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Induction of *Arabidopsis thaliana* Chitinase by Ethylene and Elicitor Treatment

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에틸렌및 Elicitor 처리에 의한 아기장대풀의 키틴 가수분해 효소 유도

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Chitinases and β-1,3-glucanases are believed to be important in defending plants against pathogens. Here, we investigated the expression of chitinase(s) in *Arabidopsis thaliana* cell suspension culture system in response to ethephon (2-chloroethyl phosphonic acid) which produces ethylene or a microbial elicitor, a bacterial pectin-degrading enzyme, α-1,4-endopolygalacturonic acid lyase (PGA lyase), treatment. Chitinase activity was measured either by radiochemical assay using ^{3}H-labeled regenerated chitin as substrate or Western blot analysis using antibody raised against tobacco chitinase(s). With 1 mg/mL of ethephon or 100 m units/mL of elicitor treatment, maximum levels of activity were reached after 48 h. We also investigated distribution of chitinase activity in seedlings, leaves, and roots of *A. thaliana* and found that roots have the highest chitinase activity.

Key words: cell suspension culture, hydrolytic enzymes, plant-microbe interaction

Plant defense against attack by pathogens is a very complex mechanism, the details of which we are just beginning to understand. This process requires gene expression in the pathogen as well as in the plant. Plants defend themselves from attempted infection by elaborating a number of diverse defense responses (Bell, 1981: Hahlbrook and Scheel, 1987). This array of defenses includes several inducible responses that are activated by microorganisms or by specific molecules (elicitors) derived from microorganisms and plant cell walls (Darvill and Albersheim, 1984). These inducible defense responses include the synthesis and accumulation of polyphenolic lignins, hydroxyproline-rich glycoproteins, hydrolytic enzymes such as chitinase and β-1,3-glucanase, and antimicrobial compounds called phytoalexins (Collinge and Slusarenko, 1987).

During the past decade, chitinases (EC3.2.1.14) (Bol et al., 1990: Bowels, 1990: Linthorst, 1991) and β-1,3-glucanases (EC3.2.1.37) (Mauch et al., 1988: Schlumbaum et al., 1986: Sela-Buurlage et al., 1993) have attracted much attention since their substrate, chitin, is a major cell wall component of many plant-pathogenic fungi. Furthermore, in vitro assays have shown that some chitinases are able to significantly inhibit the growth of various fungi, either alone or in combination with β-1,3-glucanases, with the inhibition being caused by lysis of growing hyphal tips through degradation of the cell wall (Arido et al., 1992: Mauch et al., 1988: Ordentlich et al., 1988). Moreover, recent experiments with the expression of enhanced chitinase levels in transgenic plants confirm that these enzymes also have an antifungal potential in vivo (Benhamou et al., 1993: Broglie et al., 1991). In other plant-pathogen interactions, however, enhanced chitinase levels have been reported to have no apparent effect in the infection (Neuhaus et al., 1991: Nielsen et al., 1993). Recently, chitinase has been observed to accumulate during normal development in healthy tissues (Lotan et al., 1989: Neale et al. 1990).

Arabidopsis thaliana (L.) Heynh provides an excellent experimental plant system for molecular genetics because of its remarkably small genome size, near absence of dispersed middle repetitive DNA, and short life cycle. *A. thaliana* has

recently become an interesting model host for the investigation of problems in plant pathology (Mauch-Mani and Slusarenko, 1993). As a first step in developing *A. thaliana* as a model system for studying plant-pathogen interactions, a suspension culture system was established in which putative defense responses would be induced by elicitor treatments. One major advantage of using elicitors in combination with cell cultures is that sterile, undamaged cells are treated with nonliving material, thus ensuring that the measured responses are solely due to reaction of the plant cells. Also in vitro cultures provide a homogeneous and reproducible system for induction experiments. They have been used to show the regulation of chitinase by elicitors and plant hormones (Shinshi et al., 1987), with cell suspension cultures in particular facilitating the collection and characterization of extracellular chitinases (Kragh et al., 1991). In this report, it is demonstrated that chitinases previously associated with disease resistance in other plants are induced in suspension-cultured *Arabidopsis* cells treated with the "stress"-associated hormone ethylene. and the fungal elicitor α-1,4-endopolygalacturonic acid lyase (PGA lyase).

