

Induction of tobacco genes in response to oligochitosan

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Abstract Oligochitosan has a variety of biological activities. To understand its mechanism, DDRT-PCR, reverse Northern blot and quantitative relative RT-PCR were used to identify and isolate genes whose transcription were altered in cultured *Nicotiana tabacum* (var. Samsun NN) plants that were treated with oligochitosan. Three genes whose mRNA levels significantly changed in response to oligochitosan were isolated and identified. One gene is up-regulated, and two genes are down-regulated. These genes encode a DNAJ heat shock N-terminal domain-containing protein, a histone H1 gene and a hypothetical protein, whose function is unknown. The results suggest that the usefulness of mRNA differential display technique for the detection of plant metabolic pathways affected by oligochitosan.

Keywords Differential display · RT-PCR ·
Oligochitosan · Responsive gene

Introduction

Higher plants have the ability to initiate various defence reactions such as the production of phytoalexins, antimicrobial proteins, reactive oxygen species, and reinforcement of cell walls when they are infected by

pathogens such as fungi, bacteria and viruses. If these reactions occur in a timely manner, the infection will not proceed further. However, if the defence reactions occur too late or are suppressed, the infection process will proceed successfully [1]. Thus, it is extraordinarily important for plants to detect infecting pathogens effectively and deliver such information intracellularly or intercellularly to activate their defence system.

It is believed that the detection of pathogens is mediated by chemical substances secreted/generated by the pathogens. Various types of these compounds (elicitor molecules) including oligosaccharides, (glyco)proteins, (glyco)peptides and lipids, have been shown to induce defence responses in plant cells and their involvement in the detection of (potential) pathogens in plant has been discussed [2–4].

Chitosan is the product of partial deacetylation of chitin which is homopolymer of β -1,4-linked *N*-acetyl glucosamine. They are the second most abundant polysaccharides in nature, comprising the horny substance in the exoskeletons of crabs, shrimp, and insects as well as fungi. Chitin and chitosan have been proved to be nontoxic, biodegradable, and biocompatible and are known to have various biological activities including antitumor [5, 6], antimicrobial [7, 8], antifungal [9], and immuno-enhancing effects [10, 11]. However, their high viscosity and insolubility in neutral aqueous solutions restrict their uses in vivo. Recent studies on chitosan have attracted interest for oligochitosan which is not only water-soluble, non-toxic, biocompatible, but also possesses versatile functional properties [12, 13]. Until now, however, the cellular mechanisms of oligochitosan-induced signal remained unclear. Here we have focused our attention to the effects of oligochitosan on the transcriptome and we have looked

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for genes whose expression may be influenced by oligochitosan treatment.

Differential display (DDRT-PCR) is a powerful technique used to identify and isolate genes rapidly that are differentially expressed between two cellular populations, or within a single cell type under altered conditions [14, 15]. In this paper, the DDRT-PCR technique was applied to explore how oligochitosan might affect gene expression in the *Nicotiana tabacum* (var. Samsun NN).

Materials and methods

Plant material

Tobacco plants, *N. tabacum* (var. Samsun NN), were grown in a conventional greenhouse with a day/night cycle of 14/10 h and temperatures of 26/16°C. Plants having four to six leaves were sprayed with 50 mg/l oligochitosan (produced by our laboratory) solution prepared in distilled water, and distilled water was used as the control. Leaves from soil-grown plants were harvested for RNA extraction after being treated 8 h.

Extraction of total RNA

At the end of the oligochitosan treatment, plants were washed in deionized water, and total RNA was isolated immediately from control and oligochitosan-treated samples of *N. tabacum* using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After removal of contaminating genomic DNA using the DNA-free kit (GIBCO BRL, USA), the concentration and purity of RNA samples were determined by UV absorbance spectrophotometry.

cDNA synthesis and differential display RT-PCR

Total RNA was extracted from both oligochitosan-treated and untreated *N. tabacum* leaves using TRIzol reagent (Invitrogen, USA) as described in the Protocols and Applications Guide, and was treated with RNase-free DNase I. The DD and re-amplified reaction were performed with the fluoroDD kit (Genomix, USA) according to manufacturer's instruction. One anchored primers (AP1) was used in combination with 3 mers arbitrary primers (APR1, APR2 and APR3), and products of PCR were separated in parallel by electrophoresis in 5.6% clear denaturing HR-1000 PAGE gel matrix (FL407). After electrophoresis, fluoroDD gel was analyzed on the genomixSC Fluor-

escent Imaging Scanner. M13 reverse 24-mer primer and T7 promoter 22-mer primer were used for re-amplified reaction. Reamplified cDNAs were resolved by 1.0% agarose-gel electrophoresis and then purified using Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Dalian, China).

