Exo-β-1,3-Glucanase from Yeast Inhibits *Colletotrichum lupini* and *Botrytis cinerea* Spore Germination

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Abstract

Yeast exo-β-1,3-glucanase (EXG1) was evaluated as an inhibitory agent of *Colletotrichum lupini* and *Botrytis cinerea.* Extracts obtained from yeast transformed with the *exg1* gene, expressing high levels of EXG1 activity, or control untransformed yeast cultures that lacked EXG1 activity, were added to different starting concentrations of *C. lupini* fungal spore suspensions (2.5×10^3 to 80×10^3 spores per flask), and mycelial dry weight was measured after 5 days. Inhibition of *C. lupini* mycelial growth by EXG1 compared with control extracts ranged from 41 to 20% when added to starting fungal spore concentrations of 2.5×10^3 to 80×10^3, respectively. EXG1 activity in the extracts from the transformed yeast remained high over the 5-day incubation period. Addition of the EXG1 extract after *C. lupini* spore germination resulted in lower inhibition, indicating that the EXG1 targets the β-glucan in the cell walls of the fungal spores at an early stage of germination. Furthermore, the yeast EXG1 extracts were also shown to inhibit *Botrytis cinerea* spore germination and growth. Thus, the use of the yeast *exg1* gene for protection of crops, such as lupin and pear in transgenic strategies against *C. lupini* and *B. cinerea*, respectively, could be considered.

Introduction

Anthracnose is considered as the most devastating fungal disease for lupin producers worldwide (Koch et al., 2002) as it can cause crop losses of up to 100%. *Colletotrichum lupini* causes this disease in Southern Africa (Lotter and Berger, 2005). Lupin anthracnose therefore, represents a major threat to the South African lupin industry,

which has an annual turnover of approximately 60 million rands [personal communication: Dr M Griessel, Protein Research Foundation (PRF), Rivonia, South Africa, 2003]. In turn, *Botrytis cinerea* is one of the most commonly found fungal pathogens and causes significant postharvest losses of deciduous fruit, including pear (Lennox et al., 2003), following initial infection by means of spores in the orchard.

It is well documented that plants activate a number of inducible and active defence responses following elicitor treatment or microbial attack (Broglie et al., 1986; Lawton and Lamb, 1987; Lamb et al., 1989; Vigers et al., 1991; Keen, 1992; Latunde-Dada and Lucas, 2001), e.g. pathogenesis-related (PR) proteins, such as β-endoglucanases and chitinases (Ham et al., 1991; Stinzi et al., 1993). Several *β-1,3-endoglucanase* genes from plants (E.C. 3.2.1.39) have been cloned with the aim to use them for transgenic fungal resistance (Edington et al., 1991; Chang et al., 1993). Transgenic tobacco plants expressing an acidic β-1,3-glucanase from alfalfa were reported to be resistant to *Cercospora nicotianae* (Zhu et al., 1994) and transgenic tomato resistant to Fusarium wilt was produced by transformation with a class I basic β-1,3-glucanase from tobacco (Van den Elzen et al., 1993). Transgenic tobacco plants over-expressing ß-1,3-glucanase showed enhanced resistance against certain fungal pathogens (Yoshikawa et al., 1993). Schlumbaum et al. (1986) showed that physiological concentrations of β-1,3-glucanase inhibited the growth of many pathogenic fungi.

Beta-(1,3)-exoglucanases (E.C. 3.2.1.5.8) have also been reported as inhibiting agents of fungal growth. A secretory β-1,3-exoglucanase of *Aspergillus oryzae*, was found to play a role in β-glucan utilization and an application in plant defence was suggested (Tamano et al., 2007). In addition, the *Trichoderma harzianum* AGN13.1 enzyme, an (1,3)-exoglucanase purified from culture filtrates, was highly specific for α-1,3-linkages in polysaccharides (Ait-Lahsen et al., 2001). Different rates of spore germination and inhibition were observed by the addition of the AGN13.1 enzyme to cultures of *Aspergillus niger*, *B. cinerea* and *Penicillium aurantiogriseum*, and it was suggested that the expression of the *agn13.1* gene in transgenic plants could lead to the production of plants with improved resistance to fungal pathogens. A patent for the use of an exo-glucanase from *Coniothyrium minitrans* for the improved resistance of plants against phytopathogens has also been registered (Frick et al., 2001).

