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# Transgenic alfalfa secretes a fungal endochitinase protein to the rhizosphere

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**Abstract**

Transgenic plants containing a chimeric gene construct that facilitates the exudation of proteins from roots offer novel approaches for modification of the rhizosphere and production of relatively pure recombinant proteins. The aim of this study was to develop alfalfa (*Medicago sativa* L.) plants that exude a heterologous recombinant protein into the rhizosphere. Alfalfa transformed with a fungal endochitinase (*ech42*) cDNA fused in frame to the signal peptide of a white lupin acid phosphatase and under the control of the cassava vein mosaic virus (CsVMV) promoter expressed increased chitinase activity in vegetative organs and root exudates. Chitinase activity in root exudates of transgenic alfalfa was 7.5–25.7 times higher than in the untransformed Regen-SY plants. Chitinase enzyme activity was accompanied by increased synthesis of mRNA and protein in transformed plants. By comparison, untransformed and vector only transformed plants displayed no expression of recombinant protein and mRNA. A single band of the expected molecular weight was present only in western blots of root exudates of transgenic alfalfa plants. The secreted endochitinase enzyme not only retained its lytic activity against glycol chitin but also showed antifungal activity by inhibition of spore germination of two fungal pathogens. Exudation of recombinant proteins from roots may offer alternative uses for alfalfa in the production of value-added biopharmaceuticals and may influence microbes or modify soil nutrient availability near plant roots.

## Introduction

Alfalfa (*Medicago sativa* L.) is a perennial legume widely cultivated to provide high quality forage in the form of hay, silage and to a lesser extent as a grazing crop, as well as for improving soil fertility. The availability of alfalfa lines that can be easily transformed with *Agrobacterium* and regenerated in tissue culture (Bingham, 1991), makes this plant attractive for genetic engineering and provides the opportunity to develop new uses for alfalfa with value-added products of commercial interest . Because plants can be propagated on a larger scale than other recombinant protein systems, there has been increasing interest in utilizing transgenic plants for the production of recombinant proteins of biopharmaceutical, industrial and biological value (Daniell et al., 2001; Fischer et al., 1999; Kusnadi et al., 1997). Besides, foreign proteins produced in plants appear to contain fewer contaminants and toxic substances than foreign proteins produced by microbial or animal expression systems (Fischer et al., 1999).

Plant roots release naturally as much as 20% of their assimilates as root exudates into the rhizosphere (Uren, 2001; Whipps, 1990). Root exudates contain sugars, amino acids, and enzymes, among others. Root secreted enzymes have been implicated in the mobilization of soil nutrients such as phosphorus from soils low in readily available nutrients (Marschner and Römheld, 2001; Miller et al., 2001). Although the mechanisms regulating enzyme exudation by plant

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roots are not fully understood (Walker et al., 2003), root secreted proteins contain one or more targeting domains termed signal peptides. Signal peptides are short amino acid sequences (motifs) that are located at the N-terminal region of an amino acid sequence which direct protein localization. Our laboratory recently characterized a secreted acid phosphatase (APase) enzyme (EC 3.1.3.2) from roots of white lupin (*Lupinus albus* L.) that contains a signal peptide sequence of 31-amino acids at the N-terminal region of the mature protein (Miller et al., 2001). Copious amounts of this APase are exuded from phosphorus deficient roots (Miller et al., 2001). A similar coding sequence is also involved in the secretion of APase in Indian mustard (*Brassica juncea* L.) (Haran et al., 2000).

Various groups have attempted to express and produce a wide range of recombinant proteins using transgenic tobacco, alfalfa, wheat, rice, soybean, potato, barely and canola, among others (Daniell et al., 2001; Fischer et al., 1999; Kusnadi et al., 1997). The wide utilization of plants for recombinant protein production may be hampered by difficulties in extraction and purification steps of recombinant proteins from plant tissues. Recombinant protein accumulation in plants can be enhanced by constructing vectors that contain signal peptides for targeting the recombinant protein to a particular organelle (Conrad and Fiedler, 1998; Stoger et al., 2002). One of the options for reducing purification steps in the production of plant-derived recombinant proteins has been the targeting of the recombinant protein to the endoplasmic reticulum, and hence to the apoplast, outside of the plant cell wall (Conrad and Fiedler, 1998; Fischer et al., 1999; Stoger et al., 2002). The ability to target transgene products into the rhizosphere may be a useful strategy to influence soil microbiota, cope with soil-borne disease causing organisms, and to improve acquisition of sparingly soluble plant nutrients near plant roots. This paper describes the production and characterization of transgenic alfalfa plants that express and secrete an endochitinase protein (*ech42*) from the biocontrol fungus *Trichoderma harzianum* Rifai (Hayes et al., 1994) to the alfalfa rhizosphere. Transgenic alfalfa contain the cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1996) and a signal peptide from white lupin APase (Miller et al., 2001) or the fungal endochitinase signal peptide sequence.

