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

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Abstract

The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene has recently been confirmed by *in vitro* tests to possess antifungal abilities. In this study, the *CTS1-2* gene has been evaluated for its *in planta* antifungal activity by constitutive overexpression in tobacco plants to assess its potential to increase the plant's defence against fungal pathogens. Transgenic tobacco plants, generated by *Agrobacterium*-mediated transformation, showed stable integration and inheritance of the transgene. 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, leading to severe physical damage on the hyphae. Several of the F_1 progeny lines were challenged with the fungal pathogen, *B. cinerea*, in a detached leaf infection assay, showing a decrease in susceptibility ranging from 50 to 70%. The plant lines that showed increased disease tolerance were also shown to have higher chitinase activities.

Introduction

Plants have evolved a battery of defence systems to protect themselves against constant biotic (e.g., pathogen attack) and abiotic stresses (e.g., ultraviolet 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 (Agrios, 1997).

Chitinases (EC 3.2.1.14), which are present in many 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 & Inbar, 1994, 1997; Haran et al., 1996). 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 & 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 & Jollès, 1984).

The control of fungal diseases in modern agriculture is mainly achieved by the extensive use of chemical fungicides. However, the exclusive and excessive use of fungicides is no longer a sustainable

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approach due to safety issues such as environmental concern, consumer health as well as the increasing incidence of pathogen resistance to fungicides. Therefore, a critical need exists for the development of effective, safer alternative methods for disease control in crop plants. Substitutes include the use of biological control agents and genetic engineering of plants to ultimately render them fungus resistant. Intensive activity towards the development of biocontrol agents and the genetic approach of breeding is taking place, however, commercial biocontrol products are few and breeding is expensive and time-consuming (Schickler & Chet, 1997). Genetic engineering has enabled scientists to transfer specific genes to the plant, enabling the insertion of traits for resistance to possibly render the plant resistant to fungal pathogens.

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. Success in this regard was 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 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 the yeast *Saccharomyces cerevisiae*, the 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 & 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 & Robbins, 1991).

It has been speculated by Kuranda and Robbins (1991) that similar to chitinase enzymes from other sources, the *S. cerevisiae* chitinase may also possibly exhibit some antifungal activity. It has recently been confirmed in our laboratory that the extracellular yeast chitinase has strong *in vitro* antifungal activity against *Botrytis cinerea* (unpublished data). It was also found that 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 antifungal activity of the yeast chitinase enzyme prompted the use of the *CTS1-2* gene as a candidate for overexpression in a plant expression system.

In this study, we evaluated the *S. cerevisiae* chitinase gene for its potential to enhance the plant's endogenous resistance against fungal pathogens. To this end, transgenic tobacco plants were developed that constitutively overexpressed the *S. cerevisiae* chitinase gene (*CTS1-2*) under the control of the 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.

Materials and methods

Microbial strains and culture conditions

Escherichia coli was 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). Yeast was routinely cultured on YPD medium (containing 1.2% (w/v) yeast extract, 2.5% (w/v) peptone, 1.2% (w/v) glucose). 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 distilled water 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.

DNA manipulations

Standard techniques for DNA cloning and manipulation were performed according to Sambrook et al. (1989). Restriction enzymes, Expand polymerase and T4 DNA ligase were purchased from Roche Diagnostics (Basel, Switzerland). Shrimp alkaline

phosphatase (SAP) was obtained from Amersham Biosciences (Buckinghamshire, UK). Sequencing was performed by the DNA Sequencing facility, Department of Genetics, Stellenbosch University, using an ABI PRISM^{TM} 377 automated DNA sequencer from PE Biosystems (Foster City, USA).

DNA amplifications by the polymerase chain reaction (PCR) method were done using Expand polymerase. PCR reactions were performed in 50 µl reaction mixtures, typically consisting of 1× 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 s, 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 Whatman Biometra Trio-thermoblock cycler (Göttingen, Germany).

