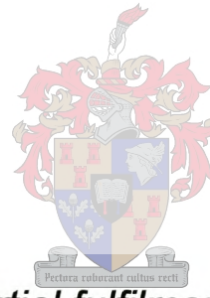
The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, as an antifungal and biocontrol agent

by

Maryke Carstens



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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 entire or in part submitted it at any university for a degree.

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SUMMARY

Fungi are an extremely diverse group of organisms and, by acting as pathogens, they can colonise various other organisms, including humans, plants and animals. The effect of this is usually detrimental, not only to agricultural crops and livestock, but also to human well-being. The extensive farming of crops and livestock requires persistent control of fungal populations, commonly through the use of chemical fungicides. However, the exclusive use of fungicides is no longer a sustainable practice, as a result of serious problems, such as increasing fungicide resistance in pathogen strains, the high costs of fungicides, as well as concern about the environment. The search by producers and scientists for alternative control measures is an ongoing process.

The fungal cell wall consists of polysaccharides that not only play a role in protection of the fungi, but also in relaying signals for the invasion and infection of susceptible hosts. Chitin, a polysaccharide composed of *N*-acteylglucosamine (GlcNAc) residues linked by β–1,4 glucosidic linkages, is one of the major components of the fungal cell wall, where it plays an important role in the apical growth of the vegetative hyphae.

Chitinases (EC 3.2.1.14) are abundant proteins produced by a variety of microorganisms and plants and are necessary for the hydrolysis of the chitin polymer. During the invasion of many plant species by a pathogen, the production of a specific group of proteins, designated pathogenesis-related (PR) proteins that include chitinases, is induced as part of their defence response. Due to the facts that pathogenic fungi contain chitin in their cell walls and that plant chitinases are induced upon pathogen attack, chitinases have been confirmed as an integral and crucial part of the plant's natural defence response. Chitinases have increasingly been targeted to upregulate plants' endogenous disease resistance mechanisms through transgenic overexpression in a variety of hosts.

Several species of fungi, including various *Trichoderma* spp., are potent biocontrol agents of plant pathogenic fungi and insects. The antagonistic activities of these biological control agents towards phytopathogens are based on the secretion of extracellular hydrolytic enzymes, such as cell wall-degrading chitinase enzymes. However, biological control is not restricted to naturally occurring biocontrol agents. Through the process of genetic transformation, other fungal or yeast species can be enhanced to produce their own chitinases or other antimicrobial substances more effectively in order to yield potent biocontrol agents.

Various types of chitinases have been applied in the production of fungal resistant plants and some research has been done on the application of chitinases, from a variety of microorganisms, as biological control agents. In contrast, very little is known about the antifungal activity of the *Saccharomyces cerevisiae* chitinase enzyme, encoded by the *CTS1-2* gene. The *CTS1–2* gene was utilised in this study as a candidate for overexpression in both yeast and plant expression systems to analyse the ability of the encoding chitinase to inhibit fungal growth.

The first objective of this study involved the high level expression and optimisation of the secretion of the *CTS1–2* gene in *S. cerevisiae* to render recombinant yeast with enhanced antifungal abilities and with possible applications as a biocontrol agent to control plant

pathogenic fungi. It was hypothesised that high-level expression and efficient secretion would be prerequisites in a biocontrol yeast strain. To this end, two strong promoters and terminators were included in the study and the secretion of the chitinase gene was evaluated by testing three different secretion signals. The secretion signals included: the native *CTS1–2* secretion signal, the *S. cerevisiae* mating pheromone α-factor (*MFα1*) secretion signal, as well as the *Trichoderma reesei* β-xylanase 2 (*XYN2*) secretion signal. The phosphoglycerate kinase 1 (*PGK1*) and alcohol dehydrogenase 2 (*ADH2*) promoters and terminators were employed to achieve high-level expression.

The results obtained from the analysis of the recombinant yeasts showed that the *PGK1* promoter-terminator constructs yielded high level *CTS1-2*-expressing and chitinase-producing strains of *S. cerevisiae* PRY488. The ability of the different secretion signals to efficiently secrete the overexpressed chitinase was analysed and it was found that the non-native secretion signals delivered significantly more protein to the extracellular environment. It was thus evident that the performance of the *MFα1* and *XYN2* secretion signals was superior to that of the native secretion signal. The antifungal activities of the recombinant chitinases produced by these constructs were tested in *in vitro* assays against *Botrytis cinerea*. The enzymes led to a significant reduction in hyphal development, caused by extreme structural damage to the hyphal tips, the hyphal cell walls as well as the ability of the fungus to form reproductive and survival structures, thereby confirming the antifungal abilities of this enzyme. The *ADH2* promoter-terminator constructs yielded *CTS1–2* transcripts, but no chitinase activity could be detected with any of these strains. The reasons for this still remain unclear.

The second objective of this study was to assess the potential of the yeast chitinase gene to upregulate defence against fungal infection *in planta*. In order to elucidate this, the *CTS1–2* gene was constitutively overexpressed in tobacco plants, targeting the chitinase both to the intra- and the extracellular environment. The results obtained showed that the transgenic tobacco lines regenerated in this study stably integrated the transgene, exhibiting transgene expression as well as the production of a biologically active yeast chitinase enzyme. The F_1 progeny were rigorously tested for resistance to *B. cinerea*, and both *in vitro* and *in planta* assays confirmed that the yeast chitinase increased the plant's tolerance to fungal infection; some of the lines showed disease resistance of 65 and 70%. The plants expressing an extracellularly targeted chitinase gene are still under evaluation. Interesting results are expected relating to the effect of the chitinase on the plant surface with regards to disease resistance to fungal pathogens.

In conclusion, the combined set of results from both the yeast and plant overexpression studies has confirmed the strong antifungal effect of yeast chitinases. The yeast *CTS1–2* chitinase could be instrumental in the development of a new generation of yeast strains with improved antifungal capabilities. This enzyme could also play an important role in genetic transformation technologies aimed at enhanced disease resistance.

OPSOMMING

Swamme omsluit 'n uiterste diverse groep organismes wat mense, plante en diere deur patogeniese aksie kan koloniseer. Die uitkoms hiervan op landbougewasse, die veebedryf en menslike gesondheid is gewoonlik skadelik. Uitgebreide gewas- en veeboerderye benodig voortdurende beheer van fungiese populasies, tipies deur van chemiese swamdoders gebruik te maak. Die uitsluitlike gebruik van swamdoders is egter nie meer 'n lewensvatbare praktyk nie, hoofsaaklik as gevolg van probleme soos die opbou van weerstand van patogeniese rasse teen swamdoders, die hoë kostes van die middels, asook besorgheid oor die omgewing. Die soektog na alternatiewe beheermaatreëls deur produsente en wetenskaplikes bly 'n aaneenlopende proses.

Die swamselwand bestaan uit polisakkariede wat nie net 'n rol in die beskerming van die swam speel nie, maar ook betrokke is in die oordrag van aanvals- en infeksieverwante seine in 'n vatbare gasheer. Chitien, 'n polisakkaried bestaande uit *N*-asetielglukosamien (GlcNAc) residu's gekoppel deur β-1,4 glukosidiese bindings, is een van die hoofkomponente van die swamselwand, waar dit 'n belangrike rol in die apikale groei van vegetatiewe hifes speel.

Chitinases (EC 3.2.1.14) is proteïene wat oorvloedig deur 'n verskeidenheid van mikroörganismes en plante geproduseer word, waar hulle vir die hidrolise van die chitien polimeer noodsaaklik is. Tydens die infeksie van verskeie plantspesies deur 'n patogeen, word die produksie van 'n spesifieke groep proteïene, die sogenaamde patogeen-verwante (PR) proteïene wat chitinases insluit, as deel van die plant se verdedigingsreaksie geïnduseer. Die feit dat patogeniese swamselwande chitien bevat en dat plantchitinases tydens infeksie geïnduseer word, het daartoe gelei dat dit bevestig is dat chitinases 'n integrale en kritiese deel van die plant se natuurlike verdedigingsreaksie uitmaak. Chitinases word toenemend geteiken in pogings om die plant se intrinsieke siekteweerstandsmeganismes te verbeter deur transgeniese ooruitdrukking daarvan in 'n verskeidenheid van gashere.

Verskeie swamspesies, insluitend verskillende *Trichoderma*-spesies, is kragtige bio-antagoniste van plantpatogeniese swamme. Die antagonistiese aksies van hierdie biologiese beheeragente teenoor fitopatogene is gebaseer op die uitskeiding van ekstrasellulêre hidrolitiese ensieme, soos die selwandverterende chitinase ensieme. Nietemin is biologiese beheer nie net tot bio-antagoniste wat natuurlik voorkom beperk nie. Deur die proses van genetiese transformasie kan ander swam- of gisspesies verbeter word om hul eie chitinases of ander antimikrobiese substanse meer effektief te produseer, wat aanleiding sal gee tot kragtige bio–antagoniste.

Verskeie tipes chitinases is al in die produksie van swambestande plante ingespan en uitgebreide navorsing is gedoen op die toepassing van 'n reeks chitinases, afkomstig van 'n verskeidenheid van mikroörganismes, as biologiese beheeragente. In teenstelling is baie min bekend oor die antifungiese aktiwiteite van die *Saccharomyces cerevisiae* chitinase ensiem, wat deur die *CTS1–2* geen ge-enkodeer word. Die *CTS1–2*-geen is in hierdie studie gebruik vir ooruitdrukking in beide gis- en plantuitdrukkingsisteme om die chitinase se vermoë om swamgroei te inhibeer, te ondersoek.

Die eerste oorkoepelende oogmerk van hierdie studie het hoë–vlak uitdrukking en optimalisering van sekresie van die *CTS1–2*-geen in *S. cerevisiae* behels, met die toekomstige doelwit om 'n rekombinante gis met verbeterde antifungiese eienskappe en met moontlike toepassings as 'n bio–antagonis teen plantpatogeniese swamme te ontwikkel. Die hipotese was dat hoë–vlak uitdrukking en voldoende sekresie voorvereistes vir 'n bio–antagonisras is. Om dié rede is twee sterk promotors en termineerders by hierdie studie ingesluit en is die sekresie van die chitinase-geen geëvalueer deur drie verskillende sekresieseine te toets. Die sekresieseine sluit in: die wilde-tipe *CTS1–2* sekresiesein, die *S. cerevisiae* paringsferomoon α-faktor (*MFα1*) sekresiesein, en die *Trichoderma reesei* β-xilanase (*XYN2*) sekresiesein. Die fosfogliseraat kinase 1 (*PGK1*) en alkohol dehidrogenase 2 (*ADH2*) promotors en termineerders is gebruik om hoë–vlak uitdrukking te dryf.

Die resultate wat vanaf die analises van die rekombinante giste verkry is, het getoon dat die *PGK1* promotor-termineerder konstrukte hoë–vlak *CTS1–2*-uitdrukkende en chitinase-produserende *S. cerevisiae* PRY488 rasse opgelewer het. Die vermoë van die verskillende sekresieseine om die ooruitgedrukte chitinase voldoende uit te skei, is geanaliseer, en daar is gevind dat die heteroloë sekresieseine aansienlik meer proteïene na die ekstrasellulêre omgewing geloods het. Dit was dus duidelik dat die *MFα1* en *XYN2* sekresieseine beter as die wilde-tipe sekresiesein presteer het. Die antifungiese aktiwiteit van die rekombinante chitinases wat deur hierdie konstrukte geproduseer is, is ook in *in vitro*toetse teen *Botryits cinerea* getoets. Die teenwoordigheid van die ensieme het gelei tot 'n aansienlike afname in hife-ontwikkeling, veroorsaak deur ekstreme strukturele skade aan die hifepunte, die hifeselwande, asook die vermoë van die swam om voortplanting- en oorlewingstrukture te vorm. Die *ADH2* promotor-termineerderkonstrukte het *CTS1–2* transkripte vertoon, maar geen chitinase-aktiwiteite kon in hierdie konstrukte waargeneem word nie. Die redes hiervoor is tot op hede onbekend.

Die tweede oogmerk van hierdie studie was om die potensiaal van die gischitinase om swaminfeksie *in planta* teë te werk, te ondersoek. Die *CTS1–2*-geen is konstitutief ooruitgedruk in tabakplante, waarin die chitinase na beide die intra- en ekstrasellulêre omgewing geteiken is. Resultate het getoon dat die geregenereerde transgeniese tabaklyne die transgeen stabiel geïntegreer het, transgeenuitdrukking vertoon en dat 'n biologies aktiewe chitinase-ensiem geproduseer is. 'n F_1-generasie is aan strawwe toetse onderwerp om weerstand teen *B. cinerea* te ondersoek. Beide die *in vitro* en *in planta* toetse het bevestig dat die gischitinase die plant se verdraagsaamheid teenoor swaminfeksie verhoog het; sommige lyne het siekteweerstand van tussen 65 en 70% getoon. Die plante wat 'n ekstrasellulêre chitinase produseer, word steeds geëvalueer. Interessante resultate word verwag aangaande die effek van die chitinase op die plant se oppervlak met betrekking tot siekteweerstand teen swampatogene.

Ten slotte, die gekombineerde stel resultate wat vanaf beide die gis- en plantuitdrukkingstudies verkry is, het die sterk antifungiese effek van gischitinases bevestig. Die gis *CTS1-2* kan instrumenteel wees in die ontwikkeling van 'n nuwe generasie gisrasse met verbeterde antifungiese eienskappe. Die ensiem kan ook 'n belangrike rol in genetiese transformasietegnologieë, wat op verbeterde siekteweerstand gemik is, speel.

BIOGRAPHICAL SKETCH

Maryke Carstens was born in Cape Town, South Africa on 18 August 1976. She matriculated at Jan van Riebeeck High School, Cape Town in 1994 and enrolled at Stellenbosch University in 1995. She obtained a BScAgric degree in Biochemistry, Microbiology and Genetics in 1998. In 1999, she enrolled at Stellenbosch University for her MScAgric degree in Wine Biotechnology at the Institute for Wine Biotechnology.

I dedicate this thesis to my parents.

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of either the journal *FEMS Yeast Research* to which Chapter 3 will be submitted for publication, or the journal *Plant Physiology*, to which Chapter 4 will be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review: Chitinases as antifungal control proteins**

Chapter 3 **Research Results: Overexpression and secretion of the *Saccharomyces cerevisiae* chitinase (*CTS1*-2) gene product from yeast**

Chapter 4 **Research Results: The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, confers antifungal activity to transgenic tobacco**

Chapter 5 **General Discussion and Conclusions**

I hereby declare that I was the primary contributor with respect to the experimental data presented in the multi-author manuscripts (Chapters 3 and 4). My supervisors, Dr. M.A. Vivier, Dr. P. van Rensburg and Prof. I.S. Pretorius, were involved in the conceptual development and continuous critical evaluation of the study.

CONTENTS

CHAPTER 3. RESEARCH RESULTS: OVEREXPRESSION AND SECRETION OF THE *SACCHAROMYCES CEREVISIAE* CHITINASE (*CTS1-2*) GENE PRODUCT FROM YEAST

CHAPTER 4. RESEARCH RESULTS: THE *SACCHAROMYCES CEREVISIAE* CHITINASE, ENCODED BY THE *CTS1-2* GENE, CONFERS ANTIFUNGAL ACTIVITY TO TRANSGENIC TOBACCO

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1. INTRODUCTION

Fungi are remarkable "pioneering" organisms that are able to grow on almost any surface. Unfortunately, fungi are also capable of colonising other organisms, such as plants, animals and humans as pathogens, leading to yield losses in agronomically important crops and livestock and contributing to human suffering. Fungi are an extremely diverse group of organisms, with approximately 250 000 species that are distributed in virtually every known ecosystem (Selitrennikoff, 2001).

The fungal cell wall not only protects the organism against hostile environments, but is also involved in relaying signals related to the invasion and infection of a susceptible plant, animal or human host. The cell wall is synthesised at each hyphal apex in a complex assembly sequence (Ruiz-Herrera, 1992) and is composed mainly of polysaccharides that constitute 80-90% of the total dry weight of the wall (Fuglsang *et al.*, 1995). One of the major components of the fungal cell wall is chitin, a polysaccharide composed of *N*-acteylglucosamine (GlcNAc) residues linked by β-1,4 glucosidic linkages. Chitin achieves a highly ordered insoluble crystalline structure comprising individual polysaccharide chains organised into microfibrils (Fuglsang *et al.*, 1995). These microfibrils are associated with other cell wall components, such as β-1,3-D-glucan, β-1,6-D-glucan and mannoprotein(s). In most fungi, β-1,3 and β-1,6 linkages dominate and the resulting mixed-linkage polymers usually interlink individual chitin microfibrils (Fuglsang *et al.*, 1995). Since chitin is the characteristic component of the fungal wall, it plays an important role in the apical growth of the vegetative hyphae, with the majority of chitin being laid down in the apex (Wessels, 1990). It is important to note that fungi have significant internal turgor pressure, therefore the slightest perturbation of the cell wall results in cell lysis (Money, 1990; Kaminskyj *et al.*, 1992; Money and Harold, 1992).

Plants are often exposed to a large and diverse number of pathogenic fungi. Unlike animals, plants do not have a clonal-selection immune system based on acquired immunity. Nevertheless, plants have evolved a complex array of chemical and enzymatic defences, both constitutive and inducible, that are not involved in the detection of the pathogen, but whose effectiveness influences pathogenesis and disease resistance (Bishop *et al.*, 2000). Plants protect themselves by re-enforcing their cell walls through polymer synthesis, producing antimicrobial compounds such as phytoalexins, small peptides and hydrolytic proteins on the cell surface. With these and other measures, such as accelerated cell death (hypersensitive response) in the areas of infection, they suppress the spread of infectious pathogens (Kitajima and Sato, 1999). These antimicrobial plant proteins that are linked to disease resistance are generally known as pathogenesis-related (PR) proteins.

PR proteins have been defined as proteins encoded by the host plant that can be induced by various types of pathogens (such as viruses, bacteria, fungi) and by the

application of chemicals that mimic the effect of pathogen infection or induce similar stresses (Bol *et al.*, 1990). These proteins were originally divided into five groups (PR-1 to 5), based on serological and amino acid sequence analyses (Van Loon, 1985). Recently, another six groups of proteins have been suggested for inclusion as PR proteins (Selitrennikoff, 2001). Each of the five classic groups of PR proteins comprise two subclasses: an acidic subclass, which is usually secreted to the extracellular space, and a basic subclass, which is found in the plant cell vacuole (Kitajima and Sato, 1999). The acidic subclass proteins are secreted to limit the pathogens that grow extracellularly, especially in the early stages of infection (Isaac, 1992). The PR proteins found in the vacuole have a different role to play in the defence mechanism. Plant cells under attack and their vacuoles are eventually ruptured due to either the hypersensitive response (Collinge and Slusarenko, 1987), or to the action of necrosis-inducing toxins and cell wall-degrading enzymes from the pathogen (Isaac, 1992). Antimicrobial compounds stored in the vacuole are thus discharged at the infection site, usually in the later stages of infection. PR proteins are very effective antifungal agents and one of the PR groups, the PR-3 group, is the subject of this thesis.

The PR-3 group consists of chitinases (EC 3.2.1.14), the enzymes necessary for the hydrolysis of the chitin polymer. Members of the chitinase gene family are found in all plants, where they are constitutively and inducibly expressed as PR proteins in tissues vulnerable to pathogen attack (Bishop *et al.*, 2000). Several lines of evidence indicate that chitinases play a direct role in plant defence by attacking fungal chitin. The relevant facts include: (i) hyphal growth can be inhibited *in vitro* by purified chitinases (Collinge *et al.*, 1993), (ii) constitutive overexpression of certain chitinases in transgenic plants can bestow increased pathogen resistance *in planta* (Broglie *et al.*, 1991; Grison *et al.*, 1996), (iii) chitinolytic breakdown products induce the production of typical defence compounds, such as phytoalexins, and they also trigger the systemic acquired resistance response (Brunner *et al.*, 1998; Bishop *et al.*, 2000).

Even though there are numerous and diverse fungal species, the cell walls of the majority share the same basic structure, in which chitin is often replaced by chitosan, the deacetylated form of the polysaccharide (Gooday, 1990). Therefore, enzymes that are effective against these structures are potentially effective against many fungi. The majority of plant chitinases (PR-3) act as endochitinases that cleave the chitin polymers randomly at internal sites, generating soluble, low molecular weight multimers of GlcNAc (Sahai and Manocha, 1993). However, some plant chitinases show exochitinase activity, which catalyses the progressive release of diacetylchitobiose from the nonreducing end of the chitin microfibril (Sahai and Manocha, 1993). The *in situ* cleavage of these chitin microfibrils results in a weakened cell wall, rendering fungal cells osmotically sensitive. These hydrolytic enzymes have also been found to be involved directly in the degradation of fungal hyphal tips, as well as in the inhibition of spore germination and germ-tube elongation (Chet and Inbar, 1994; Haran *et al.*, 1996; Chet and Inbar, 1997).

The widespread use of chemical fungicides has been the most common approach in agriculture to control fungal pathogens (Schickler and Chet, 1997). The exposure of natural habitats and human populations to harmful pesticides has reached unacceptable levels, and lately more attention has been paid to eliminating or reducing the doses of chemicals that are used (Cook and Granados, 1991). Other concerns are the high cost of fungicides and the fact that fungi are continually developing resistance to existing fungicides (Adams, 1994). The exclusive and excessive use of fungicides to control fungal pathogens is no longer a practical approach for the future.

One approach to reduce the use of chemicals is to employ breeding programmes to produce crops that are resistant to diseases. This has proven to be a successful, yet expensive and time-consuming process, complicated by the continuous evolution of new virulent strains of pathogenic microorganisms (Schickler and Chet, 1997). One of the alternative control methods for fungal pathogens involves chitinases, due to their potent antifungal activities. Studies are currently geared towards the production of transgenic resistant plant species and although this approach has not been proven on a commercial scale, excellent results have been obtained *in vitro* in glasshouse and field trail experiments.

Varied successes have been achieved by utilising chitinases in the production of fungal resistant plants. The first report was by Broglie *et al.* (1991), who constitutively overexpressed the bean chitinase *CH5B* gene in canola and tobacco plants. Relative to the control plants, there was a 44-fold increase in chitinase activity and these transformants displayed resistance to the soil-borne pathogen, *Rhizoctonia solani*. The observed resistance included a delay in the development of disease symptoms, as well as an improved ability to survive in soils infected with *Rhizoctonia*. Other researchers have also shown enhanced fungal resistance in transgenic plants overexpressing chitinase genes (Zhu *et al.*, 1994; Lin *et al.*, 1995; Terakawa *et al.*, 1997; Lorito *et al.*, 1998; Datta *et al.*, 2001).

Another approach to curb pathogens involves the development of biological control agents. Biological antagonism and mycoparasitism that occur naturally between microorganisms can be developed and exploited as a means of biological control to replace chemical fungicides (Lorito *et al.*, 1998). The antagonistic activities of biological control agents towards phytopathogens are based on the secretion of extracellular hydrolytic enzymes, including chitinases. The best known natural biocontrol fungus is *Trichoderma*. The antifungal mechanisms of the various *Trichoderma* spp. are based on the chitinase cell wall degrading enzymes (Lorito *et al.*, 1998). *Trichoderma* spp. have been used to control both soil-borne (Chet, 1987; Jin *et al.*, 1991) and leaf- and fruit-infecting plant pathogenic fungi, such as *Botrytis cinerea* (Tronsmo, 1991; Gullino, 1992; Elad *et al.*, 1993). Biological control does not only have to make use of natural biocontrol agents; through genetic engineering, other fungal or yeast species can be enhanced to produce their own chitinases or other antimicrobial substance more effectively.

1.2. SPECIFIC PROJECT AIMS

In the yeast *Saccharomyces cerevisiae*, chitin comprises only about 1% of the total cell wall, with the majority exclusively present in the primary septum of budding cells (Cabib, 1987). Chitin fulfils an essential role in cell proliferation and is needed to form the chitin ring, the primary septum and eventually the bud scar remaining on the mother cell after cytokinesis (Cabib *et al.*, 1990).

The yeast chitinase enzyme, encoded by the *CTS1-2* gene, is responsible for the breakdown of the primary chitin septum between the mother and daughter cells after cell division (Kuranda and Robbins, 1991). The lack of this enzyme results in cells with defects in cell separation, leading to cells aggregating at the septum regions and remaining as clusters (Kuranda and Robbins, 1991). Yeast chitinases are either incorporated into the cell wall, where they function in cell separation (Elango *et al.*, 1982), or are secreted into the growth medium (Kuranda and Robbins, 1991).

Similar to other chitinase enzymes, the *S. cerevisiae* chitinase may possibly exhibit some antifungal activity. It has been speculated by Kuranda and Robbins (1991) that the secreted enzyme might suppress the growth of other microorganisms through the hydrolysis of their cell wall chitin or related polysaccharides. The proposed catalytic domain of this enzyme shows 38% homology to that of the *Beta vulgaris* (sugar beet) chitinase, which is produced as a PR protein in response to pathogen invasion. The possible antifungal activity of the yeast chitinase prompted the use of the *CTS1-2* gene as a candidate for overexpression in both yeast and plant expression systems in this study.

Two overriding goals were set in this study. The first involved the optimisation of the expression and secretion of the *CTS1-2* gene in *S. cerevisiae*, to confirm the antifungal activity of this enzyme and to render a recombinant yeast with possible applications as a biocontrol agent controlling plant pathogenic fungi. *S. cerevisiae* was used as a host, since an overwhelming wealth of information on genetics, molecular biology and physiology has been accumulated on this model eukaryote (Rose and Harrison, 1989; Broach *et al.*, 1991). Also, many heterologous protein production processes are based on systems that occur in *S. cerevisiae* (Heinisch and Hollenberg, 1993; Hinnen *et al.*, 1994).

The secretion of the antimicrobial property plays a crucial role in the antagonistic activity of biocontrol agents. The hypothesis was that high-level expression and efficient secretion would be required in a biocontrol yeast strain. The secretion efficiency of chitinase was evaluated by testing three different secretion signals. Two strong promoters and terminators were also tested for high-level expression of the chitinase gene. The secretion signals used included the native secretion signal of the *CTS1-2* gene, the *S. cerevisiae* mating pheromone α1-factor (*MFα1*) secretion signal, as well as the *Trichoderma reesei* β-xylanase 2 (*XYN2*) secretion signal (La Grange *et al.*, 1996). The *MFα1* secretion signal is used widely for the secretion of heterologous proteins from

yeast (Brake, 1990), whereas the leader sequence of the *T. reesei XYN2* gene shows similarities to leader sequences of many *S. cerevisiae* genes (Kozak, 1991) and contains all the features required for processing in *S. cerevisiae* (La Grange *et al.*, 1996).

To summarise, the specific aims and approaches of this first overriding goal of the study included the following:

(i) the construction of yeast expression/secretion cassettes containing the *CTS1-2* gene fused to its native secretion signal, the *MFα1* secretion signal and the *XYN2* secretion signal, either under the control of the *PGK1* or the *ADH2* promoters and terminators;

(ii) the overexpression of the targeted chitinase gene in *S. cerevisiae* strain PRY488 through the transformation of the yeast with the various fusion cassettes;

(iii) the determination of gene expression from the various promoters and the measurement of the levels of chitinase activities of the transformed yeasts, both in the cell-associated (intracellular and periplasmic) and extracellular fractions. By comparing the levels of chitinase activities extracellularly, as well as cell-associated activities for the various gene constructs, the effectiveness of the various secretion signals will be compared;

(iv) the confirmation of antifungal activity of the secreted enzyme on spore germination and growth of the fungal pathogen, *B. cinerea*.

These approaches and their results are presented in Chapter 3 of this thesis, as well as in Addendum A of Chapter 3.

The second overriding goal of this study was to assess the potential of the yeast chitinase gene in upregulating defence against fungal infection *in planta*. To this end, the *CTS1-2* gene was constitutively overexpressed in tobacco plants. It has been shown that the majority of microbial plant pathogens grow extracellularly during at least the early stages of the infection process (Isaac, 1992). In some of the constructs, the *CTS1-2* gene was fused to the secretion signal of an antimicrobial peptide isolated from the seeds of *Mirabilis jalapa* L. (the four o'clock plant) (Cammue *et al.*, 1992). This secretion signal will target the chitinase protein to the apoplastic region to counter the initial pathogen invasion.

The specific aims and approaches of this part of the study included the following:

(i) the construction of plant expression/secretion cassettes containing the *S. cerevisiae CTS1-2* gene, either with its native secretion signal intact, or fused to the secretion signal of the antimicrobial peptide, *Mj*-AMP2, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and *Agrobacterium* octopine synthase 3' terminator;

(ii) the mobilisation of the expression cassettes into tobacco via *Agrobacterium tumefaciens* and the regeneration of transgenic tobacco lines constitutively overexpressing the yeast *CTS1-2* gene;

(iii) the analysis of the primary transformants, as well as the F_1 progeny plants for gene integration and copy number, for gene expression and chitinase activity;

(iv) the evaluation of the antifungal activities of the heterologous chitinases upon fungal infection of the transgenic lines with *B. cinerea*. The effect of the overexpressed chitinases will be determined both *in vitro* and *in planta*.

These approaches and their results are presented in Chapter 4 of this thesis, as well as in Addendum B to Chapter 4.

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CHAPTER 2

LITERATURE REVIEW:

Chitinases as antifungal control proteins

LITERATURE REVIEW

2.1. INTRODUCTION

Chitin is an abundant natural resource obtained from marine invertebrates, insects, algae and fungi; in the latter it comprises between 22% and 44% of the fungal cell wall (Muzzarelli *et al.*, 1994). The complete enzymatic hydrolysis of chitin to subsequently free *N*-acteylglucosamine (GlcNAc) residues is performed by a chitinolytic system involving chitinases, the action of which is known to be synergistic and consecutive (Shaikh and Deshpande, 1993). Chitinases are abundant proteins produced by a variety of microorganisms and plants (Blaak and Schrempf, 1995). Most of the microbial producers also contain chitin in their backbone. Plants, however, produce chitinases, but lack the chitin polymer in the plant body.

Chitinases synthesised by organisms have various functions, each uniquely adapted to the specific producer organism. In the yeast *Saccharomyces cerevisiae*, chitinases play a role in cell separation and cell division (Kuranda and Robbins, 1991). In other microorganisms, specifically fungi, chitinases are involved in morphogenesis (Sahai and Manocha, 1993), whereas in bacteria they play roles in nutrition and parasitism (Cohen-Kupiec and Chet, 1998). Several species of fungi are very potent biocontrol agents of plant pathogenic fungi and insects. These entomopathogenic and mycoparasitic fungi produce chitinases for the invasion of hyphae and the killing of host cells (Kang *et al.*, 1998). Chitinases also have been found to be involved in the defence mechanisms of plants and vertebrates (Felse and Panda, 1999; Patil *et al.*, 2000). In many plant species, the invasion of a pathogen induces the production of a specific group of proteins, designated pathogenesis-related (PR) proteins, which include chitinases, β-1,3-glucanases, proteinases, etc. (Kombrink and Somssich, 1995) as part of their defence response. The fact that pathogenic fungi contain chitin in their cell walls and that plant chitinases are induced upon pathogen attack, have confirmed chitinases and other PR proteins as integral and crucial parts of the plants' natural defence response (Cohen-Kupiec and Chet, 1998; Felse and Panda, 1999; Patil *et al.*, 2000).

Agricultural crops world-wide suffer from a vast array of fungal diseases. This leads to severe yield losses and can consequently contribute to human anguish. The high cost of chemicals and growing concern about the environment are encouraging farmers and researchers to look for alternative means to control diseases, other than the use of chemicals (Schickler and Chet, 1997). Alternatives include the use of biological control agents and the genetic improvement of transgenic crop plants through transformation technology to upregulate the plant's natural defence mechanisms. The significant roles of chitinases in natural biocontrol agents and plant defence systems have already been mentioned and motivated. In this review, attention has been focused on some general

aspects of the polymer chitin and the hydrolysing activities of chitinases of microbial and plant origin. The possible application of chitinases as antifungal agents, either in the production of biocontrol agents or in genetic transformation strategies, is also explored.

2.2. CHITIN AND CHITINASES: A general overview

Chitin, an insoluble linear β-1,4-D glycosidically linked fibre-forming homopolymer of GlcNAc units (Fig. 1), is one of the most abundant polysaccharides in nature, second only to cellulose.