## Materials and Methods

### *Constructs and plant transformation*

Two plant transformation vectors containing the coding sequence of the mature endochitinase protein, *ech42* cDNA, from the biocontrol fungus *T. harzianum* Rifai (Hayes et al., 1994; GenBank accession # l14614) were constructed. Standard molecular techniques were used for DNA manipulation (Maniatis et al., 1992). The *ech42* cDNA clone, containing a native 27-amino acid signal peptide sequence, was obtained from Dr G. Harman, Cornell University, Geneva, NY. The first vector construct was designed to contain the full-length *ech42* cDNA with its native signal peptide coding sequence. This construct was cloned by PCR amplification of the *ech42* cDNA using the forward primer 5′-ggGAATTCatgttgggcttcctcggaaa-3′ and the reverse primer 5′-ggGGATCCctagttgagaccgcttcgga-3′ (introduced restriction enzyme sites used for cloning shown in upper case). The resulting PCR product was digested with *Eco*RI and *Bam*HI and ligated to the respective sites of the plant transformation vector pILTAB357 to produce *ech42*-FSP (Figure 1A). The plant transformation vector pILTAB357, obtained from Dr C. Fauquet, Donald Danforth Plant Science Center, St. Louis, MO, contains the CsVMV promoter (Verdaguer et al., 1996) and the nopaline synthase (*nos*) 3′ terminator. The vector pILTAB357 also contains *npt*II, which confers kanamycin resistance for the selection of transformed plants, controlled by the *nos* promoter and terminator.

The second construct was designed to contain the APase signal peptide region from white lupin (*Lupinus albus* L.) (Miller et al., 2001), fused to the N-terminal region of the mature endochitinase protein. Primer sequences 5′-ggTCTAGAatgggttatagtagttttgt-3′ (forward) and 5′-ggGAATTCatccactggcttctcaataa-3′ (reverse) were used in a PCR reaction to amplify the 31-amino acid signal peptide sequence from the white lupin APase cDNA (Miller et al. 2001, GenBank accession # AF309552), and 8 extra amino acids downstream of the signal sequence (introduced *Xba*I and *Eco*RI restriction sites used for cloning shown in upper case). The PCR product was cut with *Xba*I and *Eco*RI restriction endonuclease enzymes. The coding sequence for the mature endochitinase protein (protein minus the signal sequence) was also cloned by PCR using the *ech42* cDNA as a template and the primer sequences 5′-ggGAATTCgccagtggatacgcaaacgc-3′ (for-