Subcloning and sequencing of cDNA fragments

Gel-purified cDNA fragments were subcloned into the pMD18-T vector (TaKaRa, Dalian, China) and recombinant plasmids were used to transform competent *Escherichia coli* (strain TOP 10) cells according to TOPO TA Cloning kit (Invitrogen, USA). Positive clones were collected and used for plasmid isolation. DNA inserts were sequenced using the F Primer (M13-47)CGCCAGGGTTTTCCCAGTCACGAC through ABI PRISMTM377 × L DNA Sequencer.

Analysis of the sequencing date

The homology search of genes against the NCBI Nucleotide Sequence Databases was carried out by online-based FASTA program available at National Center for Biotechnology Information (NCBI: <http://www.ncbi.nih.gov/>), Institute for Genomic Research (TIGR: <http://www.tigr.org/plantprojects.shtml>) and The Arabidopsis Information Resource (TAIR: <http://www.Arabidopsis.org/>).

Reverse Northern dot-blot and RT-PCR analysis

For Reverse Northern blotting, 100 ng of each denatured recombinant plasmids containing cDNA fragments were dotted on each Hybond-N⁺ membrane filter (Amersham Pharmacia, USA) and baked for 30 min at 80°C. As positive and loading controls, 100 ng of recombinant plasmids containing actin cDNA fragments were also applied to each membrane, following the same procedure as above. The cDNA synthesis from oligochitosan-treated and untreated *N. tabacum* respectively was carried out according to the User manual of SMARTTM RACE cDNA Amplification Kit (BD Biosciences Clontech, USA). Hybridization and labeling of cDNA probes were performed as described in the manual of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany).

For RT-PCR analysis, total RNA was purified from control and oligochitosan-treated samples of *N. tabacum* using the TRIzol reagent (Invitrogen, USA) and the recommended protocol. After DNase digestion, Reverse Transcription PCR was carried out using one

step RT-PCR kit (TaKaRa, Dalian, China) according to the manufacturer's instructions, the tobacco actin gene served as a constitutive control. (GenBank accession No. X69885). PCR reaction was carried out in a thermal cycler with a heated lid (TECHNE, England) as following: 1 cycle: 50°C for 30 min, 94°C for 2 min; 30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 2 min; 1 cycle: 72°C for 10 min; Hold at 4°C. Then, 10 µl (out of 25 µl) of each RT-PCR product were size fractionated on a 1.0% agarose gel. Gene-specific primers used in these reactions were reported in Table 1.

Results

Differentially expressed genes following oligochitosan treatment

The differential display technique was applied for the identification and isolation of genes whose transcription was positively or negatively regulated by oligochitosan. Three 5'-arbitrary primers were used for PCR amplification in combination with the 3' anchored primer (Table 1) and used to amplify cDNAs obtained by reverse transcription of total RNA from control and oligochitosan-treated *N. tabacum*. To avoid isolation of false positives, all PCR reactions were performed on two different dilutions of each cDNA sample. Only cDNAs whose levels of expression were affected by

oligochitosan in both dilutions were selected for further analysis. The fluoroDD PCR samples were separated on 5.6% clear denaturing HR-1000 PAGE gel matrix (FL407) and analyzed on the genomexSC Fluorescent Imaging Scanner. Figure 1 shows a representative gel from a differential display experiment. In total, three differentially expressed cDNA bands were detected and successfully eluted from the polyacrylamide gels, reamplified and cloned. The differential cDNA bands of DD11 (ARP1/API) and DD17 (ARP1/API) and DD44 (ARP2/API) showed an increased or decreased band intensity following oligochitosan treatment in comparison to the corresponding bands in control conditions. The lengths of the cloned cDNA fragments ranged from 376 to 608 bp (Table 2).

