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Chitosanase may enhance anti-fungal defense responses in transgenic tobacco

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ABSTRACT

Chitosanase is an enzyme, similar to chitinase, capable of hydrolyzing the β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in partially acetylated chitosan polymers found in fungal cell walls. When attacked by pathogenic fungi, many plants exploit this hydrolytic action as a component of a larger post-attack defense response, but these enzymes may also play a role in the initial plant-pathogen interaction via the generation of elicitors resulting from the hydrolysis of fungal cell walls. To gain insight into these mechanisms, a *Paenbacillus* chitosanase was cloned, sequenced, and modified for plant expression. The modified gene was delivered to tobacco (*Nicotiana tabacum* L. cv. Xanthine) leaf disks via *Agrobacterium tumenfaciens*-mediated transformation. Whole plants were regenerated from the transformed cells. The putative transformants were tested for transgene integration, transcription, and translation. Confirmed transformants were then screened for enhanced responses to a *Rhizoctonia solani* cell-wall preparation by measuring time-course production of hydrogen peroxide, phenylalanine ammonia lyase, and peroxidase. These compounds play roles at different points in a pathogenesis-related signal transduction pathway and thus allow for an initial assessment of the global defense response. Preliminary data suggest that transgenic tobacco constitutively expressing a *Paenbacillus* chitosanase may activate pathogenesis-related defense responses more quickly than wild type tobacco.

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MEET THE STUDENT-AUTHOR

Bill L. Hendrix

I received a B.S.A. degree in crop management in May 2003. When I started college, a degree in agriculture wasn't one of my plans. In fact, I was a biology major for the first two years of my college career, but when I transferred to the University of Arkansas, I started working for a professor in the Crop, Soil, and Environmental Sciences Department, Dr. James McD. Stewart. I had never worked on a farm or even seen a soybean or cotton plant up close. Needless to say I was out of my element, but I worked hard and began to enjoy the work. It didn't take long for me to realize that agriculture is applied biology and many of the things I had already learned were directly applicable to the degree. I weighed my options and decided to switch degree plans. In hindsight, that decision was a good one. Many opportunities have come my way that I would have otherwise missed. This project is one such opportunity. Over its course, I was exposed to the world of research science and realized the agricultural industry is a career niche that suits me well.

I would like to thank Drs. Stewart, Srivastava, and de Los Reyes for fielding my numerous and sometimes bothersome questions, and my wife and daughter for helping me through my research frustrations and failures.

INTRODUCTION

Chitosanase (EC 3.2.1.132) is an enzyme widely distributed in nature. Its range of cellular functions centers primarily around its ability to hydrolyze β-1,4-linkages between N-acetyl-D-glucosamine (GlnAC) and D-glucosamine (Gln) residues in partially acetylated chitosan polymers found in fungal cell walls. The related enzyme chitinase has a similar fungal cell wall-degrading capability. As a result, many scientists have made efforts to exploit its action and increase plant fungal resistance by over-expressing this enzyme in various plant systems (Punja, 2001). To date, however, there has been only one report of plant transformation with a chitosanase gene (El Quakaoui et al., 1995) and no reports describing its in-planta anti-fungal potential.

No matter which cell wall-degrading enzyme is over-expressed, the degree of increased plant fungal resistance depends on two factors: 1) The efficacy with which the enzyme can degrade cell walls and, consequently, slow the attacking fungus; and 2) the number, length, and degree of acetylation of the oligomeric-carbohydrate elicitors released from the fungal cell walls. These factors are not mutually exclusive and may work together to achieve the realized plant resistance (Lorito and Scala, 1999).

Lorito et al. (1998) reported that for chitinases the source of the enzyme may influence its efficacy in degradation of GlnAC and Gln polymers. Over the past decade, the gamut of sources of cell wall-degrading enzymes has been tested. Most plant, bacterial, and fungal chitinases have offered plants varying degrees of protection from fungal pathogens, but generally, plant-derived chitinases have conferred inadequate control while bacterial and especially fungal chitinases have typically offered higher levels of resistance.

