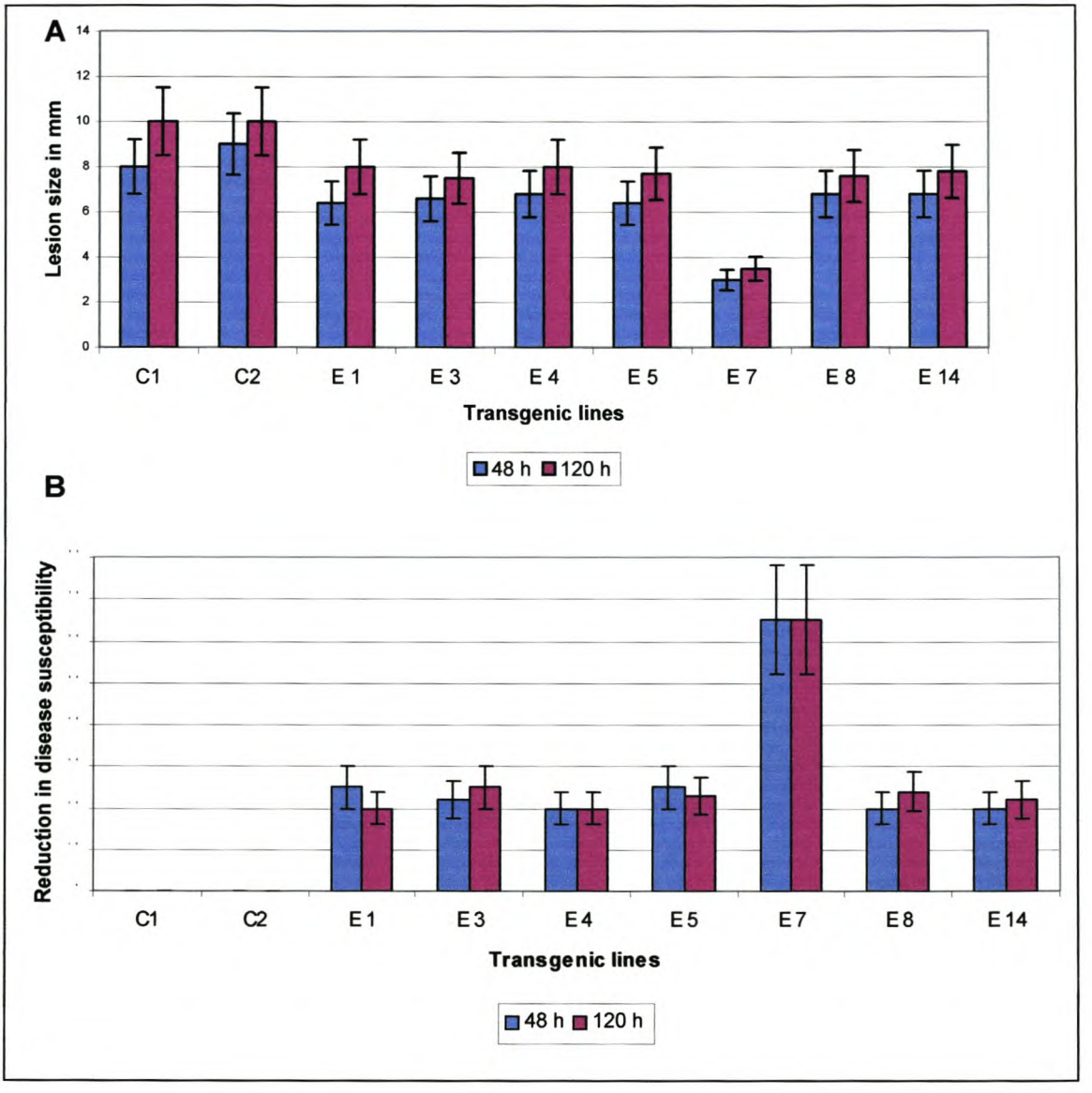


FIGURE 9: An infection study assay of *Botrytis cinerea* conducted with the tobacco lines transformed with the *EXG1* gene from *Saccharomyces cerevisiae* (transgenic lines designated E). Leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16 h light/8 h dark cycle. (**A**) The lesion sizes (in mm) recorded 48 and 120 h after leaf inoculation. The lesion sizes shown are the mean of six repetitions. (**B**) Percentage reduction in disease susceptibility was expressed as a percentage of measured lesion size, normalised against that of the untransformed control tobacco plant lines (C1 and C2). Statistical analysis was performed with GraphPadPrism; the standard deviation never exceeded 15%.