Sequencing and identification of cDNA clones

The differentially expressed cDNA fragments were cloned into the pMD18-T vector and both strands were sequenced using F Primer (M13-47) CGCCAGGGTTTTCCAGTCACGAC in ABI RISM™ 377 × L DNA Sequencer. The nucleotide sequences obtained were compared against NCBI and TIGR Nucleotide Sequence Database to identify putative

Table 1 The sequences of primers

Name of primer	Sequence
TMR-T7(dT ₁₂)API	5' acgactcactataggct(12)ga 3'
ARP ₁	5' acaatttcacacaggagactccaag 3'
ARP ₂	5' acaatttcacacaggagctagcattg 3'
ARP ₃	5' acaatttcacacaggagaccattgca 3'
M13 reverse(-48)primer	5' agcggataacaatttcacacagga 3'
T7 promoter primer	5' gtaatcagactcactatagggc 3'
Gene specific primers for actin forward	5' gatggtgtcagccactgtc 3'
Gene specific primers for actin reverse	5' atgctgctaggagccagtgc 3'
Gene specific primers for DD-11 forward	5' tgacgaacaataaagggtggca 3'
Gene specific primers for DD-11 reverse	5' atctttgaacgagcgagacg 3'
Gene specific primers for DD-17 forward	5' caagtcggaaagcagcacc 3'
Gene specific primers for DD-17 reverse	5'ggcataaaactccaaacatctc 3'
Gene specific primers for DD-44 forward	5' gaagttgtcttgggtgctgt 3'
Gene specific primers for DD-44 reverse	5' gagggattgactgagggaaa 3'

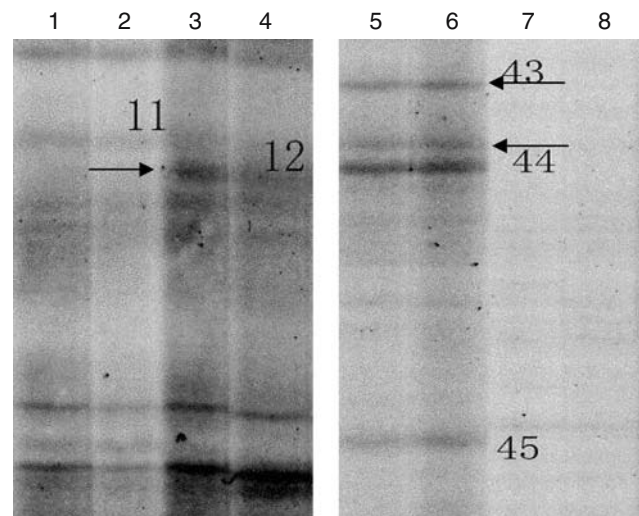


Fig. 1 Representative differential display band patterns of mRNA from control and oligochitosan-treated samples. Total RNA extracted from control and oligochitosan-treated *N. tabacum* (var. Samsun NN) plants was reverse-transcribed and amplified with the 5'-arbitrary primer and the 3'-anchored primer. Amplified cDNA fragments were separated in a 5.6% denaturing polyacrylamide gel. Arrows indicate differentially expressed cDNA fragments that were recovered from gel and analyzed further. Oligochitosan treated: lane 1, 2, 5, 6; Control: lane 3, 4, 7, 8; primer used as lane 1, 2, 3 and 4 were ARP1/API; lane 5, 6, 7 and 8 were ARP2/API

Table 2 Changes in the basal levels of differentially expressed mRNAs following oligochitosan treatment

DD product	Accession number	Length (bp)	Similarity (%)	e-value of match	Annotation of sequence match
DD11	DQ437539	523	81	5e-10	DNAJ heat shock N-terminal domain-containing protein
DD17	DQ437540	376	85	0.001	Histone H1 gene
DD44	DQ437541	608	96	0.0	Hypothetical protein

proteins that are encoded by these mRNAs. FASTA analysis indicated that the clone DD11 showed significant homology to DNAJ heat shock N-terminal domain-containing protein of *Arabidopsis thaliana* (Identities = 81%), whereas the clone DD17 showed high sequence identity with *Lycopersicon esculentum* histone H1 gene (85%), and the clone DD44 showed homology to cDNA clone KF8C.102M08 of *N. tabacum* (common tobacco) (96%), hypothetical protein of *Medicago truncatula* (71%) and *A. thaliana* (63%) as well as rice (62%) and wheat (62%) i.e. respectively.