Glucosamine oligomers, released from fungal cell walls or crab shells after hydrolysis with a chitinase or a chitosanase (or by physical means) are known elicitors of plant defense responses such as stomatal closure (Lee et al., 1999); lignification (Vander et al., 1998; Moerschbacher et al., 1988); mitogen-activated protein kinase activation (Link et al., 2002); and pathogenesis-related (PR) gene expression (Jabs et al., 1997). The degree and type of responses elicited by these molecules depend on the length; degree of acetylation (DA) (Vander et al., 1998); and number of oligomers present. These factors may be as important as enzyme efficacy for increasing plant fungal resistance; however chitinase-cleaved oligomers may differ from those cleaved by a chitosanase. If acetylated vs. non-acetylated glucosamine residues were distributed randomly along the length of a

fungal cell-wall polymer, cleavage with chitosanase would produce oligomers that vary little from those produced by chitinase, but in some fungi the distribution is not random. Fukazimo et al. (1992) found the cell wall glucosamine polymer of *Fusarium oxysporum* f. sp. *lycopersici* had a DA of approximately 25-35%. In addition, they compared the degradation products produced by chitinase and chitosanase. The chitinase produced a GlnAC–Gln dimer as the primary product, whereas the chitosanase produced a relatively larger, heterogeneous pool of products that varied in length and DA. These results indicated that the acetylated glucosamine residues are clustered along the cell wall and that a chitosanase may be more efficient for cell wall digestion. Because only over-expression of chitinases has been examined in plant systems, in vivo variability in this area has not been tested.

In this study, a newly discovered *Paenbacillus* sp. 61427 chitosanase was expressed in tobacco (*Nicotiana tabacum* L. cv. Xanthine) to investigate the in-planta anti-fungal potential of this protein and to determine whether plant-signal transduction pathways can be enhanced by its elicitor-generating action.

MATERIALS AND METHODS

Bacterial Gene Isolation

A bacterium, identified by the sequence of the 16S rRNA gene, was discovered on a chitosanase screening plate (LB agar pH 7.9 + final concentration of 0.01% w/v chitosan dissolved in 0.1N HCl overnight). The bacterium produced a clear halo around the colony, indicating the presence of strong extra-cellular chitosanase activity. The bacterium was cultured and a genomic library was constructed with pGEM®-3Zf(+) (Promega, Madison, Wisc.) in *Escherichia coli* strain DH5α. The library was screened for chitosanase activity and one colony that exhibited strong activity was selected for further experimentation (Hendrix et al., 2001). The cloned plasmid, designated pCHN1, contained an insert of approximately 8 kb.

A nucleotide-deletion experiment (Promega, Madison, Wisc.: Erase-a base kit) was performed on the insert of pCHN1. The resultant clones began to lose chitosanase activity at time point 10, indicating nucleotides important for gene expression had been removed. The clones from time point 10 to16 were sequenced to identify the open reading frame and mature protein region of the chitosanase.

Gene Modification for Plant Expression

Specific primers were designed to 1) PCR-amplify the mature protein region of the chitosanase, 2) add an *Arabidopsis* extra-cellular chitinase signal peptide (Hasselhoff, 1992), and 3) generate BamHI (5') and SalI (3') restriction sites to facilitate further cloning.

The modified chitosanase fragment along with pHPT[1], a modified pUC19 vector containing a 35S Cauliflower Mosaic Virus (35S) promoter and nopaline synthase 3' transcription terminator (nos), were digested with BamH1/SalI and BamH1/AlwnI, SalI/AlwnI, respectively. The resultant fragments were used in a trimolecular ligation. The product, pERCSN, was cloned in *E. coli* strain DH5α for further manipulation.

pERCSN was digested with XbaI to liberate the experimental construct and ligated into the binary plant-transformation vector pPZP211 (Hajdukiewicz et al., 1994), also digested with XbaI and cloned in *E. coli*. The resultant *E. coli* colonies containing the experimental vector, pPZP-ERCSN, were screened by PCR and restriction digest to confirm proper gene size and orientation.

Tobacco Transformation

pPZP-ERCSN and an empty vector control were transferred to *Agrobacterium tumenfaciens* strain GV3101 by electroporation. Sterile tobacco (*N. tabacum* cv Xanthine) leaf disks were co-cultivated with *A. tumenfaciens* for 15 min in a Murashige and Skoog (MS) salt (4.314g/L)/ 3% sucrose (pH 5.9 with 1N KOH) solution for infection. Disks were then incubated for 3 d in the dark on MS/sucrose (same as above + phytoagar) plates without antibiotics. Following the co-cultivation period, disks were washed in MS/sucrose solution containing 400 µg/ml Timetin for 3 h to kill residual A. tumenfaciens and placed on shoot-initiation medium (SIM) (MS salt 4.314g/L, 3% sucrose, 0.5mg/ml naphthaleneacetic acid, 1mg/ml 6-benzylaminopurine, 300 µg/ml kanamycin, 250 µg/ml Timetin, 0.7% phytoagar) for callus and plantlet formation. Once plantlets regenerated and were 1 to 2 cm tall, they were removed with forceps and placed in rooting medium (same as SIM medium without hormones) for root formation. After two to three weeks, rooted plants were transferred to soil and grown under typical greenhouse conditions.