Saccharomyces cerevisiae has a complex system of glucanases, encompassing several forms that are classified as exo- and endo-glucanases (Van Rensburg et al., 1997). Some of these glucanases are capable of hydrolysing β-*O*-glycosidic linkages of β-glucan chains, leading to the release of glucose and oligosaccharides. The exo-glucanases yield glucose as the end product of β-glucan hydrolysis. The endo-glucanases attack the linkages at intermediate points of the polymer chain, releasing a mixture of oligosaccharides and glucose as a minor product (Larriba et al., 1995). During the vegetative growth of yeast, several endo- and exo-β-1,3-glucanases are synthesized, some of which are secreted and remain in the cell wall, with others being released into the surrounding medium. Yeast cells possess a predominantly β-1,6-linked polymer that contains only a few β-1,3-linkages. Thus, it is believed that yeast exo-glucanases are involved in the morphogenic processes involved in controlled autolysis of β-1,3-glucan, including cell wall metabolism. Other processes that are also thought to be dependent on the actions of glucanases are apical growth, branching and budding, mating and the formation and release of ascospores.

Three exoglucanase (*Exg*) genes have been reported in *S. cerevisiae* (Larriba et al., 1995). The *Exg1* gene encodes for the major isozyme [yeast exo-β-1,3-glucanase (EXG1)] that is expressed during vegetative growth (Muthukumar et al., 1993). The other two genes have been designated *Exg2* and *Ssg1* (Larriba et al., 1995), the latter gene being synthesized only during sporulation of *S. cerevisiae* diploids (Muthukumar et al., 1993). The *exg1* gene from *S. cerevisiae* has been cloned and over-expressed in yeast (Van Rensburg et al., 1997). Exg^{+}*S. cerevisiae* strains transformed with yeast multicopy plasmids containing the *exg* gene have demonstrated a five to 20-fold increase in EXG activity compared with the exg^{+}*S. cerevisiae* wild-type strains (Nebreda et al., 1986). The yeast *exg1* gene was shown to completely inhibit the growth of a *B. cinerea* isolate from grapevine when exposed to a culture filtrate obtained from a yeast culture over-expressing the enzyme (Pretorius, personal communication; Lampbrechts et al., 1997). Cultures of the control yeast strain had no effect on the growth of the fungus. For this reason, it was proposed to investigate the potential use of the *exg1* gene for genetic modification of crop species for resistance to fungi, such as *C. lupini* and *B. cinerea*. In this report, the possible use of the yeast *exg1* gene for protection of crops, such as lupin and pear, in transgenic strategies against *C. lupini* and *B. cinerea*, respectively, is evaluated.

Materials and Methods

Yeast culture transformed with the *exg1* gene

Exg1 transformed and untransformed *S. cerevisiae* Y294 (Van Rensburg et al., 1997) cultures were obtained from the Institute for Wine Biotechnology, University of Stellenbosch, South Africa. The *exg1* gene was isolated from *S. cerevisiae* total genomic DNA and cloned into the *Escherichia coli–S. cerevisiae* shuttle plasmid YEp24 (designated pBG1), and following recombinant plasmid DNA isolation, was used to transform *S. cerevisiae* Y294 to produce Y294 (Ura^{+}). The *exg1* gene fragment contained the *exg1* gene with its native promoter and terminator sequences.

Growth curves and determination of exo-glucanase activity of *exg1* transformed and untransformed yeast cells

A 10-ml yeast peptone dextrose (YPD) culture medium containing 10 µl of 100 mg/ml ampicillin stock (Amp^{100}) was either inoculated with 5 µl of the *exg1* transformed yeast or 5 µl of the untransformed yeast. These were incubated overnight at 30°C with gentle shaking. The following day, 24 × 100 ml YPD cultures (Amp^{100}) were inoculated with either 50 µl of the *exg1* transformed or 50 µl of the untransformed yeast overnight YPD cultures and incubated at 30°C with gentle shaking. At each of the designated time points, 0, 12, 16, 20, 24, 32, 48 and 72 h after inoculation, three of the *exg1* transformed and three of the untransformed yeast cultures were harvested. Harvesting was performed by first obtaining the OD_{650} values and then removing the yeast cells by centrifugation for 5 min at 5000 ***g*** at 4°C. The supernatants were filter sterilized before being stored in sterile containers at 4°C. Growth curves for the *exg1* transformed and untransformed yeast cells were constructed by using the recorded optical density (OD_{650}) values.

