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CHITOSANASE MAY ENHANCE ANTI-FUNGAL DEFENSE RESPONSES IN TRANSGENIC TOBACCO

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Abstract:

Fungicides are expensive, dangerous, and can be harmful to the environment, but they are often necessary for profitable farming operations. New technologies may soon allow farmers to replace these chemicals with genetically engineered plants producing antifungal enzymes that degrade fungal cell walls. To explore this option, a Paenbacillus *chitosanase gene 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. The putative GMOs 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, phenyalanine ammonia lyase, and peroxidase. These compounds play roles at different points in a pathogensis-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.*

Introduction:

Fungal pathogens like *Rhizoctonia, Thielaviopsis* and *Fusarium* thrive in the soils of Arkansas and are a major threat to both emerging seedlings and established crops. For the Arkansas farmer, these and other fungal pathogens usually make fungicide application a necessary part of production. The toxicity, environmental harm, and expense that come with fungicides, though, leave many farmers looking for other options. In the future, genetically engineered crop plants that produce enzymes to degrade key structural polymers found in fungal cell walls may provide farmers with a more attractive option. One such enzyme, chitosanase, has the potential to slow or prevent fungal infection by degrading the structural chitosan found in the cell walls of many fungi. To gain insight on this possibility, a newly discovered chitosanase s from *Paenbacillus* p. 61427 was expressed in tobacco *(Nicotiana tabacum L cv. Xanthine),* and the *in planta* anti-fungal potential of this protein was examined.

Background Information:

Chitosanase is an enzyme, similar to chitinase, capable of hydrolyzing the ß-1, 4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated fungal cell wall polymer. When attacked by pathogenic fungi, many plants exploit this hydrolytic action as a component of a larger post-attack defense response (Agrios, 1997), but these enzymes may also function in pathogenesis-related (PR) signal transduction. Fungal cell wall fragments released after hydrolysis with a chitosanase or chitinase are elicitors of plant defense responses such as stomatal closure (Lee *et al.*, 1999), lignification (Vander *et al.*, 1998; Moerschbacher et *al.*, 1988), and PR gene induction (Jabs *et al.*, 1997). The responses elicited by these molecules depend on the length and degree of acetylation of the fragments released (Vander *et al.*, 1998). More specifically, long fragments or intact fungal cell walls will cause little or no reaction. Fragments that are relatively short, however, (e.g. products of chitosanase hydrolysis) are active elicitors.

Methods and Results:

Gene source

Paenbacillus sp. 61427 is a chitosanase-producing bacteria discovered in the Stewart Lab at the U. of Arkansas in 1995. To isolate this organism, many ecological niches were sampled. Samples from each niche were streaked onto agar plates with chitosan as the sole carbon source. Prospective organisms (i.e. surviving organisms) were isolated and streaked on opaque chitosan plates augmented with LB medium to verify chitosanase activity. A single bacterial isolate producing a strong extra-cellular chitosanase was selected. This bacterium was identified by its

16srRNA gene sequence as *Paenbacillus* sp. 61427 (Hendrix *et al.*, 2001).

To isolate the chitosanase gene, the *Paenbacillus* genome was fragmented and cloned in *Escherichia coli*. The *E. coli* library was screened, and a colony with chitosanase activity, termed pCHN 1, was chosen. Following further manipulation the DNA fragment cloned in pCHN I was sequenced. The sequence was electronically compared to the Genbank database to confirm the sequence and function of the gene. These comparisons revealed the *Paenbacillu*s chitosanase gene is 777bp long and highly homologous to chitosanase genes from *Bacillus circulans* and *B. ehimensis*

Gene modification

The ultimate goal was constitutive expression of this bacterial chitosanase in a plant system. Bacterial genes, though, differ in several ways from plant genes, and in their native form would not be expressed in plants. Accordingly, the *Paenbacillus* chitosanase gene was modified. Specific primers were designed that allowed a polymerase chair reaction (PCR) to amplify the mature protein region of the chitosanase gene *(csn)* and to add the nucleotide sequence of an *Arabidopsis* extracellular transport peptide. To complete the recombinant gene, the 35S Cauliflower Mosaic Virus promoter, a constitutive plant-recognized promoter, and a nopaline synthase 3' terminator were added. The final product was incorporated in the T-DNA region of a binary plant transformation vector (Figure 1).