Analysis of the Integration Locus

Transgene integration was confirmed by Southern blot and hybridization. Total DNA was isolated from the regenerated plantlets following a modified procedure reported by Zhang et al. (2000). Ten micrograms of total DNA/plant were digested with EcoRI (Fig. 1) and size-separated by electrophoresis in 1% TAE agarose gels. The digests were transferred to Hybond N+ nylon membrane (Amersham Life Science, Piscataway, N.J.) with alkali blotting. Pre-hybridization and hybridization were performed according to Ausebel et al. (1995) with a ^{32}P-dCTP random prime-labeled csn probe. The membranes were washed twice with 2x SSC/0.1% sodium

dodecyl sulfate (SDS) followed by 0.2x SSC/ 0.1% SDS at 65°C until membrane signal was low. An autoradiograph was prepared by overnight exposure of Hyperfilm (Amersham Life Science, Piscataway, N.J.) to the gel at –80°C.

Transcriptional Analysis

Chitosanase transcription was assayed by Northern blot. Total RNA was isolated from young leaves with Trizol reagent (Gibco BRL, Grand Island, N.Y.). Five micrograms of total RNA were size-separated by electrophoresis in a 1% formaldehyde-agarose gel, transferred to a Hybond N+ nylon membrane, and cross-linked with UV irradiation. Pre-hybridization and hybridization were performed at 42°C with NorthernMax hybridization buffer (Ambion, Austin, Tex.) and a ^{32}P-dCTP random prime-labeled csn probe. Washing was at 42°C, but otherwise, washes and autoradiography were performed as described above.

Recombinant Protein Assay

Protein accumulation and activity were assayed with a leaf-disk lysoplate assay using a modified protocol previously described by Grenier et al. (1990). Briefly, agar slabs were augmented with 0.05% chitosan (pH 5.0 with 1N NaOH) dissolved in 0.1N HCl and 1% Triton-X 100. Fresh leaf disks were placed on the slabs and incubated at 28°C for 1 to 3 h to allow for diffusion of the extra-cellular chitosanase into the medium. The slabs where then stained with calcoflour white to visualize zones of chitosan lysis under UV light.

In vitro Anti-fungal Assay

The anti-fungal efficacy of the native, unmodified, bacterially produced chitosanase was compared to the recombinant chitosanase to assess the effect of gene modification and plant expression on the anti-fungal activity of the protein. *Rhizoctonia solani* mycelial plugs were centered on potato dextrose agar plates and challenged by transgenic leaf disks or by chitosanase buffer (10 mM phosphate pH 6.0) containing 5 µg, 0.5 µg, 0.05 µg, and 0 µg of partially purified, native chitosanase. After overnight incubation, growth inhibition was visually assessed.

Elicitor Preparation

Elicitors were prepared from *R. solani* and *Verticillium dalihae*[2] cell walls via methods described by Ke et al. (1998). Fungi were propagated in potato-dextrose broth with shaking at 27°C for 3 d. Mycelia were pelleted by centrifugation, washed with distilled water, and suspended in 1N NaOH (1:40 w/v mycelia to solution ratio). The suspension was then autoclaved for 15 min at 121°C to lyse cells and remove proteinaceous fractions. After a brief cooling period, insoluble material was pelleted by centrifugation, and the supernatant fraction was discarded. The pellet was washed with distilled water to remove residual NaOH, then resuspended in 2% acetic acid (1:100 w/v ratio) and again autoclaved 15 min at 121°C. The slurry was filtered through two layers of miracloth to remove acid insoluble material and collected for precipitation. Cell wall preparations were precipitated with 10M NaOH by raising solution pH to approximately 10. Precipitate was collected by centrifugation, washed four times with distilled water to remove any water-soluble fractions, and air-dried overnight. Elicitors were then dissolved in distilled water adjusted to pH 4.0 with acetic acid and stored at room temperature.