Glucanase activity was determined for both the *exg1* transformed and untransformed yeast cells at each of the time points used for the construction of the growth curves, and

for the pre- and postammonium sulphate fractions when the extracts were prepared for the inhibition studies. The assay was performed over a 60-min time period with glucanase activity being determined at t_0 and t_{60} min. Glucanase assays were performed on the 20 h *exg1* transformed and untransformed yeast culture filtrates when performing the inhibition studies. Enzyme activity readings, three replicates per culture filtrate, were taken at t_0 and t_{60}. For the glucanase assay, 780 µl of culture filtrate was added to 20 µl 2 mM*p*-nitrophenyl-glucoside and 200 µl 0.05 M sodium citrate (pH 4.8). The reaction was incubated at 37°C, and at each time point, 250 µl of the reaction mix was added to 500 µl 1 M sodium carbonate to stop the reaction. These were placed on ice until the end of the assay period, after which the A_{405} values were obtained.

Preparation of *Colletotrichum lupini* and *Botrytis cinerea* fungal spores for inhibition studies

The *C. lupini* var. *setosum* [isolate SHK 788 from *Lupinus albus* plants (Lotter and Berger, 2005)] and *B. cinerea* (from pear) [SH Koch, ARC-Plant Protection Research Institute (ARC-PPRI), Pretoria, South Africa] were grown on 10 ml potato dextrose agar slants in wide neck 26 ml McCartney bottles for 10 days under 12 h fluctuating light/darkness regimes at 23°C. Five millilitres sterile distilled water were added to each fungal culture. Spores in the cultures were dislodged with a glass rod of which the one point was curved with a 45° angle. Spore suspensions were filtered through a double layer of cheesecloth. The concentration of the spore suspension was determined with a haemocytometer. The concentration of the spore suspension was adjusted to 4×10^4 condia/ml. This suspension was further diluted aseptically to 2×10^4, 1×10^4, 0.5×10^4 and 0.25×10^4 spores/ml. *Botrytis cinerea* spore suspensions were also prepared using this protocol.

Growth inhibition assay with exo-glucanase filtrates

Fifty 1-l Erlenmeyer flasks containing 50 ml 2% malt extract (Sigma) were prepared and autoclaved at 121°C for 30 min. Under sterile conditions, 25 of these flasks were inoculated with either 25 ml of the filter-sterilized filtrate obtained from the *exg1* transformed yeast cultures or with 25 ml of the filter-sterilized filtrate obtained from the untransformed yeast cultures. To each flask, 50 µl Amp (100 mg/ml stock) was added to prevent bacterial contamination of the cultures. The flasks were inoculated, in triplicate, with 1 ml of the various spore concentration suspensions used: 0, 2.5×10^3, 5×10^3, 10×10^3, 20×10^3, 40×10^3, 60×10^3 and 80×10^3 spores/ml. The flasks were incubated at room temperature (±23°C) for 5 days on a laboratory bench before harvesting. Each sample was filtered through a Whatman No. 1 filter paper disc using a Buchner funnel and suction filtration. Filtrates were discarded. Filter paper discs containing the mycelia were dried overnight at 67°C and the weight recorded in grams. The dry weights at each spore concentration to which the untransformed culture filtrate was added (control flasks) were set to 100%. Inhibition of *C. lupini* and *B. cinerea* growth at each spore concentration was calculated in relation to the growth in the control flask of that specific spore concentration. The percentage inhibition of fungal growth by the *exg1* transformed yeast extract was calculated as follows:

[mycelial dry weight (g) of fungus grown in presence of exg1 transformed yeast extract/mycelial dry weight (g) of fungus grown in presence of untransformed yeast extract] $\times$ 100 = % growth

100 − % growth = % growth inhibition.

An 80% ammonium sulphate precipitation step was performed on the culture filtrates of the *exg1* transformed and untransformed yeast cultures. A 10-ml aliquot of the *exg1* transformed yeast and untransformed yeast filtrate was kept at 4°C for determination of preammonium sulphate glucanase activity at a later stage. Culture filtrates (100 ml) were adjusted to 80% saturation with the addition of powdered ammonium sulphate. The samples were maintained at 4°C at all times and left overnight with gentle shaking. Samples were, subsequently, centrifuged at 10 000 ***g*** for 30 min at 4°C. The precipitates were resuspended in 25 ml 0.05 M sodium citrate (pH 4.8), centrifuged and the supernatants filter sterilized.