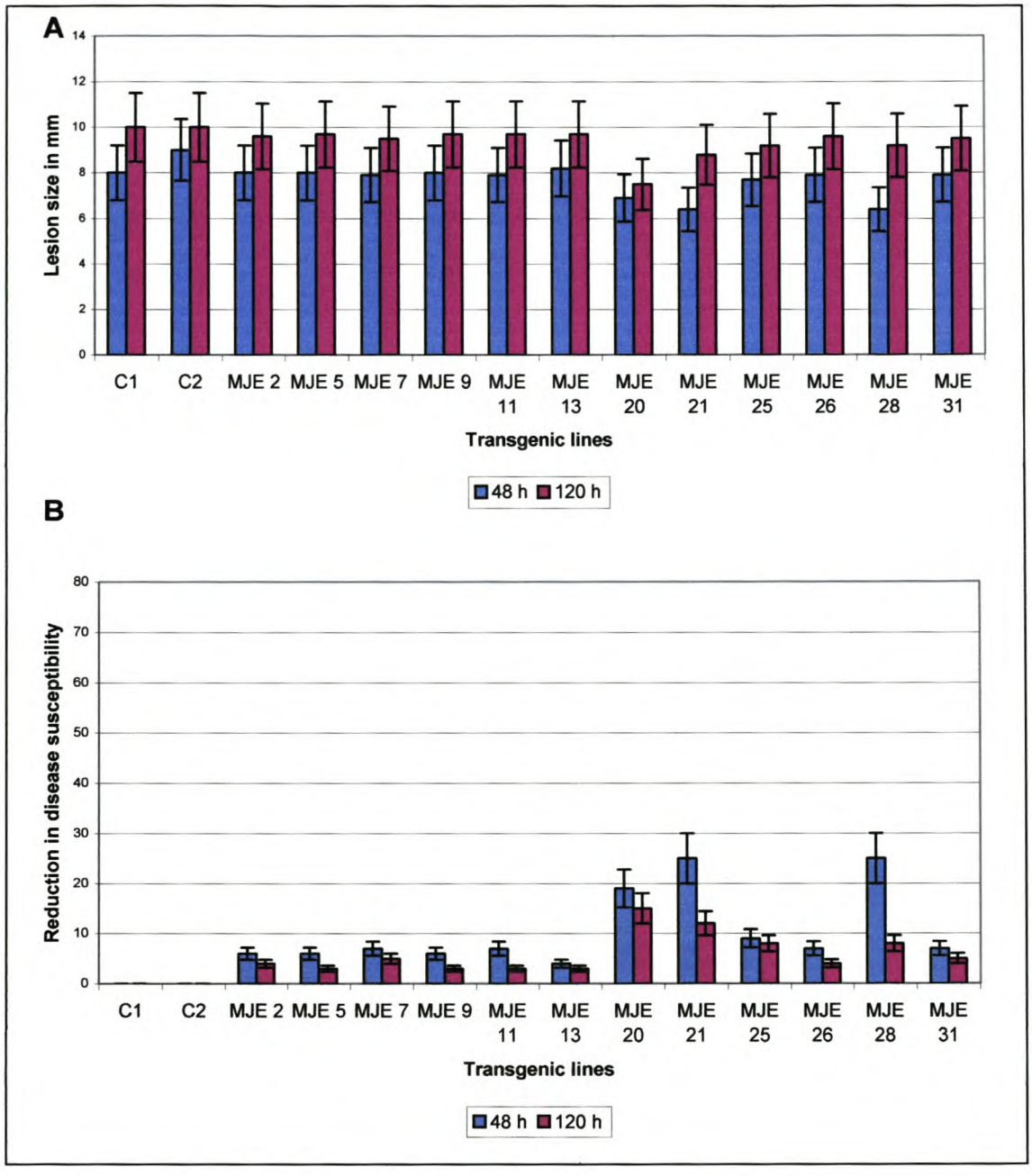
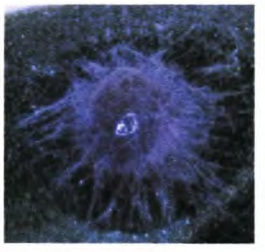
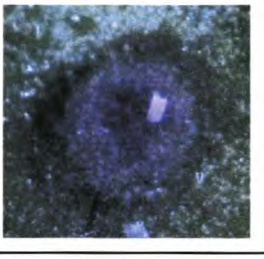
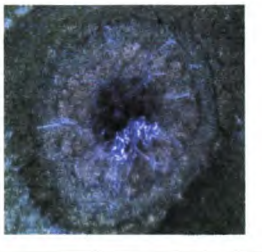