Plasmid construction

The *CTS1-2* gene was PCR amplified from *S. cerevisiae* strain DBY918 genomic DNA, using primers CTS1-2(L) (5′-GATCGGATCCGAATGTCA CTCCTTTACATC-3′; ATG underlined) and CHIT 3′ (5′-ACGTAAGCTTCGGACTCTATGAATCAATCT-3′), and sub-cloned into the pGEM T-Easy vector system (Promega; Madison, USA), producing pGEM:CTS. The inserted ∼1.7 kb fragment was excised with *Eco*RI and subcloned 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 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. The integrity of the clone was confirmed by sequence analysis.

Transformation and regeneration of tobacco plants

The plasmid pART27:CTS was mobilised from *E. coli* DH5α to *A. tumefaciens* strain EHA105 (Hood et al., 1993) by triparental mating (Goldberg & Ohman, 1984). *A. tumefaciens* harbouring the relevant plasmid was used to infect leaf discs of *Nicotiana tabacum* cv. SR-1, essentially as described by Horsch et al. (1985). After 3 weeks, the kanamycin-resistant (Km^R) tobacco shoots that developed were transferred to selective root inducing media (Horsch et al., 1985). When sufficient root formation had occurred, the plantlets were subcultured to MS-medium (Murashige & Skoog, 1962) at 28°C under a 16 h day and 8 h night regime.

Each plant line was subcultured into three *in vitro* replicates. One of these replicates was hardened off, ultimately setting seed through self-pollination. These seeds were germinated *in vitro* under selection to yield F_1 progeny. F_1 progeny plants were also subcultured into three replicates, 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.

Southern blot analysis of transgenic tobacco lines

Total DNA from the *in vitro* F_1 progeny 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 tetraacetic 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 Biosciences; Buckinghamshire, UK), as described by Sambrook et al. (1989). Lambda DNA digested with *Bst*EII was used as a molecular size marker. The *CTS1-2* gene (∼1.7 kb) was PCR-labelled as probe, using the 10× DIG dNTP labelling mixture (Roche Diagnostics; Basel, Switzerland), according to the manufacturer's specifications. Hybridisations were performed at 42°C for 16 h, using the standard DIG hybridisation buffer (Roche Diagnostics; Basel, Switzerland) containing 50% (w/v) formamide. Signal was detected according to the manufacturer's specifications.

RNA isolations and northern blot hybridisation

Total RNA was isolated from 80 to 100 mg of *in vitro* F_1 progeny leaf tissue, using the TRIzol Reagent according to the manufacturer's specifications (Invitrogen; Carlsbad, USA). Total RNA isolated

from 80 to 100 mg leaf tissue was subjected to 1.2% (w/v) formaldehyde gel electrophoresis, together with an RNA ladder (Invitrogen; Carlsbad, USA), and blotted onto Hybond-N nylon membranes (Amersham Biosciences; Buckinghamshire, UK) according to standard procedures (Sambrook et al., 1989). The DIG-labelled probe utilised for Southern blot analysis was used for RNA hybridisation at 50°C for 16 h using the standard DIG hybridisation buffer (Roche Diagnostics; Basel, Switzerland) containing 50% (w/v) formamide. Signal was detected according to manufacturer's specifications.

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 distilled water, utilising a Spectra/Por membrane 4 (Spectrum; Rancho Dominguez, USA). 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 distilled water for the endochitinase activity assay.

Endochitinase activity assays

The endochitinase 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 distilled water, were mixed with 41.67 µM 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotrioside (Sigma-Aldrich; St. Louis, USA) 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; Vermont, USA) 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 per mg of total protein.

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 (Nalge Nunc; Naperville, USA) 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) glucose) 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 48 h. The absorbancy at 595 nm (A_{595}) of the samples was determined every 24 h with the PowerWaveX Microplate Scanning Spectrophotometer (Bio-Tek Instruments; Vermont, USA), starting at 0 h. 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 growth 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. After 48 h of incubation, 10 µl of the samples in each well was microscopically analysed to determine the condition of the *B. cinerea* hyphae.