MATERIALS AND METHODS

Materials

Chemicals used for electrophoresis and protein molecular weight markers were purchased from Bio-Rad (Mississauga, Ontario, Canada). Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO).

Plant Materials and Tissue Culture

Aseptically grown *Arabidopsis thaliana* L. Co-O and cell suspension cultures prepared from those leaves were used as plant material. Leaf sections (approximately 2-3 horizontal cuttings) were cultured on Gamborg's B5 medium (Gamborg et al., 1977) (Gibco Laboratories, Grand island, NY) containing 1 mg/L of 2,4-dichlorophenoxyacetic acid solidified with 0.8% agar. Callus cultures were maintained on l-B5 plates in the dark at 24°C and subcultured every 3-4 weeks. Pieces of callus were placed in liquid l-B5 medium and maintained in the dark at 24°C on a rotary shaker at 110 rpm. Suspension cultures were started by transferring 5 ml of culture to 40 ml of fresh medium. Larger cultures were started by transferring 40 ml of a 7-day-old culture into 400 ml of fresh medium. The first experiments were conducted 8 weeks after the suspension cultures were initiated. The calli were collected in the culture flask and MS medium with vitamines and 1 μg/ml 2,4D was added. After 4 weeks, suspension cultures were well established. Thereafter, the suspension cultures were maintained by subculturing at one week interval. *Arabidopsis* seedlings were grown in a greenhouse (approximately 21°C) with a 16 h photoperiod or in a growth chamber at 20°C with a 12 h photoperiod and a light intensity of 100 $\mu E/m^2$.sec.

Ethylene and PGA lyase treatment

For ethylene treatments, plants or 4-day-old suspension cultures washed and resuspended in MS basal medium were added with 1 mg/mL solution of ethephon (2-chloroethylphosphonuc acid: Sigma) which decomposes into ethylene and phosphonic acid. The samples were then enclosed in plastic bags. Or samples were floated in small sealed and covered Petri dishes on the same solution of ethephon or water at 24°C with 16 h light/8 h darkness and then assayed for chitinase activity.

Crude PGA lyase preparations were obtained by dialyzing culture filtration of *Erwinia carotovor*a pv. carotovora (ATCC No. 495) grown on pectin-containing medium against 5 mM Tris-HCl (pH 8.5) containing 1 mM $CaCl_2$. Previous studies have demonstrated that the major, if not only, elicitors in this preparation are two isozymes of PGA lyase (Davis et al., 1984). PGA lyase activity was measured spectrometrically and preparations were filter-sterilized before being added to cultures. Experimental treatments were conducted by continuously exposing 6- or 7-day-old 400-ml cultures to elicitors as previously described for parsley cell cultures (Davis and Hahlbrook, 1987). Cells were harvested by filtration, frozen with liquid nitrogen, and stored at -70°C. All experiments were repeated at least twice: the data presented are from representative experiments unless otherwise indicated.

Chitinase Assay

The assay for chitinase utilized ^{3}H-labeled regenerated chitin as substrate, activity being measured by the release of ^{3}H-labeled product into the supernatant (Molano et al., 1977). ^{3}H-labeled chitin was produced by the acetylation of chitosan with ^{3}H-acetic anhydride (500 mCi/mmol: Amersham), which resulted in a chitin sample of specific activity 343 cpm/ μg. The assay mixture consisted of 1.05 mg

of ^{3}H-chitin, 20 mM sodium phosphate (pH 6.5), and desalted crude enzyme in a final volume of 250 μl. The reaction was stopped by addition of 250 μl of 10% (w/v) TCA after one 1 h incubation at 30°C. Subsequently, the undegraded chitin was pelleted at 1,000 g and radioactivity determined by liquid scintillation counting (LKB-WALLAC 1410) in 300 μl of the supernatant. Duplicate assay controls (minus enzyme) were included with each assay and their mean count was subtracted from all readings. Different enzyme dilutions were used for replicate experiments. Chitinase activity is described as the amount of enzyme liberating 1 DPM per second of reaction time (S^{-1}) of radioactive fragments from ^{3}H-labeled chitin under the given assay conditions, at infinite enzyme dilution. Protein content of tissue extracts was determined by the method of Bradford (1976).