Results

Growth curves and glucanase activity of *exg1* transformed and untransformed yeast

A comparative analysis of the growth curves for the *exg1* transformed and untransformed yeast cells was performed. The growth of the *exg1* transformed yeast cells plateaued at 20 h after inoculation and grew to higher cell densities than untransformed, while the growth of the untransformed yeast cells plateaued at 24 h after inoculation (data not shown). Glucanase activity assays were performed to determine at which time point the maximum glucanase activity occurred in the *exg1* transformed yeast cell cultures to be used for the *C. lupini* and *B. cinerea* spore inhibition studies by the glucanase enzyme.

Glucanase activity was present in the filtrates of the *exg1* transformed yeast cells and at much lower activity in the filtrates of the untransformed yeast cells (Fig. 1). Highest glucanase activity for the *exg1* transformed yeast cells was measured at 20 h after inoculation, which corresponded to the plateau reached in growth observed for the *exg1* transformed yeast cells at 20 h after inoculation. All further experiments were performed by growing of the yeast cultures for 20 h before harvesting of the cultures.

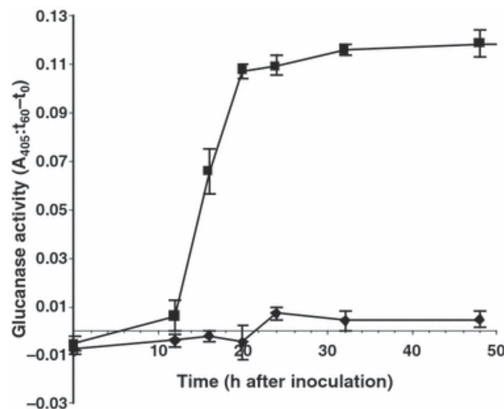

Fig. 1. Glucanase activity of the untransformed (♦) nd *exo-β-1,3-glucanase* (*exg1*) transformed yeast cultures (▪) over time. *p*-Nitrophenyl-glucoside was used as sub in the glucanase assays. Glucanase activity is represented by the mean values of th replicate reactions, and the standard deviaions are plotted as vertical bars

Inhibition of *Colletotrichum lupini* and *Botrytis cinerea* mycelial growth by glucanase filtrates

The untransformed yeast culture filtrate showed extremely low glucanase activity (ΔA_{405} = 0.005), while the *exg1* transformed yeast culture filtrate had high glucanase activity (ΔA_{405} = 0.108). Two days after inoculation, all cultures exhibited germination of the spores at all levels of spore concentrations. The *C. lupini* and *B. cinerea* mycelial dry weights were determined 5 days after inoculation (Table 1).

Table 1 Extracts from yeast transformed with *exg1* encoding an exo-β-1,3-glucanase reduce the growth of *Colletotrichum lupini* and *Botrytis cinerea* mycelial growth at a range of starting inocula

	Mycelial dry weight (g)[a]			
	Colletotrichum lupini		***Botrytis cinerea***	
Fungal inoculum (spores/ml)	**Extract from untransformed yeast**	**Extract from *exg1* transformed yeast**	**Extract from untransformed yeast**	**Extract from *exg1* transformed yeast**
2.5×10^3	0.131 ± 0.002	0.078 ± 0.001	0.134 ± 0.006	0.092 ± 0.012
5×10^3	0.274 ± 0.013	0.184 ± 0.013	0.153 ± 0.014	0.098 ± 0.005
10×10^3	0.296 ± 0.008	0.219 ± 0.007	0.167 ± 0.002	0.147 ± 0.008
20×10^3	0.325 ± 0.005	0.251 ± 0.012	0.188 ± 0.026	0.159 ± 0.005
40×10^3	0.343 ± 0.012	0.282 ± 0.014	0.206 ± 0.015	0.167 ± 0.012
60×10^3	0.355 ± 0.014	0.292 ± 0.013	nd[b]	nd
80×10^3	0.381 ± 0.016	0.309 ± 0.016	nd	nd

[a]Mycelial dry weight in grams after 5 days' growth is represented by the mean values of three replicate measurements and the standard deviations are indicated by the ± values; [b]nd, not determined.