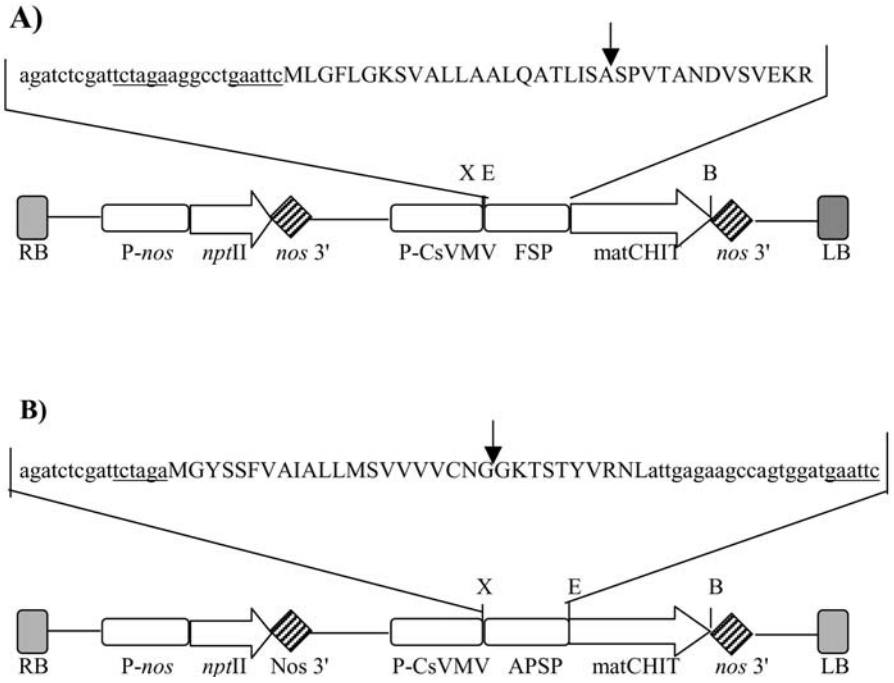


*Figure 1.* Schematic representation of plant transformation constructs containing a 1.3 kbp *Trichoderma* cDNA used in this study: (A) *ech42*-FSP, construct containing the fungal signal peptide, and (B) *ech42*-APSP, construct containing the APase signal peptide from white lupin. Upper case letters show amino acid sequences of the signal peptides, while lower case letters show unique restriction endonuclease sites (underlined) and linker sequences. Arrows indicate cleavage sites of the signal peptide sequences as predicted by PSORT analysis. Abbreviations are matCHIT, the gene encoding the mature endochitinase protein; FSP, the native signal peptide of endochitinase gene from the biocontrol fungus *T. harzianum*; APSP, signal peptide of the APase gene from white lupin; P-CsVMV, cassava vein mosaic virus promoter; *nos* 3′, nopaline synthase terminator; *npt*II, neomycin phosphotransferase II gene confering kanamycin resistance, P-*nos* , nopaline synthase promoter, LB and RB, left and right borders of the *A. tumefaciens* T-DNA. B, *Bam*HI, E, *Eco*RI, X, *Xba*I.

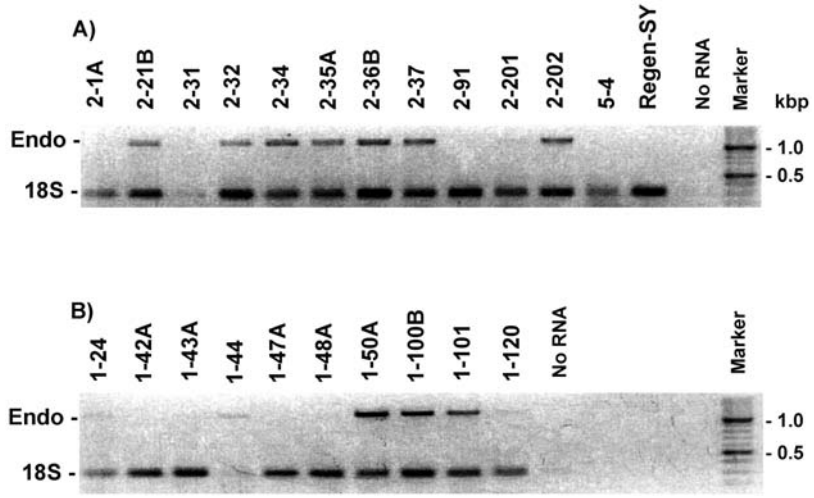


*Figure 2.* Relative RT-PCR analysis of leaf tissue confirms transcription of the fungal endochitinase transgene in transgenic alfalfa: (A) Transgenic alfalfa containing the *ech42*-APSP construct and control plants, and (B) Transgenic alfalfa containing the *ech*42-FSP construct. Control plants were Regen-SY (untransformed) and 5-4 (transformed with vector only). The 18S ribosomal gene transcription (lower band) was used as an endogenous control to evaluate endochitinase mRNA transcription (upper band) in leaf total RNA samples.

ward) and 5′-ggGGATCCctagttgagaccgcttcgga-3′ (reverse) (introduced *Eco*RI and *Bam*HI restriction endonuclease sites shown in upper case). The PCR product was cut with *Eco*RI and *Bam*HI. Digested PCR products of the signal peptide and the mature endochitinase protein coding sequences were cloned into *Xba*I and *Bam*HI cut pILTAB357 to create plant transformation vector *ech42*-APSP (Figure 1B). In addition, an empty vector (pILTAB357) construct containing only the CsVMV promoter was used to produce control transgenic plants. All PCR amplifications were done using TaqPlus® Precision PCR (Stratagene®, La Jolla, CA). All constructs were sequenced to ensure correct amplification and orientation.

