CHITINASE GENES AND GENE PRODUCTS IN PINES*

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Abstract. Chitinases are hydrolytic enzymes that are thought to play an important role in plant defense against pathogenic fungi, and potentially in guiding normal plant growth and development. We are investigating chitinase genes and gene products at the nucleic acid and protein levels. At the nucleic acid level, we have previously cloned chitinase sequences from pine using PCR, and used one of the amplification products as a probe to screen a genomic library. Based on preliminary sequence data, the genes are more similar to extracellular chitinases from herbaceous plants than to vacuolar forms. At the protein level, we found that chitosan elicits increases in both extracellular and intracellular chitinase activities in suspension cultured cells. The suspension cultures should prove useful for bulk purification of extracellular and vacuolar chitinases.

INTRODUCTION

We are studying the cellular and molecular basis for defense mechanisms in trees. By gaining insight into these defense processes, we hope to better understand tree-microbe interactions, both symbiotic and pathogenic, at the biochemical level. Chitinase activity serves as a useful marker for studying defense responses, since rapid expression of chitinase genes is often associated with disease resistance (Collinge et al. 1993). it is of interest to note that transgenic plants containing chitinase gene constructs had increased resistance to a chitin-containing root pathogen; in the same plants, the colonization of roots by a chitin-containing mycorrhizal fungus was not diminished (Vierheilig et al. 1993).

The location, and kinetics of accumulation, of chitinase gene products have profound effects on their efficacy as defensive enzymes. Chitinases are encoded by multigene families in trees (Parsons et al. 1989; Davis et al. 1991), and the regulation of individual chitinase genes can occur at many different levels. For example, mRNA and protein levels can be regulated by either synthesis or degradation. The expression of specific genes might occur in particular organs or in specific cell types. The stimulus for turning the gene "on" might be local (near a site of cellular injury) or systemic (distant from a site of injury). The protein, in turn, can be intracellular (targeted to the vacuole) or extracellular. As a step toward characterizing chitinase regulation in pines, we report in this paper that both intracellular and extracellular chitinase accumulation can be detected in pine suspension cultures, and that chitinase activity is stimulated by treatment with chitosan, a component of fungal cell walls.

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MATERIALS AND METHODS

Suspension cultured cells were initiated from callus of a single loblolly pine seedling (family 10-38), and maintained by weekly transfers to fresh medium (Lesney, 1989). The time of transfer to fresh medium was designated "day 0." Chitosan was prepared as previously reported (Lesney, 1989) and then dispensed into the suspension (-50 ug/ml final concentration) on day 1 or day 6. Using this amount of chitosan, elicitation occurred, but little or no cellular death accompanied the treatment (Lesney, 1989). Treatments were replicated three times, with each flask representing a replicate.

Twenty-four hours after chitosan addition (day 2 and day 7), cells and medium of chitosan-treated and untreated cultures were harvested separately. Cells were harvested by filtering cultures through filter paper under gentle vacuum, and were then frozen and ground in liquid nitrogen using a mortar and pestle and resuspended in 3-5 volumes of 50 mM sodium acetate (pH 5.0) containing 1% polyvinylpyrrolidone. After centrifugation (5,000 x g for 10 min), the supernatant was transferred to a fresh tube and used for determination of protein concentration (Brown et al. 1989) and for chitinase activity (Cabib 1988). The culture medium was used directly in the assays.

RESULTS AND DISCUSSION

Chitinase genes were previously cloned from pines using PCR and degenerate oligonucleotide primers (J.D., unpublished data). Partial sequence analysis indicates the pine chitinase genes we cloned are more similar in sequence to extracellular chitinases than to vacuolar chitinases. In addition, the chitinase genes we have cloned appear to belong to a medium-sized gene family (defining the gene family as consisting of members >90% identical in nucleotide sequence). We were interested in testing whether cell suspension cultures would be useful for eliciting chitinase genes, and whether suspensions might provide a convenient source of enzyme material.

For clarity of presentation, chitinase specific activity values (that is, chitinase activity / unit protein / unit time) were normalized to the specific activity present in the untreated cells on day 2 (Table 1). As such, the values represent the relative chitinase activities in the various treatments.