FIGURE 10: An infection study assay of *Botrytis cinerea* conducted with the tobacco line transformed with the *EXG1* gene from *Saccharomyces cerevisiae* under the Mj-AMP2 signal peptide (transgenic line designated MJE). Leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16 h light/8 h dark cycle. (**A**) The lesion sizes (in mm) were recorded 48 and 120 h after leaf inoculation. The lesion sizes shown are the mean of six repetitions. (**B**) Percentage reduction in disease susceptibility was expressed as a percentage of measured lesion size, normalised against that of the untransformed control tobacco plant lines (C1 and C2). Statistical analysis was performed with GraphPadPrism; the standard deviation never exceeded 15%.

The lesion sizes were normalised against those of the untransformed tobacco lines and expressed as percentage decrease in disease susceptibility (Fig. 9 and 10). The highest percentage reduction in disease susceptibility was observed for line E7 at 65%, 120 h after inoculation (Fig. 9). It was also clear that the level of resistance of several of the E lines (E3, 8, 14) increased from 48 to 120 h post-inoculation, whereas line E4 and E7 maintained the same level of resistance throughout this period.

There was also a good correlation between the observed lesion types and the increase in disease resistance. This correlation could be seen best in line E7, which had the highest disease resistance and also exhibited the most resistant lesion type (Table 5). This lesion type (Type 2) appeared slightly damp, with limited necrotic damage and a clear border between the infected and uninfected tissue. The MJE lines, however, did not show any significant reduction in disease susceptibility compared to the control (Fig. 10).

TABLE 5. Lesion types scored 48 h after inoculation of the transgenic tobacco leaves with 5000 *B. cinerea* spores and incubation at 22°C under a 16 h light/8 h dark cycle.

Line and lesion type	Lesion appearance	Line and lesion type	Lesion appearance
Tobacco control Type 9		**E3** Type 3	
E 7 Type 2		**MJE 5** Type 7	

3.4 DISCUSSION

We have investigated the protective and hydrolytic abilities of a yeast glucanase from *Saccharomyces cerevisiae* against *Botrytis cinerea* when expressed in tobacco. Glucanases have been shown to have antifungal activities as a result of fungal cell wall degradation (Jongedijk *et al.*, 1995; Masih and Paul, 2002). Moreover, glucanases form part of the plant pathogenesis-related proteins that are recruited as part of the plant's defence strategy (Leubner-Metzger and Meins, 1999; Robert *et al.*, 2001). Microscopic analysis of the crude proteins extracted from yeasts transformed with and overexpressing the *Saccharomyces EXG1* gene substantiated this antifungal phenomenon. From Figure 3, it can be seen clearly that the *EXG1*-encoded exoglucanase had a very severe and hydrolytic effect on the elongation and growth of the *B. cinerea* fungal hyphae. Fungal tip degradation and leakage of the cell material were also observed. This observed *in vitro* inhibition of *B. cinerea* hyphal growth led to the introduction of the exoglucanase-encoding gene into two genetic constructs, pEXG1 and pMJ-EXG1 (Fig. 2), to analyse its activity in the resulting transgenic tobacco lines, E and MJE respectively. The *BGL2*-encoded endoglucanase, however, did not show any significant restriction or degradation of the fungal hyphae and therefore was excluded from further analyses.