Tobacco transformation

Agrobacterium tumenfaciens is a bacterium that has evolved the ability to incorporate special regions of its DNA (T-DNA) into certain plant genomes. Tobacco leaf disks were infected with a strain of *A. tumenfaciens* carrying the recombinant vector described above. The *csn* genes along with a kanamycin resistance gene were transferred to a number of tobacco cells. To select the transformed cells, the leaf disks were subjected to kanamycin selection under tissue culture conditions. Over a period of 5-6 wks, transformed cells regenerated into transformed callus and then putative transformed plantlets (Figure 2).

Transformation confirmation

At present, plant transformation is inefficient, and GMOs are prone to problems that can negate transgene expression. To avoid these problems in our expression system, the regenerated tobacco plants were screened on three levels relative to gene expression: *csn* integration, transcription, and translation.

Transgene integration was analyzed by Southern blot and hybridization. Total DNA was isolated from the regenerated plantlets, cut with a restriction enzyme, EcoRl (Figure 1), and fragments separated by electrophoresis in an agarose gel. The fragmented DNA was denatured, transferred, and covalently bound to a nylon membrane with alkali blotting. A ^{32}P-dCTP *csn* probe was then used to detect transgene presence and copy number. The results indicate the recombinant chitosanase gene was incorporated in eight of the ten lines tested (Figure 3A).

Transgene transcription was analyzed with a Northern blot. Northern blotting is similar to the Southern procedure except that RNA, not DNA, is fixed to the nylon membrane. Total RNA was isolated, size-separated by gel electrophoresis, and transferred to a nylon membrane. The membrane was then probed with a ^{32}P-dCTP *csn* probe to detect recombinant chitosanase transcription. The results indicate seven of the lines tested were positive for *csn* transcription (Figure 3B). Transgenic lines positive both in Southern and Northern blot were selected for further experimentation or seed production.

Recombinant chitosanase translation, activity, and extracellular accumulation were assessed on agar slabs augmented with 0.05% chitosan. Fresh leaf disks were placed on the slabs, incubated, and then stained with calcoflour white to visualize lysis zones under UV light. The lysis pattern suggests that the transgenic lines accumulate active, extracellular chitosanase (Figure 3C).

In vitro antifungal assay

The anti-fungal efficacy of the bacterially produced, unmodified 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.

Rhizoetonia solani mycelia plugs were centered on potato dextrose agar plates and challenged by transgenic leaf disks or by chitosanase buffer 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. The dilutions of partially purified chitosanase inhibited growth at each concentration tested (Figure 4A), and the transgenic leafdisks from line *csn*5 inhibited growth at a level intermediate to the 0.5 to 0.05 µg level of the native chitosanase (Figure 4B). No inhibition was observed with chitosanase buffer alone or wild-type leaf disks.

In planta elictor assays

Confirmed transformants were screened for enhanced responses to a *R. 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 pathogensis-related signal transduction pathway and, thus, allow for a preliminary assessment of the global defense response.

Elicitor preparation

A non-elicitor-active solution of fungal cell wall was prepared from *R. solani* via base/acid extraction (Ke *et al.*, 1998). To gain elicitor activity, these polymeric molecules must be cleaved into shorter elicitor-active fragments by an endogenous or recombinant hydrolytic enzyme.

Systemic H_20_2 production

Production of reactive oxygen species like H_20_2 have been widely reported as one of the early events of plant defense response (e.g., Vanacker *et al.*, 2000; Hancock et *al.*, 2002). A brown, insoluble polymer forms when 3,3-diaminobenzidine (DAB) reacts with H_20_2. As a semi-quantitative assay for H_20_2, transgenic leaves were allowed to take up a solution of DAB through their petioles, exposed to the elicitor preparations, and visually assayed for the formation of the brown polymer, hence, H_20_2 production. Both csn5 and *csn*6 responded to the *R. solani* cell wall preparation with systemic production of H_20_2 at 2h and 20h. The NT line, however, showed no response to the cell wall preparation at 2h and only a slight response at 20h (Figure 5).