Leaf disks of a highly regenerable genotype selected from *Medicago sativa* cv. Regen-SY (Bingham 1991) were transformed via *Agrobacterium*-mediated transformation using procedures described by Austin et al. (1995). Tissue culture regenerated plants were grown in a sand:soil mix (1:1, v/v) in a greenhouse. Plants were watered daily with tap water and fertilized with water soluble fertilizer (20:10:20, N:P:K) when needed.

Genomic DNA from leaf tissue of untransformed as well as putatively transformed plants was isolated using standard procedures (Maniatis et al., 1992). Initial evaluation of putatively transgenic alfalfa was carried out by PCR amplification of the selectable marker gene, *npt*II, following conditions described previously (Saruul et al., 2002). Putative transformants were also verified by PCR analyses using a forward primer sequence specific for the CsVMV promoter (5′-ggaggacacatgtaaggtgg-3′) and an *ech42*-specific reverse primer (5′-cggagtagctgctccaagaa-3′). Amplification conditions were 35 cycles of 94 °C for 30 s, 54 °C for 1 min and 72 °C for 1 min followed by a final extension at 72 °C for 7 min. Because of the obligate outcrossing nature of alfalfa, all experiments were performed with primary transformants propagated from stem cuttings in vermiculite in a greenhouse. All experiments using untransformed alfalfa cv. Regen-SY were also performed from propagated cuttings. Once rooted, all plants were watered daily with tap water and fertilized once a week with a water soluble fertilizer (20:10:20, N:P;K). All experiments were done with rooted plants grown for 4 weeks in vermiculite in a greenhouse.

*Relative reverse transcription (RT)-PCR*

Total RNA was extracted from leaf and root tissues using the Trizol reagent according to the manufacturer's instructions (Life Technologies, Cleveland, OH). *ech42* mRNA expression was evaluated in a multiplex RT-PCR reaction using the access RT-PCR system (Promega, Madison, WI). To remove contaminating DNA, total RNA was incubated in 1 U of RQ1 RNase-free DNase (Promega) for 1 hour at 37 °C and was purified using the RNeasy plant mini kit (Qiagen, Valencia, CA). Primer sequences eF and eR (Bolar et al., 2000) corresponding to positions 179-198 and 1332-1350, respectively, of the *ech42* cDNA sequence were used for the analysis of endochitinase mRNA expression. The universal 18S primer pairs (Ambion, Austin, TX) were used in a multiplex RT-PCR reaction to serve as an internal control. The RT-PCR reactions (25 $\mu$L) consisted of 2.5 U AMV Reverse Transcriptase, 2.5 U *Tfl* DNA Polymerase, 1x AMV/*Tfi* reaction buffer, 0.5 mM $MgSO_4$, 250 $\mu$M dNTPS, 20 pmol of each primer, 7.5 nM universal 18S primer pairs, 17.5 nM competimers (Ambion), and 150 ng total RNA. Amplification conditions were one cycle of 48 °C for 45 min, one cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min followed by a final extension of 68 °C for 7 min. The RT-PCR products were run on a 1% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light.

*Chitinase enzyme activity assay*

Chitinase activity assays were performed using a modification of the method described by Bolar et al. (2000). Leaf or root tissues were harvested and kept on ice. Approximately 250 mg fresh plant tissues were ground in 5 mL extraction buffer containing 0.1% SDS (w/v), 10 mM EDTA, 1% (v/v) Tween-20 and 0.07% (v/v) $\beta$-mercaptoethanol in 100 mM sodium acetate (pH 5), with a mortar and pestle and centrifuged. Aliquots of the supernatant were used for the chitinase activity assay, and detection of endochitinase protein using western blotting. Total protein in tissue extracts was estimated using bovine serum albumin as a standard with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Soluble leaf and root tissue extracts were serially diluted 1:10 and 1:100 with extraction buffer. In a 96-well black-sided plate, 100 $\mu$L of tissue extract or concentrated root exudates was mixed with 50 $\mu$L substrate and incubated at 37 °C for 30 min. The

substrate solution was 0.2 mM 4-methylumbelliferyl $\beta$-D-N, N′, N″ triacetylchitotriside (Sigma, St. Louis, MO) in 3.2% (v/v) DMSO and 100 mM sodium acetate (pH 5). The reaction was stopped by adding 100 $\mu$L of 0.2 M sodium carbonate. The amount of the fluorescent 4-methylumbelliferyl (4-MU) released from the substrate was measured using a BIO-TEK® FL600 microplate fluorescence reader at an excitation wavelength of 360 nm and emission at 460 nm (Bolar et al., 2000). Microtiter plates were also photographed under a 365 nM UV light.