Chitinase activity was observed in both intracellular and extracellular fractions of untreated cultures. This is consistent with the expectation that a certain level of "constitutive" chitinase gene expression occurs in suspension cultures (Esaka et al. 1990). Given the likelihood that chitinase subcellular targeting mechanisms are similar in gymnosperms and angiosperms (Neuhaus et al. 1991), these data indicate that there is a minimum of two chitinase genes, one encoding a vacuolar enzyme and another encoding an extracellular enzyme, that are constitutively expressed in suspension cultures.

Table 1. Relative amount of chitinase activity in chitosan-treated and untreated loblolly pine suspension cultures, normalized to the specific activity of chitinase in day 2 cells. Probability of differences between chitosan treated and untreated suspensions being due to chance alone is indicated (*, $p < .05$; **, $p < .01$; ***, $p < .001$).

DAY 2 CELLS			DAY 7 CELLS		
Control	Chitosan		Control	Chitosan	
1.0	1.3	*	2.9	5.2	**
DAY 2 MEDIUM			**DAY 7 MEDIUM**		
Control	Chitosan		Control	Chitosan	
1.0	2.1	**	2.2	4.1	***

At day 7, constitutive levels of chitinase in untreated cultures had increased 2- to 3-fold over the levels found at day 2. This could reflect autoelicitation of chitinase activity by carbohydrates secreted into the medium by the cells (Lesney, 1989).

Chitosan treatment induced statistically significant increases of chitinase activity in the intracellular and extracellular compartments on both sample dates. The competence of cells to express chitinase in response to chitosan was similar (1.3 to 2.1-fold) in both day 2 and day 7 cells, but the highest chitinase activities were found in cultures treated with chitosan on day 7. This suggests that day 7 cultures are the Most desirable starting materials for bulk purification of both vacuolar and extracellular chitinase, which will be useful for antibody production.

In this paper we have described chitinase induction in loblolly pine cells in response to chitosan, a fungal cell wall component. Short-term objectives include identification of chitinase transcripts in cell suspensions. Longer-term objectives include development of a detailed understanding of the spatial and temporal expression of chitinase genes in specific tree-microbe interactions, and use of genetic transformation technology to experimentally manipulate chitinase gene expression.

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LITERATURE CITED

Brown, R.E., K.L Jarvis, and K.J. Hyland. 1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal. Biochem. 180: 136-139.

Cabib, E. 1988. Assay for chitinase using tritiated chitin. Meth. Enzymol. 161: 424-426.

Collinge D.B., K.M. Kragh, J.D. Mikkelsen, K.K. Neilsen, U. Rasmussen, and K. Vad. 1993. Plant chitinases. Plant J. 3: 31-40.

Davis, J.M., H.R.G. Clarke, H.D. Bradshaw Jr., and M.P. Gordon. 1991. Populus chitinase genes: structure, organization, and similarity of translated sequences to herbaceous plant chitinases. Plant Mol. Biol. 17: 631-639.

Esaka, M., K. Enoki, B. Kouchi, and T. Sasaki. 1990. Purification and characterization of abundant secreted protein in suspension-cultured pumpkin cells. Plant Physiol. 93: 1037-1041.

Lesney, M.S. 1989. Growth responses and lignin production in cell suspensions of *Pinus elliottii* 'elicited' by chitin, chitosan or mycelium of *Cronartium quercum* f.sp. *fusiforme*. Plant Cell Tissue Organ Culture 19: 23-31.

Neuhaus, J.-M., L. Sticher, F. Meins Jr., and T. Boller. 1991. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA 88: 10362-10366.

Parsons, T.J., H.D. Bradshaw Jr., and M.P. Gordon. 1989. Systemic accumulation of specific mRNAs in response to wounding in poplar trees. Proc. Natl. Acad. Sci. USA 86: 7895-7899.

Vierheilig **H., M.** Alt, J.-M. Neuhaus, T. Boller, and A. Wiemken. 1993. Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol. Plant-Microbe Interact. 6: 261-264.