

Research Note

Defense Response in Slash Pine: Chitosan Treatment Alters the Abundance of Specific mRNAs

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We used differential display to identify chitosan responsive cDNAs in slash pine cell cultures. Two clones that showed increased mRNA abundance had sequence similarity to genes with roles in major plant defense responses, clone 18 to cinnamic acid 4-hydroxylase, and clone 30 to chitinase.

Additional keywords: cytochrome P450, elicitation, *Pinus elliottii*.

Slash pine (*Pinus elliottii* var. *elliottii* Engelm.) is an ecologically and economically important species in the southern United States. Although genetic resistance to several pathogens has been inferred (Nelson et al. 1993), the underlying biochemistry of pine: pathogen interactions is poorly understood. Consequently, genes that condition disease resistance have not been identified in pines. Cell culture systems in conjunction with fungal cell wall elicitors have proven useful for inducing defense responses in pines and other gymnosperms (Campbell and Ellis 1992; Galliano et al. 1993; Lange et al. 1994; Lesney 1990; Popp et al., in press), and could facilitate identification and characterization of genes involved in a defense response in pines. Chitosan treated slash pine cultures showed browning of the culture medium, increased lignification, and increased activity of several enzymes including chitinase and glucanase (Lesney 1990; Popp 1993; Popp et al., in press).

The objective of the study reported here was to determine if the previously observed phenotypic changes in chitosan-treated cell cultures are associated with changes in mRNA abundance. Differential display of mRNA (Liang and Pardee 1992) was selected because it allowed screening of a large number of transcripts simultaneously, the product of the mRNA need not be known, and it allowed simultaneous selection of transcripts whose abundance appeared to increase, decrease, or remain unchanged. We used a clonal cell line in

this study because pines are genetically heterogeneous due to high levels of outcrossing (Friedman and Adams 1985). The use of seedling material could create "false positives" due to allelic differences instead of expression differences.

Cell cultures of slash pine genotype #52-56 were elicited for 24 h using chitosan (60 µg/ml final concentration, Lesney 1990). Total RNA was isolated from elicited and untreated cells using the method of Schneiderbauer et al. (1991) and then used for differential display of mRNA (Liang and Pardee 1992) according to the methods of Liang et al. (1993) with minor modifications. We used total RNA as substrate for the reverse transcriptase reaction, and substituted [α^{33} P]dATP for [α^{35} S]dATP. The University of Florida's Interdisciplinary Center for Biotechnology Research (UF/ICBR) DNA Synthesis Core supplied primers (Table 1).

A total of 63 differential products (44 from elicited RNA and 19 from control RNA) were identified on polyacrylamide gels (Fig. 1), and 39 were selected for reamplification and cloning. Reamplified samples that showed ambiguous products were discarded. Twenty three products (14 from elicited RNA and 9 from control RNA) reamplified as expected.

Table 1. Primers used for differential display

Name	Sequence 5' to 3'
Anchored primers	
71	TTTTTTTTTTTTTTTAA
72	TTTTTTTTTTTTTTTAG
73	TTTTTTTTTTTTTTTAC
74	TTTTTTTTTTTTTTTAT
75	TTTTTTTTTTTTTTTGA
76	TTTTTTTTTTTTTTTGG
77	TTTTTTTTTTTTTTTGC
78	TTTTTTTTTTTTTTTGT
79	TTTTTTTTTTTTTTTCA
80	TTTTTTTTTTTTTTTCG
81	TTTTTTTTTTTTTTTCC
82	TTTTTTTTTTTTTTTCT
Arbitrary primers	
83	GTTGCGATCC
84	CAAACGTCGG
85	AGGTGACGCT
86	GACCGCTTGT
87	AGCCAGCGAA
JOD6	GGTGCAAACG
JOD7	CGCAGCCAAG

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Genbank accession numbers UU55005-14..

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These products were cloned into pGEM-T (Promega). After ^{32}P -labeling using a random primer labeling kit (BRL), they were used as probes on Northern blots (Sambrook et al. 1989). Hybridization and washing conditions were as previously described (Church and Gilbert 1984) except that the final wash was performed at 55°C.

Cloned products fell into one of three classes, A to C (Fig. 2): class A clones (10/23; 43%) showed the expected pattern of expression on Northern blots; class B clones (7/23; 30%) were derived from elicited or control RNA and were not changed by chitosan treatment (i.e., they were false positives); and class C clones (6/23; 26%) were uninterpretable due to lack of signal on the Northern blot autoradiographs. This class could be composed of low abundance mRNAs, A/T base rich clones that were removed by washing, or cloning artifacts.

Class A and C clones were sequenced at the UF/ICBR DNA Sequencing Core, and analyzed using the facilities of the UF/ICBR Biological Computing Core. Alignments and

translations were conducted using GCG (Genetics Computer Group sequence analysis software, version 7.0 (Devereux et al. 1984), while searches were conducted using BLAST (Altschul et al. 1990). Ten clones that contained a poly-A tract and the expected 10-mer primer sequence have been submitted (Genbank accession numbers UU55005-14). Translated sequences of two submitted clones (clone 18 and clone 30) were similar to the C-terminal regions of protein sequences in the database.