Reverse Northern dot-blot and RT-PCR analysis

To confirm that the pattern of bands obtained represented true differences in gene expression and not artifacts due to the PCR amplification process, reverse Northern dot-blot analyses was performed. In this analysis, three differentially expressed cDNAs and loading controls were simultaneously examined (Fig. 2), *N. tabacum* actin was used as hybridization control. Following 8 h oligochitosan treatment, the level of expression of actin mRNA was constant as

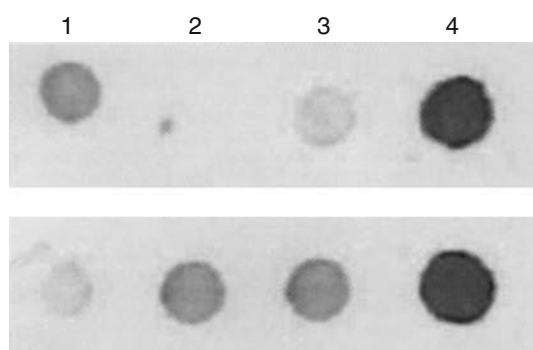


Fig. 2 Reverse Northern dot blot of differentially expressed genes. Cloned cDNA bands were amplified and aliquot of the amplified products were blotted on two membranes as described in *Materials and methods*. The membranes were hybridized with DIG-labeled cDNAs that were synthesized from total RNA prepared from control (bottom panel) and oligochitosan-treated *N. tabacum* (var. Samsun NN) leaves (top panel). Lane 1: DD11; Lane 2: DD17; Lane 3: DD44; Lane 4: *N. tabacum* (var. Samsun NN) actin as control

expected. Here, clone DD11 showed an increase in expression in leaves after oligochitosan treatment 8 h which agreed with DDRT-PCR. Clone DD17 and clone DD44 showed a decrease of expression in leaves after oligochitosan treatment 8 h. This result disagreed with DDRT-PCR, so semiquantitative RT-PCR analysis was performed. Using equal total RNA from control and oligochitosan-treated *N. tabacum* for RT-PCR, it was shown that after oligochitosan treatment the fragment of DD11 was up-regulated, and the segments of DD17 and DD44 were down-regulated truly (Fig. 3). RT-PCR analysis including oligochitosan treatment were conducted twice. All results indicated that the segments of DD17 and DD44 were surely down-regulated by oligochitosan.

Discussion

The presence of chitosan or oligochitosan triggers a wide range of cellular responses including changes in gene expression [16] and synthesis of PR protein. In this context, genes whose expression may be affected by oligochitosan were searched. For the identification and isolation of differentially expressed genes, several PCR based techniques are available [17]. DDRT-PCR offers many advantages over other methods: it is fast; it is based on simple, well established and widely accessible techniques; it has a good sensitivity to low-abundance mRNA transcripts; both induced and repressed genes can be simultaneously detected and

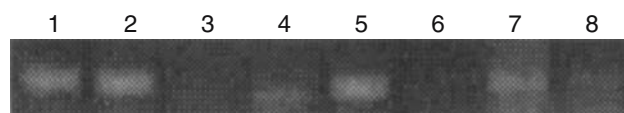


Fig. 3 Expression analysis of DD11, DD17, and DD44 by RT-PCR in control and oligochitosan-treated samples. Lane 1: actin in control samples; Lane 2: actin in oligochitosan-treated samples; Lane 3: DD-11 in control samples; Lane 4: DD-11 in oligochitosan-treated samples; Lane 5: DD-17 in control samples; Lane 6: DD-17 in oligochitosan-treated samples; Lane 7: DD-44 in control samples; Lane 8: DD-44 in oligochitosan-treated samples

more than two samples can be compared; furthermore, it requires small amounts of RNA [18].

The differential display method was used to perform a comparative gene analysis on *N. tabacum* plants that were treated by oligochitosan or distilled water. From this analysis, we identified three cDNA bands corresponding to genes that were modulated by the oligochitosan treatment. These bands were successively validated by reverse Northern dot-blot analysis and semiquantitative RT-PCR analysis, after cloning and sequencing, identified by comparison with sequences available in data banks.