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 1-month-old F_1 progeny 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 onto 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 (3 days) and 168 h (7 days) according to a 10-point lesion index scale that was developed. Lesions were photo-

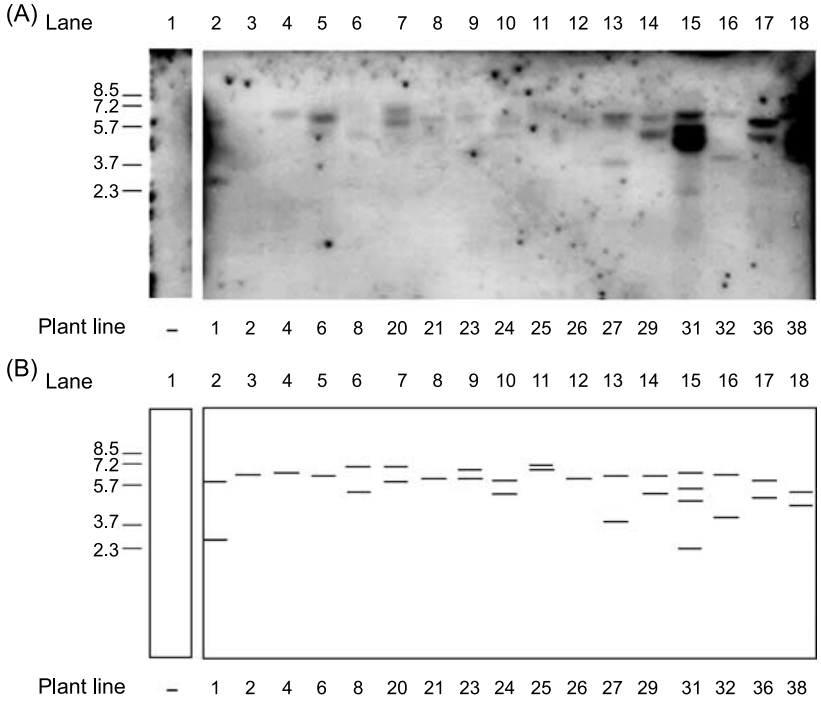


Figure 1. Southern blot analysis (A) and schematic representation of hybridisation patterns (B) of genomic DNA from F_1 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. DNA was digested with *Hpa*I and probed with the ~ 1.7 kb *CTS1-2* gene. Lane 1, untransformed tobacco DNA (negative control); lanes 2–18, digested DNA from transgenic lines. Lambda DNA digested with *Bst*EII was used as a molecular size marker and size standards are indicated in kilobases.

graphed after 72 h and again after 3 weeks. The lesions were also measured after 72 and 168 h and compared to lesions on inoculated untransformed tobacco leaves. These lesion sizes were used to calculate the percentage decrease in susceptibility of the transgenic lines.

Results

Transformation of tobacco with a yeast chitinase gene

A plant expression vector, pART27:CTS, containing the *S. cerevisiae* chitinase gene (*CTS1-2*), was introduced into *N. tabacum* via *Agrobacterium* transformation. Thirty Km^R primary transformants (T_0) were generated from approximately 40 independent inoculated leaf discs. After a 2-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 T_1 progeny plants.

Integration and expression of the CTS1-2 *gene in T_1 plant lines*

Southern-blot analysis of genomic DNA from an untransformed control and the various transgenic lines confirmed integration of the yeast chitinase gene in the T_1 progeny (Figure 1). The DNA was digested with *Hpa*I that cuts once in the middle of the *CTS1-2* gene to determine the number of integration sites of the transgene. Varying hybridisation patterns and signal intensities were observed, high intensity signals suggesting multi copy tandem integration. Therefore Southern blot analysis indicated that the copy number of the transgene varied from one to multi-copies, with the majority of transgenic lines having one copy or one site of integration.