Lignification enzymes

Lignification is another plant defense response. Lignin is a polymer that is resistant to microbial enzymatic degradation and is utilized by plants to restrict fungal movements through tissues (Agrios, 1997). Two important enzymes in the lignification pathway are phenylalanine ammonia lyase (PAL) and peroxidase (POD). Increases in the activity of these enzymes have been used as indicators of plant defense responses (Moerschbacher *et al.*, 1988; Vander *et al.*, 1998). The fungal cell wall preparation was applied exogenously to tobacco leaves. Elicitor induced PAL and POD activity was measured with a spectrophotometer at 0, 2, and 24h in transformed and non-transformed lines. Line *csn*5 and line *csn*6 showed increases in both PAL and POD rates at 2 and 24h, respectively. The PAL and POD rates for the NT line, however, remain unchanged over the course of the treatment (Figure 6). Despite minor discrepancies (i.e., elevated initial POD rates in transgenic lines), these results suggest that constitutive extracellular expression of the *Paenbacillus* sp. 61427 chitosanase may allow for enhanced plant perception of attacking fungi and thereby allow the plant to respond more quickly.

Conclusions:

In this study, the *Paenbacillus* sp. 61427 chitosanase was transcribed, translated, and transported correctly in transgenic tobacco and accumulated at concentrations inhibitory to *R. solani.* 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.

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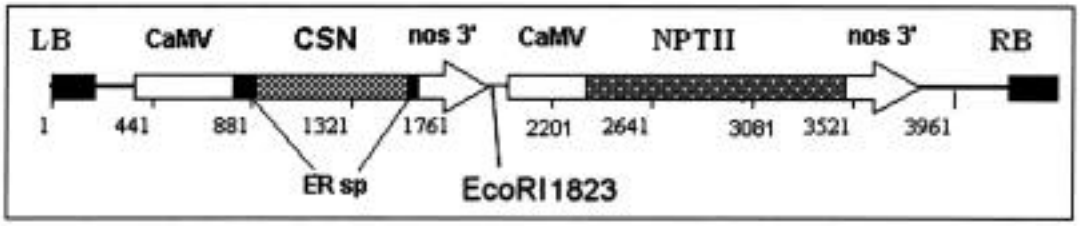

Figure 1. *Experimental T-DNA insert*. Abbreviations: **CaMV** - 35S Cauliflower Mosaic Virus promoter; **CSN** - *Paenbacillus* chitosanase mature protein gene; **ER sp** - *Arabidopsis* transport peptide; **nos 3'** -nopaline synthase terminator; **EcoRI** - digestion site; **NPTII** - kanamycin resistance gene; **LB/RB** -left/right border of T-DNA.

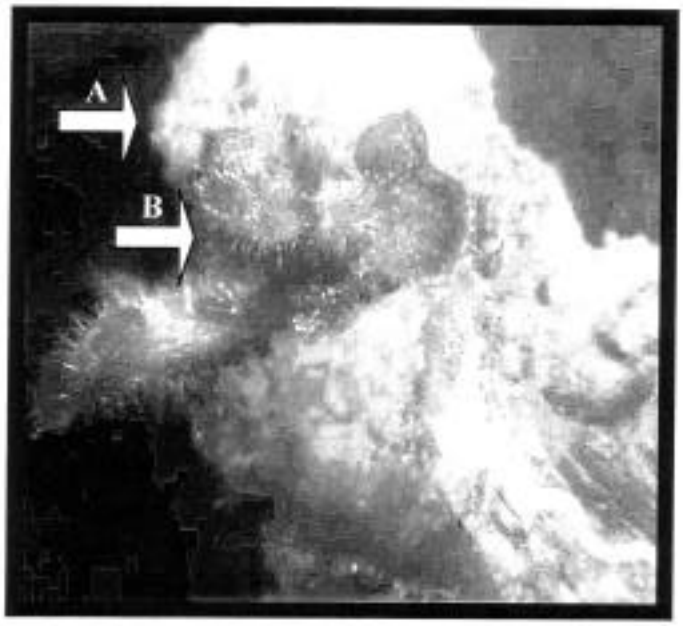

Figure 2. Tobacco leaf disk after 4 1/2 weeks of tissue culture and kanamycin selection. Callus (A) and putative transformed plantlets (B) are indicated.