At all spore concentrations, *C. lupini* mycelial growth was higher in the flasks inoculated with the untransformed yeast extract (controls) than in the flasks inoculated with the *exg1* transformed yeast extracts, i.e. extracts containing the glucanase enzyme (Table 1). The contents of the flasks were assayed for EXG1 activity at days 1, 3 and 5 after inoculation, and results showed that enzyme activity was retained (data not shown). The percentage inhibition of fungal growth (Table 2) was calculated for each spore concentration by subtracting the mycelial growth at day 5 for the spores exposed to the EXG1 extract from the growth of the same amount of spores mixed with the

control extract (calculated from data in Table 1). The percentage inhibition of *C. lupini* mycelial growth by the constant amount of glucanase enzyme (unconcentrated extract) increased with a decrease in the amount of fungal spores added to the starting inoculum, i.e. from 19% for 80×10^3 spores inoculated to 41% for 2.5×10^3 spores of *C. lupini* (Table 2, column 2).

Table 2 Inhibition of *Colletotrichum lupini* spore germination and mycelial growth by yeast exo-β-1,3-glucanase (EXG1) extracts

Spores/ml	Percentage inhibition[a,e]	Percentage inhibition[b,e]	Percentage inhibition[c,e]	Percentage inhibition[d,e]
2.5×10^3	41 ± 1.9	nd	41 ± 1.9	15 ± 1.3
5×10^3	33 ± 1.7	38 ± 1.5	25 ± 1.9	14 ± 1.9
10×10^3	26 ± 3.3	26 ± 3.8	22 ± 6.17	3 ± 4.2
20×10^3	23 ± 2.6	23 ± 2.4	17 ± 2.6	6 ± 6.8
40×10^3	18 ± 5.9	18 ± 6.2	20 ± 3.2	7 ± 6.7
60×10^3	18 ± 3.1	18 ± 2.9	nd	nd
80×10^3	19 ± 7.5	20 ± 7.1	nd	nd

Percentage inhibition after 5 days is represented by the mean values of three replicate measurements, and the standard deviations are indicated by the ± values. nd, not determined.

[a]Unconcentrated EXG1 extract; [b]unconcentrated EXG1 extract, repeat experiment of [a]; [c]ammonium sulphate-concentrated EXG1 extracts; [d]ammonium sulphate-concentrated EXG1 extracts added after spore germination; [e]percentage spore inhibition is represented by the mean values of three replicates.

In a repeat experiment, the percentage inhibition of *C. lupini* mycelial growth by the constant amount of glucanase enzyme (unconcentrated extract) increased with a decrease in the amount of fungal spores added to the starting inoculum, i.e. from 20% for 80×10^3 spores inoculated to 38% for 5×10^3 spores of *C. lupini* (Table 2, column 3).

The glucanase assay results of the pre- and postammonium sulphate fractions showed a 2.5-fold increase in the glucanase activity after ammonium sulphate precipitation of the crude culture filtrate of the *exg1* transformed yeast cells (data not shown). However, in a third experiment, the ammonium sulphate concentrated extract yielded similar results, with the inhibition ranging from 20% for 40×10^3 spores inoculated to 41% for 2.5×10^3 spores of *C. lupini* (Table 2, column 4).

A fourth experiment was performed using the *C. lupini* fungal spores where the enzyme extract was added to the spores after they had germinated (Table 2, column 5). However, inhibition of fungal mycelial growth was much lower (maximum of 15% inhibition of dry weight when added to 2.5×10^3 spores/ml) than when EXG1 extracts were added prior to germination.

Discussion

Results indicated that the percentage inhibition of *C. lupini* mycelial growth by a constant amount of unconcentrated glucanase enzyme extract remained constant (Table 2, column 2 and Table 2, column 3: from 19% for 80 × 10^3 spores inoculated to 41% for 2.5 × 10^3 spores inoculated and from 20% for 80 × 10^3 spores inoculated to 38% for 5 × 10^3 spores inoculated, respectively). The proposed reason for these results is that the glucanase enzyme will attack the β-glucan-containing fungal spores and developing mycelia, killing off some of the developing fungal material, resulting in the reduction of mycelial growth. At the lowest spore concentrations (5 × 10^3 spores/ml), growth inhibition by EXG1 was substantial (38%).

Furthermore, the ammonium sulphate-concentrated glucanase enzyme extract yielded similar results with no increase in percentage inhibition being observed despite a more concentrated enzyme extract being added to the fungal spore suspensions (Table 2, column 3: from 20% for 40 × 10^3 spores inoculated to 41% for 2.5 × 10^3 spores inoculated).Table 2 shows that the glucanase exerts its greatest effect on the fungal spores or at the early stage of germination, and following germination, the glucanase is less effective in attacking the mycelia.