Preparation of Protein Extracts and Immunoblotting

Sterile and ethephon-treated *A. thaliana* cell suspension cultures were harvested and ground to powder with a pestle in a prechilled mortar in liquid nitrogen. Chilled extraction buffer (84 mM citric acid, 30 mM Na2HPO4, at pH 2.8, containing 14 mM 2-mercaptoehanol and 6 mM ascorbic acid) were added to the powder (2 mL/g fresh weight tissue). The phosphate-citrate buffer was also used for preparation of protein extracts from vegetative tissues from *A. thaliana*. Extraction was carried out at 4°C for 60 min with continuous slow stirring. The buffer extracts were then centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Immunoblots were prepared essentially according to the procedure of Towbin et al. (1979). SDS-PAGE was performed by the method of Laemmli (1970) using 10% (w/v) polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes, blocked in PBS buffer (1XPBS=0.05 M Na phosphate, pH 7.5, 0.15 M NaCl) containing 0.1% Tween and 3% w/v nonfat dry milk, and incubated for 2 h at room temperature with the antiserum. Primary antibodies were obtained from Dr. Bernard Fritig (antiserum to tobacco chitinase, 1:1,000 dilution for immunoblot). Immunoblotting was done by using goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma). Horseradish peroxidase colorimetric reactions were performed by using dichloronaphtol as a substrate.

RESULTS

Induction of Chitinase with Ethephon Treatment

The expression of a number of plant chitinases has been induced by ethylene (Broglie et al., 1986; Memelink et al., 1990). Ethylene increases in plants are also associated with stress, including wounding and pathogen infection. Addition of

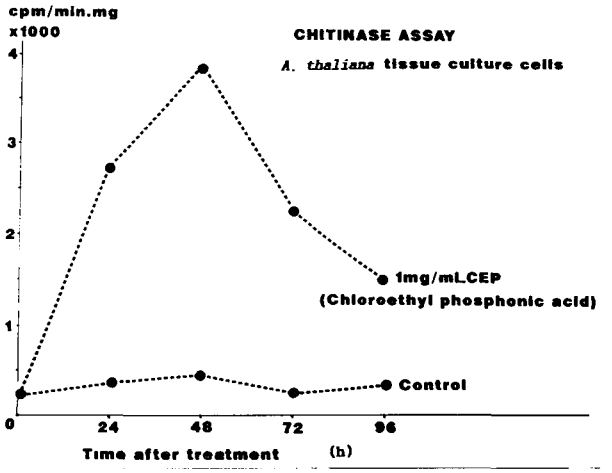

Figure 1. Chitinase induction analysis of *Arabidopsis thaliana* cell suspension culture upon a treatment of 1 mg/mL 2-chloroethyl phosphonic acid (CEP).