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 T_1 progeny (Figure 2). Varying

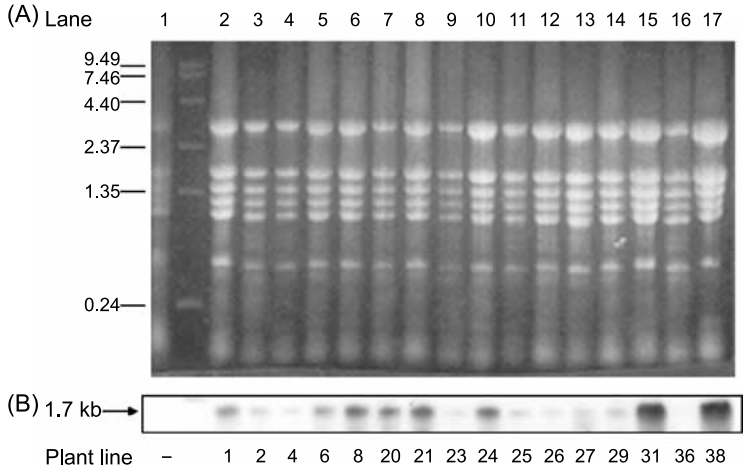


Figure 2. Total RNA separated on a formaldehyde gel (A) and northern blot analysis (B) on total RNA from T_1 tobacco lines transformed with the yeast *CTS1-2* chitinase-encoding gene. Lane 1, RNA from an untransformed plant (negative control); lanes 2–17, RNA from the various transgenic lines. RNA ladder (Invitrogen; Carlsbad, USA) was used as molecular size marker and size standards are indicated in kilobases. Detection in the northern blot was with a DIG-labelled *CTS1-2* gene probe.

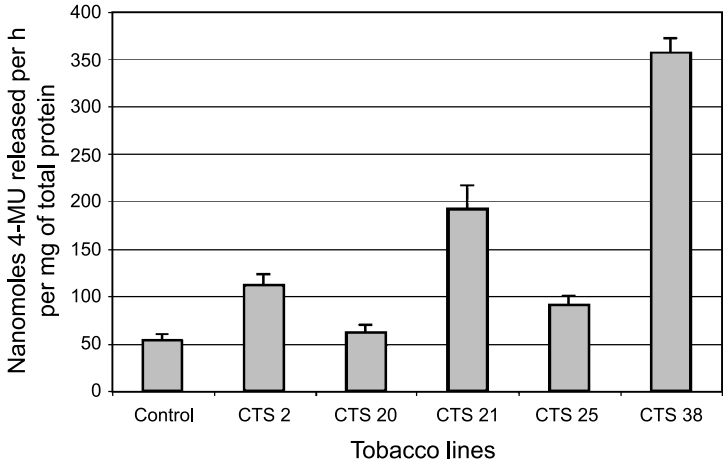


Figure 3. Endochitinase activity in leaf extracts of T_1 progeny plant lines expressing the yeast *CTS1-2* gene. Units of activity are defined as nanomoles of 4-MU released per h per mg of total protein. Error bars represent the standard deviation of six replicate assays.

intensity hybridisation signals of the expected size were detected for most of the transgenic lines, whereas no signal was observed for the RNA of the untransformed plant lines.

Endochitinase activity assays

Endochitinase activity assays performed on five of the T_1 progeny lines were used to establish the functionality as well as the levels of activity of the recombinant yeast chitinase produced (Figure 3). These assays confirmed that the yeast chitinase maintained functionality in the plant environment, with its activity increasing 2- to 7-fold when compared with the untransformed control.

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 14 lines that showed active transcription of *CTS1-2* (Figure 4(A)). Seven of the lines had fairly low percentages of growth inhibition (14–23%) against *B. cinerea*, whereas six lines exhibited growth inhibi-