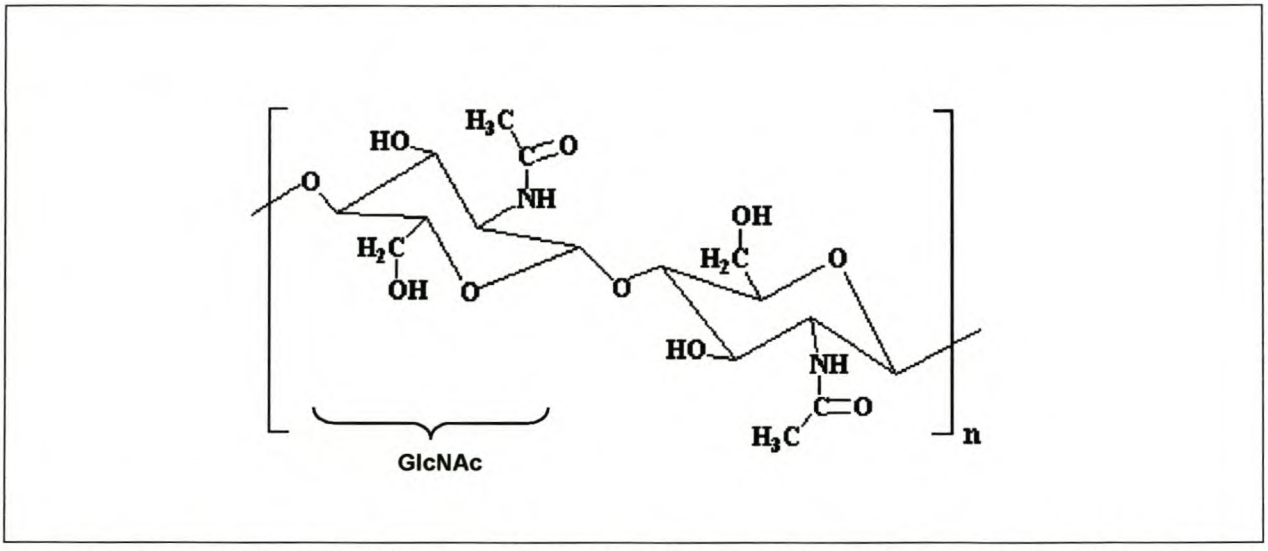

Figure 1. The structure of chitin. *N*-acetylglucosamine (GlcNAc) residues are glycosidically linked to produce chitin (adapted from Mathias, 1996).

The individual chains constituting this polymer can be seen as helixes, since each sugar is inverted with respect to its neighbours. These motifs and their arrangement lead to the formation of a more stable structure (Gooday, 1990c). The most common form of chitin is α-chitin that consists of two *N,N'*-diacetylchitobiose units of two chains in an antiparallel arrangement (Minke and Blackwell, 1978). β-chitin is less abundant and consists of one *N,N'*-diacetylchitobiose unit. These chains have a parallel arrangement that allows for more flexibility than α-chitin, but they still retain considerable strength (Lindsay and Gooday, 1985). A third form of chitin, with mixed parallel and antiparallel orientations, has also been reported by Rudall and Kenchington (1973) and has been designated γ–chitin. The structures of α- and β–chitin are shown in Fig. 2.

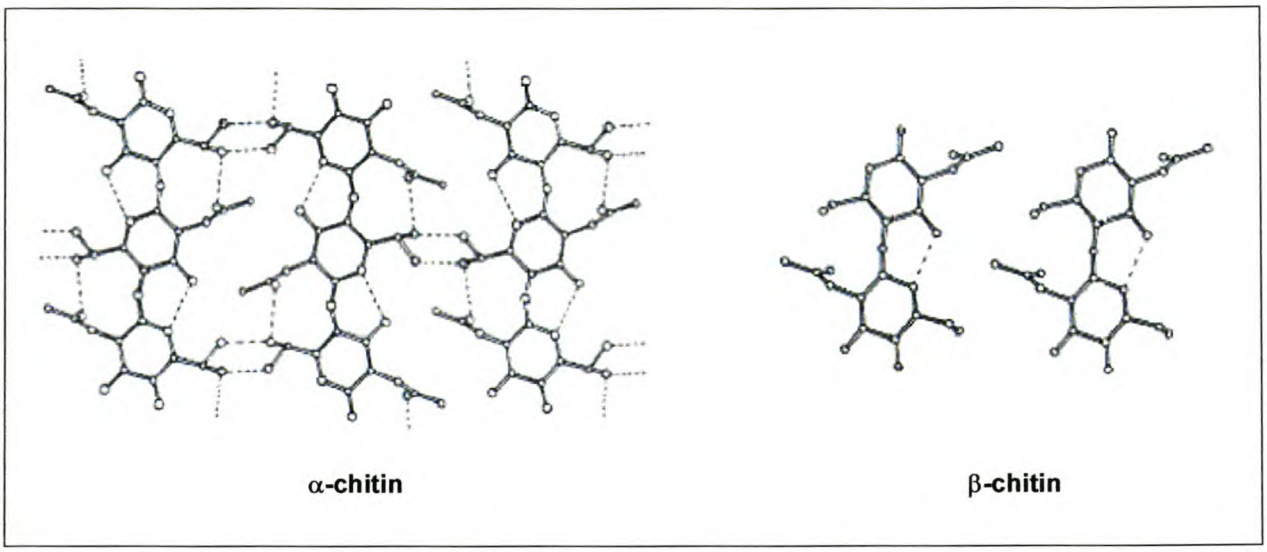

Figure 2. The antiparallel and parallel arrangement of α- and β-chitin (Blackwell, 1988).

Chitin is an important structural material and is a common constituent of insect exoskeletons, the shells of crustaceans as well as fungal cell walls. Chitin and chitosan, the latter being a deacetylated form of chitin, have important uses in the agricultural, food, cosmetic and pharmaceutical industries, as well as in medicine (Flach *et al.*, 1992). The production of chitin in the biosphere approaches 10^{10}-10^{11} ton annually (Gooday, 1990a). To reach a balanced situation, it is important that the degradation of chitin occurs at a similarly fast rate to prevent the development of a global sink of unavailable carbon and nitrogen.

Chitin is degraded via three possible mechanisms, either chitinoclastical (chitin breaking), chitinolytical (hydrolysis of β-1,4-glycosidic bonds) or through deacetylation of chitin to chitosan (Davis and Eveleigh, 1984). The mineralisation of chitin occurs primarily through microbes, with bacteria predominating in aquatic systems, whereas both fungi and bacteria are important chitin degraders in soil (Gooday, 1990a; 1993). In chitin-containing organisms, the role of the chitin degrading systems can be pathogenic, nutritive, or autolytic and morphogenetic (Gooday, 1990b). The enzymes involved in chitin degradation include chitinases, glucosaminidases, deacetylases and chitosonases; their relationship in chitin degradation is shown in Fig. 3.

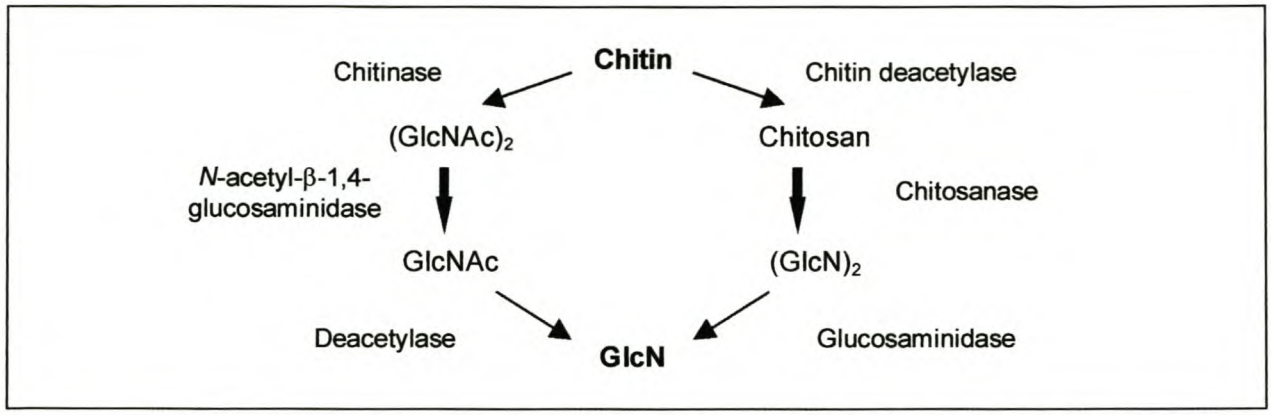

Figure 3. Possible pathways of chitin degradation. In the figure, $(GlcNAc)_2$ refers to diacetylchitobiose, GlcNAc refers to *N*-acetylglucosamine, $(GlcN)_2$ refers to chitobiose and GlcN refers to glucosamine.

Chitin-containing organisms, as well as some organisms that do not contain chitin, such as higher plants and a variety of bacteria, produce the enzyme chitinase (EC 3.2.1.14). Chitinases were first identified by Bernard (1911) (as referenced by Flach *et al.*, 1992), who identified a thermosensitive and diffusable antifungal factor from orchid bulbs. In 1929, a similar factor from snails was reported by Karrer and Hoffman (as referenced by Flach *et al.*, 1992). Chitinases are defined as enzymes that cleave a bond between the C_1 and C_4 of two consecutive GlcNAc units of chitin (Flach *et al.*, 1992). Chitinases can be classified into exochitinases (EC 3.2.1.14.) or endochitinases (EC 3.2.1.14.). The mode of action of these enzymes is shown in Fig. 4.

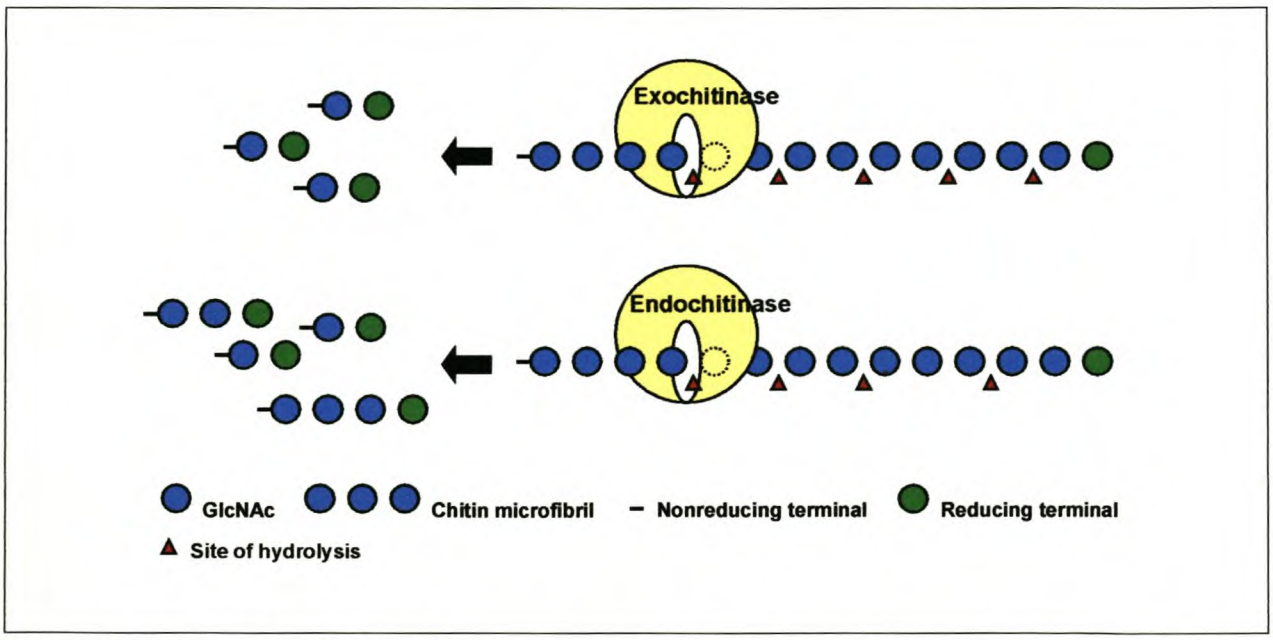

Figure 4. The mode of action of exochitinase and endochitinase enzymes (adapted from Sahai and Manocha, 1993).

Endochitinases cleave the chitin chain randomly at internal sites, generating soluble, low–molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose and the dimer diacetylchitobiose (Sahai and Manocha, 1993). Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29) (Harman *et al.*, 1993), which catalyse the progressive release of diacetylchitobiose from the nonreducing end of the chitin microfibril; and *N*-acetyl-β-1,4-glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha, 1993). Some chitinases may also display varying levels of lysozyme activity (EC 3.2.1.17) (Jollès and Jollès, 1984); exochitinases have also been reported to have transglycosidase activities (Flach *et al.*, 1992).

Six classes of chitinases have been proposed, based on sequence similarity. It has been found that if sequence similarity is high and dispersed over the entire sequence, it is indicative of folding similarity. Five of the six classes can also be grouped into two glycosyl hydrolase families, namely family 18 and 19 (Table 1) (Henrissat, 1991; Henrissat and Bairoch, 1993). Chitinases from classes I, II and IV are of plant origin and make up the family 19 of glycosyl hydrolases (Sahai and Manocha, 1993; Hamel *et al.*, 1997), which play an important role in plant defence against fungal pathogens. These classes share a homologous catalytic domain with the signal peptide found in all of them. Class III chitinases are mainly of fungal origin, although some originate from plants (Hamel *et al.*, 1997). Class III and V chitinases combined comprise family 18 of glycosyl hydrolases (Henrissat, 1991; Henrissat and Bairoch, 1993), which are structurally unrelated to family 19 (Meins *et al.*, 1992). Class V is composed mainly of bacterial chitinases, but, as in Class VI, it also includes singular examples of extraordinary plant chitinases.

Table 1. The six classes of chitinases and their groupings in the different glycosyl hydrolase families

Class	Origin	Glycosyl hydrolase family
I	Plant	19
II	Plant	19
III	Plant/Fungal	18
IV	Plant	19
V	Plant/Bacterial	18
VI	Plant	Unknown

It has been reported that chitinase gene expression in microorganisms is under the control of a repressor/inducer system in which chitin or other products of degradation act as inducers (Felse and Panda, 1999). Studies done of *Metarhizium anisopilae* in restricted cultures (a carbon-deficient medium with slow feeding of sugars) to prevent catabolic repression revealed that GlcNAc and glucosamine are the most efficient inducers of

chitinase and chitobiase respectively. Thus, the accessibility and amount of chitin, relative to other available nutrients, may control the level of chitinase through the reported repressor/inducer system (Felse and Panda, 1999).

A better knowledge of chitinases is important for several applications: they may be useful in the degradation of chitin-containing waste, they have been implicated in the biocontrol of plant pathogenic fungi, and they may be used for the chemical modification of chitin and chitosan. These practical applications have led to the extensive study of chitinase gene expression, its regulation and the cloning of chitinase genes in the search for enhanced production of the enzyme.

2.3. MICROBIAL CHITINASES

In this section, special attention will be paid to *Saccharomyces cerevisiae* chitinases. The occurrence of chitin in yeast cells will be discussed briefly to highlight its necessity in the survival of the cell. This discussion will contribute to an understanding of the function, as well as the regulation, of chitinases in yeast cells.

2.3.1. *Saccharomyces cerevisiae* chitinases

2.3.1.1. The occurrence of chitin in the yeast cell wall

Cell walls are crucial for the integrity of yeast cells; the rigid structure maintains the shape of the cell and offers protection against harmful environmental conditions (Fleet, 1991). The digestion of the cell wall in the absence of an osmotic protector leads to cell lysis, due to the high internal turgor pressure. Substances that interfere with the cell wall integrity or synthesis therefore may be considered potential antifungal agents (Kollár *et al.*, 1997). The cell wall consists mainly of carbohydrates, which occur either as free complexes or linked to proteins. The main components of the *S. cerevisiae* cell wall include β-1,3-D-glucan, β-1,6-D-glucan, mannoprotein(s) and chitin, as shown in Fig. 5.

The β-glucans play an important role in maintaining the osmotic stability and mechanical strength of the yeast cell. Eighty percent of cell wall-bound β-glucan consist of β-1,3-linked glucose residues, and the remaining 20% is accounted for by β-1,6-linked glucose units (Fleet, 1991). In *S. cerevisiae*, the alkali soluble β-1,3/β-1,6-glucan heteropolymers become alkali insoluble when linked to chitin (Hartland *et al.*, 1994). The linkages occur via β-1,4 bonds from the terminal reducing residues of chitin to the nonreducing ends of β-1,3-glucan (Kollár *et al.*, 1995). The glucans, which are interwoven with chitin fibrils, form the internal skeletal layer of the cell wall, whereas the outer layer consists of mannoproteins (Kapteyn *et al.*, 1997).

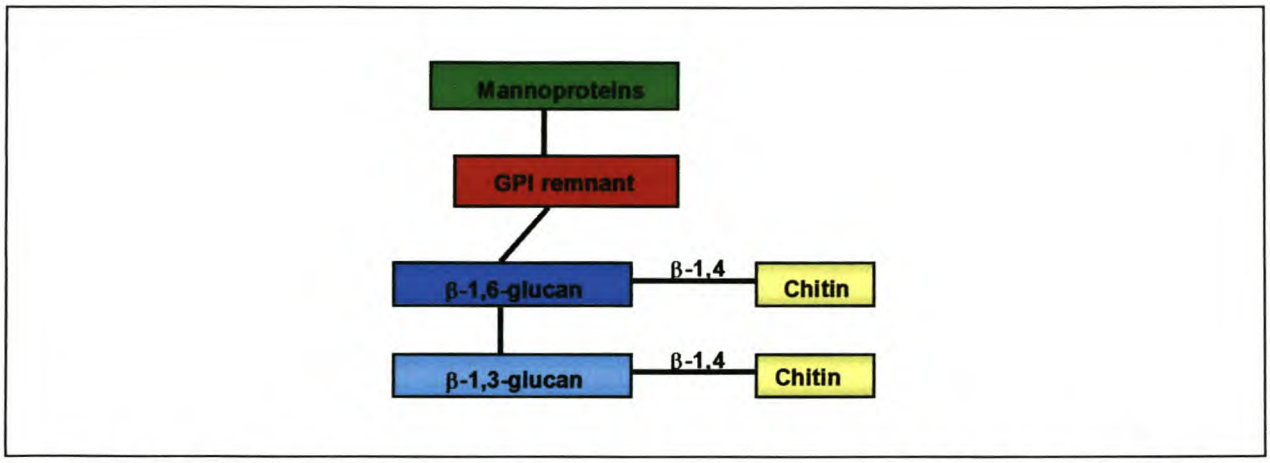

Figure 5. The yeast cell wall complex, showing the main components and their linkages (Kollár *et al,* 1997). GPI refers to glycosylphosphatidylinositol.

These mannoproteins are linked covalently to the heteropolymers of β-1,3/β-1,6-glucans via a glycosylphosphatidylinositol (GPI)-derived structure (Fig. 5) (Kapteyn *et al.*, 1996). Mannoproteins limit the permeability of the cell wall, thereby retaining the periplasmic enzymes and thus protecting the cell against degradation by enzymes from other organisms (Ram *et al.*, 1995).

Chitin and chitosan are biosynthetically related wall components of a wide variety of yeasts and fungi. In *S. cerevisiae*, chitin fulfils an essential role in cell proliferation, as it is needed to form the chitin ring, the primary septum and eventually the bud scar remaining on the mother cell after cytokinesis (Cabib *et al.*, 1990). Chitin is deposited in a highly controlled manner at the time of septation during the cell division cycle (Ballou, 1982). The formation of the primary septum in budding yeasts seems to take place in two steps (Cabib and Bowers, 1975). First, a chitin ring is deposited around the "neck" between the mother and daughter cell, onto which the primary septum is added in a centripetal fashion. Secondly, two lateral layers of gluco- and mannoproteins are added to form the mature, disk-shaped septum (Ballou, 1982). Shaw *et al.* (1991) showed that the inner layer of the cell wall consists of chitin, and that this layer is deposited in the daughter cell only after septation.

Chitin is synthesised and deposited in the cell wall through the action of chitin synthase (CS) enzymes. Three CS activities, CSI, CSII and CSIII, have been identified thus far. These three isoenzymes differ in certain properties, such as metal specificity, optimum pH and their susceptibility to inhibitors (Cabib *et al.*, 1996). CSI and CSII activities are determined only by the products of the *CHS1* and *CHS2* genes, which encode the polypeptides that contain the catalytic domain of each chitin synthase respectively.

The Chs1p is responsible for the synthesis of chitin after cell separation, during which it plays a repair function. It counterbalances the acid-induced increase in the chitinase

activity that hydrolyses the chitin in the primary septum at the end of cytokinesis (Bulawa *et al.*, 1986; Kuranda and Robbins, 1987; Cabib *et al.*, 1989; Kuranda and Robbins, 1991). Although the CSI activity represents approximately 90% of the *in vitro* measurable chitin synthase activity, its contribution to the production of chitin *in vivo* is negligible (Valdivieso *et al.*, 2000).

The Chs2p is essential for the formation of chitin at the primary septum and is thus necessary for cell division (Orlean, 1987; Shaw *et al.*, 1991). The CSIII activity is responsible for the deposition of chitin in the ring at bud emergence and along the lateral cell walls and contributes to the synthesis of most of the cell wall chitin during vegetative growth (Shaw *et al.*, 1991). The Chs3p level remains constant during cell cycle progression, but its localisation changes (Chuang and Schekman, 1996).

The mechanism of regulation of CSIII activity seems to be complex, and so far five different gene products have been related directly to it (García-Rogriguez *et al.*, 2000). In addition to Chs3p, the catalytic domain of CSIII, Chs4p and Chs7p play a direct role in the regulation of the CSIII activity, while Chs5p and Chs6p have a more general role in the intracellular trafficking of proteins (Valdivieso *et al.*, 1999). Furthermore, CS activities exhibit *in vitro* zymogenic properties, suggesting that they are regulated at a posttranslational level (Cabib *et al.*, 1996).

Chitin is not only an essential component of vegetative cell walls, but also plays an important role in spore cell walls. Spore walls of *S. cerevisiae* are made up of four layers (Briza *et al.*, 1986). The inner layers are structurally similar to vegetative cell walls, but contain mostly glucan and fewer mannan residues. The two outer layers are specific to spores and have no structural similarities to vegetative cell walls (Katohda *et al.*, 1984). These layers are responsible for the resistance to adverse conditions typically encountered by spores. The outermost layer consists of various cross-linked amino acids, which are possibly covalently linked to the second outer layer, consisting of chitosan (Briza *et al.*, 1988).

The resilience and strength of yeast cell and spore walls are the result of the covalent linkages between the different aforementioned components (Fig. 5), which are rearranged and interlinked after deposition in the walls. This leads to a continuous structure, invariably leading to strength (Kollár *et al.*, 1997).

2.3.1.2. The yeast chitinase genes, their proteins and activities

Kuranda and Robbins (1991) reported different sizes of chitinase proteins isolated from different haploid strains that all have a S288C genetic background. The *CTS1-1* gene, isolated from DBY939, encodes a low molecular weight chitinase, whereas *CTS1-2* from DBY918 encodes a chitinase with a slightly higher molecular weight. The difference in size is a result of two 15-base pair direct nucleotide repeats, which flank a serine/threonine–rich region in the protein. Thus, the two clones have only slightly

different restriction maps and differ only approximately 5% on the nucleotide level. It is likely that the two different chitinase genomic clones represent allelic variants of a unique chitinase locus, termed *CTS1* in *Saccharomyces* (Kuranda and Robbins, 1991).

The expression of *CTS1* varies throughout the cell cycle, with maximal accumulation of mRNA during the G_1 phase (Dohrmann *et al.*, 1992). The *ACE2* gene (activator of *CUP1* expression encoding metallothionein) encodes a transcription factor that activates the transcription of genes expressed in the G_1 phase of the cell cycle; hence, *CTS1* expression is controlled by the Ace2p transcription factor, whose subcellular localisation varies through the progressive stages of the cell cycle (Dohrmann *et al.*, 1992). The *ACE2* gene is expressed in the G_2 period, but the proteins remain in the cytoplasm until the late M phase. At the end of mitosis, Ace2p enters the nucleus, coinciding with *CTS1* expression, and will subsequently be degraded after activation of the target gene (Dohrmann *et al.*, 1992; O'Conalláin *et al.*, 1999).

Yeast chitinase is responsible for breaking down the chitin septum between mother and daughter cells after cell division. Strains carrying mutations in either *CTS1* or *ACE2* have defects in cell separation and the resulting haploid cells display a clumpy phenotype (Kuranda and Robbins, 1991; Dohrmann *et al.*, 1992). Since diploid cells bud in a bipolar manner, the disruption of *ACE2* results in the production of long chains of cells. These are reminiscent of the pseudohyphal structures produced by some *S. cerevisiae* strains in response to specific conditions, such as low nitrogen availability, although the cells are not elongated (King and Butler, 1998). The disruption of *ACE2* almost abolishes the production of chitinase, yet leading to no detectable effect on the levels of chitin in the cells (King and Butler, 1998). This has also been noted in a *CTS1* deletion strain (Kuranda and Robbins, 1991), suggesting that the hydrolysing activities of chitinase simply reduce the chitin chain length, but do not reduce the overall amount.

The Ace2p is homologous to the Swi5p, a zinc-finger protein required for the transcription of the *HO* endonuclease gene, which encodes the homothallic endonuclease required for mating type switching (Stillman *et al.*, 1988; Butler and Thiele, 1991). Although Ace2p and Swi5p combined are required for the maximal expression of several genes, Ace2p alone regulates the expression of *CTS1* (Dohrmann *et al.*, 1992, 1996; Toyn *et al.*, 1997).

The *CTS1-2* gene of *S. cerevisiae* encodes a mature, secreted protein of approximately 130 kDa, however the deglycosylated protein has a molecular weight of approximately 60 kDa. The protein contains four domains: a signal sequence, a catalytic domain, a serine/threonine-rich region and a carboxyl-terminal domain (Kuranda and Robbins, 1991) (Fig. 6). A function for each of the four domains has been suggested by Kuranda and Robbins (1991).

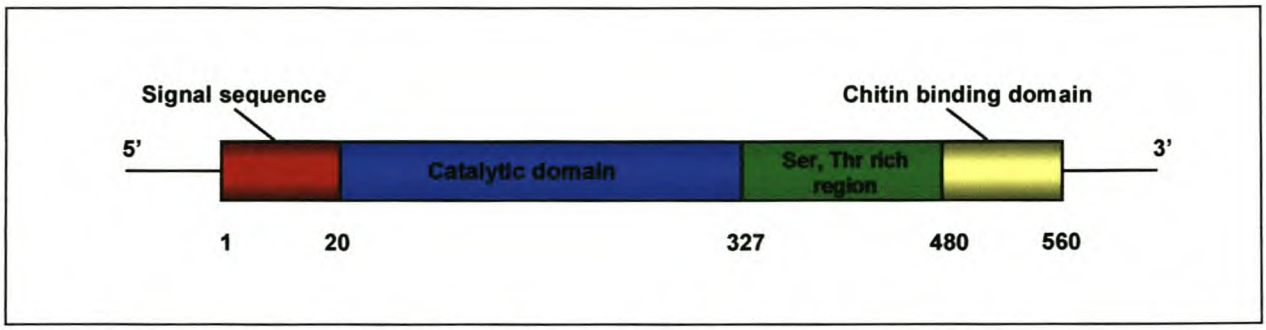

Figure 6. Schematic representation of the yeast Cts1-2 chitinase protein (adapted from Kuranda and Robbins, 1991). The numbers refer to the relevant amino acid positions on the Cts1-2p.

The first 20 amino acids serve as a typical cleavable signal sequence. Some secretory enzymes, including chitinases, have been found to be liberated from protoplasts during incubation in growth media. This is a result of the secretion of the enzyme already present in the cell, rather than the synthesis of a new enzyme. Elango *et al.* (1982) indicated that approximately 50% of the enzyme is located in the periplasmic space during growth and at least part of the remaining chitinase appears to be enclosed in vacuoles or in vesicles. In contrast to the findings with protoplasts, the chitinase secreted by intact cells into the periplasmic space does not reach the medium. The reason for this is unclear and it is speculated that hydrophobic forces may be involved (Elango *et al.*, 1982). However, it has been shown that up to 90% of chitinase activity is secreted into the growth media when cells are grown in nutrient rich medium (YPD media).

The amino acid residues between 21 and 327 comprise the proposed catalytic domain (Kuranda and Robbins, 1991). There are conserved regions between the *S. cerevisiae* chitinase, several bacterial chitinases and a mammalian lysosomal chitobiase. These regions play a role in the cleavage of the β-1,4 glycosidic bonds between adjacent GlcNAc residues, obviously playing an important role in the catalytic function of these enzymes (Jones *et al.*, 1986; Metraux *et al.*, 1989). Fig. 7 shows sequence comparisons of *S. cerevisiae* chitinases with other chitinases. This proposed catalytic domain shows 38% homology to that of the *Beta vulgaris* (sugar beet) chitinase that is produced in response to pathogen invasion.

```
                                REGION 1                                    REGION 2
S. cerevisiae (CTS1-2)     96 CTQIAEDIETCQSLGKKVLLSLGGASGSY 124 154 RPFDSAVVDGFDFDIE 169
Candida albicans (CHT1)       CSQIGSDIKSCQQQGKTILLSLGGATGNY          RPFDDAIVDGFDFDIE
C. albicans (CHT2)            CSQIGADIKTCQSLGKTVLLSLGGGVGDY          RPFDDAVVDGFDFDIE
C. albicans (CHT3)            CQTIAEDIKYCQNKGKTILLSLGGAAGSY          RPFYDAVLDGFDFDIE
Rhizopus niveus               CPTVGSDIKTCQSNGVKVLLSLGGAAGSY          RPFDDAVIDGIDLDIE
Beta vulgaris                 CNSLSSDIKTCQQAGIKVLLSIGGGAGGY          RPLGDAVLDGIDFDIE
Cicer arietinum               CTKFSPEIQACQAKGIKVLLSLGGGAGSY          RPLGDAVLDGIDFDIE
Nicotiana tabacum (acidic)    CTGLSNDIRACQNQGIKVMLSLGGGAGSY          RPLGDAVLDGIDFDIE
Nicotiana tabacum (basic)     CQQLTKSIRHCQSIGIKIMLSIGGGTPTY          RPLGDAVLDGIDFDIE
Phaseolus angularis           CNVFSDQIKECQSKDIKVLLSLGGASGSY          RPLGDAILDGVDFDIE
Cucumis sativus               CAFLSDEINSCKSQNVKVLLSIGGGAGSY          RPLGAAVLDGVDFDIE
Rhizopus oligosporus (CHI1)   CPAVGADIKKCQDKGVKVILSLGGAAGVY          RPFGDAVIDGVDLDIE
Hevea brasiliensis            CTIVSNGIRSCQIQGIKVMLSLGGGIGSY          RPLGDAVLDGIDFDIE
Rhizopus oligosporus (CHI2)   CPAVGADIKKCQDKGVKVILSLGGAAGVY          RPFGDAVIDGVDLDIE
Vitis vinifera                CTSVSTGIRNCQNRGIKVMLSIGGGAGSY          RPLGDAVLDGIDFDIE
Coccidiodes immitis           CYQIKEDLPKCKALGKTILLSLGGGADFY          RPFGEASVDGFDFDIE
Arabidopsis thaliana          CTHFGSQVKDCQSRGIKVMLSLGGGIGNY          RPLGDAVLDGIDFNIE
                              *  .   :  *:  . .::**:**.   *          **:  * :**.*::**

                              REGION 3          REGION 4
S. cerevisiae (CTS1-2)    199 AAPQCPYPD 207 220 DFAFIQFYNN 229     [100]
Candida albicans (CHT1)       AAPQCPYPD         DFAFIQFYNN         [48]
C. albicans (CHT2)            AAPQCPYPD         DFAFIQFYNN         [44]
C. albicans (CHT3)            AAPQCPYPD         DFVFIQFYNN         [44]
Rhizopus niveus               AAPQCPFPD         DFVNVQFYNN         [39]
Beta vulgaris                 AAPQCPLPD         DYVWVQFYNN         [38]
Cicer arietinum               AAPQCPYPD         DYVWVQFYNN         [38]
Nicotiana tabacum (acidic)    AAPQCPFPD         DYVWVQFYNN         [37]
Nicotiana tabacum (basic)     AAPQCPFPD         DYVWVQFYNN         [36]
Phaseolus angularis           AAPQCPIPD         DIVWVQFYNN         [36]
Cucumis sativus               AAPQCPIPD         DSVWVQFYNN         [35]
Rhizopus oligosporus (CHI1)   AAPQCPFPD         DYVNVQFYNN         [35]
Hevea brasiliensis            AAPQCPFPD         DYVWVQFYNN         [35]
Rhizopus oligosporus (CHI2)   AAPQCPFPD         DYVNVQFYNN         [35]
Vitis vinifera                AAPQCPFPD         DYVWVQFYNN         [34]
Coccidiodes immitis           AAPQCIMPD         DFIFIQFYNN         [33]
Arabidopsis thaliana          GAPQCPFPD         DYVWIQFYNN         [32]
                              :****  **         *   :*****
```

Figure 7. Sequence comparisons of *S. cerevisiae* chitinases with other chitinases. Four regions of high homology were identified by the protein-protein BLAST programme (labelled regions 1-4). Alignments were performed with the CLUSTAL W (1.8) multiple sequence alignment programme. Values in brackets are indicative of the percentage of pairwise sequence identity across the entire gene.