The tobacco transformations yielded several transgenic lines that contained copies of the transgenes in both lines E and MJE. This was confirmed by southern blot and PCR analyses (Figs 4 and 5). Transgene expression could only be confirmed in the E transgenic lines (Fig. 6), however, with no transgene expression evident in the MJE lines after both northern (results not shown) and RT-PCR analyses (Fig. 7). The E transgenic lines also showed β-glucosidase activity that confirmed the functionality of the exoglucanase protein (Table 4), whereas no significant β-glucosidase activity could be detected in the MJE transgenic lines.

The fact that no transgene expression could be detected in the MJE plant lines could be a result of mRNA degradation due to mRNA instability or the phenomenon of transgene silencing. This phenomenon is very well

documented for glucanases and several models have been proposed (Meins, 2000). One proposition is the interaction between transgenes and host genes of similar sequence frequently leads to the trans-inactivation of expression at the mRNA level in plants. According to Klebl and Tanner (1989), the *S. cerevisiae BGL2*-encoded endoglucanase is highly homologous to the tobacco glucanase, whereas the *EXG1* gene has lower homology to plant glucanases. Similarities, such as the above, could then contribute to the phenomenon of gene silencing in transgenic plants.

It is interesting to note that some of the MJE lines did show some weak antifungal activities, both in *in vitro* and *in planta* analyses, when compared to the controls, even while no expression levels could be detected. It might be possible that these lines contained the transgenes integrated in positions that naturally increased the antifungal nature of the plants without forcibly being linked to the expression of the transgenes.

From this study it was evident that elevated glucanase levels in the apoplastic environment and/or the targeting of the glucanase tot the apoplastic environment triggers a silencing mechanism in the plant, whereas cytoplasmic overexpression of the glucanase escaped this phenomenon. A possible way to overcome the silencing of the apoplastically-directed *EXG1* gene (or other glucanases typically prone to gene silencing *in planta*) could be to fuse the glucanase gene to an antifungal peptide. This possibility stems from research done by Mr A de Beer (unpublished observation) on defensins or antifungal peptides. It was found that the fusion between an antifungal peptide and *EXG1* leads to the presence of stable and active *EXG1* protein in the apoplastic space of transgenic tobacco. These results indicated that the 5' extension (the antifungal peptide-encoding sequence) protected the *EXG1*-containing transcripts from the observed mRNA instability, and subsequently from gene silencing. This gene fusion strategy might prove beneficial in these and other circumstances in which the high-level expression of glucanases is required (personal communication, Mr A de Beer, Institute for Wine Biotechnology, Stellenbosch University).

Since the overexpression of glucanases in the plant environment can lead to gene silencing at the post-transcriptional level, it is surprising that this

phenomenon did not occur in the E transgenic lines. Moreover, these plant lines showed an increase in β-glucosidase activity compared to an untransformed control. A possible explanation, although not clear from the results, could be that the copy number of the integrated *EXG1* gene and the levels of expression were lower than a certain tolerated maximum that, when exceeded, can be subjected to mRNA degradation and, thus, no transgene expression.

The inhibitory effect of the exoglucanase-expressing lines against *B. cinerea* was confirmed with a quantitative microplate assay that showed an average of 20% fungal inhibition by the transgenic exoglucanases (Fig. 8). The successful expression of *EXG1* also resulted in a significant reduction in susceptibility to *B. cinerea* infection *in planta* (Fig. 9). E7, the transgenic line showing the strongest inhibition of *B. cinerea*-induced lesion development, totally inhibited the spread of disease symptoms after 48 h (Table 5). No fungal reproductive organs were present on any of the E transgenic lines. This is important, since the conditions of the fungal infection assay were optimally suited to induce excessive infection and the formation of fungal reproductive organs. This excessive infection and formation of fungal reproductive organs were especially typical in various control plants, as well as in the MJE transgenic plants. This phenomenon of reducing the fungal inoculum over time is of great importance in the control of disease and could be seen as the primary effect of glucanases in the induced resistance strategy. The secondary mechanism of resistance would be the production of fungal elicitors through fungal degradation, thus eliciting the hypersensitive response, restricting the pathogen to the point of infection (Leubner-Metzger and Meins, 1999).