In-planta Elicitor Assay

The *R. solani* cell-wall preparation was applied to the surface of transgenic and wild-type tobacco leaves. Crude enzyme extracts (Moerschbacher et al., 1988) were taken at 0, 2, and 24 h after elicitor application, and phenylalanine ammonia lyase (PAL) and peroxidase (POD) activities were measured as described by Vander et al. (1998). All protein concentrations were estimated spectrophotometrically by the method of Bradford (1976).

Systemic H_2O_2 Production

Leaves from wild-type and transgenic plants were excised and imbibed with a 1mg/ml 3,3-diaminobenzidine (DAB) solution for 8 h. Elicitor preparations were then applied in solution through the cut petioles, and systemic H_2O_2 production was visually assayed on bleached leaves (boiled in 95% ethanol 10 min) at 0, 2, and 20 h after application as described by Orozco-Cardenas and Ryan (1999).

RESULTS AND DISCUSSION

Gene Discovery and Modification for Plant Expression

The original chitosanase-producing bacterium was identified as *Paenbacillus* sp. 61427 based on rRNA gene sequence. This bacterium produces a 259 aa, 29 kDa extra-cellular chitosanase. At the amino acid level, the *Paenbacillus* sp. 61427 chitosanase is 64% and 66% identical to *Bacillus circulans* and *B. ehimensis* chitosanases, respectively.

The *Paenbacillus* chitosanase mature protein gene was cloned and sequenced, and an *Arabidopsis* signal peptide, 35S Cauliflower Mosaic Virus promoter, and nopaline synthase terminator were added to obtain a 1676 bp experimental gene construct (Fig. 1) that was fused to the binary vector pPZP211 to obtain a 4409 bp T-DNA insert (Fig. 2).

[2] Fungi were courtesy of Dr. Craig Rothrock, University of Arkansas.

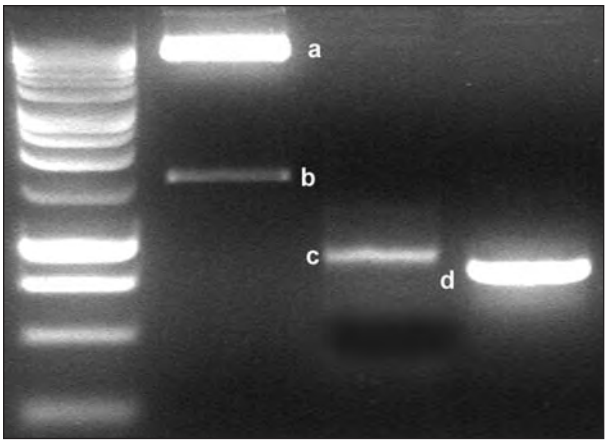

Fig. 1. 1% TAE agarose gel (EtBr stained) for confirmation of experimental vector. Ln1: 1kb ladder. Ln2 pPZPER-CSN HindIII digest; a-pPZP211 backbone 9014bp; b- experimental insert 1676bp. Ln3: PCR product, *Paenbacillus* chitosanase mature protein region + *Arabidopsis* signal peptide (ER sp); c- ER sp + csn 860bp. Ln4: PCR product, *Paenbacillus* chitosanase mature protein region; d- csn 777bp.

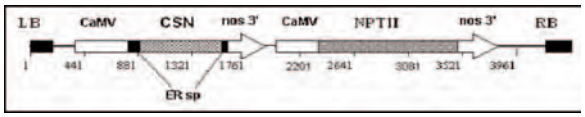

Fig. 2. 4409bp experimental T-DNA insert. Abbreviations: CaMV - 35S Cauliflower Mosaic Virus promoter; CSN - *Paenbacillus* chitosanase mature protein gene; ER sp - *Arabidopsis* signal peptide sequence; nos 3' - nopaline synthase 3' terminator sequence; EcoRI – recognition site; NPTII – neomycin phosphotransferase II gene; LB/RB – T-DNA left/right border sequences.