Approximately 152 amino acids are found in the serine/threonine–rich region. More than 50% of the amino acid residues in this region are serine or threonine, both of which can potentially act as acceptor sites for *O*–glycosylation (Kuranda and Robbins, 1991). The glycosylation of membrane and secretory proteins is the most complex post-translational modification known to occur in eukaryotes. The transfer of mannose to the hydroxy amino acids serine and threonine of the secretory proteins is catalysed by a family of protein mannosyltransferases coded for by seven genes (*PMT1*-7). *O*–glycosylation is

initiated in the endoplasmic reticulum, where mannose dolichylphosphate is the sugar donor of the reaction (Lussier *et al.*, 1995; Gentzsch and Tanner, 1996). *S. cerevisiae* chitinase carries the typical array of short mannose oligosaccharides, consisting of two to five mannose units. Protein mannosyltransferase 1 and 2 (Pmt1p and Pmt2p) are involved in the transfer of the first mannose residue to specific serine/threonine residues of this enzyme (Strahl-Bolsinger *et al.*, 1993; Lussier *et al.*, 1995). It has been shown that protein *O*-glycosylation is essential for cell wall rigidity and cell integrity (Gentzsch and Tanner, 1996) and that this protein modification is vital for *S. cerevisiae.*

The last 80 amino acids encode a noncatalytic carboxyl-terminal domain. The proposed function of this domain is a high binding affinity to chitin (Kuranda and Robbins, 1991).

The purified *S. cerevisiae* chitinase enzyme liberates oligosaccharides of various sizes from chitin, thus behaving as an endochitinase (Correa *et al.*, 1982). As found with other chitinases, this yeast chitinase is much more active on nascent chitin, *i.e.* the chitin formed in the same reaction mixture by the corresponding synthetase, than on preformed polysaccharides (Correa *et al.*, 1982).

Chitinases have two potential fates: incorporation into the cell wall or release into the growth medium. The portion of the enzyme that becomes associated with the cell wall apparently functions in cell separation. This function is suggested by the higher levels of the enzyme during the log phase in comparison to the stationary phase (Elango *et al.*, 1982). The lack of chitinase activity in the log phase results in a clear inability of the cells to separate normally; instead, the cells remain as clusters, aggregating at the septum regions (Kuranda and Robbins, 1991). The secreted *Saccharomyces* chitinase might play a dual role by also suppressing the growth of other microorganisms through the hydrolysis of cell wall chitin or related polysaccharides (Kuranda and Robbins, 1991).

Among the kinetic aspects, an unusual property of the enzyme is its low pH optimum of pH 2.5; the protein is also still relatively stable at pH 3. Another surprising fact is the easy extractability of the chitinase protein from intact cells with digitonin. Digitonin is a mild, non-ionic detergent used to solubilise receptors and permeabilise cellular and nuclear membranes (Mooney, 1988). This is due to the apparently high molecular weight of at least a part of the protein and the possibility that the cell wall acts as a barrier to substances with Mr>13 kDa. Thus, it is speculated that the chitinase is a small molecule when extracted and that it rapidly aggregates into variably sized polymers (Correa *et al.*, 1982). Correa *et al.* (1982) concluded that the chitinase enzyme is associated with mannan by means of a GlcNAc-asparagine bridge and it would appear that this sugar has some role in the aggregation process. However, in contrast to Correa *et al.* (1982), Elango *et al.* (1982) concluded that the association between the enzyme and carbohydrate (mannan) occurs before crossing the plasma membrane and that the enzyme appears to be stored in vesicles as a prelude to its secretion into the periplasmic space.

2.3.2. Other fungal chitinases

Fungal chitinases act either as endo- or exochitinases and are soluble; therefore they are probably sequestered in lysosomal vacuoles, or are membrane- or wall-bound (Flach *et al.*, 1992). Most fungal chitinase enzymes contain a signal peptide and some have a chitin-binding domain (Kuranda and Robbins, 1991), whereas others lack this domain (Hayes *et al.*, 1994). They are active at slightly acidic pH levels and have high temperature optima (Sahai and Manocha, 1993). These enzymes have a high degree of stability that may be due to glycosylation and they share high amino acid homology with class III plant chitinases (Sahai and Manocha, 1993; Limn *et al.*, 1995; Hamel *et al.*, 1997). The endochitinase of *Trichoderma harzianum* IMI206040 encoded by *ech42*, however, shares homology with bacterial chitinases (Hayes *et al.*, 1994) and with tobacco class V chitinase. This suggests that it should be included with the class V chitinases.

In *Mucor mucedo*, it has been shown (Humphreys and Gooday, 1984a) that the microsomal chitinase activity is dependent on the phospholipid environment. In contrast to cell wall-bound chitinases, membrane-bound chitinases could not be extracted by digitonin (Flach *et al.*, 1992). Humphreys and Gooday (1984b) showed that chitinase activity could be enhanced by treatment with commercial proteases. Since both chitinase and chitin synthase are membrane bound, zymogenic and activated by partial proteolysis, it was suggested that microsomal chitinases were co-regulated with chitin synthase and implicated in cell growth regulation (Manocha and Balasubramanian, 1988).

Fungal chitinases have multiple functions. They are, as already mentioned, active in fungal developmental processes, including spore germination, exponential growth and mycelial development (Gooday *et al.*, 1992), as well as in morphogenesis, since chitin is a major call wall component in fungi (Sahai and Manocha, 1993). The growth of vegetative hyphae is apical, with the majority of chitin being laid down in the apex (Wessels, 1990). To allow for the extension of these hyphae, the apical wall is maintained in a plastic state. This is due to the interplay of wall synthetic enzymes (chitin synthases) with wall lytic enzymes (chitinases) (Bartnicki-Garcia, 1973). Chitinases also play a key role in nutrition as well as in microparasitism, which is displayed by fungi such as *Trichoderma* spp. against several plant pathogenic fungi (Chet, 1987). Sahai and Manocha (1993) have listed other processes that fungal chitinases could be involved in. Some of these include the net degradation of wall polymers during starvation and septa during nuclear migration, the fusion of hyphae during anastomosis and clamp connections, and autolysis. Chitinase activity was also found to be associated with the biological activity of the *Kluyveromyces lactis* toxin (Butler *et al.*, 1991). This protein contains three subunits, namely α, β, and γ. The α subunit is an endochitinase that is required for the initial interaction of the toxin with the sensitive yeast cells.

2.3.3. Bacterial chitinases

Bacterial chitinases can act either as endo- or exochitinases. Roberts and Selitrennikoff (1988) compared the antifungal activity of plant and bacterial chitinases and found that, as expected, the plant chitinases isolated from wheat, barley and maize functioned as endochitinases. In contrast, the bacterial enzymes isolated from *Serratia marcescens*, *Streptomyces griseus* and *Pseudomonas stutzeri* were characteristically exochitinases. In addition, Yabuki *et al.* (1986) found that the chitinase enzyme of *Aeromonas hydrophila* acted in an endosplitting manner, leading to low molecular mass multimers of GlcNAc.

The chitin-binding domain of bacterial chitinases can be located either in the amino-terminal or the carboxy-terminal of the enzyme (Watanabe *et al.*, 1994). A summary of the domain structure of chitinases from *Streptomyces* identified two major classes of chitinases, with the members of each class sharing homology in the signal peptide, chitin binding and catalytic domains (Miyashita *et al.*, 1997). These enzymes can be classified as members of the class V chitinases. Most of the bacterial chitinases isolated and sequenced are included in family 18 of the glycosyl hydrolases; however, there is one report of a chitinase isolated from *S. griseus* HUT6037 that belongs to family 19 of glycosyl hydrolases (Ohno *et al.*, 1996). Until the addition of this chitinase, family 19 contained chitinases exclusively of plant origin.

Bacteria produce several chitinases to meet their nutritional needs and to hydrolyse the diversity of chitins found in nature (Svitil *et al.*, 1997). These microorganisms also play a major role in chitin mineralisation, for example in marine sediment and water (Herwig *et al.*, 1988). In bacteria, chitinases were shown to be extracellular enzymes. This was proven when *S. marcescens* was cultured in liquid media with chitin as the sole carbon source, and it was found that the chitinase was secreted into the media (Kole and Altosaar, 1985).

Bacterial chitinases can also be involved in transglycosylation reactions. The *Nocordia orientalis* chitinase (Usui *et al.*, 1987; Nanjo *et al.*, 1989), which essentially acts hydrolytically, can also catalyse transglycosylation reactions of $(GlcNAc)_4$ and $(GlcNAc)_5$. In the presence of ammonium sulphate, the enzyme converts the tetrasaccharide into $(GlcNAc)_6$ and $(GlcNAc)_2$ as the major products. It also converts the pentaoligosaccharide into $(GlcNAc)_7$ and $(GlcNAc)_3$. This method can be applied in the preparative-scale synthesis of $(GlcNAc)_{6\text{-}7}$, which are biologically active oligosaccharides of importance in biology and medicine, including the production of contact lenses and dressings for burns.

Another possible application of bacterial chitinases is the use of these enzymes against pest insects. Chitin is the major component of the lining of the digestive tract of insects. Therefore, ingestion of chitinolytic enzymes may significantly disrupt digestive physiology and can consequently reduce the growth and/or development of the insect

(Broadway *et al.*, 1995). The pH in the digestive tract of most herbivorous insects ranges from 8 to 10, while the chitinolytic enzymes that have been characterised from plants, bacteria and fungi generally function at pH 4-6 (Harman *et al.*, 1993; Sahai and Manocha, 1993). Broadway *et al.* (1995) isolated a *Streptomyces* strain that secreted chitinolytic enzymes that were active at pH 9. The active strain was identified as *S. albidoflavus*, based on its morphological and physiological characteristics and fatty acid profile. The endochitinase activity was tested and these authors were the first to demonstrate that the chitinolytic enzymes from *S. albidoflavus* had significant levels of activity under alkaline conditions.

Bifunctional chitinases, *i.e.* those with lysozyme and chitinase activities, have been described mainly in plants and are classified as class III chitinases. Two enzymes with bifunctional activity were also purified from *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997). These chitinases had antibacterial activity towards both Gram-negative and Gram-positive bacteria. This is an interesting phenomenon, since these proteins are not only antifungal, but can also inhibit bacteria.

2.4. PLANT CHITINASES

Plant chitinases are probably the most widely studied of this enzyme family (Table 2).

Table 2. Some examples of isolated plant chitinases, their origin and characteristics

Source organism	Observed phenotype/characteristics	References
Bean	Purified enzyme antifungal against *Trichoderma viride*	Schlumbaum *et al.*, 1986
Phaseolus vulgaris (bean)	Endochitinase; ethylene induced; lysozyme activity	Boller *et al.*, 1983
Bean	Chitinase-like protein; antifungal against *Rhizoctonia solani*, *Fusarium oxysporum*, *Coprinus comatus*	Ye *et al.*, 2000
Tobacco	Accumulation by environmental signals; developmental stages	Hensel *et al.*, 1999
Tobacco	Induced by various signals; antifungal against *T. viride*, *Alternaria radicina*	Melchers *et al.*, 1994
Spruce	Exochitinase; inactivates fungal elicitors	Salzer *et al.*, 1997
Grape	High levels of expression during fruit ripening	Robinson *et al.*, 1997
Hevea brasiliensis (rubber tree)	Hevamine; family 18 of glycosyl hydrolases	Bokma *et al.*, 1997

Their occurrence, expression and functions in plants are of interest, partly due to the absence of natural substrates (*i.e.* chitin) in the plant itself. Chitinases are constitutively expressed in many plants. An assortment of environmental stimuli, such as fungal challenge (Busam *et al.*, 1997a; 1997b), osmotic pressure (Yun *et al.*, 1996), and developmental stage such as fruit ripening (Clendennen and May, 1997; Robinson *et al.*,

1997), are associated with the induction of plant chitinase gene expression. Plant chitinases are small proteins (Mr between 25-40 kDa), with either very basic or very acidic isoelectric points (Flach *et al.*, 1992).

When chitinase hydrolyses a β-1,4 linkage, the GlcNAc at the reducing end contains an asymmetric C_1. The GlcNAc can exist as two different stereoisomers: configuration α, in which the hydrogen atom is above the sugar ring lane, or β, which has the OH group above the sugar ring plane. In chitin oligomers, the β configuration is normally dominant (Cohen-Kupiec and Chet, 1998).

Plant chitinases use two different hydrolytic mechanisms depending on the family of glycosyl hydrolases to which they belong. Chitinases of the family 19 of glycosyl hydrolases, which invert the anomeric configuration of the hydrolysed GlcNAc residue, are of plant origin. On the other hand, the chitinases of family 18 of glycosyl hydrolases, which retain the anomeric configuration of the hydrolysed residue, are mainly of plant and fungal origin (Iseli *et al.*, 1996). It is therefore likely that all the family 19 chitinases share the same active site structure, catalytic machinery and stereochemical outcome (Iseli *et al.*, 1996).

2.4.1. The classification of plant chitinases

Six classes (I-VI) of plant chitinases have been described, based on amino acid sequence features (Iseli *et al.*, 1996). The characteristics that determine these classes include N-terminal sequence, localisation of the enzyme, isoelectric pH, the signal peptide and inducer molecules.

Most of class I chitinases act as endochitinases, which are synthesised with a carboxy-terminal extension that targets them to the vacuole (Neuhaus *et al.*, 1991). They have a molecular mass of approximately 33 kDa and were originally found as basic chitinases (Araki and Torikata, 1995). Patil *et al.* (2000) have since subdivided them into class Ia and Ib, based on their acidic and basic nature respectively. Class I chitinases consist of a cysteine-rich amino-terminal domain linked by a short glycine/proline-rich region (signal peptide) to the catalytic domain (Perrakis *et al.*, 1993). The cysteine-rich domain, also referred to as the wheat-germ agglutinin (WGA) domain, is important for the binding of chitin and for substrate affinity, but not for catalytic activity (Iseli *et al.*, 1993). It has been suggested that the class I chitinase is the product of a gene encoding the hevein gene. The latter is a single–domain protein that corresponds to one of the WGA domains (Araki and Torikata, 1995). This indicates that the class I chitinase is restricted to plant sources.

Class II chitinases, found mainly in dicotyledonous species, but also in fungi and bacteria, have molecular masses of approximately 28 kDa and predominantly acidic pK_a values (Araki and Torikata, 1995). They share homology with class I chitinases, but lack the cysteine-rich domain and the carboxy-terminal vacuolar targeting signal. This

indicates that these chitinases do not bind chitin and are secreted in the apoplast, where they act as exochitinases (Shinshi *et al.*, 1990; Patil *et al.*, 2000).

Class III chitinases exhibit the same molecular mass as class II chitinases, but differ structurally from classes I and II. They are bifunctional enzymes with lysozyme and chitinase activities (Gerhardt *et al.*, 1997) and do not share any sequence similarities with the chitinases from other classes (Araki and Torikata, 1995).

The class IV chitinases, which are also found mainly in dicotyledonous species, comprise a group of acidic and basic extracellular chitinases that share 41%-47% sequence homology with class I chitinases in the catalytic domain and also contain cysteine-rich regions resembling chitin-binding domains. Class IV chitinases are smaller, however, because of deletions in both of these domains (Collinge *et al.*, 1993).

Classes V and VI include singular examples of extraordinary chitinases (Iseli *et al.*, 1996). Other proteins with endochitinase activity that share some similarities with bacterial exochitinases, yet are unrelated to the previously mentioned plant chitinases, were classified as class V chitinases (Gerhardt *et al.*, 1997).

Araki and Torikata (1995) analysed the phylogenetic relationships of plant chitinases besides the established classification of domain structure and carboxy-terminal extension sequences, thereby describing a new structural classification of chitinases by protein genealogies (Fig. 8). Two phylogenetic lineages were found by these authors in the protein genealogy of class I and class II chitinases. They identified high and low molecular weight type chitinases. The high molecular weight subclasses of class I (class I-H) and class II (class II-H) showed no genetic distance. However, the low molecular weight subclasses (class I-L and class II-L) showed independent lineages. Araki and Torikata (1995) then concluded that the genes of low molecular weight subclasses evolved from the high molecular weight subclass genes.

The classification of plant chitinases according to phylogenetics is shown in Fig. 8.

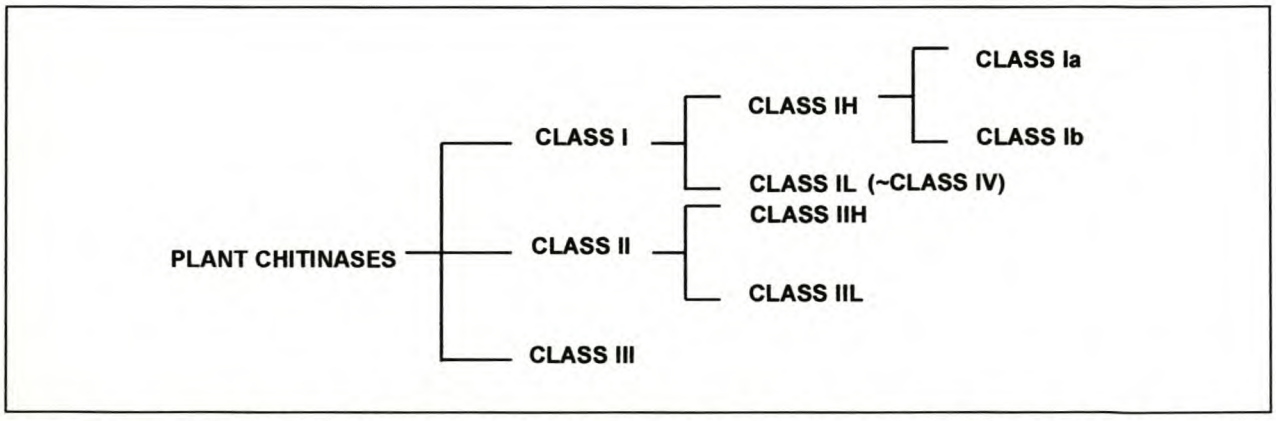

Figure 8. Classification of plant chitinases according to phylogenetic linkages between the different classes (Araki and Torikata, 1995).

Class I comprises class I-H and class I-L subclasses. Class I-H is further divided into class Ia, which contains a carboxy-terminal extension sequence, and class Ib (Flach *et al.*, 1992). Class II consists of class II-H and class II-L. As mentioned previously, there is no genetic difference between class II-H and the main structure of class I-H. The reported class IV chitinase (Collinge *et al.*, 1993) corresponds to class I-L in this classification.

The relationship between these classes and related genes were studied and discussed by Araki and Torikata (1995). This is summarised in Fig. 9. According to this hypothesis, an ancestral single domain gene evolved into a double domain through gene duplication, ultimately yielding the class II-H gene (Araki and Torikata, 1995). The class II-H gene evolved into the class I-H gene through the incorporation of the amino-terminal domain gene. This pathway is supported by the presence of direct repeats at the 5' and 3' flanking regions of the class I chitinase gene (Shinshi *et al.*, 1990). As the trees of the main structure of class I chitinase and the amino-terminal domain are similar, there is no genetic distance before and after the incorporation of the amino-terminal domain gene. The class I-H and class II-H genes then evolved into the class I-L and class II-L genes, respectively, by means of an independent pathway. The class III chitinase gene may have evolved from a different ancestral gene by convergent evolution (Araki and Torikata, 1995).

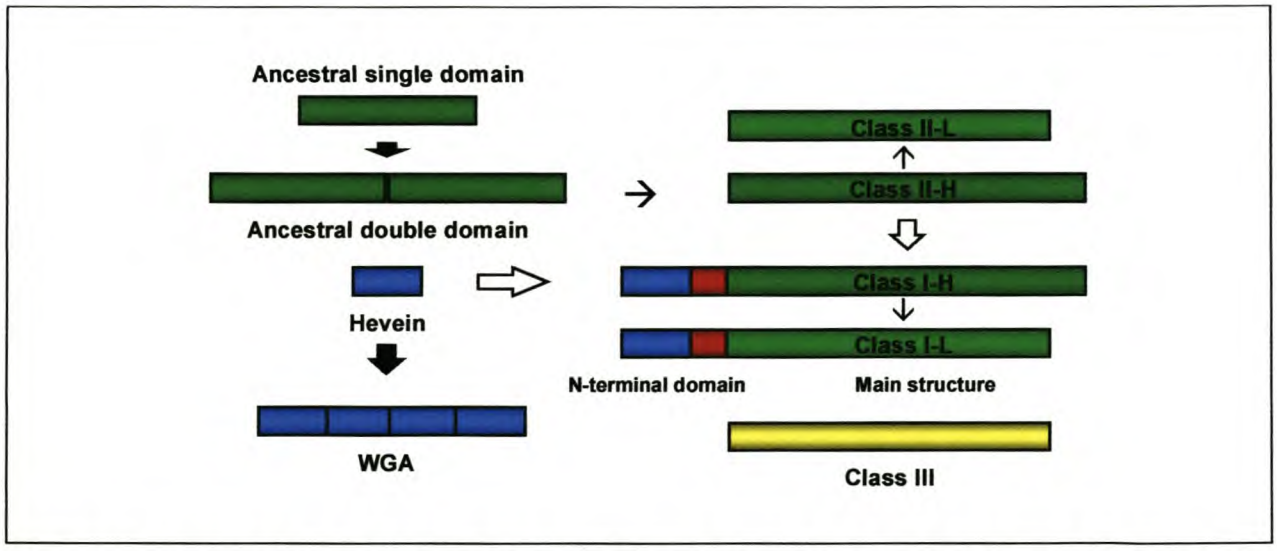

Figure 9. Scheme of the molecular evolution of plant chitinase and related genes (adapted from Araki and Torikata, 1995). Solid, open and thin arrows indicate gene duplication, gene recombination and evolution, respectively.

2.4.2. Chitinases and their role in plant defence

Throughout their life cycle, plants have to react to various threats from the outside environment. As plants are sessile, they have developed a range of strategies, collectively known as defence responses, to protect themselves against biotic and abiotic stresses.

Given favourable environmental conditions, the progress of plant diseases is determined by interactions between the plant and the pathogen. A successful plant defence response is based on an effective surveillance system that enables the early recognition of the threat and, as a consequence, the activation of defence-specific processes that act to prevent further pathogen development. In turn, successful pathogens need to neutralise the plant's resistance strategy.

Plant-pathogen (microbial) interactions can be divided into three categories, depending on their outcome (Dempsey *et al.*, 1998). Most frequently, a non-host interaction occurs, during which the plant exhibits no response to the pathogen and the pathogen fails to colonise the plant. Alternatively, the plant actively resists infection by activating numerous defences that prevent pathogen replication and/or spread. There are at least six clearly defined types of resistance: parasite-specific, cultivar-specific, non-host or basic, organ-specific, age-related and induced (localised or systemic) resistance (Heath, 1996). The third and least common plant-pathogen interaction is characterised by susceptibility, in which the plant is colonised by the pathogen and disease symptoms generally develop (Dempsey *et al.*, 1998). In this case, the pathogen overcomes the host defences and initiates attack under the prevailing physiological and environmental conditions (Prusky, 1996). The more common pathogens that attack plants include bacteria, viruses and pathogenic fungi. The aforementioned interactions between plants and pathogens are of fundamental interest, as a better understanding of the processes of disease development may eventually lead to more rational and effective control methods (Lucas, 1998a).

Plant defence systems can be classified as either passive (constitutive) or active (inducible), depending on whether they are pre-existing features of the plant, or are switched on after challenge (Lucas, 1998a). These categories can be further subdivided into structural and biochemical mechanisms. The structural mechanisms act as physical barriers and inhibit the pathogen from gaining entrance to and spreading through the plant. The biochemical reactions that take place in the cells and tissues of the plant produce substances that are either toxic to the pathogen, or create conditions that inhibit the growth of the pathogen (Agrios, 1997).

Passive defence mechanisms

The passive anatomical features characteristic of a plant species represent highly effective obstacles to penetration by most microorganisms (Lucas, 1998a). Some structural defences are present in the plant even before the pathogen comes into contact with it. These structures include: the quality and amount of wax and cuticle that cover the epidermal cells, the structure of cell walls, the size, location and shapes of stomata and lenticels, and the presence of tissues made of thick-walled cells that hinder the advance of the pathogen (Agrios, 1997). These structures often play a role in the formation of a

water-repelling surface, which may reduce infection. They also increase resistance to infection through the inhibition of direct penetration or the spread of the pathogen (Agrios, 1997). Considerable evidence points to the cell wall as the primary site of resistance when pathogens try to penetrate directly into the epidermal cells (Heath, 2000a). Every plant cell is itself surrounded by a cell wall that acts as a substantial obstacle. When this is impregnated with chemical substances that are harmful to microorganisms, it forms an even more effective barrier against pathogens.

Plants synthesise a vast array of preformed inhibitors, many of which are toxic to potential pathogens and pests. Secondary metabolites, such as phenols, alkaloids, glycosides, saponins, tannins and resin, all possess antibiotic properties and may therefore contribute to resistance (Lucas, 1998a; Heath, 2000a). These inhibitors in the plant tissues often occur as non-toxic precursors, which are converted to active forms following cell damage or exposure to certain enzymes. Plants also contain preformed proteins and peptides that are potential deterrents against microbial infection and which may determine the host range of some fungal pathogens (Heath, 2000a). Although these changes are induced by infection, they still involve the conversion of preformed compounds that are distinct from defence responses involving active host metabolism (Lucas, 1998a).

Plants exude a variety of substances through their surface into their surrounding environment. These plant defence proteins include: plant defensins (antimicrobial peptides), lectins, toxic cell wall proteins, hydrolytic enzymes such as glucanases, chitinases and lysozyme, and various classes of enzyme inhibitors. The latter group shows activity against degradative and microbial enzymes produced during tissue colonisation (Agrios, 1997; Lucas, 1998a; Strange, 1998).

Another group of enzymes produced in response to infection are putative antiviral proteins with ribonuclease and protease activities (Lusso and Kuc, 1995). Antiviral proteins inhibit protein synthesis by affecting ribosomes in the host cells, consequently blocking virus replication. These inhibitors are described as ribosome-inactivating proteins or RIPs (Lucas, 1998a).

The distinction between passive and active resistance mechanisms is not absolute and the regulation of these defence proteins may vary from tissue to tissue. Currently, there is considerable interest in plant defence proteins, as many of their respective genes have been isolated and sequenced. The isolation and sequencing of these genes provides the opportunity to engineer transgenic plants expressing such proteins to increase resistance to both pathogens and pests.

Active defence mechanisms

After the pathogen has penetrated the preformed defence structures, plants usually respond by forming an array of structures to protect the plant from further colonisation.

These include the histological defence structures, in which the tissues surrounding the infection site are involved, as well as the cytoplasmic defence reactions that involve the cytoplasm of the cells under attack. The histological defence structures include the formation of cork layers, abscission layers and tyloses and the deposition of gums (Agrios, 1997), all acting to isolate the point of infection from the rest of the plant.

Another defence mechanism of cellular defence structures involves alterations in the appearance or properties of the cell walls of invaded cells. By fortifying their cell walls, plants increase their resistance to degradation, thereby reducing penetration and further colonisation by the pathogen. Changes in the cell wall composition typically include cross-linking of structural proteins, such as hydroxyproline-rich glycoproteins (HRGP), proline-rich proteins (PRP) and glycine-rich proteins (GRP), the deposition of callose, silica and papilla, as well as lignification (Kuc, 1997; Grant and Mansfield, 1999; Heath, 2000a). Following infection, HRGPs accumulate in the host cell walls and may contribute to resistance by trapping pathogen cells or acting as structural barriers, as well as play a role in lignin deposition (Lucas, 1998a).

The initiation of a resistance response requires the perception of signal molecules, either synthesised by the invading organism or released from the plant cell walls. These signal molecules have collectively been termed elicitors (Wojtaszek, 1997). The recognition of the elicitors by the host plant leads to the activation of a cascade of biochemical reactions in the attacked and surrounding cells, giving rise to new or altered cell functions that lead to the formation of activated defence-related compounds (Agrios, 1997). Some of these reactions include: ion fluxes (H^+ and Ca^{2+} influx, Cl^- and K^+ efflux) across the plant plasma membrane, the phosphorylation and dephosphorylation of specific proteins involving MAP kinases, the generation of highly reactive oxygen species (oxidative burst), the transcriptional activation of numerous defence genes, the induction of phytoalexins, localised cell death at the infection sites (*h*ypersensitive *r*esponse, HR) and the induction of *l*ocal and *s*ystemic *a*cquired *r*esistance (LAR and SAR respectively) in distal plant organs (Bent, 1996; Crute and Pink, 1996; Hammond-Kosack and Jones, 1996; Knogge, 1996).

Plant cells typically (although not always) respond to elicitors with an oxidative burst during which reactive oxygen species (ROS), such as H_2O_2, O_2- and OH, are usually generated extracellularly (Bolwell, 1999). A local oxidative burst is one of the earliest events correlated with plant resistance at the site of pathogen infection. The ROS are not only directly toxic to microbes, but could be involved in the cell death pathway leading to the HR (Shah, 1997). During an oxidative burst, a rapid accumulation of hydrogen peroxide can occur, resulting in simultaneous crosslinking of structural proteins of the cell wall, rendering it more resistant to attack by the pathogen's enzymes (Strange, 1998). Some evidence suggests that these reactive oxygen species might have a signalling role

in salicylic acid (SA) accumulation; the latter has been linked directly to the spread of the defence response in the observed SAR response (Shah, 1997).

Plants also defend themselves through the production of antimicrobial substances in the attacked cells. Some of these include pathogenesis-related (PR) proteins, which will be discussed later, phytoalexins, simple phenolic compounds, toxic phenols and phenol-oxidising enzymes (Agrios, 1997). Phytoalexins are low-molecular weight, broad-spectrum antimicrobial compounds that accumulate in plants as a result of infection or stress. This accumulation is frequently associated with the HR (Strange, 1998). Phytoalexins are biocides, affecting fungi, bacteria, nematodes, higher animals and plants. Despite their broad spectrum of activity, organisms vary widely in their sensitivity to phytoalexins (Strange, 1998). The impact of phytoalexins on resistance depends on the rate at which they are synthesised, the speed with which they are degraded by the microorganism and/or plant, as well as their toxicity to the organism (Kuc, 1997). Phytoalexin-based strategies for improving disease resistance have been proposed, such as the production of new phytoalexins in transgenic plants (Dempsey *et al.*, 1998).

Certain abundant phenolic compounds that are toxic to pathogens are produced and accumulated at a faster rate after infection. These include chlorogenic acid, caffeic acid and ferulic acid. Plants also contain nontoxic glycosides that consist of a sugar moiety joined to a phenolic molecule. Some pathogens produce glycosidases to hydrolyse these complex molecules, resulting in the release of phenolic compounds that are toxic to the pathogen (Agrios, 1997). Polyphenol oxidases are phenol-oxidising enzymes that are involved in the oxidation process of phenolic compounds, yielding quinones. Quinones are often more toxic to microorganisms than the original phenol. Another phenol-oxidising enzyme, peroxidase, not only oxidises phenolics, but also produces antimicrobial hydrogen peroxide (Agrios, 1997).

The HR is the most common expression of host as well as non-host resistance. The result of non-host resistance is the resistance of an entire plant species to a specific pathogen (Heath, 2000a). Challenged cells, and sometimes those in their immediate vicinity, become apoptotic, leading to necrosis, which is associated with pathogen limitation as well as defence gene activation (Goodman and Novacky, 1994; Strange, 1998). The HR is thought to be responsible for providing resistance by limiting the growth of the pathogen, especially obligate parasites, since they require living host cells to grow. For non-biotrophic pathogens that do not require their host cells to stay alive, cell death alone cannot restrict pathogen growth. To overcome this, the HR encompasses both cell death and defence gene expression (Heath, 2000b).

The cells surrounding the necrotic lesions as a result of HR are known as the zone of LAR (Hammond-Kosack and Jones, 1996). These local responses often trigger non–specific resistance throughout the plant, the well-known SAR response. The latter provides significant and durable protection in distant, uninfected plant parts against

infection by a broad range of pathogens (Sticher *et al.*, 1997). Systemically protected plants often contain high levels of the PR proteins (see later sections). The accumulation of SA that accompanies SAR has been shown to be essential for SAR signalling (Gaffney *et al.*, 1993). Evidence has accumulated that demonstrates that jasmonic acid and ethylene are also important signals in the induction of systemic defence resonses (Pieterse and Van Loon, 1999).

The response of plants to pathogens is multifaceted and the timing of the response is critical in the determination of resistance and susceptibility. The rapid and timely activation of defence systems appear to slow the progress of the invader, giving the plant time to express systems that first confine and then kill the pathogen. The complex relationship between the different components of the responses and the factors that regulate the timing of these responses are not well defined yet and remain important foci in the study of resistance in plants (Kuc, 1997; Lucas, 1998a). In this review, only chitinases as part of the PR proteins will be discussed in this regard.