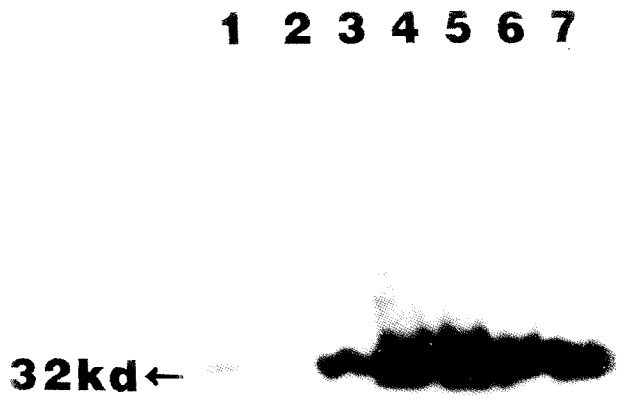

Figure 2. Western blot analysis for chitinase from *Arabidopsis thaliana* cell suspension culture. Antiserum to tobacco chitinase was used as antiserum. Lane 1, total cell extract from tobacco plants: Lane 2, empty: Lane 3, *Arabidopsis thaliana* (Co-O) cell suspension culture without CEP treatment: Lanes 4-7, 24, 48, 72 or 96 h, respectively, after CEP treatment.

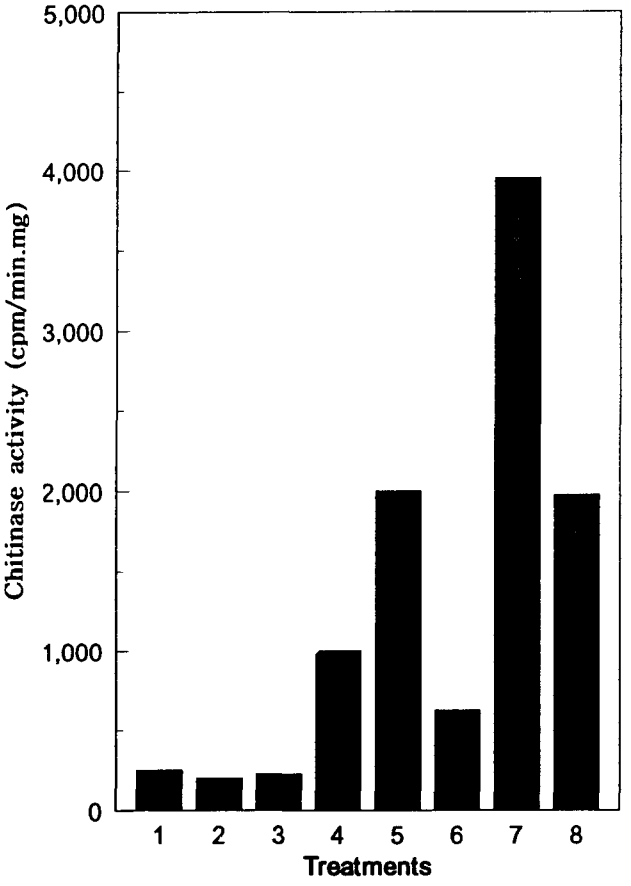

Figure 3. Distribution of chitinase in the seeds, seedlings, leaves, and roots of *Arabidopsis thaliana* compared to ethylene and PGA lyase treatments in cell suspension cultures. Results represent mean number of three replicates. Lane 1, seeds: 2, imbibed seeds: 3, seedlings (4 or 5 days after germination): 4, leaves (4 weeks after germination): 5, roots (4 weeks after germination): 6, cell suspension culture without treatment: 7, with ethephon treatment: 8, with PGA lyase treatment.

exogenous ethylene is possible through the use of ethylene gas directly or ethylene releasing compounds such as 2-chloroethylphosphonic acid (CEP). To test whether the *Arabidopsis* chitinase responds to ethylene, *Arabidopsis* plants were treated with 1 mg/mL ethephon solution. Control plants were sprayed with water. After 12, 24, 48, 72 or 96 h, respectively, cells were harvested and assayed for chitinase activity The chitinase activity reached maximum level after 48 h (Figure 1).

In Western blot analysis, the antiserum detected 2 or 3 proteins from tobacco plants, however, only one protein of approximately 32 kd was detected in *Arabidopsis thaliana* cell suspension cultures (Figure 2). The result of Western blot analysis coincided with that of chitinase induction assay by the radiochemical method. That is, after 48 h incubation in liquid medium containing 1 mg/mL ethephon, callus in suspension cultures had the highest chitinase specific activity (more than 20-fold induction of the basal level: lane 5 of Figure 2).