Another interesting observation is the fact that, in yeast, the exoglucanases normally exhibit fungal inhibition (Masih and Paul, 2002), whereas in plants, the endoglucanases are part of the plant's defence mechanism and inhibit fungal growth (Leubner-Metzger and Meins, 1999). The possibility then exists for a yeast endo- and exoglucanase to be overexpressed together in a plant to enhance the already antifungal actions of the plant endoglucanases.

In conclusion, the overall data presented here confirm and extend the observation that the overexpression of antifungal proteins is a feasible approach for enhancing fungal resistance in economically important crop plants. A reduction in *B. cinerea* susceptibility achieved in transgenic tobacco lines overexpressing the *EXG1*-encoded exoglucanase from *S. cerevisiae*, was shown to result from the stable integration and expression of the gene. Although the expression of the glucanase gene clearly resulted in partial reduction in susceptibility rather than immunity to infection by *B. cinerea*, the observed delay in symptom development and apparent tolerance to infection in the more resistant plant line (E7) are expected to provide sufficient protection for the survival of the tobacco plant following natural *B. cinerea* infection under glasshouse conditions. If required, overall levels of partial resistance might be further enhanced by specifically expressing this hydrolytic enzyme in those tissues and cellular compartments that are predominantly invaded by the fungi of interest and/or by adding a variety of other genes encoding proteins with known antifungal activities.

Caution should, however, be taken with regard to the targeted location of the protein or the levels of expression of a glucanase, since it has been reported and confirmed in this study that glucanase is a sensitive protein for transgene silencing. Therefore, it is important to look for ways to overcome or avoid this problem, since single gene-encoding antifungal hydrolytic proteins, such as glucanases, are a very effective and logical way of enhancing disease resistance in plants.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

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Most agricultural and horticultural crop species all over the world suffer from a vast array of fungal disease, which cause severe yield losses. Single dominant genes providing immunity to specific fungi have been identified but these genes are often easily overcome by rapidly evolving new fungal races (Jongedijk *et al.*, 1995). Today, however, continuous accumulation of race-specific resistance genes in commercial varieties remains the major method of achieving sufficient resistance to a wide range of fungal pathogens. Economically-viable levels of durable resistance in crop plants against a relatively broad range of fungi might be achieved by recently described molecular approaches. These approaches include the expression of fungal avirulence genes to provoke non-specific, hypersensitive resistance genes, the expression of genes involved in the synthesis of phytoalexins toxic to fungi, and the expression of genes encoding inhibitors of fungal enzymes or known antifungal proteins (Lamb *et al.*, 1992).

The focus of this thesis falls broadly on these molecular approaches. The aim was to evaluate yeast glucanases for their antifungal characteristics, specifically when introduced and overexpressed in transgenic tobacco as a model system. Plants utilise endogenous glucanases, as part of the broader pathogenesis-related (PR) proteins, in their pathogen defence responses. Since plant glucanases are constitutively expressed at low levels, overexpression of the endogenous or heterologous glucanases could increase the plant's defence against pathogens. This approach is not novel, since several examples already exist of glucanases from plant, fungi and yeast being used and shown to have antifungal effects. The yeast *Saccharomyces cerevisiae* contains both endo- and exoglucanase activities and this study is the first report of the evaluation of the *EXG1* and *BGL2* genes from *Saccharomyces* as possible antifungal genes in the plant environment.

To this end, two plant expression vectors were designed and transformed into tobacco as a model system. The first of these constructs was

pEXG1, containing the *EXG1* glucanase-encoding gene from *Saccharomyces cerevisiae* under the control of a strong constitutive promoter. pMJE was the second construct, in which the *EXG1* gene was fused to the signal sequence of Mj-AMP2 from *Mirabilis jalapa*. This construct was prepared to direct the *EXG1* gene product to the apoplastic region of the plant cell, where the protein can be situated ideally to confront the fungus before it reaches the plant cell itself.