Chitinases as pathogenesis-related proteins

The PR proteins were first detected and described in the early 1970s in tobacco leaves reacting hypersensitively to the tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). PR proteins are a structurally diverse group of plant proteins that are toxic to invading microbial pathogens. They are relatively small and stable proteins that exist intracellularly, in plant cells, as well as apoplastically (Agrios, 1997; Lucas, 1998a). These proteins are widely distributed in trace amounts in plants, but are produced in much higher concentrations following pathogen attack or stress (Agrios, 1997). On the genetic level this translates to PR proteins being produced constitutively and being inducible under certain circumstances. PR proteins have very characteristic physicochemical properties, which aid in their detection and isolation. Specifically, they are very stable at low pHs, at which they remain soluble and reactive; they are relatively resistant to the action of both endogenous and exogenous proteolytic enzymes; they are monomers with rather low molecular weights (8-50 kDa); and they are localised in compartments such as the vacuole, cell wall and/or the apoplast (Stintzi *et al.*, 1993).

PR proteins were initially classified into five families, based on their function, amino acid sequence and physical properties, such as electophoretic mobility, size and acidity (Agrios, 1997; Lucas, 1998a). Each of the five classic PR protein families generally consists of two subclasses: an acidic protein that is usually secreted to the extracellular space, and a basic subclass that is usually transported to the vacuole by a signal sequence located at the C-terminus end (Kitajima and Sato, 1999). More recently, at least five additional families of PR proteins were identified (Dempsey *et al.*, 1998) (Table 3). The induction of PR gene expression during pathogen infection is mediated by various signalling molecules. Salicylic acid and reactive oxygen mediate the expression of acidic

PR genes (Ryals *et al.*, 1996), whereas the expression of basic PR genes is mediated by gaseous phytohormone ethylene and methyl jasmonate (Xu *et al.*, 1994). In the following section, only the five major PR protein groups will be discussed, taking the extensively studied tobacco PR proteins as the model system.

Table 3. The different groups of pathogenesis-related proteins and their characteristics

Group	Protein type	Localisation	Biological activity	References
PR-1	Related to glioma	Acidic: extracellular; basic: intracellular	Antifungal	Stintzi *et al.*, 1993
PR-2	β-1,3-glucanases	Acidic: extracellular; basic: intracellular	Antifungal	Meins *et al.*, 1992; Côté *et al.*, 1991
PR-3	Chitinases class I, II, V	Acidic: extracellular; intracellular	Antifungal	Majeau *et al.*, 1990
PR-4	Wheatwins; Hevein; Chitin-binding proteins	Acidic: extracellular	Antifungal	Van Damme *et al.*, 1999
PR-5	Osmotins; Thaumatin-like	Neutral: extracellular; basic: intracellular	Antifungal	Dore *et al.*, 1991; Grosset *et al.*, 1990
PR-6	Proteinase inhibitors	Intracellular	Unknown	Dempsey *et al.*, 1998
PR-7	Proteinases	Unknown	Unknown	Dempsey *et al.*, 1998
PR-8	Chitinases class III, with lysozyme activity	Intracellular; extracellular	Antifungal	Dempsey *et al.*, 1998
PR-9	Peroxidases	Extracellular	Antimicrobial compound production	Caruso *et al.*, 1999
PR-10	Low Mr proteins; novel sequences	Intracellular		Dempsey *et al.*, 1998

Classification of PR proteins

The PR-1 group consists of four members, of which three are acidic and one basic. Their molecular masses vary from 15 to 17 kDa. All four of these members are localised extracellularly and some have antifungal activity (Stintzi *et al.*, 1993).

The PR-2 group consists of β-1,3-glucanases. They are usually monomers with molecular masses in the range of 25-35 kDa. The majority are endoglucanases, producing oligomers of two to six glucose units from laminarin (an almost unbranched β-1,3-glucan) or from other β-1,3-glucans (Meins *et al.*, 1992). Stintzi *et al.* (1993) have isolated five distinct members of the glucanase group: four acidic and one basic enzyme. The acidic enzymes are extracellular, whereas the basic enzyme occurs in the vacuole (Côté *et al.*, 1991).

Chitinases make up the PR-3 group. These enzymes are usually monomers of 25-35 kDa. Many purified plant endochitinases possess some degree of lysozyme activity. Consequently, these enzymes can hydrolyse the β-1,4-linkages between *N*-acetylmuramic

acid and GlcNAc in bacterial peptidoglycan (Majeau *et al.*, 1990). These proteins are either situated in the vacuole or occur extracellularly (Stintzi *et al.*, 1993).

The PR-4 group consists of four proteins isolated from tobacco mosaic virus (TMV)-infected leaves (Kauffmann *et al.*, 1990). These acidic proteins are all extracellular and are serologically related to each other, but do not show a serological relationship to other PR proteins or PR-groups (Stintzi *et al.*, 1993). Van Damme *et al.* (1999) isolated a hevein-like, chitin–binding protein from mature elderberry fruits. This protein is synthesised as a chimeric precursor consisting of an N-terminal chitin-binding domain and an unrelated C-terminal domain. The N-terminal domain has high sequence homology with the N-terminal of PR-4 proteins, whereas the C–terminus is more closely related to that of class V chitinases, which belong to the PR-3 group. These authors concluded that this gene could be considered a hybrid between a PR-4 and a class V chitinase (PR-3 group) gene.

The PR-5 group, also known as thaumatin-like proteins, consists of two neutral, extracellular proteins found in the apoplast (Kauffmann *et al.*, 1990; Dore *et al.*, 1991) and two basic counterparts, identified as osmotins. These basic proteins are localised in the vacuolar compartment (Grosset *et al.*, 1990). Stintzi *et al.* (1993) showed that several members of the PR-5 group had differential antifungal activities with specificity for different fungal species (Vigers *et al.*, 1992). It has been shown that the PR-5 group inhibits hyphal growth and mediates hyphal and spore lysis in *in vitro* assays (Vigers *et al.*, 1992).

Role of chitinases in plants

As mentioned before, PR proteins are induced in the presence of pathogens and pathogen extracts and also due to stress. Chitinase induction can either be local or systemic. Vögeli-Lange *et al.* (1988) showed that the local induction seems to be correlated with an HR in the plant. Moreover, chitinase activity has been observed in the systemic resistance induced by localised induction in cucumber leaves (Schlumbaum and Boller, 1988) and tobacco (Tuzun *et al.*, 1989).

The effect of plant chitinases on pathogens could also be indirect, through the induction of other plant defence mechanisms. The activities of plant chitinases and β-1,3-glucanases are directly antimicrobial through the hydrolysis of the structural components of the cell walls of microbes. They are also indirectly antimicrobial, by modulating the release of oligosaccharides that are active elicitors of defence responses, effectively leading to the amplification of defence responses (Lamb *et al.*, 1989; Ryan and Farmer, 1991). One of the defence genes influenced by these PR proteins is phenylalanine ammonia-lyase (PAL) that is induced by their actions. The PAL enzyme is responsible for the conversion of phenylalanine, an important precursor of the synthesis of phenolic compounds to *trans*-cinnamic acid. From there it enters the pathway for biosynthesis of

phenylpropanoid compounds, which include various groups of phytoalexins and the precursors of structural defence molecules (Lucas, 1998a).

Chitinases do not only enhance the plant's defence system against pathogen attack, but could also play a role in the survival of the plant during extreme temperatures. Many freeze-tolerant plants accumulate antifreeze proteins (AFPs) during cold acclimation (Griffith and Ewart, 1995). These AFPs inhibit the growth and recrystallisation of the intercellular ice crystals via hydrogen bonds and/or Van der Waals interactions (Ewart *et al.*, 1999). AFPs are not unique proteins (Hon *et al.*, 1995); they are rather similar to the PR proteins that are normally secreted in response to infection by pathogens (Stintzi *et al.*, 1993). Hon *et al.* (1995) identified the AFPs of winter rye (*Secale cereale*) as two glucanases, two thaumatin-like proteins and two chitinases. The chitinases exhibited both endochitinase and antifreeze activity, subsequently proving that they are dual–function enzymes (Hon *et al.*, 1995). Yeh *et al.* (2000) speculated on the origin of the antifreeze activities in the PR proteins. They suggested that the two winter rye chitinase AFPs might have acquired antifreeze activity through the evolution of gene sequences that encode variants of chitinases that have both ice-binding and catalytic domains. These authors also suggested another possibility, namely that the mature amino acid sequence of the chitinases is chemically modified at cold temperatures, thereby allowing the proteins to bind to ice.

Chitinases play a role during plant development; it is evident that chitinase production is observed not only after infection or stress, but that constitutive or developmentally regulated levels also occur (Majeau *et al.*, 1990). Collinge *et al.* (1993) suggested that chitinases could play important roles during early embryo development and potentially also in other developmental processes. This is presumably due to the fact that plant cells contain substrates for chitinase that are not chitin *per se*, but may resemble chitin structurally.

Robinson *et al.* (1997) studied the occurrence and role of PR proteins in fruits, specifically grapes. Grapes are nonclimacteric fruit that exhibit a double sigmoidal growth curve (Coombe, 1976). The first growth phase is due to cell division and subsequently to cell enlargement (Harris *et al.*, 1968). Thereafter, the fruit goes through a short period of little or no growth, durimg which metabolic reorganisation occurs to commence the ripening process. The second growth, or ripening, phase follows, during which berry volume increases due to cell expansion coinciding with berry softening. The onset of this second growth phase is referred to as *veráison*, which is a viticultural term used to describe the point at which various developmental events are initiated. These include the accumulation of sugars, a decrease in organic acids, colour development, berry expansion and softening (Coombe, 1973).

Prior to *veráison*, no chitinase activity is detected in the berries, but the activity increases sharply at veráison and continues to accumulate throughout ripening (Robinson

et al., 1997). In this case, the induction is linked more to developmental control than to pathogen attack. Robinson *et al.* (1997) also suggest an alternative explanation, namely that PR proteins may have a critical role in normal growth and development and that the constitutive expression in certain tissues may be unrelated to pathogen resistance.

Some grape PR proteins, including chitinases, have been implicated in haze formation in white wines. The quality of white wines can be lowered considerably by the appearance of sediment after bottling. These precipitates commonly are the result of denatured grape proteins that survive the vinification process and are carried through into the wine (Waters *et al.*, 1992). These proteins have been identified by Waters *et al.* (1996, 1998) as PR proteins, of which the most abundant proteins were thaumatin-like proteins and chitinases. As mentioned previously, the expression of PR proteins is often induced systemically as a response to stress, wounding and pathogen attack (Stintzi *et al.*, 1993). However, Pocock *et al.* (2000) showed that wounding by mechanical harvesting and the effect of water stress on vines have little influence on the production of PR proteins. All PR proteins, including chitinases and thaumatin-like proteins, are acid-soluble and resistant to protease degradation (Linhorst, 1991). Strategies to remove the haze-forming proteins based on proteolysis are therefore probably futile. Potential solutions include the manipulation of viticultural practices that influence the synthesis of these proteins in grape berries (Robinson *et al.*, 1997), or the genetic engineering of grapevines to lower the expression of the genes encoding the specific PR proteins involved in wine haze formation (Waters *et al.*, 1998). However, the effect that this approach will have on the plant's natural defence system is unknown.

Chitinases also play a role in mycorrhizal interactions (Flach *et al.*, 1992). The constitutively expressed chitinases, localised in the apoplastic space of the host root, degrade part of the chitinous elicitors produced by the mycorrhizal fungi. This occurs while the elicitors are transversing across the cell wall before they interact with the specific receptors in the plasma membrane. This inactivation of the fungal chitin-derived elicitors might be one of the preconditions required to create a compatible interaction between plant and fungus in the mycorrhizial symbiosis (Cohen-Kupiec and Chet, 1998).

Collectively seen, it is evident that plant chitinases are important in several aspects of plant growth, development and defence. These proteins have been linked to several strategies to engineer disease resistance in plants and some aspects of the upregulation of diseases resistance in plants will be discussed in this regard.

2.5. THE APPLICATION OF CHITINASES AS ANTIFUNGAL AGENTS

2.5.1. Introduction

Field-grown crops are permanently endangered by an array of fungal pathogens, and extensive farming requires the persistent control of fungal pathogen populations. Although plant pathogenic fungi include various species with diverse characteristics, only *Botrytis cinerea* will be discussed as an example of these fungal pathogens.

B. cinerea is one of the most important plant pathogens and has a wide host range, including fruit species, ornamental plants and salad vegetables (Groves *et al.*, 1988). This fungus, which is the causal organism of *Botrytis* bunch rot of grapes (Nair and Hill, 1992), is perhaps the most common and best known fungal pathogen and has been the centre of mycological research for a number of years. In the grapevine industries, *Botrytis* bunch rot causes serious reduction in the quantity and quality of the crop. The reduction in yield may be associated with the loss of juice and the desiccation of the berries, or with the premature drop of bunches due to stalk rot. In table grape production, a substantial loss of fruit quality occurs in the vineyard, in storage or during transit. In the wine industry, the modified chemical composition of the diseased berries leads to serious qualitative damage (Bulit and Dubos, 1988). *B. cinerea* also penetrates floral parts (petals, stigmas, styles) and remains dormant until it resumes activity and invades the fruit later in the season or during ripening (Prusky, 1996).

The control of fungal disease in modern agricultural practices is achieved mainly by the extensive use of chemical fungicides (Schickler and Chet, 1997). A number of fungicides, including systemic fungicides such as benzimidazoles and dicarboximides (Gullino, 1992a), have been used widely to control diseases caused by *B. cinerea*. Systemic fungicides are taken up by the host plant and transported in the plant via the xylem and phloem (Causier *et al.*, 1994). Therefore, the active substance is not removed by rainfall; the treatment is curative and the vegetation formed after the treatment is also protected. However, the spectrum of activity is often selective and requires the combination of several active compounds (Busam *et al.*, 1997a). Another group of fungicides used extensively are the non–systemic fungicides. These are broad spectrum and non-specific in their action against plant pathogenic fungi. They are also known collectively as surface fungicides, since they are applied to the plant surface and are not taken up into the plant system (Gullino, 1992a). These fungicides need to be applied frequently throughout the season, depending on fungal pressure.

A serious problem of pesticide resistance has arisen as a result of the use of systemic botryticides (Adams, 1994), causing the resistance to both benzimidazoles and dicarboximides to be widespread in *B. cinerea* on grapevine, vegetable and ornamental crops (Gullino and Garibaldi, 1986). Although chemicals still play a major role in the

control of *Botrytis* spp. on most crops, it is clear that, in many cases, their use leads to practical problems of various types. For this reason, the exclusive use of fungicides can no longer be considered to be a workable approach.

As argued in the previous sections, chitinases and their encoding genes might prove to be critical to the alternative approaches required by modern agriculture. The progress made with these initiatives will be discussed in the following sections.

2.5.2. Chitinases as biological control agents

In a broad definition, the term biological control has been applied to cover the use of any organism to control a pathogen. A clearer definition of biological control is: the reduction in attack of a crop species by a pathogen using another living organism or organisms (Lucas, 1998b). A biocontrol agent must comply with a certain set of criteria before being released on the market. These include that the biocontrol agent must be grown in large quantities fairly easily; the antagonist must be able to survive and remain active in a range of environmental conditions, both prior and after application; the biocontrol material needs to be genetically stable and non-toxic to animals, plants and the environment, yet must still provide good control of the target pathogen; and finally, the biocontrol product should at least equal the efficiency and ease of the use of existing fungicides at the same price (Whipps and McQuilken, 1993).

The biological antagonism that occurs naturally between microorganisms potentially can be developed as a means of biological control to replace chemical fungicides. The filamentous fungi belonging to the *Trichoderma* spp. are mycoparasites of plant pathogens and have been accepted as one of the most potent agents for the biocontrol of plant diseases (Margolles-Clark *et al.*, 1996). However, the molecular basis of biocontrol is not understood clearly. Proposed mechanisms of antagonism resulting in biocontrol are antibiosis (Ghisalberti and Sivasithamparam, 1991), competition (Chet, 1987), production of cell wall-degrading enzymes (Chet, 1987; Schirmböck *et al.*, 1994), or a combination of these antagonistic activities. Mycoparasitism is a complex process in which a *Trichoderma* spp. grows chemotropically toward its host and coils around and attaches to the host hyphae, sometimes penetrating them (Chet, 1987). During the later stages of the parasitic process partial degradation of the host cell wall is normally observed and the expression of extracellular cell wall-degrading enzymes is assumed to be involved in this process, (Margolles-Clark *et al.*, 1996). Together with other hydrolases, chitinolytic enzymes and glucanases are considered to play a major role in biocontrol through their actions on the cell wall constituents of the target fungi (Chet, 1987). These enzymes act synergistically in combination (Lorito *et al.*, 1993b) and are not toxic to vertebrates or plants, which do not contain substrate polymers.

In field trials, biological or integrated control (a combination of biological and chemical control) using the *Trichoderma* spp. generally resulted in significant, but inconsistent, control. According to Gullino (1992b), commercially useful biological control could be obtained in situations in which disease incidence is low, but in which unacceptable levels of disease can occur under more severe conditions. Elad *et al.* (1993) recommends the alternation of fungicide and *Trichoderma* sprays for the control of *B. cinerea* on grapes and other crops.

Antifungal application of yeast chitinases as biocontrol agents

S. cerevisiae is regarded as a GRAS (*g*enerally *r*ecognised *a*s *s*afe) organism and has been used successfully in the baking and brewing industries for many years. An overwhelming wealth of information on the molecular biology, genetics and physiology of this model eukaryote has been accumulated (Rose and Harrison, 1989; Broach *et al.*, 1991), making this species the best-characterised eukaryotic system today. More than a thousand genes have been characterised (Heinisch and Hollenberg, 1993) and the entire genome has been sequenced (Goffeau *et al.*, 1996). Therefore, the availability of the relevant structural genes and their use in genetic engineering constitute a fascinating approach to the creation of microbial strains that can produce large amounts of valuable proteins of good quality. Due to the obvious advantages and the various possibilities of influencing the metabolic flux to improve production yields (Hinnen *et al.*, 1994), many heterologous protein production processes are based on the systems in *S. cerevisiae* (Heinisch and Hollenberg, 1993; Hinnen *et al.*, 1994). In order to render a possible biocontrol agent, proteins such as chitinases and other pathogenesis-related proteins are ideal candidates to be overexpressed in this well-established system.

Kuranda and Robbins (1987) isolated and cloned the gene for *S. cerevisiae* endochitinase (*CTS1*) into a *Schizosaccharomyces pombe/S. cerevisiae* shuttle vector. *S. pombe* did not show any chitinase activity before the transformation. The transformation of this organism with the *CTS1* clone resulted in the appearance of an approximate five to 13-fold increase in chitinase activity. The highest levels of chitinase activity were observed in the logarithmic phase, during which localised chitin synthesis is important in the formation of septa, marking the point of separation between the mother and daughter cells during budding (Kuranda and Robbins, 1987). Due to the antifungal activity of chitinases, the overexpression of *CTS1* in *S. pombe* or in *S. cerevisiae* is an attractive approach in the biological control of plant pathogenic fungi.

In the grapevine industry, the use of *S. cerevisiae* strains as biocontrol agents should be approached with caution. Recent reports suggested that certain strains of *S. cerevisiae* (*S.c.* sp., SCPP and Σ1278b) could penetrate *Vitis* plants, resulting in delayed growth or even death of the plantlets (Gognies *et al.*, 2001). In order to explain the mechanism of this attack, the authors hypothesised that the yeast strains' aggressiveness was related to

their ability to develop mycelial forms. Previously it had been postulated that the switch between a cellular and a filamentous form contributes to the virulence of several fungal pathogens (Madhani *et al.*, 1999). In the observed strains of *S. cerevisiae* (SCPP and *S.c.* sp), differences in the levels of aggressiveness were correlated with the strains' abilities to form pseudohyphae. The authors concluded that the differences between the phytopathogenicity of the different strains were due to their filamentous state, but that their pectolytic activities were also required to invade grapevine tissues. It was also suggested that yeast strains capable of pseudohyphal growth might secrete lytic enzymes, such as endopolygalacturonase that could be involved in the hydrolysis of the plant cell wall (Gognies *et al.*, 2001). Although these factors are important to keep in mind, a more detailed analysis of a larger collection of yeast strains is necessary before the pathogenic status of *S. cerevisiae* on *V. vinifera* can be assumed to be conclusive.

Antifungal application of other fungal chitinases as biocontrol agents

Trichoderma spp. have been used to control both soil-borne (Chet, 1987; Jin *et al.*, 1991) as well as leaf- and fruit-infecting plant pathogenic fungi (Tronsmo, 1991; Gullino, 1992b; Elad *et al.*, 1993). *Trichoderma* produces many chitinolytic enzymes, which may include five to seven distinct enzymes, depending on the strain (Haran *et al.*, 1995). In the best-characterised *Trichoderma* isolate (isolate TM), the system is composed of four endochitinases (Chit31p, Chit33p, Chit42p and Chit52p), two *N*-acetyl-β-1,4-glucosaminidases (Chit73p and Chit102p) and one exochitinase (Chit40p) (Haran *et al.*, 1996). *Trichoderma* endochitinases tend to be more effective in controlling fungi than those found in plants or other fungi (Harman *et al.*, 1993; Lorito *et al.*, 1993a). These enzymes inhibit spore germination, as well as the hyphal elongation of various fungal pathogens *in vitro* (Lorito *et al.*, 1993a; Ali, 1998). They are also involved directly in other effects on fungal hyphae, including swelling, branching, vacuolisation and necrosis (Lorito *et al.*, 1993a). The different components of the chitinolytic system of *Trichoderma* involve complementary modes of action of the component enzymes, but the entire system is required for maximum efficacy (Lorito *et al.*, 1993b).

The most interesting individual enzyme of the chitinolytic system is the 42 kDa endochitinase. This enzyme hydrolyses *B. cinerea* cell walls *in vitro*, resulting in the bursting of hyphal tips (Di Pietro *et al.*, 1993), inhibition of spore germination of *B. cinerea* conidia, as well as germ-tube elongation of various fungi (Lorito *et al.*, 1993b; Schirmböck *et al.*, 1994). The corresponding gene in *T. harzianum* IMI206040, *ech42*, is induced strongly during fungus-fungus interactions and during growth in the presence of colloidal chitin or the mycelia of several fungi as the sole carbon source (Carsolio *et al.*, 1994; García *et al.*, 1994). The *ech42* gene expression is repressed by glucose, but is induced by nutritional stress conditions and exposure to light (Carsolio *et al.*, 1994; García *et al.*, 1994).

Hayes *et al.* (1994) isolated the gene encoding the endochitinase, *ThEn-42*, from strain P1 of *T. harzianum*. The cellulolytic filamentous fungi *T. reesei* is not suitable for the biocontrol of plant pathogens, but has been used extensively for the industrial production of hydrolases because of its high protein secretion capacity. *T. reesei* has also been used successfully to produce heterologous proteins, such as antibody fragments, using the strong *cbh1* (cellobiohydrolase I) promoter (Nyyssönen *et al.*, 1993). Margolles-Clark *et al.* (1996) overexpressed the *ThEn-42* gene in *T. reesei* under the control of the *cbh1* promoter and found that the large amounts of cellulases produced by the fungus cause the heterologous enzyme mixture to be effective against a broad spectrum of plant pathogenic fungi. This is especially important when targeting oomycetes, which have cellulose as a cell wall component and lack appreciable amounts of chitin.

The antifungal activity of the chitinolytic enzymes of *T. harzianum* P1 has been tested against various fungal strains. Most notably, the endochitinases caused morphological effects, including the lysis of the germ tubes, spores and mycelia of the fungi (Lorito *et al.*, 1993b).

Antifungal application of bacterial chitinases as biocontrol agents

Some bacterial species have an antagonistic effect on fungi and may also play a role in biological control. Although many strains of *Streptomyces* produce chitinases, their chitinase production differs greatly. Mahadevan and Crawford (1997) studied the physiology of *S. lydicus* WYEC108. This strain was selected because of its very high chitinase production and its potential role as a biocontrol agent (Yuan and Crawford, 1994). This organism is active against fungi (Suh, 1992) and it has been shown that *S. lydicus* WYEC108 produces a very high level of chitinase activity when grown in the presence of fungal cell wall chitin, specifically with chitins present from the walls of an *Aphanomyces* sp. and of *Pythium ultimum* (Yuan and Crawford, 1994). The chitinases produced were active against the fungi *R. solani* and *P. ultimum*, as measured by the release of the sugars from their cell walls (Mahadevan and Crawford, 1997).

The bacteria *Serratia marcescens* produce five chitinolytic proteins with subunit molecular weights of 21, 36, 48, 52 and 57 kDa respectively, and of which all are extracellular enzymes. Jones *et al.* (1986) isolated and characterised two genes from *Serratia* that encode chitinases, namely *ChiA* and *ChiB*, which showed no detectable homology to each other. The *ChiA* gene has an N-terminal signal peptide typical of genes encoding secreted bacterial proteins and codes for a protein with a molecular weight of 57 kDa, whereas DNA sequence analysis of *ChiB* predicted a 52 kDa mature protein (Harpster and Dunsmuir, 1989).

Attempts have been made to transform these *S. marcescens* genes (*ChiA* and *ChiB*) into *E. coli* and then to transform them back into *S. marcescens* on a high-copy-number plasmid. The objective was to produce a high chitinase-yielding strain for the enhanced

bioconversion of shellfish waste. *E. coli* containing the *S. marcescens* chitinase genes was also used as an effective biocontrol agent for various fungal diseases of plants (Gooday, 1990b). The same *ChiA* gene was introduced into the mycoparasitic fungus, *T. harzianum*, to give rise to transformants with a remarkable advantage in controlling *Sclerotium rolfsii* compared with the wild-type (Haran *et al.*, 1993). Furthermore, cosmids that carry chitinase genes from *S. marcescens* were mobilised into *Pseudomonas* strains, which inhibited the growth of *R. solani* and reduced the occurence of disease induced by *Fusarium oxysporum*, which causes dry-rot of potatoes (Sundheim, 1992).

Another bacterial chitinase gene (*chiA*) that codes for an endochitinase in *Enterobacter agglomerans*, could also have an antifungal application. The transformed *E. coli* carrying *chiA* was able to secrete the enzyme and the growth of *R. solani* on plates was inhibited by the transformed strain, indicating that this strain could be used as a potential biocontrol agent (Leonid *et al.*, 1997).

2.5.3. The use of chitinases in genetic transformation strategies to upregulate plant defence systems

Although intensive efforts go towards the development of biological control agents, few products of this type are available commercially. Newly developed technology for the identification, isolation and transfer of specific genes has enabled the insertion of genes encoding for resistance, without interfering with the intrinsic properties of the acceptor plant. Therefore, great effort is being put into identifying and isolating genes that, upon transfer, may render plants resistant to fungi. Much attention has been given to the genes encoding toxic compounds, such as thionins and lectins (Sahai and Manocha, 1993), as well as enzymes involved in direct inhibitory effects on fungi, one of which is chitinase (Schickler and Chet, 1997). Moreover, the currently available techniques for the transformation of plant species could answer questions regarding the precise role of different chitinases in plant defence by enabling relevant experiments.

Fungal chitinases used in genetic transformation strategies

Lorito *et al.* (1998) constitutively expressed the *ThEn-42* gene (Hayes *et al.*, 1994) from strain P1 of *T. harzianum* in tobacco and potato. Transgenic lines showed a high level and a broad range of resistance against both soil-borne and foliar pathogens. The resistance to *R. solani*, *B. cinerea, Alternaria alternata* and *A. solani* was genetically stable and transferred to the progeny. This high level of resistance represents a major improvement in comparison to results from the transgenic expression of other chitinase genes, *i.e.* from plants or bacteria. This improvement is in accordance with the finding that endochitinases and other cell wall–degrading enzymes from *Trichoderma* have a much stronger antifungal activity against a wide range of phytopathogens, compared to other chitinolytic enzymes

(Lorito *et al.*, 1993b). Lorito *et al.* (1998) concluded that this work discovered a new source of genes that can be used either singly or in synergistic combinations to provide protection against a wide range of fungal pathogens.

In addition, Brants and Earle (2001) showed that cell suspensions and calli, obtained from tobacco plants transformed with an endochitinase-encoding cDNA of the *ThEn-42* gene (Hayes *et al.*, 1994) under the control of the double CaMV 35S promoter, a leader sequence for enhanced gene expression and a *T. harzianum* secretion signal peptide, had high endochitinase activity. Endochitinase activity was detected in the medium from transgenic suspensions and also in the medium surrounding the calli. It was concluded that the fungal enzyme is secreted, proving that the fungal signal peptide in the cDNA construct operates in plant cells. The culture medium (after concentration) also inhibited germination of *Penicillium digitatum* spores.

The genes encoding for chitinases I and II (*chiI and chiII*, respectively) of *Rhizopus oligosporus* were isolated and sequenced (Yanai *et al.*, 1992). In contrast to those of *S. cerevisiae*, it was found that the *Rhizopus* proteins comprise five domains, the fifth being an additional C-terminal domain. Chitinase I is produced during the late stage of growth, when *Rhizopus* typically autolyses (Yanai *et al.*, 1992). This protein therefore has excellent chitinolytic activity against the cell walls of *R. oligosorus*, as well as those of phytopathogenic fungi. Terakawa *et al.* (1997) introduced the *chiI* gene into tobacco to develop disease-resistant plants by gene transfer. A fungal infection assay on the leaves of the transgenic plants with the discomycete pathogens, *Sclerotinia sclerotiorum* and *B. cinerea*, revealed that the disease symptoms observed were remarkably suppressed when compared to the control leaves. Terakawa *et al.* (1997) were the first to report on the expression of a fungal chitinase gene in transgenic plants and the protection it confers against fungal pathogenesis. They concluded that this gene could be useful in engineering crops with enhanced protection activity against fungal pathogens.

Bacterial chitinases used in genetic transformation strategies

Plant transformation studies were conducted by Jones *et al.* (1988) to express the *S. marcescens ChiA* gene in tobacco. Under the control of the ribulose biphosphate carboxylase (*rbcS)* promoter, the heterologous ChiAp accumulated to 0.25% of the total leaf protein. The transformed plants also exhibited significantly higher chitinase activities relative to the controls. The successful introduction of *ChiA* into tobacco has also been shown using other promoters, such as those of the cauliflower mosaic virus (CaMV) 35S and the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* (Lund *et al.*, 1989; Nagel *et al.*, 1990; Lund and Dunsmuir, 1992).

Plant chitinases used in genetic transformation strategies

Chitinase genes of plant origin have been introduced heterologously into various plant species (Table 4).

Table 4. Some examples of transformed plant chitinases, their origin and characteristics

Source organism	Transformation host	Observed phenotype/characteristics	References
Bean:			
1. *Ch5B*	Tobacco and Rape	Resistant to *Rhizoctonia solani*	Broglie *et al.*, 1991
Tobacco:			
1. Tobacco chitinase	Tobacco	Resistant to *R. solani*	Vierheilig *et al.*, 1993; 1995
2. Class I chitinase	*Nicotiana sylvestris*	Slightly increased resistance to *Cercopsora nicotinae*	Neuhaus *et al.*, 1991
Rice:			
1. *RCH10*	Tobacco	Regulation by fungal elicitors	Zhu *et al.*, 1993
2. *RC7*	Indica rice	Resistant to *R. solani*	Datta *et al.*, 2001
3. *RCC2*	Chrysanthemum	Resistant to *Botrytis cinerea*	Takatsu *et al.*, 1999
4. *Chi11*	Rice	Resistant to *R. solani*	Lin *et al.*, 1995
Other chitinases:			
1. Pine (*Pschi 4*)	Tobacco	Chitosan inducible	Wu *et al.*, 1997
2. Peanut (*A.h.Chi2;1*)	Tobacco	Enhanced by fungal spores	Kellmann *et al.*, 1996
3. Sugar beet (*SE2*)	Tobacco	Transgenic plants not appreciably protected from *C. nicotinea*	Nielsen *et al.*, 1993

Broglie *et al.* (1991) were the first to assess the role of transformed plant chitinases in resistance to fungal pathogens. The timing of the natural host defence mechanisms was modified from temporal to constitutive expression by transforming the bean chitinase (*CH5B*) gene into tobacco plants under the control of the CaMV 35S constitutive promoter. The transformed plants showed increased chitinase activity: a two- to four-fold increase in the roots and a 23- to 44-fold increase in the leaves was observed when compared to the control plants. To determine the susceptibility of these transgenic tobacco plants to fungal attack, homozygous progeny were grown in the presence of the soil-borne phytopathogenic fungus, *R. solani*. The transgenic tobacco plants expressing high levels of chitinases grew faster, lost at least three-fold less root weight and had a lower seedling mortality rate relative to the control seedlings. The 35S-*CH5B* construct was also transformed into canola plants grown in soil infested with *R. solani*. The extent of the infection was lowered considerably and was restricted mainly to the root cortex. The hyphae that developed on the transgenic plants appeared physically damaged and suffered increased vacuolisation and cell lysis in comparison to the metabolically–active fungi found on the control plants (Benhamou *et al.*, 1993). The extent of the disease resistance observed in the transgenic tobacco and canola plants varied with the amount of fungal inoculum used, which is a typical characteristic of quantitative resistance (Broglie *et al.*, 1991). The delay in the appearance of symptoms, as well as the lower severity of disease, may contribute to the survival of young seedlings during the critical period of

establishment in the field, when they are most susceptible to attack by soil-borne pathogens.