Transformation and Expression Confirmation

Numerous tobacco plantlets were regenerated, but gene transfer was tested in only 10 lines. Eight lines were confirmed transformed with varying copy number by Southern hybridization (Fig. 3A), and seven of the lines had high levels of csn mRNA as evidenced by Northern blot (Fig. 3B). Lines csn4, 5, 9, 10, and 11 were confirmed transformed by both Northern and Southern blots and were used for further experimentation or seed production. In addition, line csn3 was allowed to set seed to explore the possibility of gene reactivation in the segregating progeny of this multi-copy-silenced transgenic line. Before any line was used in an experiment, accumulation of active extra-cellular chitosanase was tested with a leaf-disk lysoplate assay (Fig. 3C).

Growth Inhibition of R. solani

Many times, bacterial proteins expressed in plant systems are heavily glycosylated or may be otherwise rendered inactive or less efficacious. To assess the effect of the gene modifications and plant expression on our bacterial protein, the fungal pathogen *R. solani* was challenged by the native and recombinant chitosanase. The partially purified native chitosanase dilutions inhibited growth at each concentration tested (Fig. 4A), and the transgenic leaf-disks from line csn5 inhibited growth at a level intermediate to the 0.5 to 0.05 μg level of the native chitosanase (Fig. 4B). No inhibition was observed with chitosanase buffer or wild-type leaf disks. These results indicated the recombinant chitosanase is expressed in our transgenic tobacco with in-planta activity levels sufficient for further experimentation.

In-planta Elicitor Assay

Lines csn5, csn6, and a non-transformed (NT) line (Fig. 3C) were assayed for a response to the *R. solani* cell-wall preparations. The cell walls were prepared to eliminate the small water-soluble oligomers known to elicit plant defense responses (Barber et al., 1994; Vander et al., 1998). As such, these polymeric glucosamine macromolecules required cleavage to produce soluble fragments capable of diffusing into plant tissues and triggering a plant defense response. The intent was to assay for enhanced plant perception of a simulated fungal attack in which wounding and extraneous proteinaceous elicitors were absent.

Line csn5 and line csn6 showed increases in both PAL and POD rates at 2 and 24 h. The PAL and POD rates for the NT line, however, remained unchanged over the course of the treatment (Fig. 5). POD activity in the transgenic lines was initially much higher than that of the NT line. We believe these results stem primarily from growing conditions. The transgenic lines were grown in small magenta boxes as a matter of convenience. The plants, though, had become quite large at the time of the experiments and were prone to moisture stress. While not visibly stressed at elicitor application, peroxidase levels may have been elevated due to an earlier moisture-stress response. The NT lines were grown under the same conditions but were much smaller and were never visibly moisture stressed. As such, they had normal POD activity at elicitor application. Despite minor discrepancies, these results suggest that constitutive extra-cellular expression of the *Paenbacillus* chitosanase may allow for enhanced plant perception of attacking fungi and thereby allow the plant to respond more quickly.

Systemic H_2O_2 Production

Lines csn5, csn6, and an NT line were evaluated for elicited H_2O_2 production in response to *R. solani* cell-wall preparations. Again, efforts were made to minimize wounding in order to assess only signal transduction events originating from elicitors released by the hydroly-