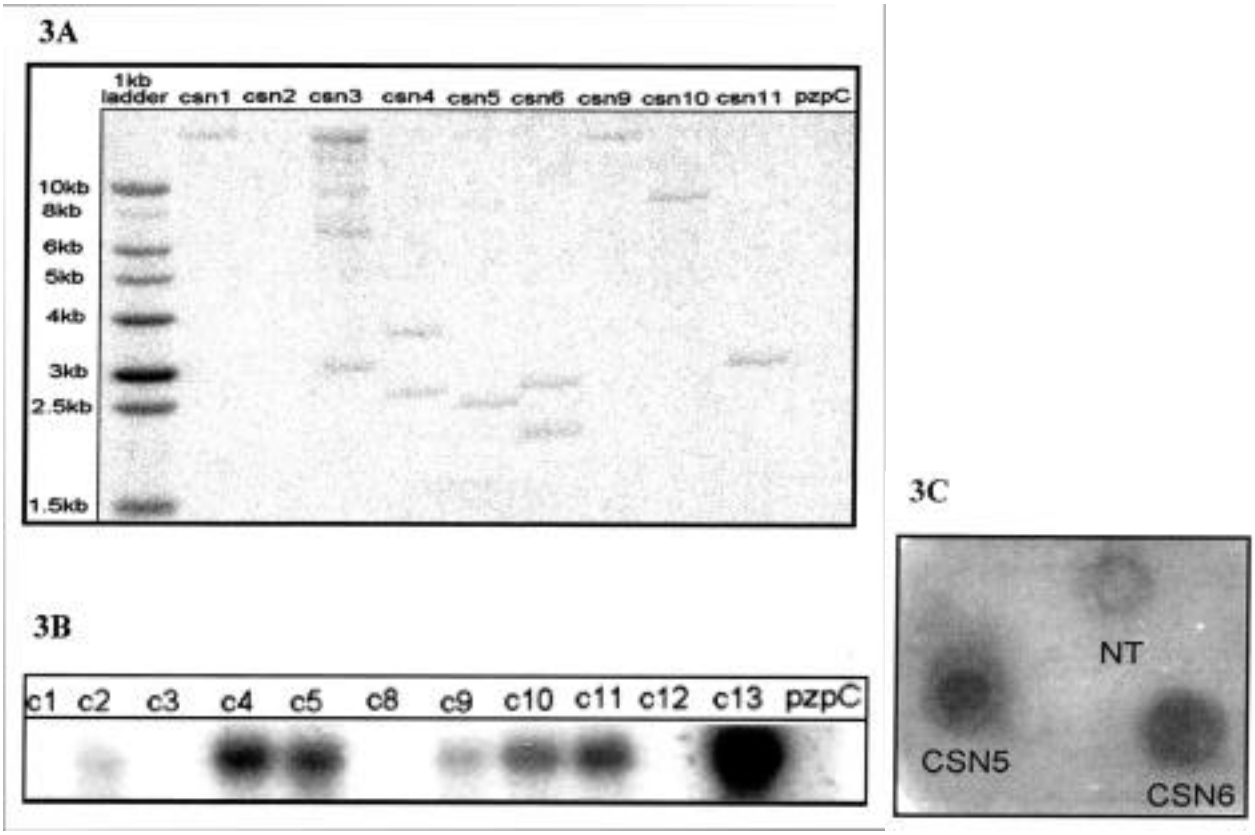

Figure 3A: Southern blot of ten transgenic lines and empty vector control (pzpC). One band = one copy of transgene. **B**: Northern blot of 11 csn lines and empty vector control. **C:** Example of leaf disk lysoplate assay confirming accumulation of active, extracellular chitosanase in two transgenic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