Lin *et al.* (1995) took the same approach and overexpressed a rice chitinase gene (*Chi11*) under the control of the CaMV 35S promoter in rice. This high level, constitutive expression of chitinases in plants could potentially improve resistance to fungal attack in two ways. Firstly, it attacks the fungal cell walls directly and secondly, it releases oligo-GlcNAc residues that function as elicitors for the activation of defence-related responses in plant cells. The resistance of the transgenic rice progeny was determined against the sheath blight pathogen, *R. solani*, and it was found that the degree of resistance corresponded to the level of chitinase expression. During the antifungal assay, lesions appeared on both the transgenic and the control rice plants. However, the number and size of lesions were smaller and confined to the lower half of the transformants' sheath, whereas the lesions spread to the upper half and covered a larger area of the control plants. Recently, more genetic transformation strategies have been employed to manage rice sheath blight disease. A PR-3 rice chitinase gene (*RC7*), isolated from *R. solani*-infected rice plants, was introduced into various indica-type (Southern long-grain) rice cultivars (Datta *et al.*, 2001). Different levels of chitinase proteins were synthesised constitutively by the transformants and the progeny from these transgenic plants showed different levels of enhanced resistance when challenged with *R. solani*.

The CaMV 35S promoter was also employed by Rohini and Rao (2001) for the constitutive expression of transgenes. Tobacco chitinase and neomycin phosphotransferase (*npt* II) genes driven by this promoter were transformed into peanut (*Arachis hypogaea* L.) plants. Protein extracts from the transgenic plants showed varied, but enhanced, levels of chitinase activity in comparison to the untransformed control plants. Small-scale tests showed an increased ability of these plants to resist the fungal pathogen *Cercospora arachidicola*. This fungus is a major pathogen of peanut, being highly virulent in the Indian cultivars, in which it causes local necrotic lesions on leaves that are typical of leaf spot or tikka disease. The intensity of the disease symptoms on the transgenics was variable and correlated with the chitinase activities.

The resistance evaluation of chitinase-expressing transgenic plants was taken one step further by Grison *et al.* (1996). *Brassica napus*, commonly known as oilseed rape, was transformed with a tomato chitinase gene, also under the control of the CaMV 35S promoter. The authors challenged the third-generation progeny of these transgenic plants with three different fungal pathogens, namely *Cylindrosporium concentricum*, *Phoma lingam* and *S. sclerotiorum*, in field trials at two different geographical sites. The mechanism of protection observed involved both a delay in the appearance of symptoms and a reduction in lesion numbers. The transgenic genotypes showed different degrees of protection against all three fungal pathogens at the two field sites. Symptom reduction was exhibited in all cases, ranging roughly from 23 to 79%.

The observed variable antifungal effect of chitinase is problematic, however. The success of the engineered defence mechanism depends not only on the type of chitinase, but also on the fungus tested. This was shown in *in vitro* assays, in which a class I chitinase from *Arabidopsis* was effective against *T. reesei*, but not against commercially important pathogens, such as *F. oxysporum*, *A. solani*, *S. rolfsii* or *Phytophora megasperma* (Verburg and Huynh, 1991; Graham and Sticklen, 1994). Moreover, not all transformed plant lines with high levels of chitinase activity exhibit the expected increase in resistance to fungal pathogens. Neuhaus *et al.* (1991) introduced a gene for a tobacco class I chitinase regulated by the CaMV 35S promoter into *N. sylvestris*. Most of the transformants accumulated very high levels of chitinases, but when challenged with *Cercospora nicotianae*, a major fungal pathogen of tobacco, disease symptoms were reduced only slightly. Nielsen *et al.* (1993) also found that transgenic plants bearing the class III chitinase gene (*SE2*) from sugar beet were not protected appreciably from infection by *C. nicotianae*. However, these observations are not unique to chitinases in engineered resistance and merely emphasise the fact that manipulated resistance is more complex than mere overexpression of a certain defence gene.

It has been found *in vitro* that chitinase is much more effective when used in combination with a β-1,3-glucanase (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). Zhu *et al.* (1994) used this approach to enhance protection against fungal attack by constitutive co-expression of the rice basic chitinase (*RCH10*) and the alfalfa acidic β-1,3-glucanase (*AGLU1*) genes. Markedly higher protection was observed in plants expressing both aforementioned transgenes when challenged with *C. nicotianae*, in comparison to plants expressing each transgene alone. The protective effects involved a delay in the appearance of the first visible lesions and a subsequent reduction in both the size and number of the lesions.

Genetic engineering offers the opportunity to complement conventional breeding strategies for further crop improvement, as there is an increasing demand for higher yields in food crops. Chitinases have been shown to be an integral component of the plant's defence response to fungal attack. In most cases, plants that have been transformed with chitinase genes show improved resistance to phytopathogenic fungi. Although a great deal of knowledge has been gathered pertaining to the functions and different classes of chitinases and PR proteins, the relative activities of these proteins against specific pathogens have been elucidated only partially. Therefore, it is necessary to evaluate each combination of plant, pathogen and introduced gene for enhanced resistance, as well as plant viability and productivity, to ultimately achieve modern biotechnology-based crop improvement.

2.6. FUTURE TRENDS

Some important successes have been achieved utilising chitinases in the biocontrol of plant pathogens, as well as in the development of transgenic plants. However, chitinases also can be employed in human health care, such as for making ophthalmic preparations with microbicides and chitinases (Patil *et al.*, 2000). Previously, attention was focused on isolating chitinase-encoding genes and classifying them. The present research into the cloning of chitinase genes is directed mainly toward the study of gene regulation and catalytic activity. The understanding of the biochemistry of chitinolytic enzymes, including the signalling pathways for induction, together with an elucidation of the responsive elements in their genes (Schickler and Chet, 1997), will make them more useful in a variety of processes in the near future (Patil *et al.*, 2000).

Felse and Panda (1999) believe the importance of the stability of cloned chitinase genes in microorganisms has largely been ignored, hence a considerable amount of research on the stability of plasmids is yet to be done. This is of great significance and importance if the transformed microorganisms are destined for industrial use. However, through the integration of the chitinase gene into the genome of a microorganism, the instabilty of plasmids can be overcome, resulting in microorganisms that would be more applicable to the industrial sector.

The techniques used to develop transgenic plants have improved dramatically during the last decade. Concurrently, numerous genes encoding antimicrobial compounds have been identified (Dempsey *et al.*, 1998) and their antimicrobial properties not only have been studied *in vitro*, but also *in vivo* with transgenic plants (Fritig *et al.*, 1998). This has allowed the development of new disease- and pest-resistant crops, some of which are now available commercially (Dempsey *et al.*, 1998).

The identification of novel antimicrobial and pesticidal compounds and proteins, as well as an improved understanding of the pathways employed to induce their synthesis, should provide additional tools for engineering resistance. Thus, the evolving understanding of the mechanisms by which resistance is activated should continue to provide new opportunities to improve existing methods and to create novel strategies for engineering durable, broad-spectrum disease resistance in plants.

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CHAPTER 3

Overexpression and secretion of the *Saccharomyces cerevisiae* chitinase (*CTS1*-2) gene product from yeast

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RESEARCH RESULTS

Overexpression and secretion of the *Saccharomyces cerevisiae* chitinase (*CTS1*-2) gene product from yeast

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Plant protection is a major challenge to modern agriculture worldwide, with fungi being one of the leading causes of considerable yield losses. Chemical fungicides have been used extensively to control fungal diseases. Public concern over the harmful effects of these fungicides on the environment and on human health, as well as pathogen resistance to chemical fungicides, have intensified the search for alternative control measures. Biological control agents are promising alternatives to chemicals in the control of plant pests. Chitin constitutes a major component of fungal cell walls and is of extreme importance in fungal growth and development. This has emphasised the roles of chitinolytic enzymes (chitinases) as antimicrobial substances in biological control agents and as defence proteins in transgenic plants. The *Saccharomyces cerevisiae* chitinase (*CTS1-2*) gene, fused to different extracellular targeting signals, was overexpressed in *S. cerevisiae* to yield recombinant yeasts with possible applications as biocontrol agents of plant pathogenic fungi. The extracellular targeting sequences compared in this study include the native *CTS1-2* secretion signal, the leader sequence of the precursor of the yeast mating pheromone α-factor (*MFα1*), and the leader sequence of the *Trichoderma reesei* β-xylanase 2 (*XYN2*) gene. These secretion signals, fused to the *CTS1-2* gene, were under the control of the yeast phosphoglycerate kinase 1 (*PGK1*) promoter and terminator on episomal plasmids. Yeast transformation rendered recombinant yeasts, which were analysed for transgene expression by Northern blot analysis. Endochitinase assays were conducted to confirm the activities of the overexpressed proteins, in the process also evaluating the secretion efficiency from the various extracellular targeting signals used. The assays indicated that both the *XYN2* and *MFα1* secretion signals secreted the chitinase protein more optimally than the native *CTS1-2* signal. Crude protein extracts from the culture media of the various transformed yeast strains were used to study the effect of the chitinase protein on a fungal pathogen. Preliminary results showed that the overexpression of the chitinase had dramatic effects on spore germination and hyphal growth in *Botrytis cinerea*.

3.1. INTRODUCTION

Chitin is an unbranched homopolymer of *N*-acetylglucosamine (GlcNAc) residues linked by β-1,4 bonds. Chitin microfibrils are major structural components of the shells or

cuticles of arthropods, the cell walls of most filamentous fungi and some algae, molluscs, nematodes, worms and many other types of organisms. However, this highly abundant polymer is absent from plant cells (Cabib, 1987; Gooday, 1990). In the yeast *Saccharomyces cerevisiae*, short chitin microfibrils comprise only about 1% of the total cell wall and are situated almost exclusively in the primary septum of budding cells, of which it seems to be the sole component (Cabib, 1987).

Chitinases (EC 3.2.1.14), which hydrolyse the β-1,4 linkages in the insoluble chitin microfibril, are found in a variety of organisms, including plants, yeasts, most fungi and some prokaryotes (Cohen, 1993). In *S. cerevisiae*, this enzyme is involved in cell division and cell separation (Kuranda and Robbins, 1991). The yeast cells are unable to separate normally if they lack chitinase activity in the logarithmic phase, and instead they aggregate at the septum regions and remain as clusters (Kuranda and Robbins, 1991).

Despite these important functions, chitinases are generally more renowned for their antifungal activities. Although chitin does not exist in plant cells, chitinases have been purified from a large number of plants encompassing diverse taxonomic groups. In plants, invasion by a pathogen induces the production of pathogenesis-related (PR) proteins, such as chitinases, β-1,3-glucanases, proteinases, etc. (Kombrink and Somssich, 1995). Since pathogenic fungi and insects contain chitin in their protective covers, induction of chitinases in plants is considered a major defence response (Patil *et al.*, 2000). It has been shown that chitinases degrade fungal cell walls, inhibit fungal growth by lyses of the hyphal tips (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988), as well as associate with hyphal walls *in planta* (Benhamou, 1995).

Chitinases not only play an important role in the defence mechanisms of plants, but also in the mycoparasitic processes of fungi. Mycoparasitic and antagonistic fungi have been studied to develop a biological alternative to chemical fungicides (Lorito *et al.*, 1998). The antagonistic activity of biological control agents towards phytopathogens is based on the secretion of extracellular lytic enzymes. The antifungal mechanism of *Trichoderma*, an extensively studied and widely used biocontrol fungus, relies on chitinase cell wall–degrading enzymes (Lorito *et al.*, 1998) and has been used to control a variety of plant pathogens (Chet, 1987; Jin *et al.*, 1991; Tronsmo, 1991; Gullino, 1992; Elad *et al.*, 1993). In addition to the already widely explored *Trichoderma* spp. as natural biocontrol agents (Lorito *et al.*, 1998), other fungal or yeast species could also be manipulated in this regard through genetic engineering.

In this study, high–level expression and efficient secretion of the *S. cerevisiae CTS1–2* gene product from a laboratory yeast strain was attempted. Previously, the *S. cerevisiae* chitinase gene had been cloned and overexpressed in *Schizosaccharomyces pombe*, resulting in a five- to 13-fold increase in chitinase activity (Kuranda and Robbins, 1987). The efficient secretion of the chitinase protein is of extreme importance, since the yeast transformants will be evaluated for their possible use as antifungal biocontrol agents. A comparative study of the native *CTS1-2* secretion signal, the *S. cerevisiae* mating pheromone α1-factor (*MFα1*) secretion signal,

as well as the *T. reesei* β-xylanase 2 (*XYN2*) secretion signal (La Grange *et al.*, 1996), was conducted to ultimately determine the effectiveness of these secretion signals to deliver the *CTS1-2* gene product extracellularly. High-level expression of the *CTS1-2* gene was attempted by employing the constitutive *PGK1* promoter and terminator on multicopy plasmids. Preliminary results showed that the chitinase enzyme, under the control of the *PGK1* promoter and terminator, had inhibition effects on the spore germination and hyphal growth of *B. cinerea*, confirming the strong antifungal effect of this yeast chitinase. These promising results might lead to the development of a biocontrol yeast strain, in which antagonism is based on chitinase activity.

3.2. MATERIALS AND METHODS

3.2.1. Microbial strains and culture conditions

The sources and relevant genotypes of the bacterial and yeast strains are listed in Table 1. *Escherichia coli* transformants were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) supplemented with ampicillin (100 μg/ml). All media compositions are indicated as weight per volume (w/v), unless otherwise stated. Yeasts were routinely cultured on YPD medium (containing 1.2% yeast extract, 2.5% peptone, 1.2% glucose). Yeast transformants were grown on selective, buffered synthetic SCD medium, containing 0.67% yeast nitrogen base without amino acids (Difco), supplemented with the required amino acids except uracil, 20 mM succinate (pH 6.0) and 2% glucose (SCD^{-Ura}). Solid media contained 2% agar. Bacteria and yeasts were cultured routinely at 37°C and 30°C respectively.

B. cinerea spores were obtained from the Department of Plant Pathology, Stellenbosch University. Fungal cultures were obtained by germinating spores on apricot halves canned in natural juice. Before inoculation, the apricot halves were rinsed with sterile dH_2O and blotted on sterile filter paper. Each apricot half was placed in a tissue culture petri dish and was subsequently inoculated with a few spores of *B. cinerea*. The plates were incubated in the dark at 25°C until sporulation occurred.

Table 1. Strains and plasmids used in this study

Strain or plasmid(s)	Relevant genotype or insert	Source or reference
E. coli strains		
DH5α	supE44 *lac*U169[*ϕ80lacZ*M15) *hsd*R17*rec*A1*gyr* A96*thi*-1*rel* A1]	Life technologies (GIBCO/BRL)
Yeast strains		
PRY488	MATa his3-Δ200 leu2-3, 112, trp1-Δ1 ura3-52 Gal^+ cts::TRP1	P.W. Robbins
DBY918	MATα his4-38	D. Botstein
Plasmids		
pBluescript SK(+)	Amp^R	Stratagene
pJC1	Amp^R *URA3* $PGK1_{PT}$	Crous *et al.*, 1995
pJC-H	Amp^R *URA3* $PGK1_{PT}$ without *Hin*dIII restriction sites	This study
pDLG4	Amp^R *URA3* $ADH2_P$-$MF\alpha1_S$-$ADH2_T$	La Grange *et al.*, 1997
pBSCTS	pBluescript SK(+) Amp^R *CTS1-2*	This study
pBSXYN	pBluescript SK(+) Amp^R $XYN2_S$	D.C. la Grange (unpublished)
pBSXYN:CTS	Amp^R $XYN2_S$ *CTS1-2*	This study
pJC-H:CTS	Amp^R *URA3* $PGK1_P$-*CTS1-2*-$PGK1_T$	This study
pJC-H:MFα	Amp^R *URA3* $PGK1_P$-$MF\alpha1_S$-$PGK1_T$	This study
pJC-H:MFα-CTS	Amp^R *URA3* $PGK1_P$-$MF\alpha1_S$-*CTS1-2*-$PGK1_T$	This study
pJC-H:XYN-CTS	Amp^R *URA3* $PGK1_P$-$XYN2_S$-*CTS1-2*-$PGK1_T$	This study

3.2.2. DNA manipulations and construction of plasmids

All plasmids used in this study are listed in Table 1 and the PCR primers and their applications are listed in Table 2. Standard molecular techniques were applied, as described by Sambrook *et al.* (1989). Restriction enzymes, Expand Polymerase and T4 DNA Ligase were purchased from Roche Diagnostics. Klenow fragment of *E. coli* DNA polymerase I and shrimp alkaline phosphatase (SAP) were obtained from Amersham Life Sciences. Sequencing was performed by the DNA Sequencing facility, Department of Genetics, Stellenbosch University, using an ABI PRISM™ 377 automated DNA sequencer from PE Biosystems.

PCR amplifications were done using Expand Polymerase (Roche Diagnostics) in 50 µl reaction mixtures, typically consisting of 1x Expand Polymerase PCR buffer (without $MgCl_2$), 200 µM dNTPs, 200 nM of each primer, 5 ng template DNA and $MgCl_2$ added to the optimal concentration. Typical amplification conditions included an initial DNA denaturation step at 95°C for 2 min, followed by cycles of denaturation at 95°C for 10 sec, primer annealing according to the specific primer melting temperatures, and elongation at 72°C, allowing 1 min per 1 kb amplified. Reactions proceeded for 30 cycles in a Biometra Trio-thermoblock cycler.

Table 2. Primer pairs and sequences used in this study

Primer	Sequence	Paired with	Template	Product
CTSEC 5'	5'-ATGCGAATTCAATAATAGAAT GTCACTCCT-3'; *Eco*RI-site underlined	CHIT 3'	*S. cerevisiae* strain DBY918 genomic DNA	1.69 kb of *CTS1-2* gene including secretion signal
CHIT 3'	5'-ACGTAAGCTTCGGACTCTATG AATCAATCT-3'; *Hin*dIII-site underlined	CTSEC 5'	*S. cerevisiae* strain DBY918 genomic DNA	1.69 kb of *CTS1-2* gene including secretion signal
CHIT 5'	5'-ACGTAAGCTTCTTTTGATAGGT CTGCTAACAC-3'; *Hin*dIII-site underlined	CHIT 3'	*S. cerevisiae* strain DBY918 genomic DNA	1.62 kb of *CTS1-2* gene excluding ATG and secretion signal
XYNCTS 5'	5'-GATCTCGCGATTTGATAGGTC TGCTAACAG-3'; *Nru*I-site underlined	XYNCTS 3'	*S. cerevisiae* strain DBY918 genomic DNA	1.62 kb of *CTS1-2* gene excluding ATG and secretion signal
XYNCTS 3'	5'-GATCTCGCGACGGACTCTAT GAATCAATCT-3'; *Nru*I-site underlined	XYNCTS 5'	*S. cerevisiae* strain DBY918 genomic DNA	1.62 kb of *CTS1-2* gene excluding ATG and secretion signal
MFα1 5'(L)	5'-GATCGGATCCATGAGATTTCC TTCAATTTTTACTGCAG-3'; *Bam*HI-site underlined	None	Sequencing primer for *MFα1S*-constructs	

The two *Hin*dIII restriction enzyme sites of pJC1 were blunt–ended with DNA polymerase I (Klenow fragment) and ligated to remove these sites, thereby yielding pJC-H. The unsuccessful digestion of pJC-H with *Hin*dIII confirmed the removal of these sites. The *S. cerevisiae CTS1-2* gene, including its ATG and native secretion signal, was PCR amplified using primers CTSEC 5' and CHIT 3' (Table 2) from *S. cerevisiae* strain DBY918 genomic DNA. This PCR product (~1.71 kb) was sub-cloned into pBluescript SK(+) at the *Eco*RI-*Hin*dIII sites, generating pBSCTS. The PCR fragment was excised at the *Eco*RI-*Xho*I sites of pBSCTS and cloned into the corresponding sites of pJC-H, yielding the clone pJC-H:CTS. An *Eco*RI-*Xho*I fragment, containing the *S. cerevisiae MFα1S* (~260 bp) followed directly by a *Hin*dIII restriction site, was recovered from pDLG4 (kindly provided by D.C. la Grange) and was cloned into the corresponding sites of pJC-H, constructing pJC-H:MFα. The *S. cerevisiae CTS1-2* gene, excluding its ATG and native secretion signal (~1.65 kb), was PCR amplified using primers CHIT 5' and CHIT 3' (Table 2) from *S. cerevisiae* strain DBY918 genomic DNA, and was cloned into the *Hin*dIII site of pJC-H:MFα, yielding the clone pJC-H:MFα-CTS. The *S. cerevisiae CTS1-2*, gene without its ATG and native secretion signal (~1.65 kb), was PCR amplified using primers XYNCTS 5' and XYNCTS 3' (Table 2) and was cloned into the SAP-treated *Nru*I site of pBSXYN (kindly provided by D.C. la Grange), generating the sub-clone pBSXYN:CTS. The ~1.78 kb fragment containing the *T. reesei XYN2S* (~120 bp) and *CTS1-2* was excised from pBSXYN:CTS at the *Eco*RI-*Bgl*II sites, and was cloned into the corresponding sites of pJC-H, constructing pJC-H:XYN-CTS. All constructs were confirmed by sequencing.

3.2.3. Yeast transformation

S. cerevisiae laboratory strain PRY488, with its natural chitinase activity disrupted, was transformed with episomally-derived plasmid DNA by the lithium acetate dimethylsulfoxide (DMSO) method described by Hill *et al.* (1991). The yeast expression cassettes pJC-H:CTS, pJC-H:MFα-CTS and pJC-H:XYN-CTS are multi-copy *E. coli–S. cerevisiae* shuttle vectors and were maintained as autonomous circular minichromosomes in the recombinant yeast cells.

3.2.4. RNA isolation and Northern blot analysis

Yeast cells transformed with plasmids containing the $PGK1_{PT}$ were grown in the selective medium SCD^{-Ura}. The cells were cultured overnight to an OD_{600} of 1 at 30°C. Total RNA was isolated from 5 ml cultures as described by Ausubel *et al.* (1987). RNA (10 μg) from each culture was subjected to 1.2% (w/v) formaldehyde gel electrophoresis together with an RNA ladder (Life Technologies (GIBCO/BRL) and blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) according to standard procedures (Sambrook *et al.*, 1989). The *CTS1-2* gene (~1.69 kb) was PCR-labelled as probe DNA, using the 10x DIG dNTP labelling mixture (Roche Diagnostics) according to the manufacturer's specifications. Hybridisations were performed at 50°C for 16 h, using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications.

3.2.5. Endochitinase activity assays

The endochitinase activity assay was adapted from Kuranda and Robbins (1991). All constructs containing the $PGK1_{PT}$ were grown in SCD^{-Ura} as selective media or in YPD during the assays. Six individual transformants of each construct were assayed in duplicate. The untransformed *S. cerevisiae* strain DBY918 was employed as a positive control.

The recombinant yeasts were grown overnight in 5 ml of selective media, whereas DBY918 was grown overnight in 5 ml of YPD as starter cultures. Cells from the overnight cultures were centrifuged, washed with fresh YPD, and then diluted to a final OD_{660} of 0.25 in 20 ml of YPD. The cultures were incubated at 30°C with agitation. The activity assays were conducted every 2.5 h, while also monitoring the growth of the culture by determining the OD_{660} values. The cells (1 ml) were removed and centrifuged to yield the supernatant, which was assayed as the extracellular fraction. The cell pellet was washed in 1 ml dH_2O and resuspended in 400 μl dH_20. Glass beads (0.45-0.55 mm diameter, Sigma) were added to a level of approximately 200 μl and the suspension was incubated on ice for 5 min. The cells were mixed vigorously for 2 min, incubated on ice for 5 min and vigorously vortexed for another minute. The broken cell slurry was

recovered to be assayed as the combined periplasmic and intracellular fraction (designated the cell-associated fraction).

Samples (200 µl) of either the supernatant or the broken cell slurry were mixed with 40 µl of 41.67 µM 4-methylumbelliferyl β-D-*N,N',N''*-triacetylchitotrioside (Sigma) in a final volume of 240 µl and incubated for 1 h at 30°C. A 0.48 M sodium citrate buffer, pH 3.0, was used to make up the substrate stock. The reaction was stopped by the addition of 0.42 M glycine, NaOH buffer, pH 10.4 in a final volume of 1.5 ml. Cell debris was removed by centrifugation, and the liberated 4-methylumbelliferone (4-MU) was measured with a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.) at an excitation wavelength of 350 nm and an emission wavelength of 440 nm. Units of activity were defined as nanomoles of 4-MU released per h.

3.2.6. Antifungal activity assays

The recombinant yeasts were grown overnight in 50 ml of the respective selective media. The cells were centrifuged and the supernatants removed, of which 15 ml of each was concentrated through Centriprep™ (Millipore) centrifugation devices with protein exclusion membranes of 30 kDa. The retentate, which was used in the assay, had a final volume of approximately 1 ml and contained all proteins larger than 30 kDa. All the procedures involving Millipore centrifugation columns were done according to the manufacture's guidelines.

B. cinerea spores, at a concentration of 2×10^3 spores in 100 µl half strength potato dextrose broth (PDB) (containing 10% infusion from potato and 1% dextrose), were mixed with 50 µl of the respective retentates in a 500 µl microcentrifuge tube. The samples were incubated in the dark at 25°C for 4 days. The antifungal effects of the proteins were observed microscopically and photographed every 24 hours. The negative control consisted of *B. cinerea* spores in half-strength PDB, mixed with 50 µl retentate of yeast transformed with pJC–H.

3.3. RESULTS

3.3.1. Construction of chitinase yeast expression vectors and yeast transformation

The *S. cerevisiae CTS1-2* gene was fused to either its own secretion signal or the *MFα1* or *XYN2* secretion signals under the control of the constitutive *PGK1* promoter and terminator. A schematic representation of these constructs is given in Figure 1. All the clones were transformed separately into a *S. cerevisiae* laboratory strain lacking endogenous chitinase activity.

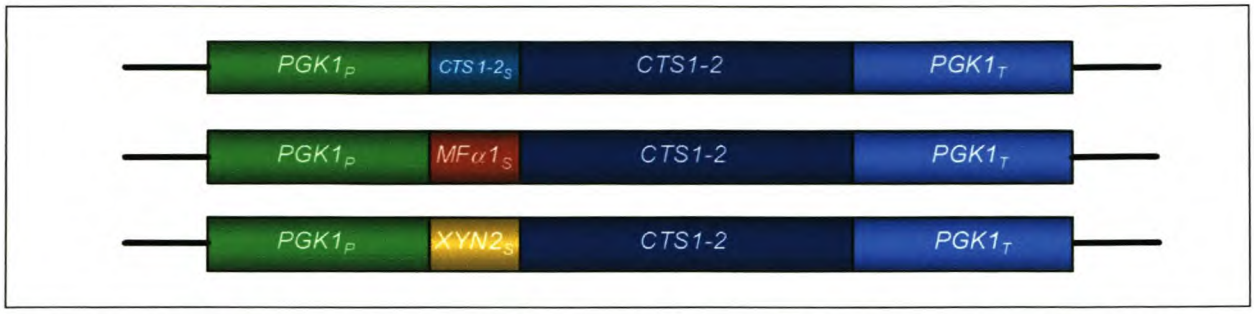

Figure 1. Schematic representation of the constructs used for yeast transformation with the yeast *CST1-2* chitinase gene. *PGK1*$_P$, phosphoglycerate kinase 1 promoter; *PGK1*$_T$, phosphoglycerate kinase 1 terminator; *CTS1-2*$_S$, native yeast *CTS1-2* gene secretion signal; *MFα1*$_S$, yeast mating pheromone α-factor; *XYN2*$_S$, the leader sequence of the *Thrichoderma reesei* β-xylanase 2 gene; *CTS1*-2, yeast chitinase gene. The backbone plasmid for these constructs is pJC-H (this study).

3.3.2. Northern blot hybridisation

Northern blot analysis revealed the presence of the *CTS1-2* transcript in the recombinant *S. cerevisiae* strains (Figure 2). The detected signals corresponded to the expected sizes when compared to the RNA ladder of Life Technologies (GIBCO/BRL). No chitinase transcripts were detected in the RNA isolated from the yeast strain or a recombinant strain transformed with the backbone of the yeast expression vector, pJC-H.

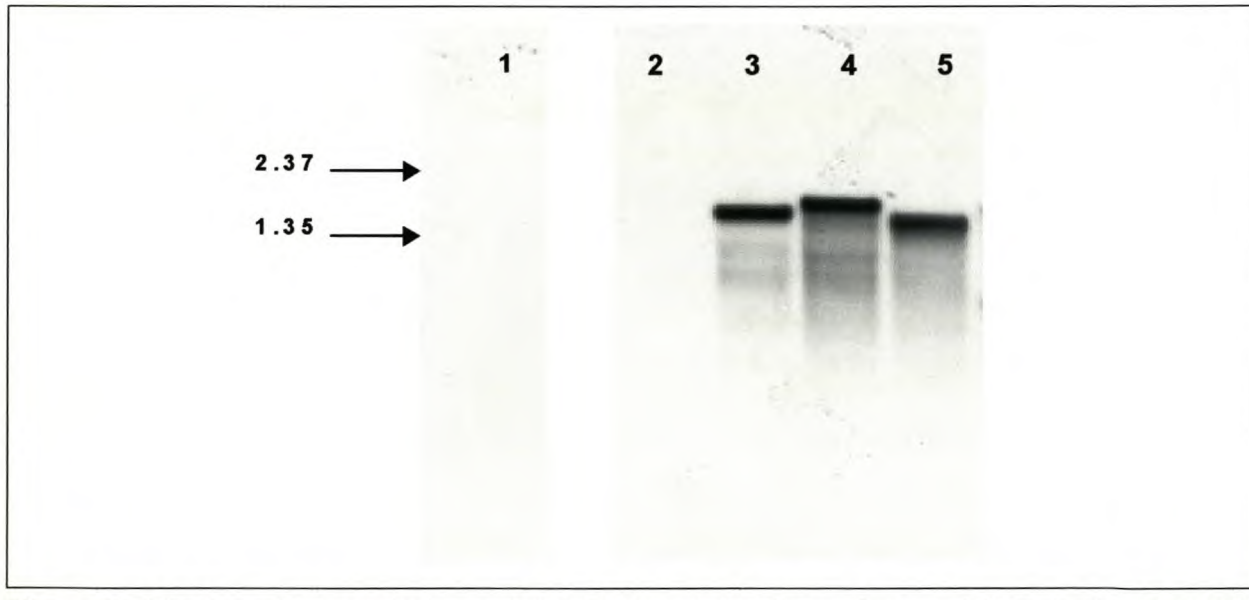

Figure 2. Northern blot analysis on total RNA isolated from *S. cerevisiae* strains PRY488 (negative control, lane 1) and PRY488 transformed with pJC–H (plasmid control, lane 2), pJC-H:CTS containing the *CTS1–2* gene fused to its native secretion signal (lane 3), pJC-H:MFα-CTS containing the *CTS1–2* gene fused to the *MFα1* secretion signal (lane 4) and pJC-H:XYN-CTS containing the *CTS1–2* gene fused to the *XYN2* secretion signal (lane 5). An RNA ladder was used as a molecular size standard and the sizes are indicated in kilobases. The *CTS1–2* gene was used as a DIG-labelled probe for hybridisation.

3.3.3. Endochitinase activity assays

The chitinase-producing yeast strains, under the control of the *PGK1* promoter and terminator, were analysed for their ability to secrete biologically active endochitinases over a 14.5 h period (Figure 3), while also documenting the various growth curves of the strains (Figures 3 and 4). Both the cell-associated and extracellular activity was determined for six individual transformants containing either the plasmid backbone (as a control) or the various chitinase constructs.

The control showed constant low levels of chitinase activity throughout the various stages of the growth curve. These values were used to normalise the chitinase activity units obtained for the various recombinant strains. The control as well as various chitinase-overexpressing strains had virtually identical growth curves over the 14.5 h monitoring period (Figure 4). From the chitinase activity assays on the recombinant strains, it was evident that total chitinase production was very similar for the strains containing the *MFα1* and *XYN2* secretion signals differing in only 20 units, especially during the logarithmic growth phase (Table 3, for values normalised against the control). These values were 381 and 362 units more for the *MFα1* and *XYN2* secretion signals respectively, than those of the strains containing the native *CTS1-2* secretion signal. The chitinase production (measured by total chitinase activity in all fractions tested) followed the growth curves closely (Figure 3), as would be expected, since the same regulatory units were used for all the constructs.

When the cell-associated fractions were assayed, very similar production curves were observed with all the recombinant chitinase-expressing strains (Figure 3). The total cell-associated chitinase activities normalised against the control, once again had lower values for the construct harbouring the *CTS1-2* secretion signal.