Lampbrechts et al. (1997) reported 100% growth inhibition of a grapevine isolate of *B. cinerea* by *exg1* transformed yeast extracts. An experiment to find the effect of the glucanase enzyme on *B. cinerea* spore germination and mycelial growth was thus performed in order to investigate whether similar results could be obtained. The *B. cinerea* study yielded similar results to those obtained for the *C. lupini* inhibition studies. As observed for *C. lupini*, at all spore concentrations, *B. cinerea* mycelial growth was higher in the flasks inoculated with the untransformed yeast extract than in the flasks inoculated with the *exg1* transformed yeast extracts, i.e. extracts containing the glucanase enzyme (Table 1). The percentage inhibition of *B. cinerea* mycelial growth by the constant amount of glucanase enzyme increased with a decrease in the amount of fungal spores added to the starting inoculum, i.e. from 19% for 40 × 10^3 spores inoculated to 32% for 2.5 × 10^3 spores of *B. cinerea* (calculated from data in Table 1). These results are similar to those obtained for the inhibition of *C. lupini* mycelial growth by *exg1* transformed yeast culture filtrate (Table 1) when similar concentrations of the enzyme was added to the spores. Results obtained in this study with *B. cinerea* did not correlate with the data of Pretorius (personal communication) and Lampbrechts et al. (1997), who found 100% inhibition of *B. cinerea* growth by *exg1* yeast extracts. This may be attributed to the differences in the properties of the fungal isolates, as the *B. cinerea* isolate used in this study was from pear, whereas the *B. cinerea* isolate used by Van Rensburg et al. (1997) was from grape.

Certain fungi that are sensitive to ß-1,3-glucanases and chitinases become resistant to these enzymes after several hours of exposure to these enzymes (Ludwig and Boller, 1990). This is an indication that the fungi are able to adapt to the presence of these enzymes. A similar phenomenon could have been observed in this study. The EXG1 extract was added to the fungal spores and left to incubate for 5 days, during which enzyme activity was still retained, and could have resulted in sufficient time for the fungus to adapt to the presence of the enzyme.

Another possible explanation for the levels of inhibition obtained in this study not exceeding 41% could be that the fungus secreted an inhibitor of the EXG1. It is known that fungi can secrete proteins that have the ability to inhibit the action of plant enzymes, such as the ß-1,3-glucanases (Ham et al., 1997). For biotechnological applications, it is of great importance to determine whether the target pathogen produces an inhibitor of the proposed antifungal protein to be expressed in the transgenic plant.

It can be concluded from the presented data that exposure of fungal spores of *C. lupini* and *B. cinerea* to yeast exo-glucanase results in the inhibition of spore germination and mycelial growth. Expression of the yeast *exg1* gene in transgenic plants at the site of spore germination of pathogenic fungi, such as *C. lupini* and *B. cinerea*, for example, the leaf surfaces of lupin plants or the immature flowers or skin of pear fruit; has promise as a biotechnological tool for enhanced protection of crop plants against phytopathogenic fungi.

References

Ait-Lahsen H, Soler A, Rey M, de la Cruz J, Monte E, Llobell A. (2001) An antifungal exo-α-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. *Appl Environ Microb* **67**:5833–5839.

Broglie KE, Gaynor JJ, Broglie RM. (1986) Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. *Proc Natl Acad Sci USA* **83**: 6820–6824.

Chang MM, Culley DE, Hadiwiger LA. (1993) Nucleotide sequence of a pea (*Pisum sativum*) β-1,3-glucanase gene. *Plant Physiol* **101**:1121–1122.

Edington BV, Lamb CJ, Dixon RA. (1991) cDNA cloning and characterization of a putative 1,3-ß-glucanase transcript induced by fungal elicitor in bean cell suspension cultures. *Plant Mol Biol* **16**:81–94.

Frick MM, Huang TY, Cheng KJ et al.*An isolated polypeptide (I) possessing beta-(1,3)-exoglucanase activity for improvement of plant resistance to fungal pathogens and to promote growth. Accession number AAE 10698, GeneSeq™ on STN®*. Karlsruhe, Germany, Derwent World Patent Index, 2001.