*Collection of root exudates*

Four weeks after cuttings had been rooted in vermiculite, plants were rinsed with distilled water, blotted dry and placed in a 50 mL sterile centrifuge tube with 15 mL filter-sterilized medium. The liquid medium consisted of Murashige and Skoog basal salt mixture (Sigma) amended with 15 g/L sucrose (MSS) (Borisjuk et al., 1999), and a Complete®mini tablet protease inhibitor for every 100 mL of solution (Roche, Indianapolis, IN). Root exudates were collected for 3 h at room temperature with constant shaking and then the root-bathing medium was filtered through Whatman paper to remove debris and concentrated with a 15 mL capacity ultrafiltration unit with a 10 kDa cutoff (Amicon, Beverly, MA). Root exudates samples were adjusted to a 10 × concentration. Root dry weight of plants used for collection of root exudates was determined after drying in a 60 °C oven for 72 hours.

*SDS-PAGE analysis*

For western blots, tissue extracts or concentrated root exudates were resolved on a 10% (w/v) SDS-polyacrylamide gel (PAGE) (Laemmli, 1970). SDS-PAGE gels were electroblotted to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), and probed with 1:2000 diluted anti*ech*42 serum (De La Cruz et al., 1992) and detected using the ECL Western Blot Detection Kit (Amersham). The anti*ech*42 serum was provided by Dr. A. Llobell of Centro de Investigaciones Científicas Isla de la Cartuja, Spain.

*Bioassay for antifungal activity*

To detect lytic activity of root exudates, concentrated root exudate samples were run on a native 10% (w/v) PAGE (Laemmli, 1970) that incorporated glycol chitin (0.01% w/v; Sigma) as a substrate for chitinase (Trudel and Asselin, 1989; Pozo et al., 1998). After electrophoresis, gels were incubated in 100 mM sodium acetate (pH 5) for 4 h at 37 °C, and then transferred to freshly prepared brightener solution (0.01%, w/v, calcofluor M2R in 500 mM Tris-HCl, pH 8.9) for 5 min (Trudel and Asselin, 1989), rinsed in distilled water for 1 h at room temperature and photographed under a 365 nM UV light.

Two alfalfa fungal pathogens, *Phoma medicaginis* Malbr. & Roum. var *medicaginis* and *Colletotrichum trifolii* Bain. & Essary, were grown on PDA (Difco Laboratories, Detroit, MI) plates at room temperature for approximately 7 days before the start of the bioassay. For spore collection, the plates were flooded with sterile distilled water and scraped with a wire loop. Spore concentration was adjusted to 10,000 spores/mL with sterile distilled water. In a 12-well plate, 250 $\mu$L of spore suspension was mixed with 50 $\mu$L of concentrated root exudate from transgenic plants and 300 $\mu$L of sterile MSS medium in each well. Control wells contained 250 $\mu$L of spore suspension, 50 $\mu$L of concentrated root exudate from untransformed Regen-SY plants and 300 $\mu$L of sterile MSS medium. Plates were incubated at room temperature for 24 h and the number of spores forming germ tubes (germinated spores) and those that did not germinate were counted under an inverted microscope. In each sample, approximately 200–500 spores were counted. The experiment was done twice and each sample was duplicated in each experiment.

## Results

*Characterization of transgenic alfalfa expressing endochitinase cDNA from* Trichoderma

PSORT analysis (http://psort.nibb.ac.jp/form.html) predicted that the N-terminal sequences of the *ech*42 constructs containing either the fungal signal peptide (*ech42*-FSP), or the APase signal peptide (*ech42*-APSP) from white lupin contain signal peptides that can be cleaved from the mature protein (Figure 1). According to PSORT, the degree of certainty for directing the mature endochitinase protein for secretion to the outside of the cell was 0.504 and 0.820 for the fungal and plant APase signal peptides, respectively.