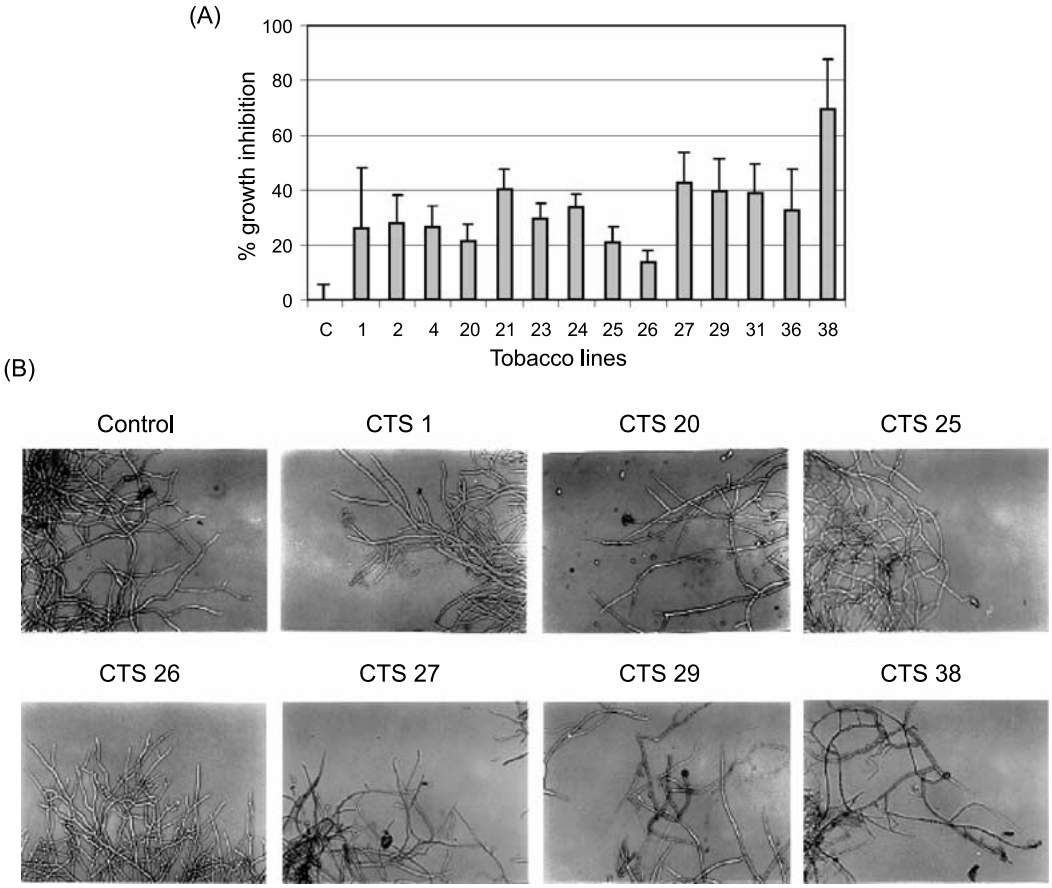


Figure 4. The effect of the yeast *CTS1-2* chitinase protein in leaf extracts from T_1 progeny transgenic tobacco plants on growth and spore germination of *B. cinerea* after 48 h of incubation. (A) Percentage growth inhibition is defined as 100× the ratio of the corrected A_{595} of the control plant minus the corrected A_{595} of the sample over the corrected A_{595} of the control plant. (B) Microscopic analyses of the appearance of the *B. cinerea* hyphae after incubation.

tion from 30 to 45%. One line (CTS 38) approached 70% growth inhibition of *Botrytis* under the conditions tested (Figure 4(A)).

The effect of the crude protein extracts on spore germination and hyphal development of *B. cinerea* was microscopically observed after 48 h of incubation (Figure 4(B)). In the case of the untransformed control plant, the spore germination and hyphal growth appeared normal, leading to high fungal biomass. Tobacco lines CTS 25 and 26, which had low percentages of growth inhibition (14 and 21%, respectively), also showed the formation of high fungal biomass. Lower fungal biomass was observed for lines CTS 1 and 20, however, the hyphae did show normal elongation which would eventually lead to high biomass. In contrast, structural and physiological damage to the hyphae was observed in the case of lines CTS 27, 29 and 38, which was shown to have high percentage growth inhibition (Figure 4(A)).