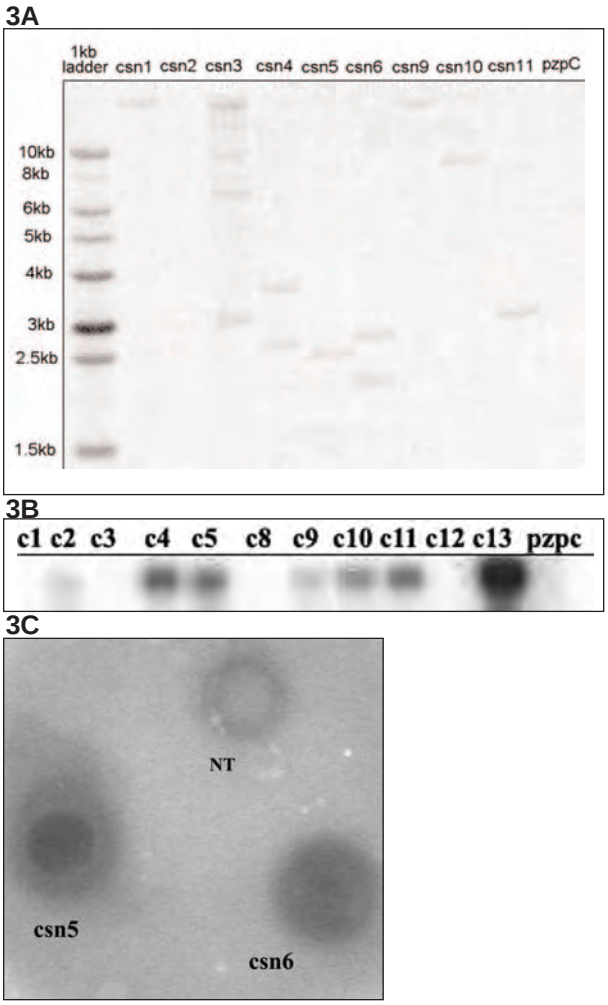

Fig. 3. Confirmation of transformation. A: Southern blot of ten transgenic lines and empty vector control (pzpC). One band = one copy of transgene B: Northern blot of 11 csn lines and empty vector control C: Example of leaf-disk lysoplate assay confirming accumulation of active, extracellular chitosanase in two transgenic lines.

sis of the applied fungal cell-wall preparation. Production of reactive oxygen species like H_2O_2 has been widely reported as one of the early events of plant defense response (Hancock et al. 2002; Vanacker et al., 2000). DAB forms a brown, insoluble polymer in the presence of H_2O_2 and peroxidase. As a semi-quantitative assay for H_2O_2, the leaves were allowed to take up a DAB solution through their petioles, exposed to the elicitor preparations, and visually assayed for the formation of the brown polymer and, hence, H_2O_2 production. Lines csn5 and csn6 both responded to the *R. solani* cell-wall preparation with systemic production of H_2O_2 at 2 and 20 h. The NT line, however, showed no response to the cell-wall preparation at 2 h and only a slight response at 20 h (Fig. 6). These results provide further evidence that

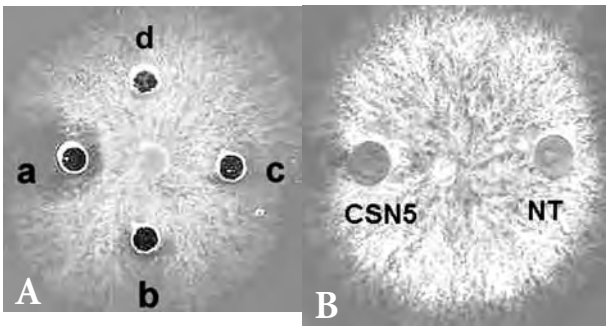

Fig. 4. Comparison of anti-fungal activity of the (A) native *Paenbacillus* chitosanase at 5 µg (a), 0.5 µg (b), 0.05 µg (c), and 0 µg (d) of total protein and (B) recombinant chitosanase from transformed leaf disks. The single copy line, CSN5, inhibited *R. solani* at levels near that of 0.05µg (c) of native protein.

transgenic tobacco constitutively expressing chitosanase may be capable of faster responses to attacking fungi than NT lines are.

In this study, the *Paenbacillus* chitosanase was transcribed, translated, and transported correctly in transgenic tobacco. Additionally, this enzyme enhanced the ability of transgenic tobacco to respond to fungal cell-wall-derived elicitors by cleaving these macromolecules into small fragments active as elicitors. This action may be able to increase plant fungal resistance by both lowering fungal infection efficacy and decreasing the time required for defense-gene induction.

ACKNOWLEDGMENTS

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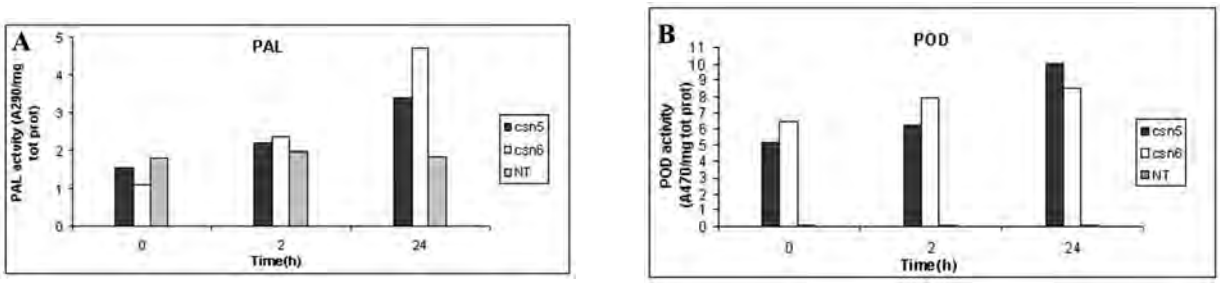

Fig. 5. Time-course changes in (A) PAL and (B) POD production in elicitor-treated tobacco leaves.