Kinetics of Chitinase Induction by Ethylene and Fungal Elicitor

Previous studies have demonstrated that phenylpropanoid biosynthesis is often induced in plant cell cultures treated with elicitors (Ebel et al., 1984: Cramer et al., 1985). To determine whether such is the case for *Arabidopsis*, Co-O cell cultures were treated with the microbial elicitor, the bacterial pectin-degrading enzyme PGA lyase. Cell cultures treated with crude PGA lyase preparations had increased levels of chitinase activity. The analyses of the induction kinetics of chitinase activities in cell cultures treated with 50 or 100 m units/mL of PGA lyase is shown in Figure 3. As a control the cell cultures were treated with heat-inactivated PGA lyase preparations.

Concominant with the induction of enzymes involved in phenylpropanoid biosynthesis, elicitor-treated cells became brown and showed a decreased growth rate, as determined by measuring fresh weights of cells 48 h after treatment (data not shown). Induction kinetics of chitinase enzyme activity was similar in elicitor-treated cell suspension cultures of *A. thaliana* (Figures 1, 2 and 3), maximum levels of activity being reached after 48 hrs of exposure to ethylene and elicitor. In both experiments, the hydrolase activity began to increase between 4 and 8 h after addition of ethephon or elicitor, and remained high from 24 to 48 h.

DISCUSSION

Ethylene induction of chitinase was the first indication of the possible defense role of this hydrolase (Abeles, 1970), although the influence of other plant hormones during chitinase induction (Shinshi et al., 1987) and ethylene-independent pathogen-mediated chitinase induction (Mauch et al., 1984), suggest the involvement of other signals in a complex regulatory pathway.

Abundant expression of the chitinase(s) in roots (Figure 3) is consistent with the role of chitinases in plant defense. Roots are vulnerable to infecrtion by numerous soil-borne pathogens. Opportunities for invasion by these pathogens are created by the constant wounding young roots suffer as they move

through the soil and by their lack of lignified structural barriers. Thus, as a defense against invasion, healthy roots may constitutively express high levels of hydrolytic enzymes such as chitinases and β-1,3-glucanases (Shinshi et al., 1987; Memclink et al., 1990). Lotan et al. (1989) suggest that genes involved in the normal physiology could have evolved to have an additional function in plant defense. Future experiments will address the functional role(s) of chitinase(s) by characterising individual chitinase or its gene.

In summary, the results of this study demonstrated that the response of *Arabidopsis* cell cultures to elicitor treatment was similar to that observed in other plant cell culture systems (Davis and Hahlbrook, 1987). This would suggest that the regulation of putative defense responses of *Arabidopsis* is similar, if not identical, to the regulation of defense genes in other plant species.

적 요

키틴 가수분해 효소와 베타-1,3-글루카네이즈는 식물체의 주요 방어효소로 여겨지고 있다. 본 논문에서는 아기장대풀의 잎조직으로부터 고정된 현탁배양주를 만들고 이어서 에틸렌과 유인제 (elicitor) 처리에 의한 키틴 가수분해 효소 유도양상을 분석하였다. 키틴 가수분해 효소활성은 ^{3}H으로 표지된 키토산을 기질로 한 radiochemical 분석방법이나 담배식물체의 키틴 가수분해 효소를 대상으로 얻어진 항체를 이용하여 Western 분석방법을 사용하였다. 이 결과 에틸렌과 유인제 처리 공히 48시간후에 가장 활성이 높게 나타남을 관찰하였다. 또한 씨앗, 새싹, 식물체의 잎, 뿌리 등에서의 활성을 조사하여 뿌리에서 키틴 가수분해 효소 활성이 높음을 확인하였다.