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 10-point lesion index scale was developed to categorise the lesions (Figure 5). 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)

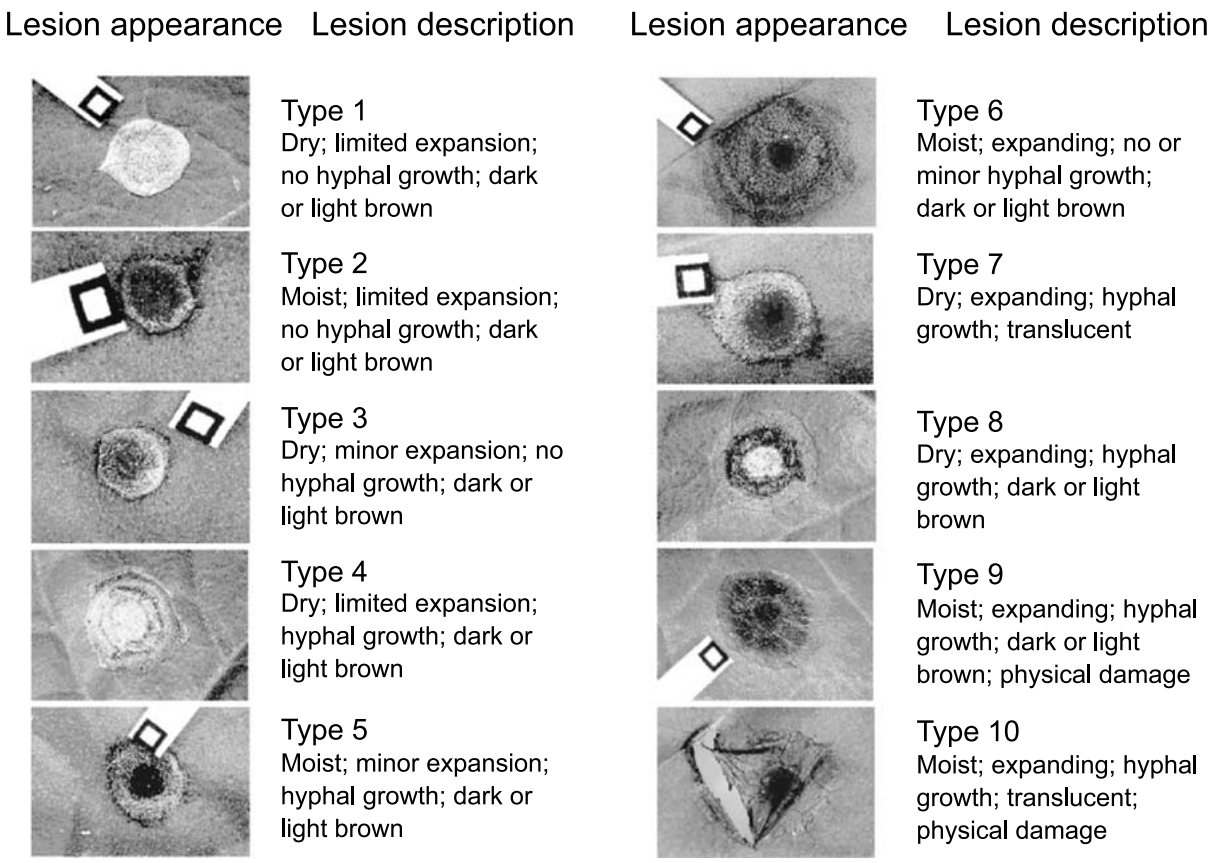


Figure 5. Lesion index scale and lesion descriptions used to define tolerance and susceptibility after infection of tobacco with *B. cinerea*. Type 1 lesions indicate the most tolerant phenotype, whereas type 10 represents the most susceptible phenotype.

(Figure 5). 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 2-fold reduction in lesion size when compared to the untransformed control (Figure 6(A)). 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.