Tobacco was transformed successfully with these constructs, rendering transgenic lines E and MJE, containing pEXG1 and pMJ-EXG1 respectively. The transgene integration of both constructs could be detected readily. Transgene expression could, however, only be detected in the E transgenic tobacco lines. The successful expression of *EXG1* led to a significant decrease in susceptibility to *B. cinerea* infection in most of the transgenic lines. These assays were performed both *in vitro*, with leaf extracts from the transgenic lines, and in a detached leaf assay to assess the *in planta* activity. In both these assays, a good correlation could be drawn between the levels of glucanase activity observed in the transgenic lines, the decrease in susceptibility when challenged with the fungal pathogen and the morphological and physiological effects on the *Botrytis* hyphae in the presence of the recombinant protein. This confirms the antifungal activities of glucanases and, specifically, yeast exoglucanases.

No transgene expression could be detected with the transgenic tobacco transformed with pMJE. A possible explanation could be the incidence of induced mRNA degradation in response to a gene silencing mechanism. Frequent reports of the gene silencing of glucanases in transgenic systems have been documented previously (Holtorf *et al.*, 1999). Interactions between transgenes and host genes of similar sequence in plants frequently lead to the transinactivation of expression at the mRNA level (Meins, 2000). Further investigations into this matter are necessary to determine the exact cause of the lack of expression of this gene.

Many papers and reviews have discussed the advantages of using glucanases for plant protection because these enzymes are fungicidal, part of the plant's defence system and non-toxic to plants, animals and higher vertebrates. Transgenic expression of glucanase genes in plants has been

shown to improve disease resistance in various crops. However, the level and the spectrum of resistance obtained have not so far supported the development of this technology for the production of new disease-resistant varieties suitable for commercial agriculture. One of the main limitations has been the relatively high level of transgene silencing occurring in transgenic trials, as was mentioned above. Secondly, the relatively low level of resistance obtained with a single glucanase gene, which has resulted in the need to use gene combinations, is also a limiting factor. This strategy may not be attractive, however, since a higher degree of genetic modification of the plant may substantially increase the costs of product development, complicate and delay the registration process in some countries, and be in contrast with the public's feelings about accepting genetically modified crops. Thirdly, there are almost no reports of glucanase-transgenic plants showing resistance to several fungi, indicating that the genes and enzymes used thus far have a narrow spectrum of antifungal activity. This spectrum may represent a major limitation in protecting crops that are susceptible to more than one pathogen and makes this transformation-based strategy unsuitable to substitute or be integrated with the application of chemical fungicides, which have a relatively broad spectrum of activity.

An alternative strategy is to use glucanases in a biological control system. It has been shown that the successful biocontrol organism, *Trichoderma harzianum*, secretes the proteins β-1,3-glucanase and chitinase. These proteins have been suggested to be key enzymes in the lysis of phytopathogenic fungal cell walls during mycoparasitic action (De la Cruz *et al.*, 1995). Another suggestion is to transform the yeast that occurs naturally on the plant, with the β-glucanases thereby increasing the levels of β-glucanase secretion. The idea is then to spray the plant with a suspension of this recombinant yeast species. This strategy can take advantage of the hydrolytic action of glucanases, without risking gene silencing in a transgenic plant environment.

Furthermore, it has been shown that a fusion between an antifungal peptide and the 5' end of the *EXG1* gene may lead to the presence of stable

and active EXG1-protein. This is proposed to be due to the protecting role of the 5' extension present in the fusion product (Mr A. de Beer, Institute for Wine Biotechnology, Stellenbosch University, South Africa, personal communication).

In conclusion, this study showed that *S. cerevisiae EXG1*-encoded exoglucanases can be used in genetic transformation schemes and are active in plants when occurring intracellularly. It also showed that a single gene introduced into a plant genome could have a significant effect on the disease susceptibility of the plant. Furthermore, this study has shown that yeast glucanases, like other heterologous glucanases, are susceptible to mRNA degradation and/or gene silencing. Careful planning, however, can minimise the risk of gene silencing and, in doing so, benefit from the many advantages of using single gene-encoded glucanases in transgenic crops. Ultimately, this work has resulted in valuable information regarding the use of *S. cerevisiae* glucanases in a genetic transformation scheme.

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