Chitinase assays on the extracellular fractions of the various recombinant strains showed clearly that the majority of the chitinase proteins are efficiently secreted by all three secretion signals tested (Table 3 and Figure 3). The *MFα1* and *XYN2* secretion signals were more efficient than the *CTS1-2* signal, however. The former two signals both delivered 61% of the total chitinase activity to the extracellular environment in the logarithmic growth phase, whereas the *CTS1-2* signal secreted only 53% of the chitinase activity in the corresponding period (Table 3). The percentages of activity in the extracellular fractions were highest for all the secretion signals in the early logarithmic and early stationary phases of the growth curve (Table 3), reaching levels of 71 and 74% for the *MFα1* and *XYN2* secretion signals respectively. From the production curves in Figure 3, it is clear that the *MFα1* and *XYN2* secretion signals reach and maintain a high ratio of secretion early in the active growth phases.

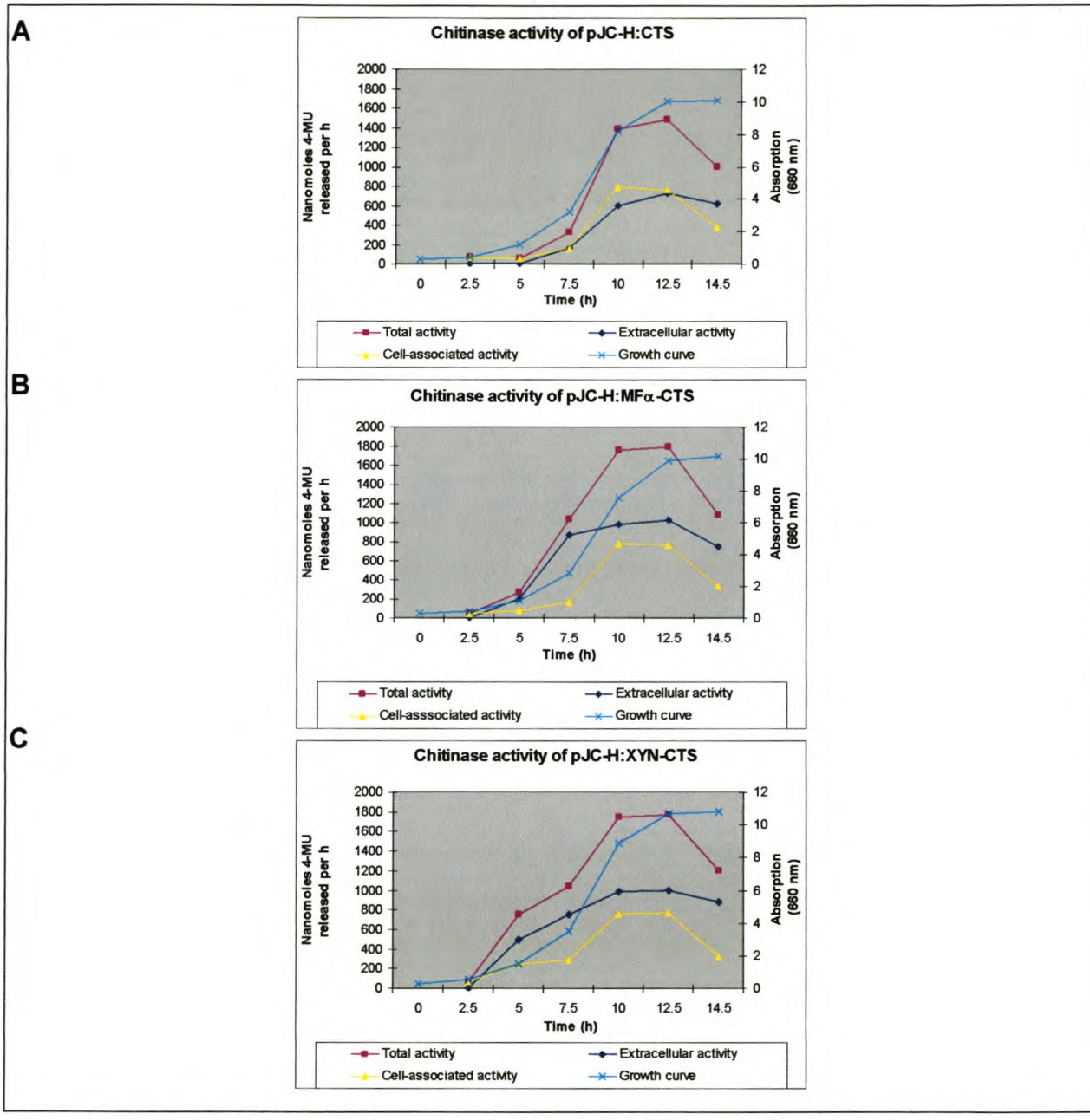

Figure 3. Growth and endochitinase activity of the cell-associated and extracellular fractions of recombinant *S. cerevisiae* strain PRY448 in YPD media. PRY488 transformed with pJC-H:CTS containing the *CTS1-2* gene and its native secretion signal (**A**), pJC-H:MFα-CTS containing the *CTS1-2* gene fused to the *MFα1* secretion signal (**B**) and pJC-H:XYN-CTS containing the *CTS1-2* gene fused to the *XYN2* secretion signal (**C**). All fusion constructs were under the control of the *PGK1* promoter and terminator. Units of activity were defined as nanomoles of 4-MU released per h. Standard deviation never exceeded 25% in the endochitinase activity curves and never exceeded 1% in the growth curves.

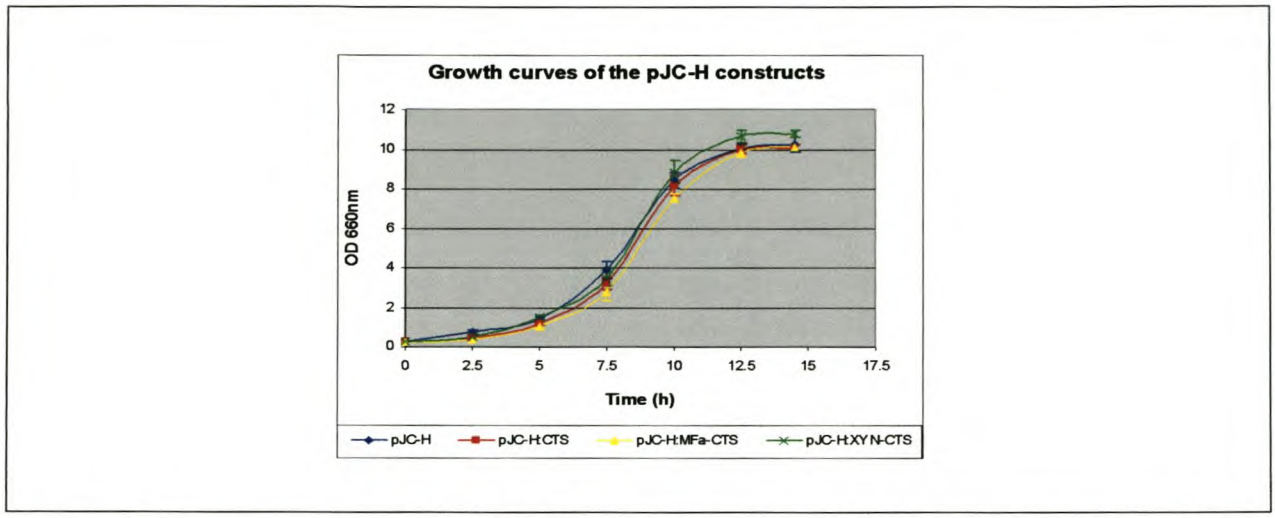

Figure 4. Growth curves of recombinant *S. cerevisiae* strain PRY448 in YPD media. PRY488 transformed with pJC-H:CTS containing the *CTS1-2* gene and its native secretion signal, pJC-H:MFα-CTS containing the *CTS1-2* gene fused to the *MFα1* secretion signal and pJC-H:XYN-CTS containing the *CTS1-2* gene fused to the *XYN2* secretion signal. All fusion constructs were under the control of the *PGK1* promoter and terminator.

Table 3. Total chitinase activity of recombinant yeasts expressed as % cell-associated and % extracellular activity after 5 h, 10 h and 14.5 h. Standard deviation never exceeded 25%

	Total chitinase activity (nanomoles 4-MU released per h) ± stdev	% Cell-associated activity	% Extracellular activity
Time 5 h:			
pJC-H:CTS	59 ± 12	42	58
pJC-H:MFα-CTS	267 ± 64	29	71
pJC-H:XYN-CTS	746 ± 186	32	68
Time 10 h:			
pJC-H:CTS	1380 ± 345	47	53
pJC-H:MFα-CTS	1761 ± 0.5	39	61
pJC-H:XYN-CTS	1742 ± 0.2	39	61
Time 14.5 h:			
pJC-H:CTS	991 ± 248	33	67
pJC-H:MFα-CTS	1082 ± 178	29	71
pJC-H:XYN-CTS	1201 ± 288	26	74

3.3.4. Antifungal activity assay of yeast chitinase

Partially purified chitinase preparations from the supernatants of the transformants containing pJC-H, pJC-H:CTS, pJC-H:MFα-CTS and pJC-H:XYN-CTS were utilised in a microscopic analysis of fungal inhibition on *B. cinerea* spore germination and hyphal growth (Figure 5).

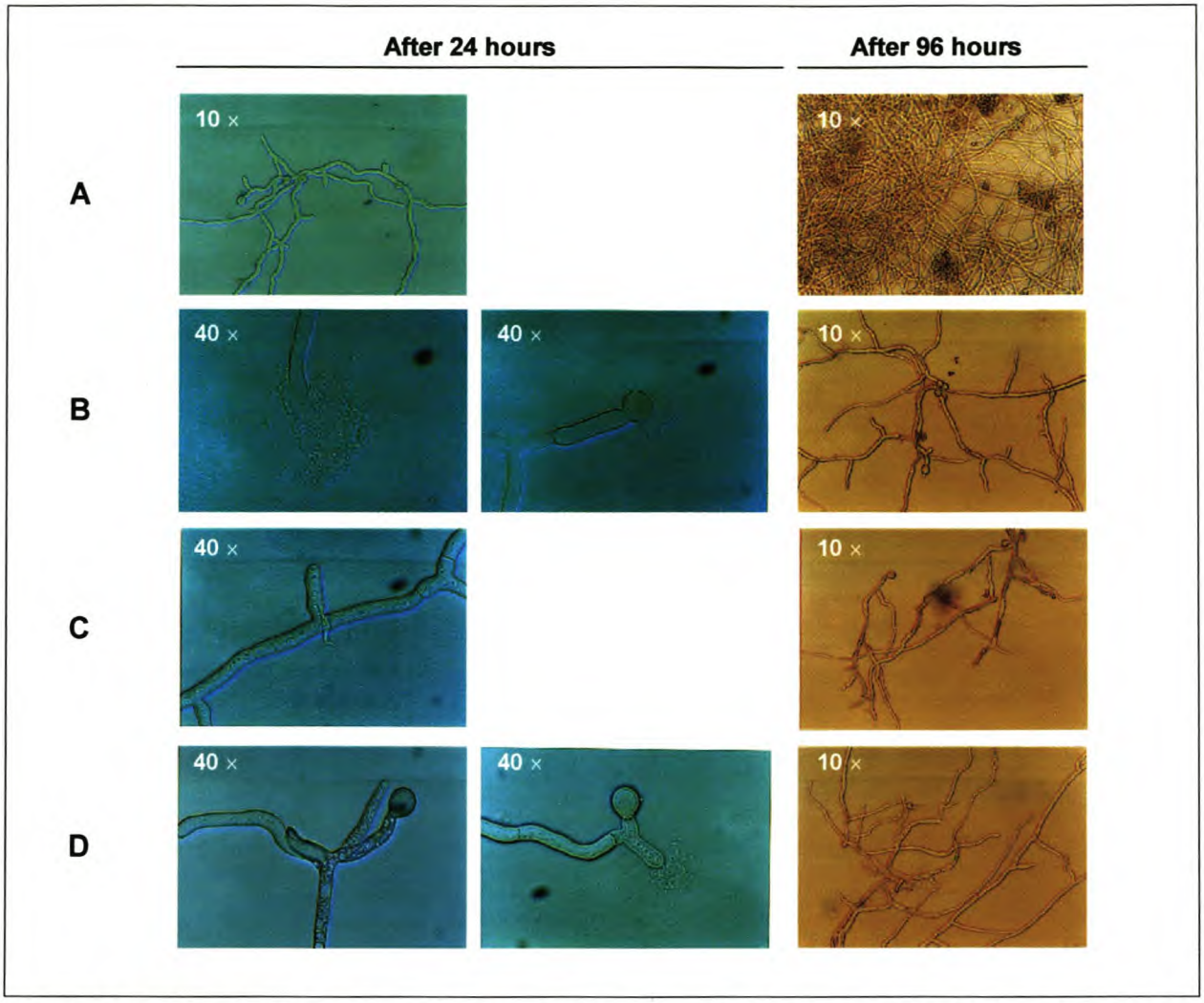

Figure 5. The effect of yeast chitinase proteins secreted by recombinant *S. cerevisiae* on *B. cinerea* spore germination and hyphal growth in a microscopic analysis. Supernatants of pJC-H (control, **A**), pJC-H:CTS containing the *CTS1-2* gene and its native secretion signal (**B**), pJC-H:MFα-CTS containing the *CTS1-2* gene fused to the *MFα1* secretion signal (**C**) and pJC-H:XYN-CTS containing the *CTS1-2* gene fused to the *XYN2* secretion signal (**D**) were used in the antifungal activity assay. All secretion constructs were under the control of the *PGK1* promoter and terminator. The 10× magnification and the 40× magnification are represented by 10× and 40× respectively. Photographs were taken after 24 and 96 h respectively.

In the control (Figure 5A), the *B. cinerea* spore germination and hyphal growth appeared normal, leading to a high fungal biomass after 96 h of incubation. In contrast, *B. cinerea* spore disruption and leakage as well as degradation of fungal hyphal tips, were observed microscopically after 24 h of incubation with the recombinant chitinase proteins (Figure 5B and D). Large vesicles also appeared in some of the hyphal cells (Figure 5D). It was clear that certain segments of the hyphae have been emptied due to the leakage of the cytoplasm (Figure 5C). This structural and physiological damage to the spores and hyphae led to much-reduced hyphal growth and overall biomass production compared to the control.

3.4. DISCUSSION

The search for fungal control alternatives has increased over the years, with the concern for the environment and the enhanced resistance of fungi to chemical fungicides being the driving force. The results presented here describe the construction of *S. cerevisiae* strains overexpressing the chitinase gene (*CTS1-2)* fused to different secretion signals. The ultimate goal is potentially to develop a biocontrol yeast strain; in this study, however, high-level expression, as well as efficient secretion of this antifungal protein, was the target as a first step towards this goal.

The transcription of the fusion proteins, consisting of the *CTS1-2* gene fused to either its native secretion signal, the *MFα1* or the *XYN2* secretion signal, was driven efficiently by the *PGK1* promoter and terminator. From Northern blot analysis and chitinase activity assays, it was confirmed that the gene constructs led to active transcription and translation of the chitinase genes and that the various secretion signals all functioned in delivering the chitinase proteins to the extracellular environment. When chitinase production was monitored as the recombinant yeasts progressed through the various stages of their growth curves, it was evident that total chitinase production did not vary extensively between the various yeast strains. This confirmed that a good test system has been developed to evaluate the efficiency of the different secretion signals.

By analysing the various contributions of the extracellular and cell-associated fractions to the total chitinase activity of the recombinant strains, it became evident that the yeast chitinase could be secreted more optimally by heterologous secretion signals. Both the *MFα1* and the *XYN2* secretion signals were more efficient than the native *CTS1-2* signal, leading to higher percentages of the chitinase occurring extracellularly, as well as initiating and maintaining high levels of secretion earlier in the growth curve. From these activity assays, it was also evident that all three secretion signals continued to secrete the chitinases in the stationary phase and that activity accumulation occurred as a result of the stability of the proteins.

The high chitinase activities present in the extracellular fractions of the various recombinant yeasts prompted a preliminary study on the effect of the chitinases on the fungal growth of *B. cinerea* (Figure 5). The supernatants of the recombinant yeasts induced severe morphological and physiological changes in the hyphae, leading to coagulation and destruction of the fungal protoplasm. The chitinolytic activity of the chitinase enzymes hydrolysed the chitin present in the fungal cell wall, ultimately leading to the degradation of the hyphal tips and the leakage of cellular components or cytoplasm. Large vesicles appeared in certain cells of the hyphae, which is indicative of stress conditions. The general effect of these hydrolysing enzymes on the growth of the fungi led to a significant reduction in the biomass of the hyphae, thus also reducing the potential to produce large quantities of spores and survival structures.

In conclusion, three chitinase gene cassettes fused to either the native secretion signal, the *MFα1* secretion signal or the *XYN2* secretion signal under the control of the

PGK1 promoter and terminator were transcribed successfully in the laboratory strain PRY488 of *S. cerervisiae*. We could determine that the high-level expression and the secretion of a biologically-active endochitinase enzyme were found to be equally optimal by the *MFα1* or the *XYN2* secretion signal, both being more efficient than the native chitinase secretion signal. It was found that the overexpressed chitinase enzymes had a severely detrimental effect on the growth and survival of an important plant pathogen under *in vitro* conditions. All of these results combined have established a base from which yeast chitinase production and secretion could be optimised further, perhaps leading to the development of a biocontrol yeast strain for the control of fungal pathogens. It is imperative to continue with this research to evaluate the chitinase gene constructs in other (non-laboratory) yeast strains, as well as under normal *in vivo* infection conditions.

3.5. ACKNOWLEDGEMENTS

We are grateful to D.C. la Grange for supplying the pDLG1, pDLG4, and pBSXYN plasmids, and to Emden Viljoen, who constructed the pJC-H plasmid. The authors would also like to thank the Institute for Plant Biotechnology, Stellenbosch University, for the use of their FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.). This work was funded by the South African Wine Industry (Winetech) and the National Research Foundation (NRF) of South Africa.

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ADDENDUM A

CONSTRUCTION, TRANSFORMATION AND EVALUATION OF THE *CTS1–2* SECRETION CONSTRUCTS UNDER CONTROL OF THE *ADH2* PROMOTER AND TERMINATOR

A.1 Introduction

It was hypothesised that high-level expression and efficient secretion would be required in a biocontrol yeast strain. The secretion efficiency of the chitinase enzyme was evaluated by testing the previously mentioned secretion signals. To obtain high-level expression of the chitinase enzyme, two promoters were tested for their *CTS1-2* gene expression levels. The results, which were obtained by the constructs under the control of the yeast phosphoglycerate kinase 1 (*PGK1*) promoter and terminator, were discussed in Chapter 3. High-level expression of the chitinase secretion constructs were also tested under the control of the yeast alcohol dehydrogenase 2 (*ADH2*) promoter and terminator.

A.2 Bacterial strains and culture conditions

The sources and relevant genotypes of the bacterial and yeast strains are listed in Table A.1. *E. coli* transformants were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) supplemented with ampicillin (100 μg/ml). All media compositions are indicated as weight per volume (w/v), unless otherwise stated. Yeasts were cultured routinely on YPDL medium (containing 1.2% yeast extract, 2.5% peptone, 0.8% glucose). Yeast transformants were grown on selective, buffered synthetic medium SCDL containing 0.67% yeast nitrogen base without amino acids (Difco), supplemented with the required amino acids except uracil, 20 mM succinate (pH 6.0) and 0.8% glucose ($SCDL^{-Ura}$). Solid media contained 2% agar. Bacteria and yeasts were cultured routinely at 37°C and 30°C respectively.

A.3 Construction of plasmids

*Eco*RI-*Xho*I fragments, comprising the different secretion signals and *CTS1-2*, were excised from the clones pJC-H:CTS, pJC-H:MFα-CTS and pJC-H:XYN-CTS (previously described in Chapter 3). These fragments were cloned separately into the corresponding sites of pDLG1 (kindly provided by D.C. la Grange), yielding the clones pDLG:CTS, pDLG:MFα-CTS and pDLG:XYN-CTS, respectively (Figure A.1). All constructs were confirmed by sequencing.

Table A.1 Strains and plasmids used in this study

Strain or plasmid(s)	Relevant genotype or insert	Source or reference
E. coli strains		
DH5α	supE44 *lac*U169[*ϕ80lac*ZM15) *hsd*R17*rec*A1*gyr* A96*thi*-1*rel* A1]	Life technologies (GIBCO/BRL)
Yeast strains		
PRY488	MATa his3-Δ200 leu2-3, 112, trp1-Δ1 ura3-52 Gal^+ cts::TRP1	P.W. Robbins
DBY918	MATα his4-38	D. Botstein
Plasmids		
pBluescript SK(+)	Amp^R	Stratagene
pJC1	Amp^R *URA3 PGK1*$_{PT}$	Crous *et al.*, 1995
pJC-H	Amp^R *URA3 PGK1*$_{PT}$ without *Hin*dIII restriction sites	This study
pDLG1	Amp^R *URA3 ADH2*$_{PT}$	La Grange *et al.*, 1996
pDLG4	Amp^R *URA3 ADH2*$_P$-*MFα1*$_S$-*ADH2*$_T$	La Grange *et al.*, 1997
pBSCTS	pBluescript SK(+) Amp^R *CTS1-2*	This study, Chapter 3
pBSXYN	pBluescript SK(+) Amp^R *XYN2*$_S$	D.C. la Grange (unpublished)
pBSXYN:CTS	Amp^R *XYN2*$_S$ *CTS1-2*	This study, Chapter 3
pDLG:CTS	Amp^R *URA3 ADH2*$_P$-*CTS1-2*-*ADH2*$_T$	This study
pDLG:MFα-CTS	Amp^R *URA3 ADH2*$_P$-*MFα1*$_S$-*CTS1-2*-*ADH2*$_T$	This study
pDLG:XYN-CTS	Amp^R *URA3 ADH2*$_P$-*XYN2*$_S$-*CTS1-2*-*ADH2*$_T$	This study

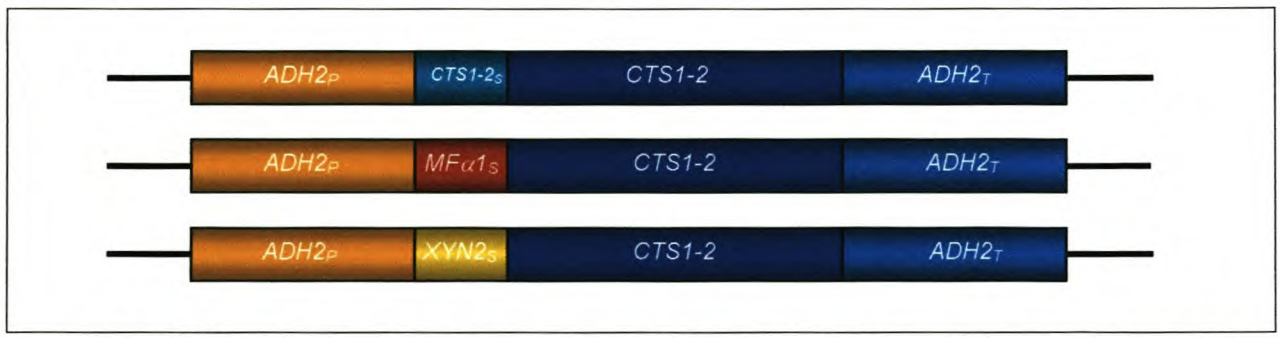

Figure A.1 Schematic representation of the constructs used for yeast transformation in this study. *ADH2*$_P$, alcohol dehydrogenase 2 promoter; *ADH2*$_T$, alcohol dehydrogenase 2 terminator; *CTS1-2*$_S$, native yeast *CTS1-2* gene secretion signal; *MFα1*$_S$, yeast mating pheromone α-factor; *XYN2*$_S$, the leader sequence of the *Thrichoderma reesei* β-xylanase 2 gene; *CTS1*-2, yeast chitinase gene. The backbone plasmid for these constructs is pDLG1 (La Grange *et al.*, 1996).

A.4 Yeast transformation

S. cerevisiae laboratory strain PRY488, with its natural chitinase activity disrupted, was transformed with episomally-derived plasmid DNA by the lithium acetate dimethylsulphoxide (DMSO) method described by Hill *et al.* (1991). The plasmids pDLG:CTS, pDLG:MFα-CTS and pDLG:XYN-CTS are multi-copy *E. coli-S. cerevisiae* shuttle vectors and were maintained as autonomous circular minichromosomes in the recombinant yeast cells.

A.5 RNA isolation and Northern blot hybridisation

Yeast cells transformed with these plasmids were grown in $SCDL^{-Ura}$ to eliminate glucose repression of the promoter. The cells were cultured overnight to an OD_{600} of 1 at 30°C. Total RNA was isolated from the 5 ml cultures as described by Ausubel *et al.* (1987). RNA (10 μg) from each culture was subjected to 1.2% (w/v) formaldehyde gel electrophoresis together with an RNA ladder (Life Technologies (GIBCO/BRL)) and blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) according to standard procedures (Sambrook *et al.*, 1989). The *CTS1-2* gene (~1.69 kb) was PCR-labelled as probe DNA, using the 10x DIG dNTP labelling mixture (Roche Diagnostics) according to the manufacturer's specifications. Hybridisations were performed at 50°C for 16 h using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications.

The presence of the *CTS1-2* transcript in the recombinant *S. cerevisiae* strains was revealed by Northern blot analysis (Figure A.2). The detected signals corresponded to the expected sizes when compared to the RNA ladder of Life Technologies (GIBCO/BRL). No chitinase transcripts were detected in the RNA isolated from the yeast strain or a recombinant strain transformed with the backbone of the yeast expression vector, pDLG1. Transcription from the *ADH2* is almost undetectable when *S. cerevisiae* is grown on fermentable sugars such as glucose, but it is derepressed to a level representing about 1% of the total soluble cellular protein when the yeast is grown on fermentable sugars at concentrations of less than 1% or on nonfermentable carbon sources (Romanos *et al.*, 1992).

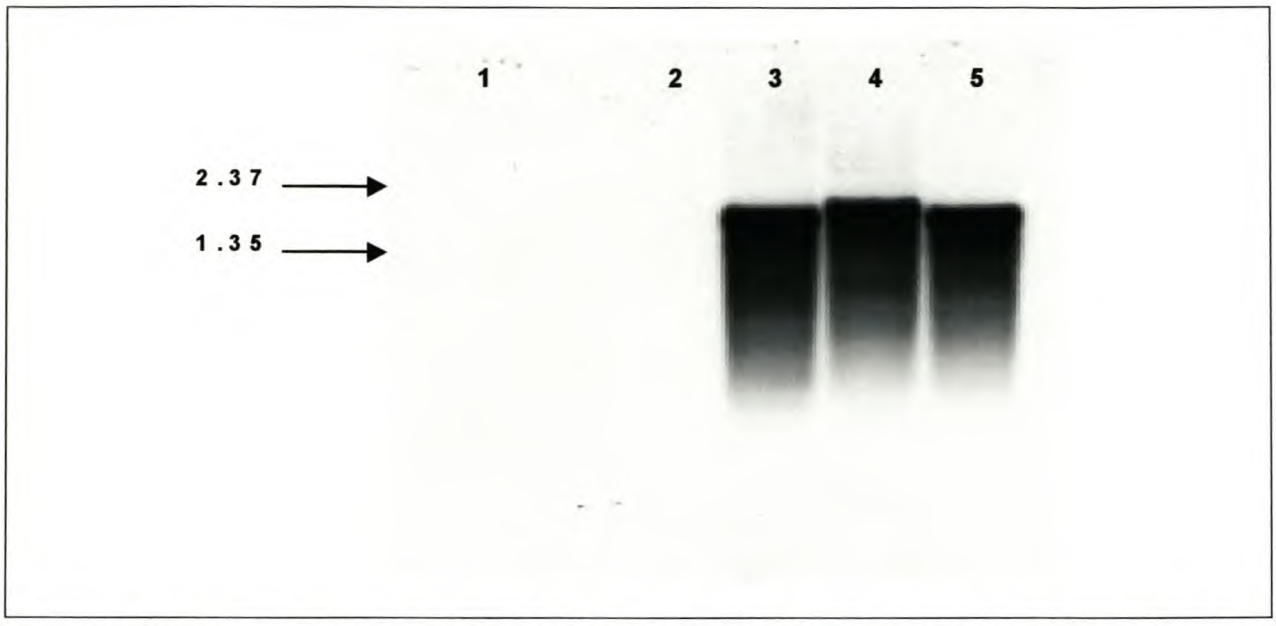

Figure A.2 Northern blot analysis of the total RNA isolated from *S. cerevisiae* PRY488 (control, lane 1) and *S. cerevisiae* PRY488 transformed with pDLG1 (control, lane 2), pDLG:CTS containing the *CTS1–2* gene fused to its native secretion signal (lane 3), pDLG:MFα–CTS containing the *CTS1–2* gene fused to the *MFα1* secretion signal (lane 4), pDLG:XYN–CTS containing the *CTS1–2* gene fused to the *XYN2* secretion signal (lane 5). All the pDLG-constructs were under the control of the *ADH2* promoter and terminator. Molecular size standards are indicated in kilobases and the *CTS1–2* gene (~1.69 kb) was used as a DIG-labelled probe.

A.6 Endochitinase activity assays

The endochitinase activity assay was adapted from Kuranda and Robbins (1991). The constructs under the control of the $ADH2_{PT}$ were grown in $SCDL^{-Ura}$ as selective media or in YPLD during the assays. Six individual transformants of each construct were assayed in duplicate. The untransformed *S. cerevisiae* strain, DBY918, was employed as a positive control.

The recombinant yeasts were grown overnight in 5 ml of selective media, whereas DBY918 was grown overnight in 5 ml of YPD, overnight as starter cultures. Cells from the overnight cultures were centrifuged, washed with fresh YPLD medium, and then diluted to a final OD_{660} of 0.25 in 20 ml of YPLD. The cultures were incubated at 30°C with agitation. The activity assays were conducted every 2.5 h, while also monitoring the growth of the culture by determining the OD_{660} values. The cells (1 ml) were removed and centrifuged to yield the supernatant, which was assayed as the extracellular fraction. The cell pellet was washed with 1 ml dH_2O and resuspended in 400 µl dH_20. Glass beads (0.45-0.55 mm diameter, Sigma) were added to a level of approximately 200 µl and the suspension was incubated on ice for 5 min. The cells were mixed vigorously for 2 min, incubated on ice for 5 min and vigorously vortexed for another minute. The broken cell slurry was recovered to be assayed as the combined periplasmic and intracellular fraction (designated cell-associated fraction).

A sample (200 µl) of either the supernatant or the broken cell slurry was mixed with 40 µl of 41.67 µM 4-methylumbelliferyl β-D-*N*,*N'*,*N''*-triacetylchitotrioside (Sigma) in a final volume of 240 µl and incubated for 1 h at 30°C. A 0.48 M sodium citrate buffer, pH 3.0, was used to make up the substrate stock. The reaction was stopped by the addition of 0.42 M glycine, NaOH buffer, pH 10.4 in a final volume of 1.5 ml. Cell debris was removed by centrifugation, and the liberated 4-methylumbelliferone (4-MU) was measured with a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.) at an excitation wavelength of 350 nm and an emission wavelength of 440 nm. Units of activity were defined as nanomoles of 4-MU released per h.

The results obtained in the endochitinase activity assays of the pDLG, pDLG:CTS, pDLG:MFα-CTS and pDLG:XYN-CTS constructs are extremely puzzling. During these assays, the same substrate, buffers and conditions were applied as in the assays used for the pJC-H constructs. The only varying factor was the media used, differing only in the amount of glucose present, *i.e.* 0.8% glucose versus 2% glucose. Low levels of chitinase activity were found in the intracellular fractions of the constructs, as well as in the positive assay control (results not shown), which was indicative of a working substrate and assay method. In opposition, no chitinase activity could be established for the constructs or the positive control in the extracellular fractions utilising the same substrate, buffers and conditions as the intracellular fractions (results not shown). Although the buffers, substrate and media were varied, activity still could not be determined (results not shown). The assays, on both the positive controls as well as on these constructs, were attempted seven times.

The transcription of these fusion proteins was successful, as is apparent in Figure A.2. However, no significant conclusions can be drawn from these assay results. An alternative assay method should be employed to determine if the high-level expression of a biologically active endochitinase enzyme and the secretion thereof actually do occur.

In conclusion, the comparison between the *PGK1* and *ADH2* promoters to determine which delivered higher expression levels of the chitinase enzyme was unsuccessful.