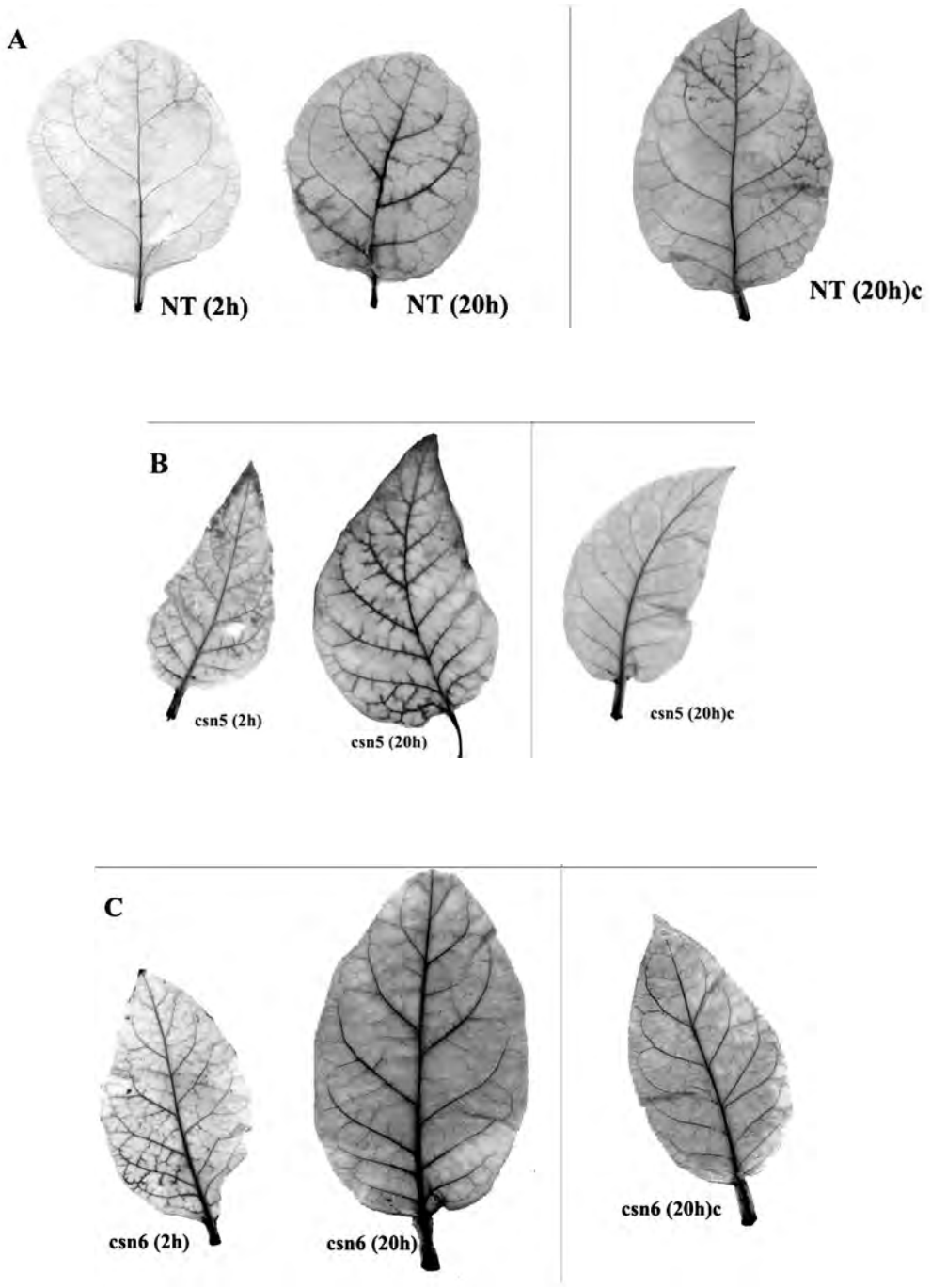

Fig. 6. Time-course changes in brown coloration due to DAB staining (H_2O_2 production) in the (A) NT, (B) csn5, and (C) csn6 lines at 2 and 20 h after treatment and at 20 h with DAB but no elicitor treatment [(20 h)c].