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REFERENCES

Abeles FB (1990) Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol **60**: 129-134

Arido M, Ludwig A, Boller T, Bonfante P (1992) Inhibition of fungal growth by plant chitinases and β-1,3-glucanases. Protoplasma **171**: 34-43

Bell AA (1981) Biochemical mechanisms of disease resistance. Annu Rev Plant Physiol **32**: 21-81

Benhamou N, Broglie K, Chet I, Broglie R (1993) Cytology of infection of 35S-bean chitinase transgenic canola plants by *Rhizoctonia solani*: cytochemical aspects of chitin breakdown in vivo. Plant J **4**: 295-305

Bol JF, Linthorst HJ, Cornelissen BJ (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopath **28**: 113-138

Bowles DJ (1990) Defense-related proteins in higher plants. Annu Rev Biochem **59**: 873-907

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal Biochem **72**: 248-254

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254**: 1194-1197

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 USA **83**: 6820-6824

Collinge DB, Slusarenko AJ (1987) Plant gene expression in response to pathogens. Plant Mol biol **9**: 389-410

Cramer CL, Bell JN, Ryder TB, Bailey JA, Schuch W, Bolwell GP, Robbins MP, Dixon RA, Lamb CJ (1985) Coordinated synthesis of phytoalexin biosynthetic enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.). EMBO **4**: 285-289

Davis KR, Lyon GD, Darvill AG, Albersheim P (1984) Host-pathogen interactions. XXV Plant cell wall fragments. Plant Physiol **74**: 52-60

Davis KR, Hahlbrock K (1987) Induction of defense responses in cultured parsley cells by plant cell wall fragments. Plant Physiol **85**: 1286-1290

Darvill AG, Albersheim P (1984) Phytoalexins and their elicitors-a defense against microbial infection in plants. Annu Rev Plant Physiol **35**: 243-275

Ebel J, Schmidt WE, Loyal R (1984) Phytoalexin synthesis in soybean cells: Elicitor induction of phenylalanine ammonia-lyase and chalcone synthase mRNAs and correlation with phytoalexin accumulation. Arch Biochem Biophys **232**: 240-248

Gamborg OL, Shyluk JP, Brar DS, Constabel F (1977) Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci Lett **10**: 67-74

Hahlbrock K, Scheel D (1987) Biochemical responses of plants to pathogens. Plant Mol Biol **9**: 389-410

Kragh KM, Jacobsen S, Mikkelsen JD, Nielson KA (1991) Purification and characterization of three chitinases and one β-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci **76**: 65-77

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci **10**: 123-150

Lotan T, Ori N, Fluhr R (1989) Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell **1**: 881-887

Mauch F, Hadwinger LA, Boller T (1984) Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol **76**: 607-611

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88**: 936-942

Mauch-Mani B, Slusarenko AJ (1993) *Arabidopsis* as a model host for studying plant-pathogen interactions. Trends Microbiol **1**: 265-270

Memelink J, Linthorst HJM, Schilperoot RA, Hoge JHC (1990) Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol Biol **14**: 119-126

Molano J, Duran A, Cabib A (1977) A rapid and sensitive assay for chitinase using tritiated chitin. Anals Biochem **83**: 648-656

Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Ry Meeks-Wagner D, Peacock WJ, Dennis ES (1990) Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell **2**: 673-684

Neuhaus J-M, Ahl-Goy P, Hinz U, Flores S, Meins F Jr (1991) High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris*. Susceptibility of transgenic plants to *Cercospora nicotianae* infection. Plant Mol Biol **16**: 141-151

Nielsen KK, mikkelsen JD, Kragh KM, Bojsen K (1993) An acidic class III chitinase in sugar beet: induction by *Cercospora belticola*, characterization and expression in transgenic tobacco plants. Mol Plant-Microbe Interact **6**: 495-506

Ordentich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium* rolfsii. Phytopathology **78**: 84-88

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

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, van den Elzen PMJ, Cornelissen BJC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol **101**: 857-863

Shinshi H, Mohnen D, Meins FJr (1987) Regulation of a plant pathogenesis-related enzyme: inhibition of chitinases and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA **84**: 89-93

Towbin H, Staelhelin T, Gordon T (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76**: 4350-4354

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