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CHAPTER 4

The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, confers antifungal activity to transgenic tobacco

To be submitted for publication in *Plant Physiology*

RESEARCH RESULTS

The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, confers antifungal activity to transgenic tobacco

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A critical need exists for the development of effective, safer alternative methods for disease control in crop plants. Plants defend themselves against pathogens by a combination of pre-existing and inducible defence strategies. Upon interaction with the pathogen, plants initiate these defence strategies, one of which includes a dramatic increase of chitinase activity. Chitinases catalyse the hydrolysis of chitin, a substance absent from plants, but a major component of the cell walls of most fungi. Chitinases have been shown to be part of the group of pathogenesis-related (PR) proteins which are actively involved in plant defence mechanisms. The *Saccharomyces cerevisiae* chitinase gene (*CTS1-2*) was constitutively overexpressed in tobacco plants to assess its potential to increase the plant's defence against fungal pathogens. The transgenic tobacco plants, generated by *Agrobacterium*-mediated transformation, showed stable integration and inheritance of the transgenes. Northern blot analyses conducted on the transgenic tobacco plants confirmed transgene expression. Leaf extracts from the transgenic lines inhibited *Botrytis cinerea* spore germination and hyphal growth by up to 70% in a quantitative *in vitro* assay. Several of the F_1 progeny lines were challenged with the fungal pathogen, *B. cinerea*, in a detached leaf infection assay, showing disease resistance from 50 to 70%. The plant lines that showed increased disease tolerance were also shown to have higher chitinase activities.

4.1. INTRODUCTION

Plants have evolved a battery of defence systems to protect themselves against constant biotic (e.g. pathogen attack) and abiotic stresses (e.g. UV light, mechanical wounding). One of these defence strategies includes the synthesis of an array of proteins whose apparent function is to restrict the growth of an invading pathogen (Bowles, 1990). These proteins include pathogenesis-related (PR) proteins, such as chitinases, which are expressed constitutively in tissues vulnerable to pathogen attack (Bishop *et al.*, 2000). When an attack occurs, they are highly expressed in and around the infected cells.

Chitinases (EC 3.2.1.14), which are present in all plant species, hydrolyse the β-1,4 linkages of the *N*-acetylglucosamine (GlcNAc) homopolymer of chitin, which is a major structural component in the cell walls of most filamentous fungi, but is, however, absent

from plant cells (Cabib, 1987; Gooday, 1990). Chitinases are also found in a variety of other organisms, including most fungi, yeasts and some prokaryotes (Cohen, 1993). Due to their hydrolytic ability, chitinases are able to inhibit the growth of fungal pathogens by inhibiting spore germination and germ-tube elongation, degrading the cell walls, as well as inhibiting growth at the hyphal tips (Chet and Inbar, 1994; Haran *et al.*, 1996; Chet and Inbar, 1997). In addition to this direct action, chitinolytic breakdown products induce the production of defence compounds, such as phytoalexins, and systemic acquired resistance by acting as elicitors (Ren and West, 1992; Bishop *et al.*, 2000). It has been found that some plant chitinases may also display varying levels of lysozyme (EC 3.2.1.17) activity and thus may be involved in conferring resistance to bacterial pathogens (Jollès and Jollès, 1984).

The antifungal activities of chitinases render these proteins ideal candidates for the enhanced natural resistance of plants through constitutive overexpression of these normally inducible defence genes. Successes in this regard were first reported by Broglie *et al.* (1991) who generated transgenic tobacco and canola plants utilising the cauliflower mosaic virus (CaMV) 35S promoter and the bean *CH5B* gene. Chitinase activity in the transformants increased 44-fold in the leaves, relative to the control plants. The transformants showed delayed development of disease symptoms, as well as an improved ability to survive in soil infected with *Rhizoctonia solani*. Other studies have also revealed that transgenic plants expressing chitinase genes showed enhanced resistance to fungal pathogens (Zhu *et al.*, 1994; Lin *et al.*, 1995; Terakawa *et al.*, 1997; Lorito *et al.*, 1998; Datta *et al.*, 2001).

In this study, we evaluated a yeast chitinase gene for its potential to enhance the plants' endogenous resistance against fungal pathogens. To this end, transgenic tobacco plants were developed that constitutively overexpress the *S. cerevisiae* chitinase gene (*CTS1-2*) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The *in planta* enzyme activity and antifungal effect of the *CTS1-2* gene against *B. cinerea* were investigated and it was confirmed that the yeast chitinase has strong antifungal activity against *B. cinerea* in the transgenic F_1 progeny plants.

4.2. MATERIALS AND METHODS

4.2.1. Microbial strains and culture conditions

The sources and relevant genotypes of microbial strains, as well as the plasmids used in this study, are listed in Table 1. *Escherichia coli* transformants were grown at 37°C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), or in LB medium supplemented with either ampicillin (100 µg/ml) or kanamycin (20 µg/ml), for the selection of transformants. *Agrobacterium tumefaciens* strains were cultured routinely at 28°C in YEP media (containing 1% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) sodium chloride). Solid media contained 2% (w/v) agar. *B. cinerea* spores were obtained from the

Department of Plant Pathology, Stellenbosch University. Fungal cultures were obtained by germinating spores on apricot halves. Before inoculation, the apricot halves, canned in natural juice, were rinsed with sterile dH_2O and blotted dry on sterile filter paper. Each apricot half was placed in a tissue culture petri dish and was subsequently inoculated with a few spores of *B. cinerea*. The plates were incubated in the dark at 25°C until sporulation occurred.

Table 1. Strains and plasmids used in this study

Strain or plasmid(s)	Relevant feature or insert	Source or reference
***E. coli* strains**		
DH5α	supE44 *lac*U169[*ϕ80lac*ZM15) *hsd*R17*rec*A1*gyr* A96*thi*-1*rel* A1]	Life technologies (GIBCO/BRL)
Helper plasmid strain	*E. coli* C199 containing helper plasmid pRK2013	Goldberg and Ohman, 1984
***A. tumefaciens* strains**		
EHA105	Disarmed, succinomopine-type strain	Hood *et al.*, 1993
Plasmids		
pGEM T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega
pART7	Cloning vector	Gleave, 1992
pART27	Binary plant expression vector, kanamycin-resistant marker	Gleave, 1992
pGEM:CTS	1.69 kb *CTS1-2* PCR product in pGEM T-Easy	This study
pART7:CTS	1.7 kb PCR fragment in pART7	This study
pART27:CTS	3.9 kb *Not*I cassette in pART27	This study
pART27:MJ-CTS	4.0 kb *Not*I cassette in pART27	This study, see Addendum B

4.2.2. DNA manipulations

Standard techniques for DNA cloning were performed according to Sambrook *et al.* (1989). Restriction enzymes, Expand polymerase and T4 DNA ligase were purchased from Roche Diagnostics. Shrimp alkaline phosphatase (SAP) was obtained from Amersham Life Sciences. Sequencing was performed by the DNA Sequencing facility, Department of Genetics, Stellenbosch University, using an ABI PRISM™ 377 automated DNA sequencer from PE Biosystems.

PCR amplifications were done using Expand polymerase. PCR reactions were performed in 50 µl reaction mixtures, typically consisting of 1x Expand Polymerase PCR buffer (without $MgCl_2$), 200 µM dNTPs, 200 nM of each primer, 5 ng template DNA and $MgCl_2$ added to the optimal concentration. Typical amplification conditions included an initial DNA denaturation step at 95°C for 2 minutes, followed by cycles of denaturation at 95°C for 10 seconds, primer annealing according to the specific primer melting temperatures, and elongation at 72°C, allowing 1 minute per 1 kb amplified. Reactions proceeded for 30 cycles in a Biometra Trio-thermoblock cycler.

4.2.3. Plasmid construction

The *CTS1-2* gene was PCR amplified from *S. cerevisiae* strain DBY918 genomic DNA, using primers CTS1-2 (L) (5'-GATCGGATCCGAATGTCACTCCTTTACATC-3') and CHIT3' (5'-ACGTAAGCTTCGGACTCTATGAATCAATCT-3'), and sub-cloned into the pGEM T-Easy vector system (Promega), producing pGEM:CTS. The ~1.7 kb fragment was excised with *Eco*RI and cloned into the corresponding site of pART7, generating pART7:CTS (Gleave, 1992). After the correct orientation was confirmed by digestion with *Not*I and *Pst*I, a 3.9 kb *Not*I *CTS1-2* cassette under control of the cauliflower mosaic virus (CaMV) 35S promoter and *Agrobacterium* octopine synthase 3' terminator was in turn isolated from this plasmid. This cassette was cloned into the corresponding SAP-treated *Not*I site of pART27 (Gleave, 1992), yielding the clone pART27:CTS (Fig. 1). A second clone, pART27:MJ-CTS, was also constructed (see addendum B). The integrity of all clones was confirmed by sequence analysis.

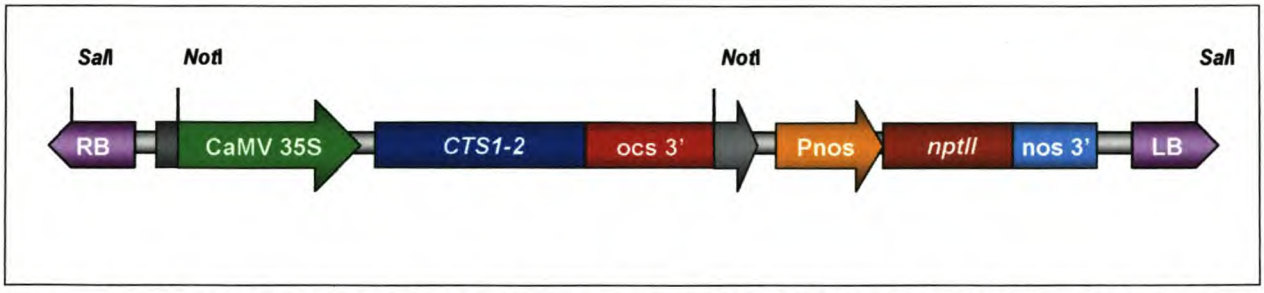


Figure 1. Schematic representation of the pART27:CTS construct containing the 3.9 kb *CTS1-2* cassette used for plant transformation in this study. RB, right border of T-DNA; CaMV 35S, cauliflower mosaic virus promoter; *CTS1-2*, yeast chitinase gene; ocs 3', octopine synthase 3' terminator; Pnos, nopaline synthase promoter; *nptII*, neomycin phosphotransferase (kanamycin resistance gene); nos 3', nopaline synthase terminator; LB, left border of T-DNA.

4.2.4. Transformation and regeneration of tobacco plants

The plasmid pART27:CTS was mobilised from *E. coli* DH5α to *A. tumefaciens* strain EHA105 by triparental mating (Goldberg and Ohman, 1984). *A. tumefaciens* harbouring the relevant plasmid was used to infect leaf discs of *N. tabacum* cv. SR-1, essentially as described by Horsch *et al.* (1985). Briefly, *A. tumefaciens* was cultured overnight at 28°C to an OD_{600} of 0.8 in selective media. The cells were spun down (7000 rpm for 5 min.) and redissolved in media without any antibiotics. Approximately 40 leaf discs from *in vitro* tobacco plants were suspended in the bacterial culture for 20 min., blotted slightly on sterile Whatman filter paper (Whatman International Ltd.) and placed on co-cultivation media (regeneration medium without cefotaxime or antibiotics, Table 2) for 3 days in the dark at 22°C. The leaf discs were subsequently washed with dH_2O containing cefotaxime (400 mg/l) and then transferred to selective medium (regeneration medium, Table 2) and incubated at 28°C with 16-h day and 8-h dark cycles. After three weeks, the kanamycin-resistant tobacco shoots that developed were transferred to

selective root inducing media (rooting medium, Table 2). When sufficient root formation had occurred, the plantlets were subcultured to maintenance medium (Table 2) at 28°C under a 16-h day and 8-h night regime.

Table 2. Media used for the cultivation of plant material

Organic constituent	Regeneration	Rooting	Maintenance
Difco-agar (g/l)	8	8	8
myo-inositol (mg/l)	100	100	100
Nicotinic acid (mg/l)	1	1	1
Pyridoxin HCl (mg/l)	1	1	1
Thiamin HCl (mg/l)	10	10	10
6-benzylaminopurine (μM)	10	0	0
β-naphtoxyacetic acid (μM)	0	0.5	0
Sucrose (g/l)	30	15	15
Cefotaxime (mg/l)	400	400	0
Kanamycin (mg/l)	120	120	0
pH (with 1 M KOH)	5.8	5.8	5.8

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

Each plant line was subcultured into three *in vitro* copies. One of these copies was hardened off, ultimately setting seed through self–pollination. These seeds were germinated *in vitro* under selection to yield the F_1 progeny. The F_1 progeny plants were also subcultured into three copies, one of which was hardened off. The hardened–off tobacco plants were grown in chambers maintained at 25°C under a 16–h day and 8–h night regime.

4.2.5. Southern blot analysis of transgenic tobacco lines

Total DNA from the *in vitro* leaf tissue was isolated according to McGarvey and Kaper (1991). The amount of leaf material was increased to 40-50 mg and the necessary volume adjustments were made. An extraction buffer, consisting of 3% (w/v) cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylene-diamine tetra-acetic acid (EDTA) and 1 M Tris-HCl (pH 8), was used for the extraction. The DNA samples were digested with 10 units of *Hpa*I, subjected to 0.8% (w/v) agarose gel electrophoresis and blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech), as described by Sambrook *et al.* (1989). Lambda DNA digested with *Bst*EII was used as a molecular size marker. The *CTS1-2* gene (~1.69 kb) was PCR-labelled as probe, using the 10x DIG dNTP labelling mixture (Roche Diagnostics), according to the manufacturer's specifications. Hybridisations were performed at 42°C for 16 h, using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications.

4.2.6. RNA isolations and Northern blot hybridisation

Total RNA was isolated from 80-100 mg of *in vitro* leaf tissue, using the TRIzol Reagent according to the manufacturer's specifications (Life Technologies (GIBCO/BRL)). Total RNA isolated from 80-100 mg of leaf tissue was subjected to 1.2% (w/v) formaldehyde gel electrophoresis, together with an RNA ladder (Life Technologies (GIBCO/BRL)), and blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) according to standard procedure (Sambrook *et al.*, 1989). The DIG-labelled probe utilised for Southern blot analysis was used for the hybridisation to the RNA at 50°C for 16 h using the standard DIG hybridisation buffer (Roche Diagnostics) containing 50% (w/v) formamide. Signal detection proceeded according to the manufacturer's specifications.

4.2.7. Preparation of crude protein extracts from transgenic tobacco lines

Total proteins were extracted from 3 g of leaf tissue from hardened–off F_1 progeny transgenic plants. The extraction buffer consisted of 1 M NaCl, 0.1 M sodium acetate (pH 6.0), 1% polyvinylpyrrolidone and 10 mM β-mercaptoethanol. The proteins were extracted overnight at 4°C with steady agitation. The samples were centrifuged to remove insoluble debris and the supernatant was dialysed overnight against dH_2O, utilising a Spectra/Por membrane 4 (Spectrum). The crude protein extracts were subjected to freeze-drying, and subsequently dissolved in 25 mM sodium citrate (pH 5.0) buffer for the antifungal activity assay, or dH_2O for the endochitinase activity assay.

4.2.8. Endochitinase activity assays

The chitinase activities of the transgenic lines were determined using the method of Kuranda and Robbins (1991). Specifically, 200 µl of the crude protein samples (5 mg/ml), dissolved in dH_2O, were mixed with 41.67 µM 4-methylumbelliferyl β-D-*N,N',N''*-triacetylchitotrioside (Sigma) in a final volume of 240 µl and incubated for 1 h at 30°C. A 0.48 M sodium citrate buffer, pH 3.0, was used to make up the substrate stock. The reaction was stopped by the addition of 0.42 M glycine, NaOH buffer, pH 10.4 in a final volume of 1.5 ml. A time zero reading was used to normalise the values obtained from the leaf extracts. The liberated 4-methylumbelliferone (4-MU) was measured with a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.) at an excitation wavelength of 350 nm and an emission wavelength of 440 nm. Units of activity were defined as nanomoles of 4-MU released per hour per mg of total protein.

4.2.9. Antifungal inhibition activity assays on crude protein extracts

A microtiter plate inhibition assay was adapted from Ludwig and Boller (1990) and was used to quantitatively determine the antifungal effect of the crude proteins in the leaf extracts from the transgenic lines on *B. cinerea* spore germination and fungal growth. The assays were performed in 96–well microtiter plates (Nunc) in a final volume of

100 µl. The crude protein samples (5 mg/ml) constituted 50 µl and was added to 50 µl of Potato Dextrose Broth (PDB) (containing 20% (w/v) infusion from potato, 2% (w/v) dextrose) and *Botrytis* spores to a concentration of 4×10^4 spores per ml. As a background control, 50 µl of the 25 mM sodium citrate (pH 5.0) buffer was added to 50 µl of spore-containing PDB. The plates were incubated at 25°C for two days. The OD_{595} of the samples was determined every 24 hours with the PowerWaveX Microplate Scanning Spectrophotometer (Bio-Tek Instruments, Inc.), starting at 0 hours. The time zero values were used to normalise the 24- and 48–h values. These values are referred to as corrected A_{595} values. Percentage inhibition is defined as 100× the ratio of the corrected A_{595} of the control plant minus the corrected A_{595} of the sample over the corrected A_{595} of the control plant.

4.2.10. Fungal inhibition studies on detached leaves

B. cinerea spores were harvested from sporulating cultures and suspended in sterile grape juice at a density of 2.5×10^6 spores per ml. The leaves of one month-old F_1 generation hardened–off plants were inserted into Magenta containers with 0.8% (w/v) water agar for a detached leaf infection study. Three leaves per plant from 14 plants were inoculated. Two aliquots of 2 µl of the spore suspension were dropped on the upper surface of a leaf. The spore viability was tested by plating 5×10^3 spores onto water agar plates. These leaves were maintained at 22°C and 100% humidity for 3 weeks. The disease lesions were scored after 72 h and 168 h (7 days) according to a 10–point lesion index scale that was developed. Lesions were photographed after 72 h and again after three weeks. The lesions were also measured after 72 h and 168 h and compared to lesions on inoculated untransformed tobacco leaves. These lesion sizes were used to calculate the percentage disease resistance of the transgenic lines.

4.3. RESULTS

4.3.1. Transformation of tobacco with a yeast chitinase gene

A plant expression vector, pART27:CTS, containing the *S. cerevisiae* chitinase gene (*CTS1-2)* (Fig. 1), was introduced into *N. tabacum* via *Agrobacterium* transformation. Thirty Km^R primary transformants (F_0) were generated from approximately 40 independent inoculated leaf discs. After a two-month *in vitro* growth period, the regenerated plants exhibited normal morphology and were hardened off. These plants showed healthy growth, flowered and set seed through self-pollination. These seeds were germinated under selection to yield F_1 progeny plants.

4.3.2. Integration and expression of the *CTS1-2* gene in primary (F_0) and F_1 plant lines

Southern blot analysis of genomic DNA from an untransformed control and the various transgenic lines confirmed the integration of the yeast chitinase gene in the F_0 and F_1 progeny (Figs. 2 and 3 respectively). The DNA was digested with an enzyme that cuts once in the middle of the gene to determine the copy number of the transgene. Therefore, two signal bands are indicative of one copy of the transgene. The Southern blot analysis indicated that the copy number of the transgene varied from one to two, with the majority of transgenic lines having two copies.

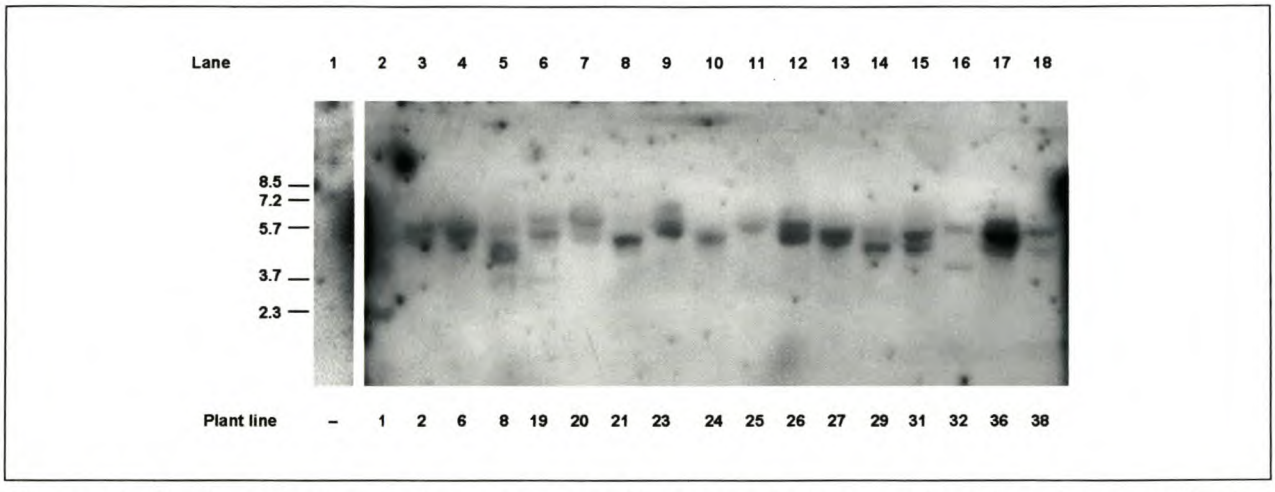


Figure 2. Southern blot analysis on genomic DNA from F_0 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. DNA was digested with *Hpa*I and probed with the ~1.69 kb *CTS1-2* gene. Lane 1, untransformed plant DNA (negative control); lanes 2-18, digested DNA from transgenic lines. Molecular size standards are indicated in kilobases.

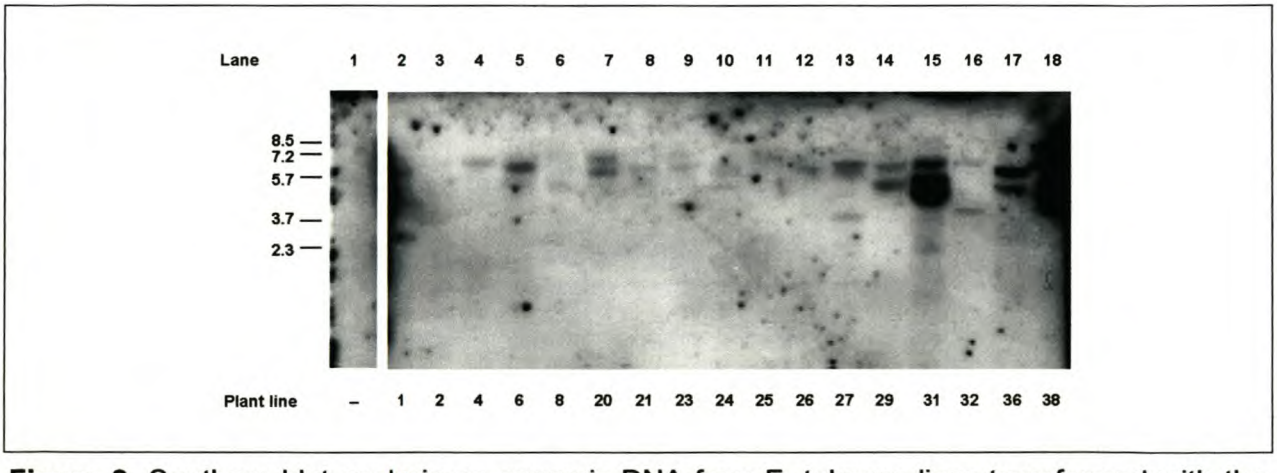


Figure 3. Southern blot analysis on genomic DNA from F_1 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. DNA was digested with *Hpa*I and probed with the ~1.69 kb *CTS1-2* gene. Lane 1, untransformed plant DNA (negative control); lanes 2-18, digested DNA from transgenic lines. Molecular size standards are indicated in kilobases.

Northern blot analysis of the total RNA isolated from an untransformed control and the various transgenic lines confirmed the expression of the yeast chitinase gene in the F_0 and F_1 progeny (Figs. 4 and 5, respectively). Hybridisation signals of the expected sizes were detected for most of the transgenic lines, whereas no signal was observed for the RNA of the untransformed plant lines.

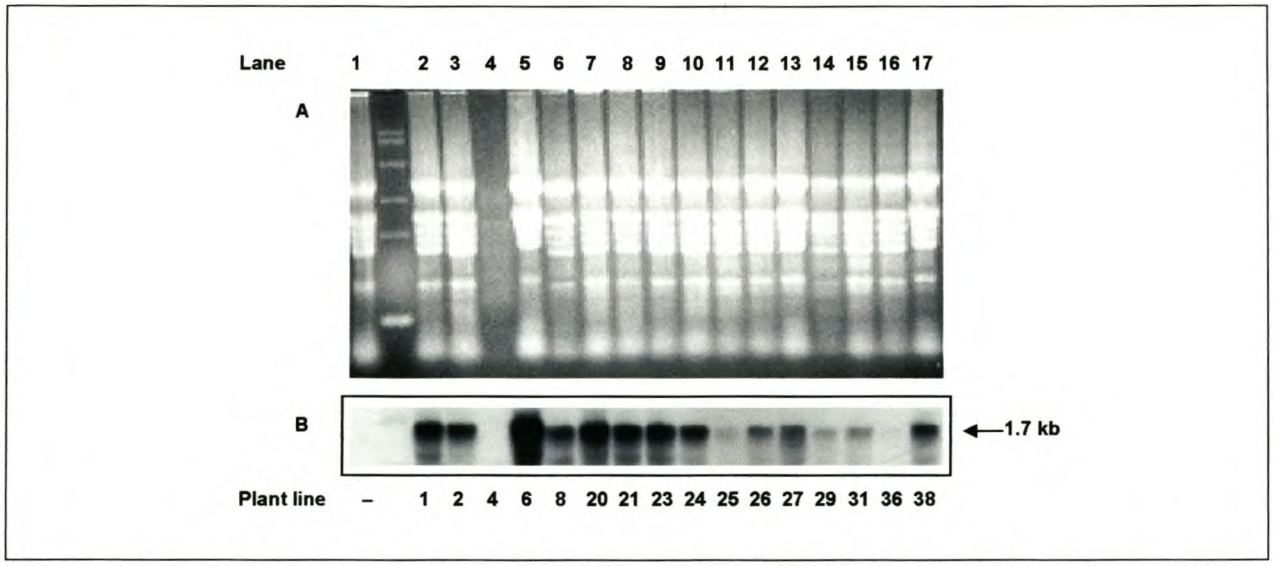


Figure 4. Total RNA separated on a formamide gel (**A**) and Northern blot analysis (**B**) on total RNA from F_0 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. Lane 1, RNA from untransformed plant (negative control); lanes 2-17, RNA from the various transgenic lines. Detection in the Northern blot was with a DIG-labelled *CTS1-2* gene probe.

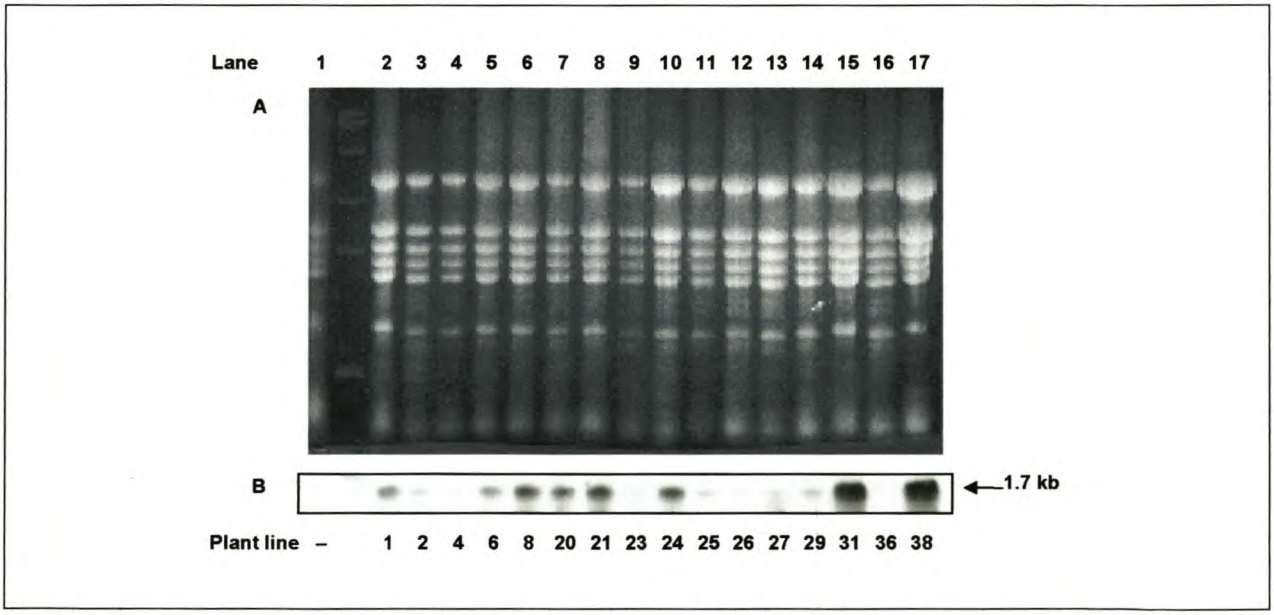


Figure 5. Total RNA separated on a formamide gel (**A**) and Northern blot analysis (**B**) on total RNA from F_1 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. Lane 1, RNA from untransformed plant (negative control); lanes 2-17, RNA from the various transgenic lines. Detection in the Northern blot was with a DIG-labelled *CTS1-2* gene probe.

4.3.3. Endochitinase activity assays

Endochitinase activity assays performed on a few of the F_1 progeny lines were used to establish the functionality as well as the levels of activity of the recombinant yeast chitinase produced (Fig. 6). These assays confirmed that the yeast chitinase maintained functionality in the foreign plant environment, with its activity increasing two- to seven-fold when compared with the untransformed control.

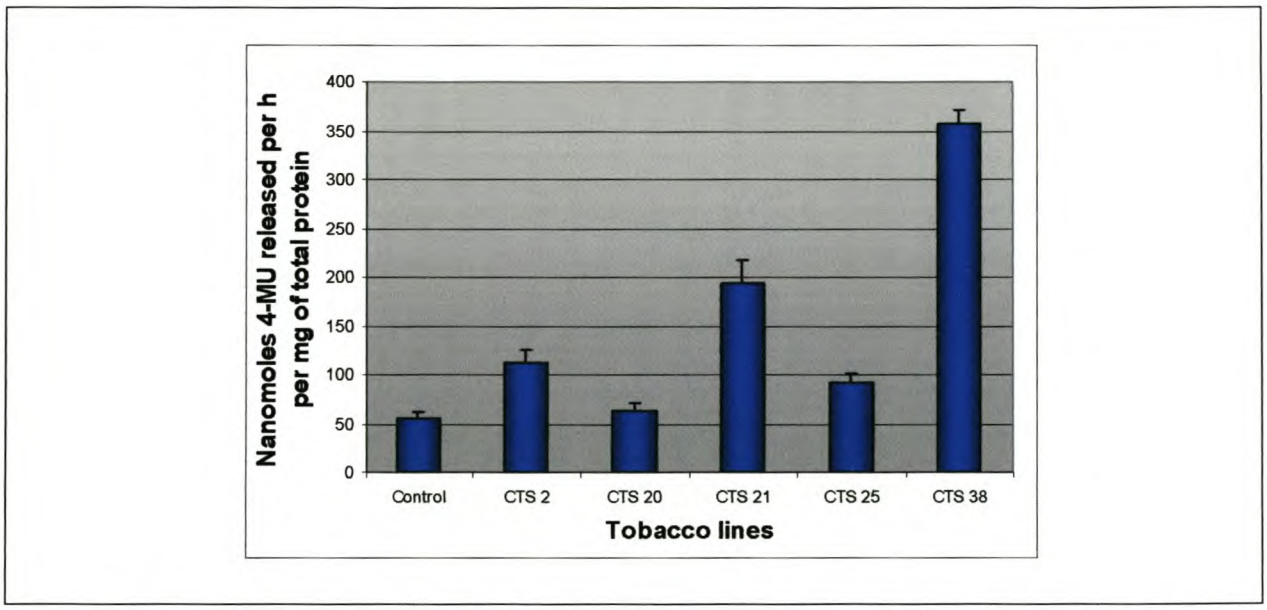


Figure 6. Endochitinase activity in leaf extracts of F_1 progeny plant lines expressing the yeast *CTS1-2* gene. Units of activity are defined as nanomoles of 4-methylumbelliferone (4-MU) released per hour per mg of total protein. Error bars represent the standard deviation of six replicate assays.

4.3.4. Antifungal Inhibition Activity Assays on Crude Protein Extracts

Microtiter plate assays to quantitatively establish the fungal inhibition of the yeast chitinase present in the leaf extracts of the transgenic lines were conducted on crude protein extracts from the various lines that showed active transcription of *CTS1-2* (Fig. 7). Seven of the lines had fairly low percentages of growth inhibition (17-23%) against *B. cinerea*, whereas six lines exhibited growth inhibition from 30 to 45%. One line (line 38) approached 70% growth inhibition of *Botrytis* under the conditions tested (Fig. 7).