The lesion sizes were subsequently normalised against that of the untransformed tobacco lines and expressed as percentage decrease in susceptibility (Figure 6(B)). The highest percentage decrease in susceptibility was observed for lines 21, 24, 27 and 38, which were at 60–70% 7 days (168 h) after inoculation (Figure 7(B)). It was clear that the levels of susceptibility of several of the lines (21, 23, 27 and 31) decreased from day 4 to 7 post-inoculation, whereas the opposite occurred in lines 1, 2, 4, 20 and 25 (Figure 6(B)). Lines 26, 29 and 36 showed unchanged low levels of susceptibility over the 168 h incubation period. Good correlation could be drawn with the observed lesion types, characterised with the index in Figure 5, and the deduced susceptibilities, that is, lines 21 and 38, which had the most significant decrease in susceptibility, also exhibited very resistant type 2 and 3 lesions (Figure 7).

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 over time, measuring 9.5 mm after 72 h of incubation. Physical damage also appeared on the leaf tissue and the lesion was described as a type 10 lesion (Figure 7). 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 7).

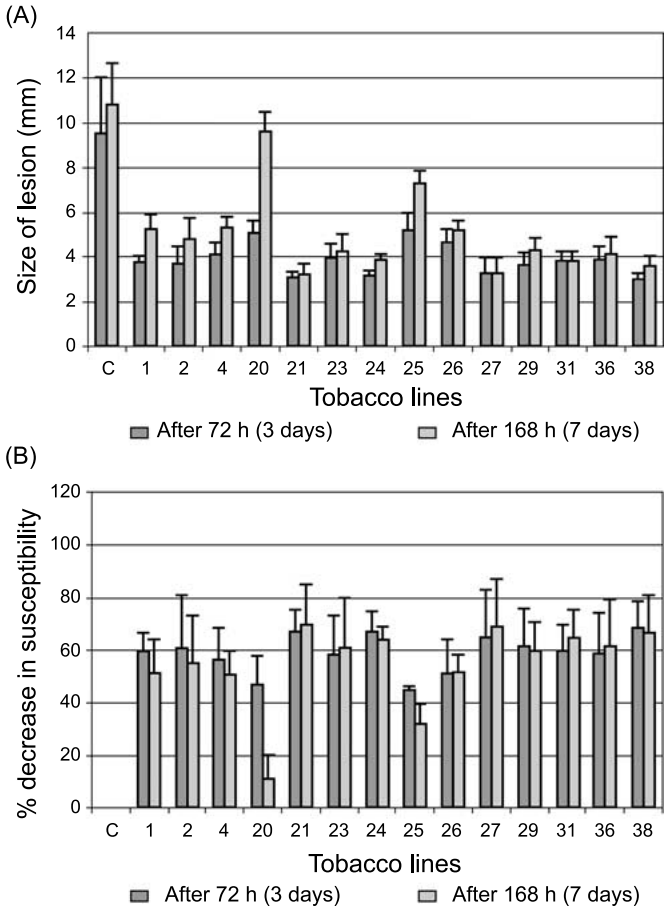


Figure 6. Decrease in susceptibility to *B. cinerea* of detached leaves of T_1 progeny transgenic tobacco lines expressing the yeast *CTS1-2* gene. (A) Size of lesions (mm) produced on the leaves of the controls and the different transgenic tobacco lines 72 h (3 days) and 168 h (7 days) after inoculation. Error bars represent standard deviation from six replicate assays. (B) The susceptibility to *B. cinerea* of controls and different tobacco transgenic lines 72 and 168 h after inoculation. It is expressed as a percentage of the decrease in susceptibility normalised against the tolerance of the control plants, which was taken as 0%.

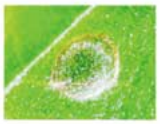
After 3 weeks, all the leaves were inspected for the formation of fungal survival structures. Due to the very high inoculum and extremely favourable conditions, the level of pathogen infection was severe in susceptible hosts. The pathogen was able to form a large number of reproductive organs and survival structures on the leaves of the untransformed control plants (Figure 7). In contrast, the most tolerant 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 (Figure 7). Tobacco line CTS 20, which exhibited severe type 8 lesions, had the lowest measured percentage reduction in susceptibility measured. After 3 weeks, clear survival structures were also present on the leaves on this line (Figure 7).