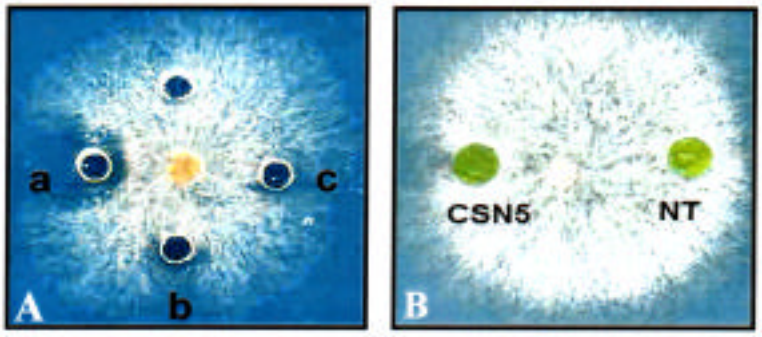

Figure 4. Comparison of anti-fungal activity of (A) the native *Paenbacillus* chitosanase at 5µg(a), 0.5µg(b), and 0.05µg (c) of total protein and (B) recombinant chitosanase from transformed leaf disks. The single copy line, CSN5, inhibited *R. solani* at levels near that of 0.05µg(c) of native protein.

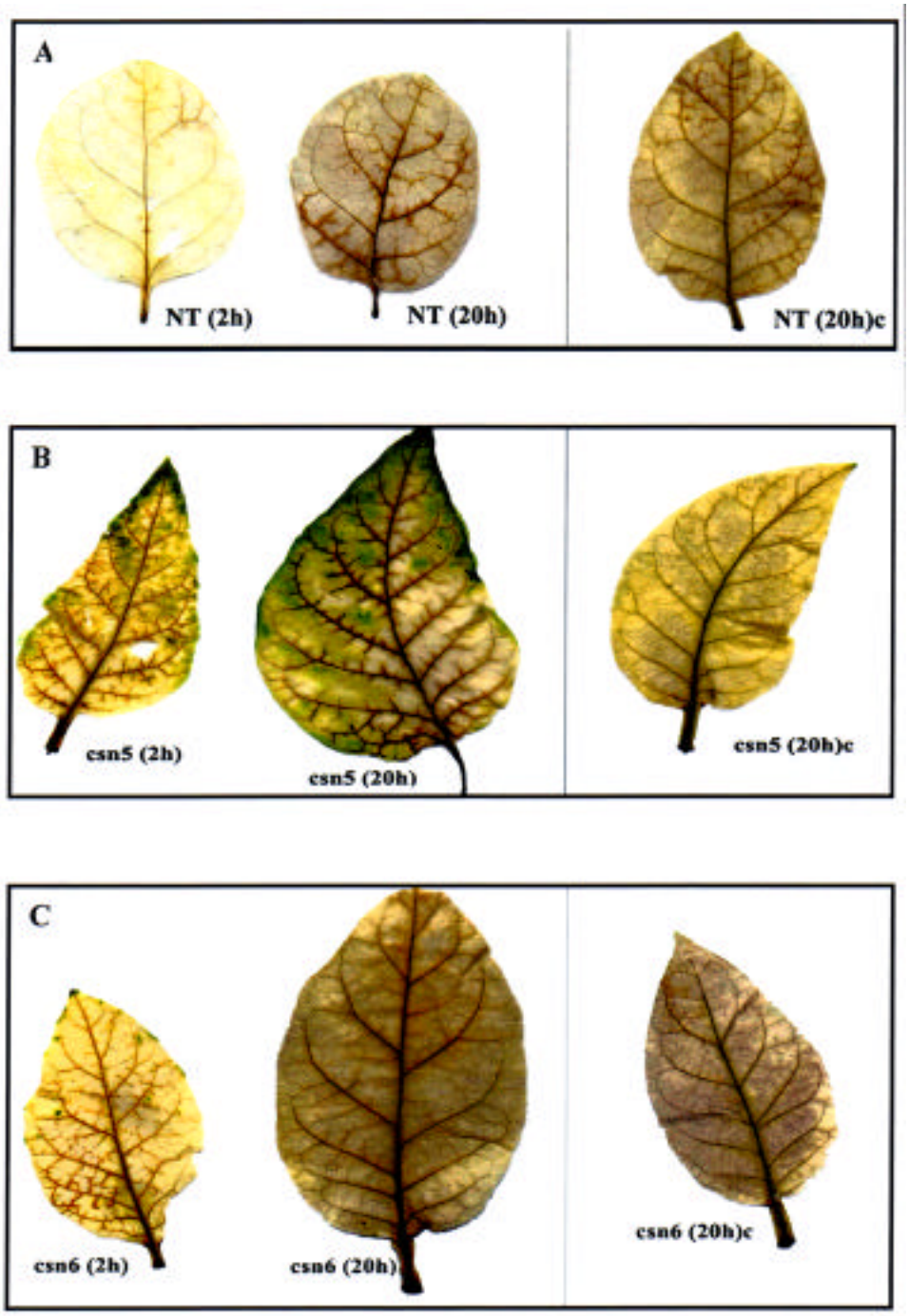