The first fragment (DD11) identified in our analysis is up-regulated by oligochitosan: it encodes the DnaJ heat shock N-terminal domain-containing protein, a member of the DnaJ-like protein family regulated the chaperone function of Hsp70 proteins in the folding of proteins and the assembly of protein complexes within subcompartments of the eukaryotic cell, which occurs through direct interaction of different Hsp70 and DnaJ-like protein pairs that appear to be specifically adapted to each other [19]. DnaJ-like proteins from tobacco, *A. thaliana* [20] and tomato [21] were found to specifically interact with NSm (non-structural protein encoded by the M RNA segment), suggesting a possible involvement of a DnaJ-like protein in tomato spotted wilt virus (TSWV) movement. The plant induced resistance to virus by oligochitosan treatment has been reported [22] the fact that the expression level of the DnaJ-like protein gene is increased in oligochitosan-treated plants suggests that the plant induced resistance to virus by oligochitosan may involve Interactions between the virus movement protein and plant proteins.

The other segment (DD17) identified in our analysis, is down-regulated by oligochitosan. The gene has nucleic acid sequence similarity with *L. esculentum* H1 histone, a linker histone. Histone H1 molecules exhibit striking sequence divergence. Despite this divergence, histone H1 molecules have maintained three distinct regions. These consist of an amino-terminal 'nose', a conserved central globular domain and a positively charged carboxy-terminal tail. H1 histone has been found in the interior of the 30 nm fiber [23] and is presumed to function in chromatin compaction. Eukaryotic chromatin structure is not static; structural changes resulting in altered chromatin states occur to process genetic information [24]. Chromatin state has been shown to alter the function of the genome. Histones, including H1, provide the framework for proper transcription, and depending upon the gene in question, may repress or induce transcription [25–28]. Variants of

H1 histone, which occur in different species, cell types or developmental stages, have been shown to have differing roles in transcriptional repression [29]. Research indicated that the expression of H1 histone could be induced by gibberellins in the leaf of the gibberellin-deficient tomato, and the increased H1 histone could correlate with enhanced DNA replication in leaf tissue [30]. The increase of H1 histone transcripts were also detected in young anthers with pollen mother cells, which suggested that H1 histone may be involved in regulating the pollen dedifferentiation [31]. The regulation of H1 histone is essential for development of the male gametophytic cell [32] and coleoptiles of etiolated wheat seedlings [33]. It was found that H1 histone is important for the DNA methylation in *Arabidopsis* [34]. Then the H1 histone mRNA decrease in the present of oligochitosan suggests that the H1 histone may be part of a global mechanism by which oligochitosan controls the transcriptional state of a set of genes.

The third gene (DD44) identified in our analysis is a hypothetical protein. The homologs of DD44 are widespread among plants including *A. thaliana*, *Solanum tuberosum*, *L. esculentum*, *Ocimum basilicum*, *Solanum chacoense*, *Ipomoea nil*, *Picea glauca*, *Picea sitchensis*, *Cucumis melo*, *Nuphar advena*, *Prunus pers.*, and expressed in various tissues and organs including leaf, root, shoot, flower buds, flower, fruit mesocarp, and peltate glandular trichome i.e., in *A. thaliana*, the homolog of D44 has been expressed in vitro, whose locus number is AT5G25360. This expressed protein is consisted of 170 aa and its pI is 7.2472, MW is 19131 Da. Although certain characters of this protein have been investigated, there are many aspects about this protein including the cellular component, molecular function and biological process still unknown. Then exact function of this protein in induced resistance to stress by oligochitosan is uncertain.

In conclusion, although the results in this paper do not depict an exhaustive survey of all possible changes of gene expression in *N. tabacum* induced by oligochitosan treatment, they suggest that several novel oligochitosan responsive genes or possible pathways are not currently recognized. Furthermore, the sequencing of these fragments provides a valuable addition to the identification of the complete tobacco transcriptome and new molecular resources for investigating stress in this tobacco species. These results also showed that the differential display technique appears to be a very useful tool in the identification of the plant metabolic pathways affected by oligochitosan.

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