The correlations between disease incidence and the severity thereof could be linked to the levels of chitinase activity measured as well as the percentage growth inhibition in several of the transgenic lines.

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 & Chet, 1998;

Tobacco line	Lesions after 72 h	Lesion size and type	Fungal survival structures	Leaves after 3 weeks	% growth inhibition	Endochitinase 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

* Units of activity were defined as nanomoles of 4-MU released per hour per mg of total protein

Figure 7. The correlation between chitinase activity, appearance and development of lesions, the formation of survival structures and the percentage growth inhibition in transgenic tobacco lines expressing the *S. cerevisiae CTS1-2* chitinase gene. The control line represents an untransformed tobacco line and CTS 20 a highly susceptible transgenic line. The CTS 21 and 38 lines are indicative of very tolerant lines with corresponding high chitinase activities.

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 in this case we have 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 T_1 generation with an indistinguishable phenotype compared to the untransformed control. These lines were confirmed to have integrated the transgene into their genome (Figure 1). Multiple bands in Southern blots could be attributed to possible rearrangements or truncation of the transgene, and these were inherited from the primary transformants to the T_1 progeny.

Northern blot analysis also confirmed that transcription of the *CTS1-2* gene had occurred efficiently (Figure 2). It is known that the position of integration has an influence on the expression of the introduced gene as is apparent from plant lines with the same copy number, having varying levels of expression (Figure 1, plant lines 20 and 27). High expression levels were observed for multi-copy plant lines 31 and 38, however multi-copy plant line 36 had very low levels of expression (Figures 1 and 2). Low levels of expression were also observed in plant lines 2, 6 and 26 owing to the possibility of the rearrangement or truncation of the transgene.

The transcripts yielded active yeast chitinase proteins that exhibited significant increases in chitinase activity in some of the transgenic lines when compared to untransformed controls (Figure 3). In some cases correlations could be found between the transcription levels and the observed endochitinase enzyme activity. Both plant lines 2 and 25 showed low levels of expression (Figure 2), low levels of endochitinase activity (Figure 3) and were also more susceptible to

fungal attack, whereas plant lines 21 and 38 had high expression levels (Figure 2), correspondingly high levels of endochitinsase activity (Figure 3) and showed less susceptibility to *B. cinerea* infection. However, plant line 20 exhibited relatively high levels of expression (Figure 2) and low levels of endochitinase activity (Figure 3), whereas plant line 27 demonstrated low levels of expression but relatively high fungal resistance. This phenomenon could be due to post-transcriptional modification or other unidentified aspects linked with the transformation event itself.

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. These assays were performed with high spore inoculations and under conditions favouring fungal growth. 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 (Figure 4(A)). The results obtained with the *in planta* infection studies mirrored the tendencies seen in the *in vitro* assay. 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 high percentages of decreased disease susceptibility, as calculated from normalised lesion size measurements. Most of the transgenic lines showed decreased susceptibility above 60%, reaching 70% in several lines (Figure 6). These values were obtained after 168 h of incubation post-inoculation, but typically led to a complete stop in infection, since, even after 3 weeks of incubation, the lesions had not increased or spread in the most tolerant transgenic lines (Figure 7).

The effect of the crude protein extracts on the survival of *B. cinerea* was microscopically analysed (Figure 4(B)). The chitinolytic activity of the chitinase enzymes present in the protein extracts hydrolysed the chitin present in the fungal cell walls, thereby inducing severe morphological and physiological changes in the hyphae. The general effect of the hydrolysing enzymes on the survival 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.

The strong correlation found between the levels of endochitinase activity and the results of the *in vitro* and *in planta* fungal inhibition assays in the transgenic lines confirmed that the inhibitions observed were 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 will hopefully add the yeast chitinase gene to the list of PR chitinases that are useful in genetic manipulation strategies.

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