Ham KS, Kauffmann S, Albersheim P, Darvill AG(1991) Host-Pathogen Interactions XXXIX. A soybean PR protein with ß-1,3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal walls. *Molec Plant-Micr Interact* **4**:545–552.

Ham KS, Wu SC, Darvill AG, Albersheim P. (1997) Fungal pathogens secrete an inhibitor protein that distinguishes isoforms of plant pathogenesis-related endo-ß-1,3-glucanases. *Plant J* **11**: 169–179.

Keen NT. (1992) The molecular biology of disease resistance. *Plant Mol Biol* **19**:109–122.

Koch SH, Ghebremariam DS, Swart WJ. (2002) Susceptibility of lupin cultivars to South African isolates of *Colletotrichum gloeosporioides* associated with lupin anthracnose. *Afr Plant Prot* **8**:51–56.

Lamb CJ, Lawton MA, Dron M, Dixon RA. (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56**:215–224.

Lampbrechts MG, Vivier MA, Pretorius IS. (1997) *From the vineyard to the winebarrel: MUCI, the yeast's answer to a changing environment*. Grahamstown, South Africa, 2nd Conference on Biotechnology and Development in Southern Africa.

Larriba G, Andaluz E, Cueva R, Basco RD. (1995) Molecular biology of yeast exoglucanases. *FEMS Microbiol Lett* **125**:121–126.

Latunde-Dada A, Lucas JA. (2001) The plant defense activator acibenzolar-S-methyl primes cowpea [*Vigna unguilata* (L.) Walp] seedlings for rapid induction of resistance. *Physiol Molec Plant Pathol* **58**:199–208.

Lawton MA, Lamb CJ. (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. *Molec Cell Biol* **7**:335–341.

Lennox CL, Spotts RA, Cervantes LA. (2003) Populations of *Botrytis cinerea* and *Penicillium* spp. on pear fruit, and in orchards and packinghouses, and their relationship to postharvest decay. *Plant Dis* **87**:639–644.

Lotter HC, Berger DK. (2005) Anthracnose of lupins in South Africa in caused by *Colletotrichum lupini* var. *setosum*. *Australas Plant Pathol* **34**:385–392.

Ludwig A, Boller T. (1990) A method for the study of fungal growth inhibition by plant proteins. *FEMS Microbiol Lett* **69**:61–66.

Muthukumar G, Suhng SH, Magee PT, Jewell RD, Primerano DA. (1993) The *Saccharomyces cerevisiae* SPR1 gene encodes a sporulation-specific exo-1,3-beta-glucanase which contributes to ascospore thermoresistance. *J Bacteriol* **175**:386–394.

Nebreda AR, Villa TG, Villanueva JR, del Rey F. (1986) Cloning of genes related to exo-beta-glucanase production in *Saccharomyces cerevisiae*: characterization of an exo-beta-glucanase structural gene. *Gene* **47**:245–259.

Schlumbaum A, Mauch F, Vögeli U, Boller T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**:365–367.

Stinzi A, Heitz T, Prasad V et al. (1993) Plant pathogenesis-related proteins and their role in defense against pathogens. *Biochimie* **75**:687–706.

Tamano K, Satoh Y, Ishii T et al. (2007) The β-1,3-exoglucanase gene *exgA* (*exg1*) of *Aspergillus oryzae* is required to catabolize extracellular glucan, and is induced in

growth on a solid surface. *Biosci Biotech Biochem* **71**:926–934.

Van den Elzen PJM, Jongedijk E, Melchers LS, Cornelissen BJC. (1993) Virus and fungal resistance: from laboratory to field. *Phil Trans Royal Soc London* **342**:271–278.

Van Rensburg P, van Zyl WH, Pretorius IS. (1997) Over-expression of the *Saccharomyces cerevisiae* exo-β-1,3-glucanase gene together with the *Bacillus subtilis* endo-β-1,3-1,4-glucanase gene and the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase gene in yeast. *J Biotechnol* **55**:43–53.

Vigers AJ, Roberts WK, Selitrennikoff CP. (1991) A new family of antifungal proteins. *Molec Plant-Micr Interact* **4**:315–323.

Yoshikawa M, Tsuda M, Takeuchi Y. (1993) Resistance to fungal diseases in transgenic tobacco plants expressing phytoalexin elicitor-releasing factor, ß-1,3-glucanase, from soybean. *Naturwissenschaften* **80**:417–420.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ. (1994) Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. *Bio/Technology* **12**:807–812.