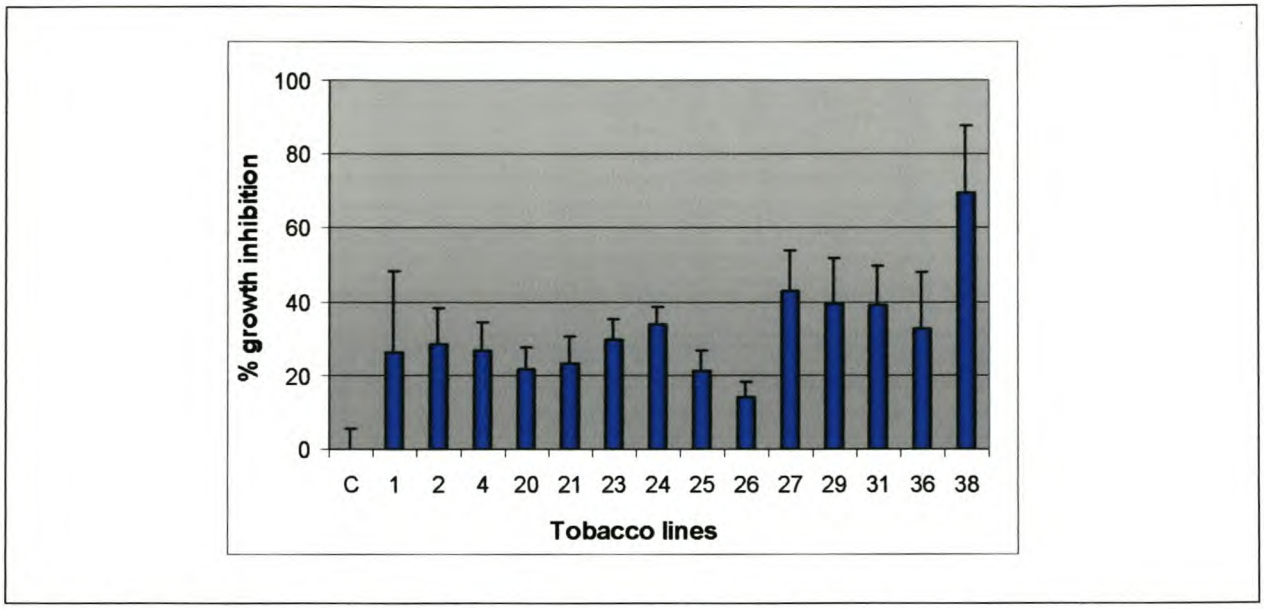


Figure 7. The effect of the yeast *CTS1-2* chitinase protein in leaf extracts from F_1 progeny transgenic plants on growth and spore germination of *Botrytis cinerea* after 48 h of incubation. Percentage inhibition is defined as 100× the ratio of the corrected A_{595} of the control plant minus the corrected A_{595} of the sample over the corrected A_{595} of the control plant.

4.3.5. Fungal inhibition studies on detached leaves

In order to describe the lesions found on the control and transgenic plants following fungal infection in a detached leaf assay, the different types of lesion were photographed and described. A ten-point lesion index scale was developed to categorise the lesions (Fig. 8). The detached leaves were inoculated with high spore concentrations and incubated under conditions favourable for disease development. The spore viability of the *Botrytis* spore suspension used was in excess of 99%.

The tissue damage caused by *B. cinerea* on the untransformed control tobacco leaves was severe and actively-spreading lesions developed (type 10 lesions) (Fig. 8). Fourteen transgenic lines expressing the *CTS1-2* were used in this assay, and the results indicated that the transgenic tobacco plants were highly tolerant of this pathogen. All the tobacco lines, except lines 20 and 25, showed at least a two-fold reduction in lesion size when compared to the untransformed control (Fig. 9A). The lesion types of the more tolerant lines were characterised with the index in Fig. 8 and were typically type 2 or 3 lesions (Fig. 10). After 72 h, the control lesions were typically three times the size of the lesions present on most of the transgenic lines. Although these lesions on the transgenic lines usually increased slightly after 168 h of incubation, none reached the same size as the control.

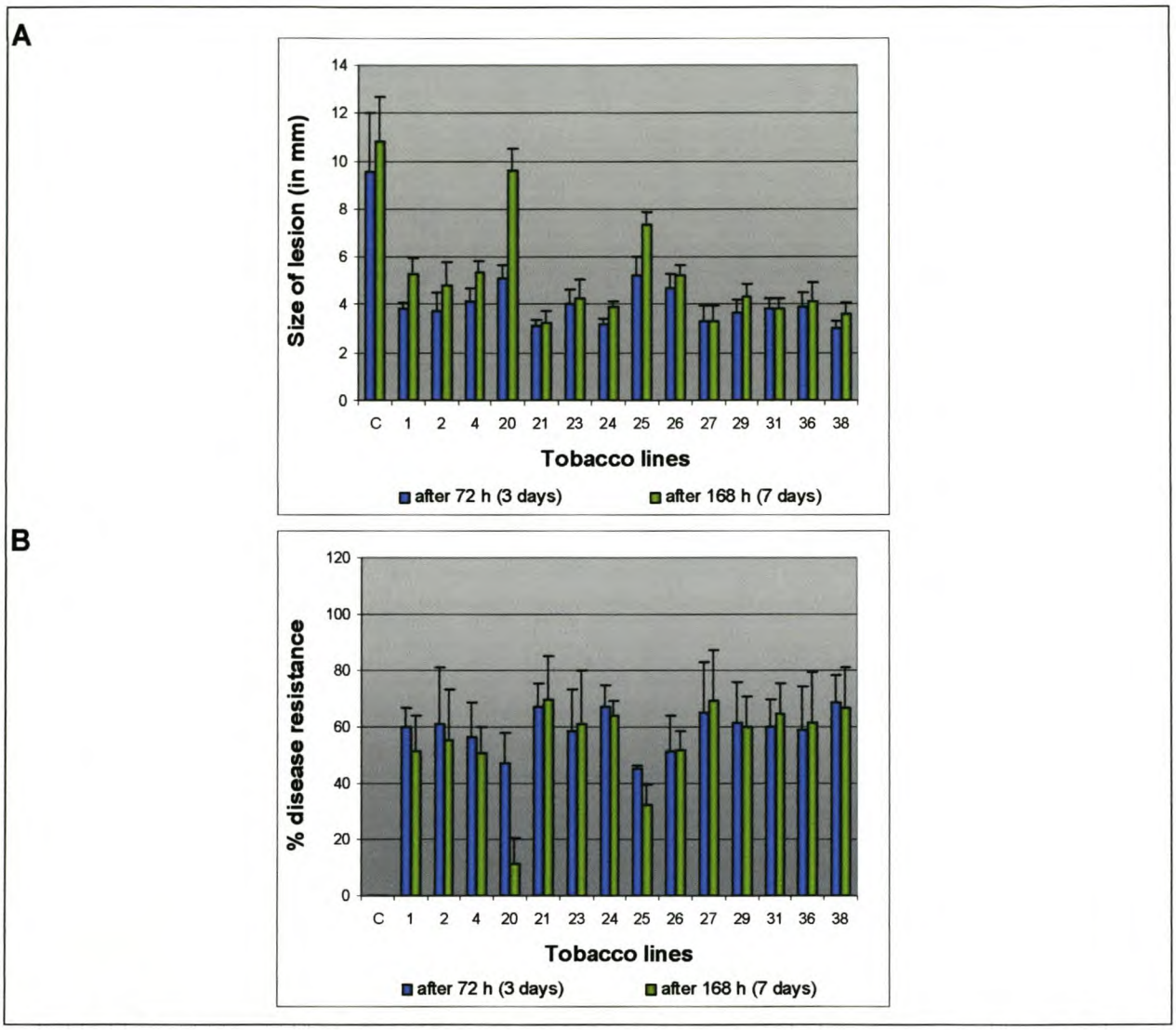


Figure 9. Resistance to *Botrytis cinerea* of detached leaves of F_1 progeny transgenic tobacco lines expressing the yeast *CTS1-2* gene. (**A**) Size of lesions (mm) produced on the leaves of the controls and the different transgenic tobacco lines 72 h (3 days) and 168 h (7 days) after inoculation. Error bars represent standard deviation from six replicate assays. (**B**) Percentage disease resistance of *B. cinerea* of controls and different tobacco transgenic lines 72 h and 168 h after inoculation. It is expressed as a percentage of the disease resistance present on the control plant, which was taken as 0%.

The infection process typically caused lesions at the infection site on all the lines 48 h after inoculation. In the case of the control plant, the lesions expanded rapidly in the following hours, measuring 9.5 mm after 72 h of incubation. The lesions developed into moist, irregular, translucent spots without clearly definable edges. Physical damage also appeared on the leaf tissue and the lesion was described as a type 10 lesion (Fig. 10). On the plant lines that showed good reduction in lesion sizes, the appearance of the lesions started to change after 48-72 h of incubation, typically leading to the formation of dry, necrotic layers that restricted fungal growth and the spread of the lesions.

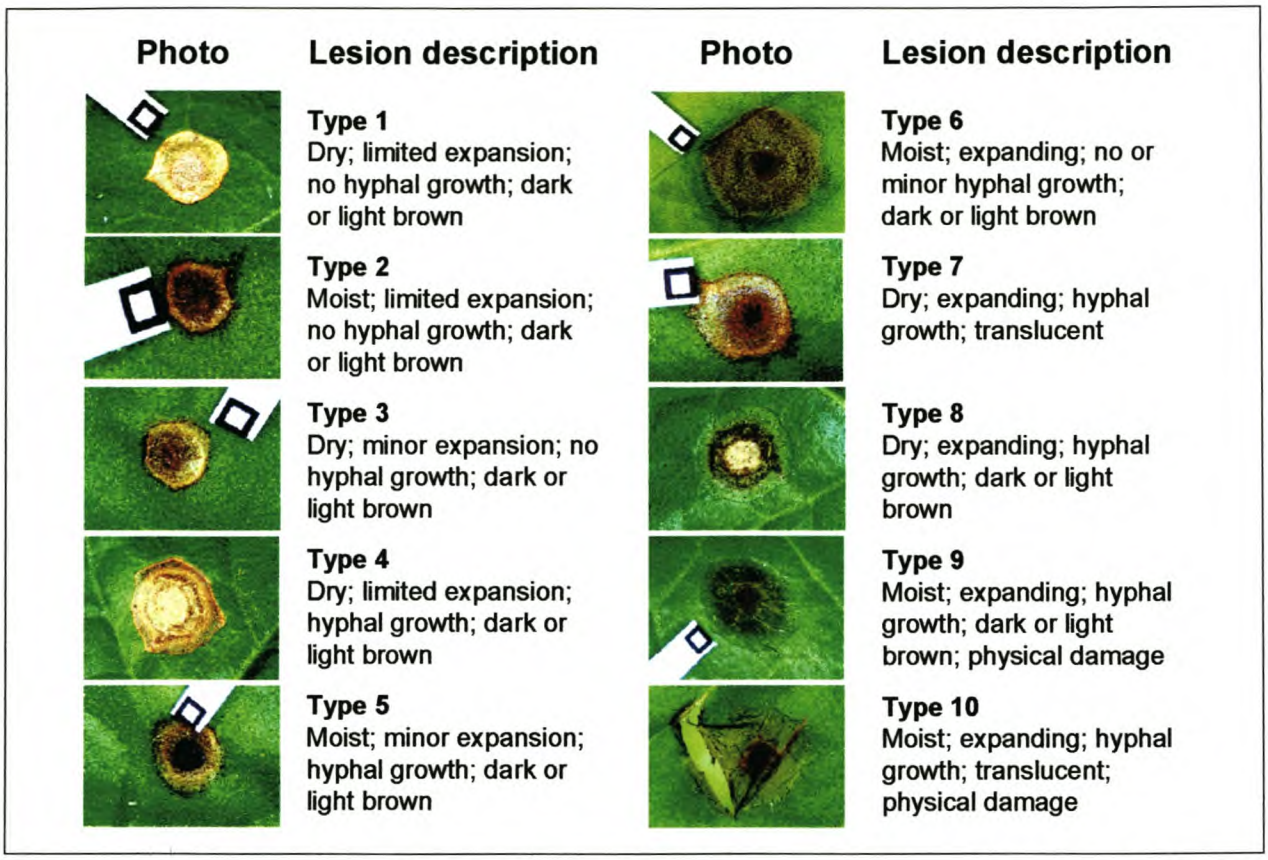


Figure 8. Lesion index scale and lesion descriptions used to define resistance and susceptibility after infection of tobacco with *Botrytis cinerea*. Type 1 lesions indicate the most resistant phenotype, whereas type 10 represents the most susceptible.

The lesion sizes were subsequently normalised against that of the untransformed tobacco lines and expressed as percentage disease resistance (Fig. 9B). The highest percentage disease resistance was observed for lines 21, 24, 27 and 38, which were at 60-70% seven days (168 h) after inoculation (Fig. 9B). It was clear that the levels of resistance of several of the lines (21, 23, 27 and 31) increased from day four to seven post-inoculation, whereas the opposite occurred in lines 1, 2, 4, 20 and 25 (Fig. 9B). Lines 26, 29 and 36 showed unchanged high levels of resistance over the 168 h incubation period. Good correlation with the observed lesion types could be drawn with the deduced disease resistances, *i.e.* lines 21 and 38, which had the highest disease resistances, also exhibited very resistant type 2 lesions (Fig. 10).

After three weeks, all the leaves were inspected for the formation of fungal survival structures. Due to the very high inoculum and extremely favourable conditions, the level of pathogen infection was severe, causing excessive physical damage to the untransformed control leaves (Fig. 10). The pathogen was also able to form a large number of reproductive organs and survival structures. In contrast, the most resistant lines seemed to have curbed the infections completely, since no reproductive organs or survival structures were visible on these leaves. Moreover, the leaves were still green and healthy and showed no signs of further disease development. Tobacco line CTS 20, which exhibited severe type 8 lesions, had the lowest percentage of disease resistance measured. After three weeks, clear survival structures were also present on the leaves on this line (Fig. 10).

The correlations between disease incidence and the severity thereof could be linked to the levels of chitinase activity measured in several of the transgenic lines (Fig. 10).

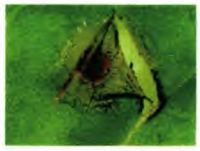
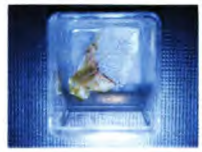
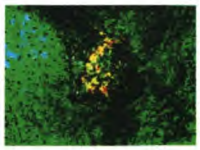
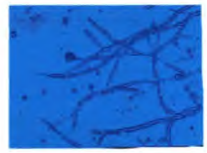
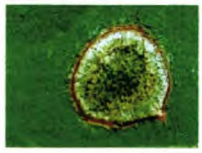
Tobacco line	Photo after 72 h	Lesion size and type	Fungal survival structures	Photo after 3 weeks	% Growth inhibition	*B. cinerea* photo	Chitinase activity
Control		9.5 mm Type 10	Yes		0		55.28
CTS 20		5 mm Type 8	Yes		21		63.35
CTS 21		3 mm Type 3	No	Not determined	41		193.43
CTS 38		3 mm Type 2	No		70		357.39

Figure 10. Fungal infection assays of F_1 progeny transgenic tobacco plants with *Botrytis cinerea*. The lesions were measured, described and photographed 72 h after inoculation. The description of the lesion types is represented in Figure 8. The formation of fungal survival structures was determined and photographed three weeks after inoculation. Units of endochitinase acitivity are defined as nanomoles of 4-methylumbelliferone (4-MU) released per h per mg of total protein.

4.4. DISCUSSION

Transformation of tobacco with a yeast chitinase

Chitinases have been studied well and have been shown to play an integral part in the plant's innate resistance to pathogens (Cohen-Kupiec and Chet, 1998; Strange, 1998). These hydrolytic enzymes form part of the PR proteins in plants and, as such, have been the target of numerous biotechnological projects to evaluate their potential to increase the plant's resistance mechanism through genetic transformation technologies (Zhu *et al.*, 1994; Lin *et al.*, 1995; Terakawa *et al.*, 1997; Lorito *et al.*, 1998; Datta *et al.*, 2001). This study had a similar aim, but tested a previously unexploited chitinase gene for this purpose. Here, the *CTS1-2* gene from *S. cerevisiae* was subcloned into a plant expression vector conferring constitutive expression in all parts of the transformed tobacco plants that were used as transformation hosts.

Evaluation of transgenic tobacco for the presence of active yeast chitinase

Several transgenic lines were obtained that exhibited normal growth, seed formation and ultimately seed germination and that led to an F_1 generation with an indistinguishable phenotype compared to the untransformed control. These lines were confirmed to have integrated the transgene stably into the tobacco genome. Northern blot analysis also confirmed that transcription of the *CTS1-2* gene had occurred efficiently. These transcripts yielded active yeast chitinase proteins that exhibited significant increases in chitinase activity in some of the transgenic lines when compared to the untransformed control lines.

Evaluation of the antifungal activity of the yeast chitinase as a PR protein

The potential value of the heterologously expressed yeast chitinases as PR proteins was determined by *in vitro* as well as *in planta* fungal inhibition studies. The *in vitro* assays relied on the antifungal activity of the chitinase proteins present in leaf extracts of the transgenic lines expressing the yeast chitinase gene. This assay confirmed a strong antifungal activity for these proteins, leading to fungal growth inhibitions of 25-70% in the various transgenic lines. The assays were performed with high spore inoculations and under conditions favouring fungal growth. The good fungal inhibitions observed are therefore all the more promising. The results obtained with the *in planta* infection studies mirrored the tendencies seen in the *in vitro* assay. The detached leaf assay once again favours fungal growth and severe disease development, since high spore concentrations are inoculated onto the leaves in sterile grape juice under conditions of high humidity. The severity of the infections on the untransformed control plants confirmed the favourable infection conditions. Notwithstanding these harsh conditions, several of the transgenic lines exhibited very good percentages of disease resistance, as calculated from normalised lesion size measurements. Most of the

transgenic lines showed disease resistance above 60%, reaching 70% in several lines. These values were obtained after 168 h of incubation post inoculation, but typically led to a complete halt in infection, since, even after three weeks of incubation, the lesions had not increased or spread in the most resistant transgenic lines.

An excellent correlation was found between the levels of chitinase activity in the transgenic lines and the results of the *in vitro* and *in planta* fungal inhibition assays. This confirmed that the good inhibitions that were observed are linked directly to the presence of the heterologously overexpressed yeast chitinase protein. These promising results indicate that the *S. cerevisiae CTS1-2* gene is an excellent candidate to be used for the production of active antifungal proteins in a heterologous plant system. This work and further studies that are underway will hopefully add the yeast chitinase gene to the list of PR chitinases that are useful for genetic manipulation strategies.

4.5. ACKNOWLEDGEMENTS

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Table B.1 Strains and plasmids used in this study

Strain or plasmid(s)	Relevant feature or insert	Source or reference
E. coli strains		
DH5α	supE44 *lac*U169[*ϕ80lac*ZM15) *hsd*R17*rec*A1*gyr* A96*thi*-1*rel* A1]	Life technologies (GIBCO/BRL)
Helper plasmid strain	*E. coli* C199 containing helper plasmid pRK2013	Goldberg and Ohman, 1984
A. tumefaciens strains		
EHA105	Disarmed, succinomopine-type strain	Hood *et al.*, 1993
Plasmids		
pGEM T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega
pUC18	Cloning vector	Life technologies (GIBCO/BRL)
pFAJ3068	Plant expression vector	Prof. W.F. Broekaert
pART7	Cloning vector	Gleave, 1992
pART27	Binary plant expression vector	Gleave, 1992
pGEM-MJ	160 bp PCR product from pFAJ3068	This study
pUC18-MJ	160 bp PCR product from pGEM-MJ	This study
pUC18-MJ-CTS	1.69 kb *CTS1-2* PCR product in pUC18-MJ	This study
pART7:MJ-CTS	1.86 kb fragment from pUC18-MJ-CTS in pART7	This study
pART27:MJ-CTS	4.0 kb *Not*I cassette in pART27	This study

B.4 Plasmid construction

The signal peptide, consisting of a fusion between the tobacco mosaic virus (TMV) 5' leader sequence and the *Mj*-AMP2 signal peptide, was PCR amplified using primers Hs-AFP1-5' and MJ-3' (Table B.2), using as template pFAJ3068, which was kindly provided by Prof. W.F. Broekaert. The 160 bp PCR product was sub-cloned into the pGEM T-Easy vector system (Promega), producing pGEM-MJ. The 160–bp fragment was excised from pGEM-MJ at the *Eco*RI and *Bam*HI sites and sub-cloned into the corresponding sites in pUC18 (Life Technologies (GIBCO/BRL)), yielding pUC18-MJ. The *CTS1-2* gene was PCR amplified using primers CTS1-2 PI-2 (L) and CHIT 3' (Table B.2) from *S. cerevisiae* strain DBY918 genomic DNA. The ~1.69 kb PCR product was cloned into the pUC18-MJ at the *Bam*HI and *Hin*dIII sites, constructing pUC18-MJ-CTS. A ~1.85 kb fragment, including the secretion signal and the *CTS1-2* gene, was excised from pUC18-MJ-CTS at the *Eco*RI and *Hin*dIII sites and sub-cloned into pART7 (Gleave, 1992) at the corresponding sites, generating pART7:MJ-CTS. A 4.0 kb *Not*I MJ-CTS cassette under control of the cauliflower mosaic virus (CaMV) 35S promoter and *Agrobacterium* octopine synthase 3' terminator was in turn isolated from pART7:MJ-CTS. This cassette was cloned into the corresponding SAP-treated *Not*I site of pART27 (Gleave, 1992), yielding the clone pART27:MJ-CTS (Fig. B.1). This clone was confirmed through DNA sequencing.

ADDENDUM B

CONSTRUCTION AND TRANSFORMATION OF A PLANT EXPRESSION CASSETTE IN WHICH THE YEAST *CTS1-2* GENE IS TARGETED TO THE APOPLAST

B.1 Introduction

The subcellular location of defence compounds in the plant could influence the effectiveness of the plant's defence mechanism. The majority of microbial plant pathogens grow extracellularly, at least during the early stages of the infection process (Isaac, 1992). Therefore, during this stage the pathogen can only be countered by extracellular defence compounds.

The *CTS1-2* gene was fused to the secretion signal of an antimicrobial peptide isolated from the seeds of *Mirabilis jalapa* L., designated *Mj*-AMP2 (Cammue *et al.*, 1992), to direct the chitinase protein to the apoplastic region to counter the initial pathogen invasion. This secretion signal was previously used to direct proteins to the extracellular environment in tobacco plants (De Bolle *et al.*, 1996). These authors confirmed the correct targeting of the proteins to the apoplast by processing and localisation analyses.

B.2 Bacterial strains and culture conditions

The sources and relevant genotypes of the bacterial strains, as well as the plasmids used in this study, are listed in Table B.1. *E. coli* transformants were grown at 37°C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), or in LB supplemented with either ampicillin (100 µg/ml) or kanamycin (20 µg/ml), for the selection of transformants. *A. tumefaciens* strains were cultured routinely at 28°C in YEP media (containing 1% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) sodium chloride). The solid media contained 2% (w/v) agar.

B.3 DNA manipulations

Standard techniques for DNA cloning were performed according to Sambrook *et al.* (1989). Restriction enzymes, Expand Polymerase and T4 DNA Ligase were purchased from Roche Diagnostics. Shrimp alkaline phosphatase (SAP) was purchased from Amersham Life Sciences. Sequencing was performed by the DNA Sequencing facility, Department of Genetics, Stellenbosch University, using an ABI PRISM™ 377 automated DNA sequencer from PE Biosystems. PCR amplifications were performed as described previously in Chapter 4.

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Table B.2 Primer pairs and sequences used in this study

Primer	Sequence	Paired with	Template	Product
CTS1-2 PI-2 (L)	5'-GATCGGATCCTCACTCCTTT ACATCATTCTT-3'; *Bam*HI-site underlined	CHIT 3'	*S. cerevisiae* strain DBY918 genomic DNA	1.69 kb of *CTS1-2* gene
CHIT 3'	5'-ACGTAAGCTTCGGACTCTA TGAATCAATCT-3'; *Hind*III-site underlined	CTS1-2 PI-2 (L)	*S. cerevisiae* strain DBY918 genomic DNA	1.69 kb of *CTS1-2* gene
Hs-AFP1-5'	5'-GCCGCTCGAGTATTTTTACA ATTACCAAC-3'; *Xho*I-site underlined	MJ-3'	pFAJ3068	Signal peptide from *Mj*-AMP2
MJ-3'	5'-GATCGGATCCTGCTTCTAG CATGCCGGACATGGC-3'; *Bam*HI-site underlined	HsAFP1-5'	pFAJ3068	Signal peptide from *Mj*-AMP2

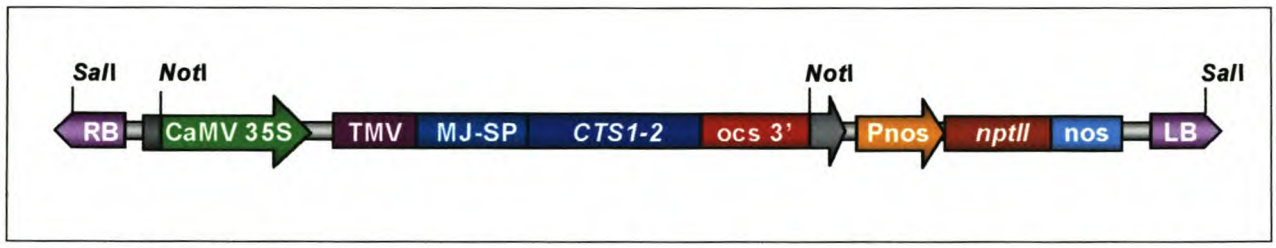

Figure B.1 Schematic representation of the pART27:MJ-CTS construct used for plant transformation, containing the 4.0 kb *CTS1-2* cassette, in which the *CTS1-2* gene is fused to an extracellular targeting signal. RB, right border of T-DNA; CaMV 35S, cauliflower mosaic virus promoter; TMV, tobacco mosaic virus 5' leader sequence; MJ-SP, secretion signal from *Mj-AMP2*; *CTS1*-2, yeast chitinase gene; ocs 3', octopine synthase 3' terminator; Pnos, nopaline synthase promoter; *nptII*, neomycin phosphotransferase (kanamycin resistance gene); nos 3', nopaline synthase terminator; LB, left border of T-DNA.

B.5 Transformation of tobacco plants

Nicotiana tabacum cv. SR-1 was transformed with pART27:MJ-CTS, as previously described in Chapter 4. The *nptII* gene confers the kanamycin resistant (Km^R) phenotype to the transformed plant cells. Thirty Km^R primary transformants (F_0) were generated from approximately forty independent leaf segments. The plantlets have not yet reached a sufficient size to be tested for integration and expression of the transgene. Assays will be conducted once the integration of the transgene and the transgene expression of these plants are established. These assays include the establishment of endochitinase activity, antifungal inhibition activity assays on crude protein extracts of the transgenic plants, as well as fungal inhibition studies on the detached leaves. Further studies on these plantlets will be conducted as soon as enough plant material is available.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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5.1. GENERAL DISCUSSION AND CONCLUSIONS

The ability to add a gene or genes to the genome of a specific plant species became a reality with the development of transformation technology for plants in 1985 (Horsch *et al.*, 1985). This, as with any important technological development, was a watershed event in the plant sciences. The 16 years that have passed since then have seen a dramatic change in the way complex problems are unravelled in the study of plants. One of the aspects of plant growth and development that has benefited greatly from genetic transformation technology is the disease resistance mechanism of plants. A wealth of knowledge on how plants defend themselves has become known by the identification, cloning and characterisation of genes involved in the complex defence pathways typical of plants. Plant molecular biology, together with other disciplines, has confirmed or disproved favourite assumptions regarding defence, as well as led to the inference of new hypotheses or working models. A classical example is the confirmation on genetic and gene level of the gene-for-gene hypothesis, proposed by Flor in 1956 (Flor, 1956), which describes the dynamic interaction between host and pathogen and the genetic basis of the outcome of such an interaction.

The aim of this thesis involved the activities of a class of proteins that has gained recognition for its important roles during plant defence, mostly against fungal pathogens. These proteins are the chitinases that occur in all plant species and in several other life forms (Sahai and Manocha, 1993; Blaak and Schrempf, 1995; Kombrink and Somssich, 1995). Their substrates, chitin and related substances, are absent from the plant body, however, but are otherwise recognised as extremely abundant polymers, also occurring in fungal hyphae and spore walls (Muzzarelli *et al.*, 1994). The chitinases have several important functions in the variety of organisms in which they occur, but the focus of this thesis fell on their antifungal activities as part of the pathogenesis-related (PR) proteins that act as inducible defence proteins and elicitors of defence messages in plants (Kombrink and Somssich, 1995). The chitinase proteins have also been the subject of numerous genetic manipulation strategies, not only to increase their levels in plants as a defence strategy (Zhu *et al.*, 1994; Lin *et al.*, 1995; Terakawa *et al.*, 1997; Lorito *et al.*, 1998; Datta *et al.*, 2001), but also in the study of their antagonistic mechanisms that are critical to the success of mycoparasites, such as *Trichoderma* spp., as biocontrol agents (Lorito *et al.*, 1998).

A two-pronged approach was taken with the *CTS1-2* chitinase gene from the yeast *Saccharomyces cerevisiae.* It has been speculated that this chitinase may exhibit antifungal activities and therefore it was overexpressed in both yeast and in a plant environment to evaluate its antifungal abilities and its possible applications in disease management.

The first approach presented in this thesis is the overexpression and secretion of the yeast *CTS1-2* gene from *S. cerevisiae* to ultimately confirm the antifungal activity of

the yeast chitinase. The chitinase-encoding gene, fused to either its native secretion signal, or to the *MFα1* or *Trichoderma reesei* xylanase (*XYN2*) secretion signals, was placed under the control of two strong promoter-terminator combinations on episomal plasmids. The *PGK1* promoter-terminator constructs yielded high-level *CTS1-2* expression and chitinase-producing strains of *S. cerevisiae* PRY488, a laboratory strain that lacked native chitinase activity (Chapter 3 of this thesis). The other promoter-terminator constructs, employing the *ADH2* regulatory sequences, yielded *CTS1-2* transcripts, but no chitinase activity could be detected in any of the strains. The constructs were all verified by sequence analysis and, up to now, no logical explanation could be provided for these results (presented in Addendum A of this thesis).

The *PGK1*-driven constructs were analysed for the ability of the various signal peptides to efficiently secrete the overexpressed chitinase. It was evident that the non-native signal sequences both performed superiorly to the native *CTS1-2* secretion signal, since the former two delivered significantly more of the protein to the extracellular environment. This is a valid conclusion, since the overall production and level of chitinase activity in the various strains were virtually identical. The high-level of expression and efficient secretion of the yeast chitinase were evaluated, since the possibility exist to develop strains of *S. cerevisiae* as excellent biocontrol agents against fungal pathogens, or to develop other organisms that express and produce the yeast chitinases efficiently. The preliminary *in vitro* assays against *Botrytis cinerea* revealed a significant reduction in hyphal development, caused by extreme structural damage to the hyphal tips, the hyphal cell walls as well as the ability of the fungus to form reproductive and survival structures, thereby confirming the strong antifungal activity of this enzyme.

The second approach involved the heterologous expression of the *CTS1-2* gene from a plant vector under the control of a strong, constitutive promoter. Tobacco transformation yielded several transgenic lines that stably incorporated the transgene and exhibited expression of the *CTS1-2* gene as well as production of active yeast chitinases. An F_1 progeny yielded normal plants with growth behaviour and phenotype matching that of the untransformed control. These plant lines were tested rigorously for resistance to *B. cinerea* as an example of an economically important pathogen. Both *in vitro* and *in planta* assays confirmed that the yeast chitinase increased the plant's tolerance to fungal infection; some of the lines showed disease resistances between of 65 and 70%.

In addition to these transgenic lines containing the chitinase in the intracellular environment, the *CTS1-2* gene was also fused to a signal peptide of the *Mj-AMP2* gene, encoding a plant defensin from *Mirabilis jalapa*. This signal will target the chitinase to the apoplastic environment. The gene was also expressed under a strong, constitutive promoter (Addendum B of this thesis). Although transformations have been performed, the regenerated transgenic lines are still under evaluation and undoubtedly will yield interesting results regarding the effect of the chitinase on the plant surface in relation to disease resistance against fungal pathogens.

In conclusion, the combined set of results from both the yeast and plant overexpression studies have confirmed the strong antifungal effect of yeast chitinases. The plant expression studies showed that the yeast chitinase can be expressed in a heterologous plant system, leading to disease resistance levels of up to 70% under the conditions tested. The yeast *CTS1-2* chitinase gene could be instrumental in the development of a new generation of yeast strains with improved antifungal capabilities and/or could well become an important non-plant PR protein in genetic transformation technologies aimed at enhanced disease resistance.

5.2. LITERATURE CITED

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