Clone 18 showed increased transcript abundance in elicited cells (Fig. 2), and the translated sequence showed 68% identity to a cytochrome P450 enzyme, cinnamic acid 4-hydroxylase (C4H) from sunflower (Fig. 3A; Teutsch et al. 1993, Bolwell et al 1994). C4H catalyzes the hydroxylation of *trans*-cinnamic acid to *trans-p*-coumaric acid in the phenyl-

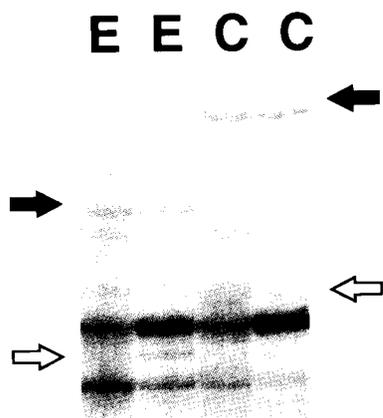


Fig. 1. A portion of a differential display autoradiograph showing the differences between lanes derived from elicited cell RNA and control cell RNA. Solid arrows identify products selected for further analysis, while hollow arrows identify nonreproducible products (not selected). Arrows to the left identify products unique to elicited cell RNA, and those to the right, products unique to control cell RNA. E = lane derived from elicited cell RNA, C = lane derived from control cell RNA. All lanes were amplified using the same primer pair.

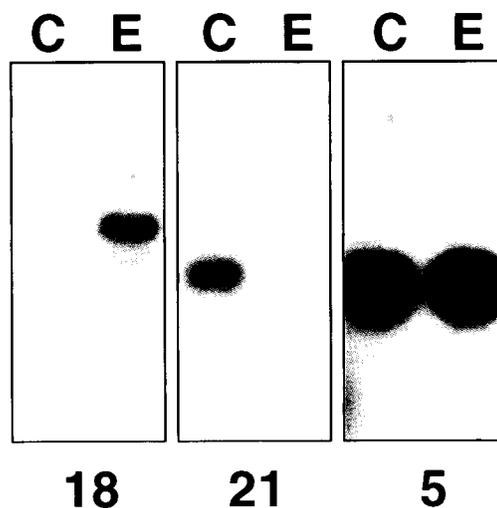


Fig. 2. Northern blots using differentially displayed products as probes. Left panel, class A clone selected from elicited lane showing the expected increase in transcript abundance in elicited cells. Center panel, class A clone selected from control lane showing the expected decrease in expression in elicited cells. Right panel, class B clone selected from elicited lane showing no change in transcript abundance, i.e., a false positive. E = elicited cell RNA, C = control cell RNA. The number below the panel is the number of the clone used as probe.

A

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Ht  ITIGRLVQNFELLPPPGQSKIDTDEKGGQFSLHILKHSTIVAKPRSF
Pc  IVIGRLVQNFELLPPPGQSKIDTAEKGGQFSLQILKHSTIVCKPRSL
18  VTVGRLQLQNFELLPPPGKSKVDVSDKGGQFSLPILNHTLLVAKPRLPASS
    ---GRL-QNFELLPPPG-SK-D---KGGQFSL-IL-H---V-KPR--
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B

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NtE NDAVEDRIGYYRRYCGMLNVAPGENLDCYNQRNFGQG
NtV DSRVQDRIGFYRRYCSIIGVSPGDNLDCGNQRSFGNGLLVDTM
30  GDRQQGRIGFYQRYCSLLGVDTGSNLDCQNQKHF
    -----RIG-Y-RYC--L-V--G-NLDC-NQ--F
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Fig. 3. A, Alignment of translated clone 18 with Ht (*Helianthus tuberosus* *trans*-cinnamate 4-monooxygenase; Teutsch et al. 1993) and Pc (*Petroselinum crispum* *trans*-cinnamate 4-monooxygenase; Logeman et al. 1995). **B,** Alignment of translated clone 30 with NtE (*Nicotiana tabacum* extracellular chitinase PR-Q; Payne et al. 1990) and NtV (*Nicotiana tabacum* vacuolar chitinase CHN50 (Shinshi et al. 1990). Residues conserved in all sequences are shown as capital letters in the bottom line.

propanoid pathway (Dixon and Paiva 1995), several products of which may be associated with pine:fungal pathogen interactions. These compounds include lignin in pines (Rowan 1970) and pine cell cultures (Lesney 1989; Lesney 1990; Popp 1993, Popp et al., in press), stilbenes in infected tissues of pines (Jorgensen 1961) and pine cell cultures (Lange et al. 1994), and pigments, probably anthocyanins, at sites of presumed fungal interaction (Lundquist and Miller 1984; Miller et al. 1976).

Clone 30 (Fig. 3B, Payne et al. 1990; Shinshi et al. 1990) was induced by chitosan treatment (data not shown). Translated clone 30 showed 53% and 65% identity to extracellular and vacuolar chitinases, respectively, from tobacco (Fig. 3B). The translation product lacks the vacuolar targeting signal that is present in tobacco vacuolar chitinase (Neuhaus et al. 1991), suggesting the clone 30 gene product is an extracellular chitinase. Chitinases and other hydrolytic enzymes are recognized as pathogen-related proteins, and are induced during defense responses in many higher plants (Collinge et al. 1993; Stintzi et al. 1993). Secreted chitinase activity increased in loblolly pine cell cultures after chitosan treatment (Popp et al., in press), indicating chitinases are likely to be involved in pine:fungal interactions as well.

Differential display was used to clone elicitor responsive cDNAs in slash pine, demonstrating that changes in mRNA abundance are associated with elicitation in this species. Our results suggest that there are approximately as many transcripts induced by chitosan as suppressed, indicating elicitor treatment does not simply halt cellular function and kill the cells. We found that pine cells show a chitosan-induced increase in certain gene products associated with inducible defense responses in angiosperms. These clones can now be used as molecular probes to determine the extent to which these genes are involved in previously described defense responses in pines and other gymnosperms.

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