Figure 5. Time-course changes in brown coloration due to DAB staining (H_20_2 production) in the (A) NT, (B) csn5, and (C) csn6 lines at 2h and 20h after treatment and at 20h with DAB but no elicitor treatment [(20h)c].

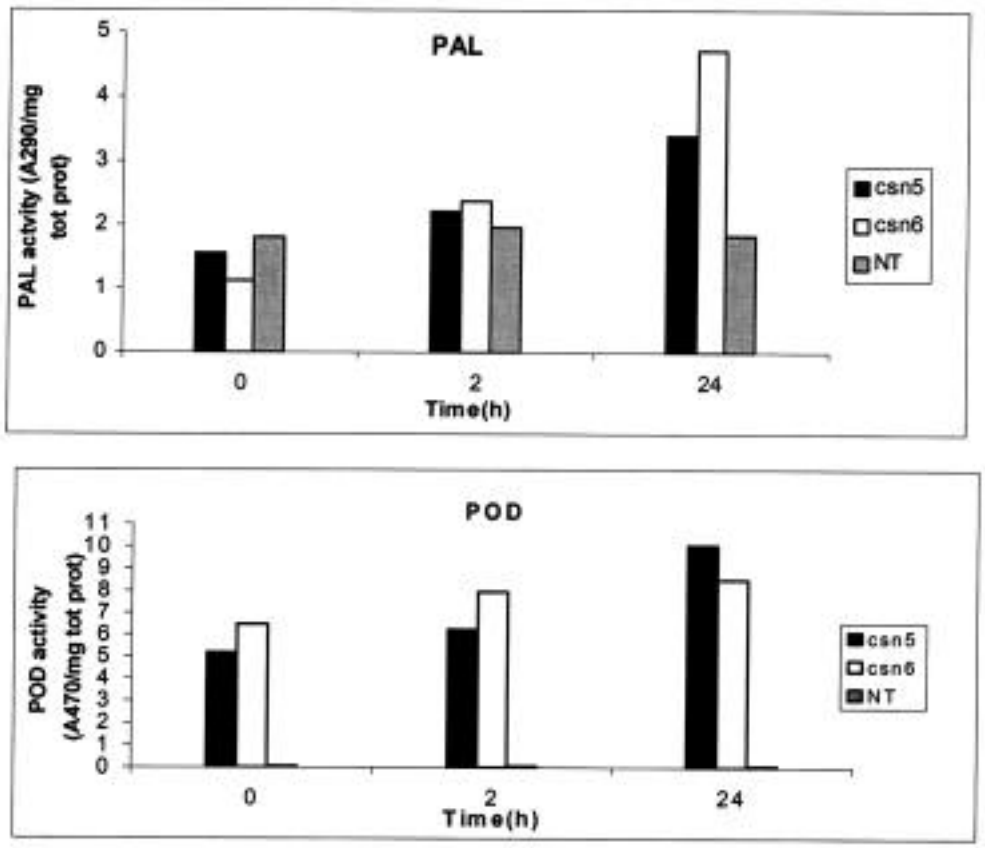

Figure 6. Time-course changes in PAL (A) and POD (B) production in elicitor treated tobacco leaves. Data represent average values from two independent experiments.