The *ech42*-FSP and *ech42*-APSP constructs were used to transform alfalfa via *Agrobacterium*-mediated transformation (Figure 1). Following transformation, 14 putatively transformed alfalfa plants were regener-

ated containing the *ech42*-FSP construct, while 24 putatively transformed plants were regenerated with the *ech42*-APSP construct. The presence of the expression cassettes was verified by PCR amplification of portions of both the *npt*II gene and the CsVMV::*ech42* transgenes. The expected PCR products were visualized in 12 and 22 of the putatively transformed plants produced using the *ech42*-FSP and the *ech42*-APSP constructs, respectively, indicating that these plants contained the promoter, the respective signal peptide sequences and the endochitinase gene. There were 34 regenerated plants produced using the empty vector construct, but only six were screened by PCR for the presence of the *npt*II gene. The untransformed Regen-SY and empty vector transformed plants did not contain the fungal endochitinase transgene.

Genomic DNA from leaf tissue was digested with the restriction enzyme *Eco*RI and hybridized with an approximately a 1.3 kbp *ech42* cDNA probe. The *ech42* transgene contains a single *Eco*RI restriction site in the 5′ region. The hybridizing bands in DNA blots should therefore be bordered at one end by an *Eco*RI site in the transgene and at the other end by another *Eco*RI site in the chromosome near the site of insertion, and thus may indicate the number of transgene insertions. From the number of unique banding patterns, it appears that there were 11 and 13 independently transformed alfalfa lines with the e*ch42*-FSP and e*ch42*-APSP constructs, respectively, with 1-5 T-DNA insertions (data not shown). Vegetative cuttings made from primary transformants were subsequently evaluated for transgene transcription and expression using RT-PCR analysis, enzyme activity assays, and western blotting.

Endochitinase mRNA transcript level in leaf and root tissue of alfalfa plants was examined by using relative RT-PCR and the 18S ribosomal gene product as an internal standard. Eleven independently transformed lines containing the *ech42*-APSP construct and 10 independently transformed lines containing the *ech42*-FSP construct were analyzed. As expected, the untransformed control and all of the *ech42*-FSP and *ech42*-APSP transgenic alfalfa lines produced an RT-PCR product for the 18S ribosomal gene (lower band in Figure 2). *ech42* mRNA transcripts were detected in leaves (Figure 2) and were also present in root tissues (data not shown) of most transgenic lines containing the *ech42*-APSP and *ech42*-FSP constructs. When transcripts were present, *ech42* mRNA transcripts were at relatively high levels in leaves compared to root tissues. In contrast, no *ech42* mRNA transcript was observed in plant samples of untransformed Regen-SY control plants and transformed plants with empty vector construct (Figure 2).

*Chitinase enzyme activity*

Chitinase activity was evaluated in leaf, root tissue and root exudates of selected transgenic lines that showed *ech42* mRNA transcript accumulation. In addition, leaf, root tissue and root exudates from plants of the untransformed Regen-SY and vector only transformants were analyzed for comparison. Chitinase activity in leaf and root tissues of the untransformed Regen-SY and vector only transformant was very low (Figure 3A). In contrast, all of the transgenic alfalfa lines tested that expressed the *ech42* transgene showed significantly higher chitinase activity than control plants (untransformed Regen-SY and vector only transformant).

Concentrated root exudates from roots of transgenic lines and control plants were evaluated for chitinase activity. Very low chitinase activity was present in the root exudates of control untransformed Regen-SY and the transformed plant with the vector only construct (Figures 3B and 3C). Concentrated root exudates from transgenic lines showed 7.5–25.7 times higher chitinase activity than root exudates from the untransformed Regen-SY plants.

*Immunoblot analysis*

Endochitinase polypeptide expression in shoot and leaf tissue extracts of alfalfa plants was evaluated by western blot. The *Trichoderma* endochitinase protein was translated efficiently in transgenic alfalfa leaf and root tissues, as a band of the expected size protein, about 42 kDa, was detected in all transgenic plant tissues analyzed (Figures 4A and 4B). No *ech42* protein signal was detected in leaf and root tissue protein extracts of untransformed Regen-SY plants or plants transformed with the vector only construct. At comparable total soluble protein concentrations, as measured by the BioRad protein assay, the amount of endochitinase polypeptides from leaf tissues that reacted with the anti*ech42* serum was considerably greater than that from root tissues of the same transgenic line. These observations were consistent with the steady state *ech42* mRNA abundance and enzyme activity observed in Figures 2 and 3.

Immunoblots of concentrated root exudates showed the expected endochitinase-specific band only from

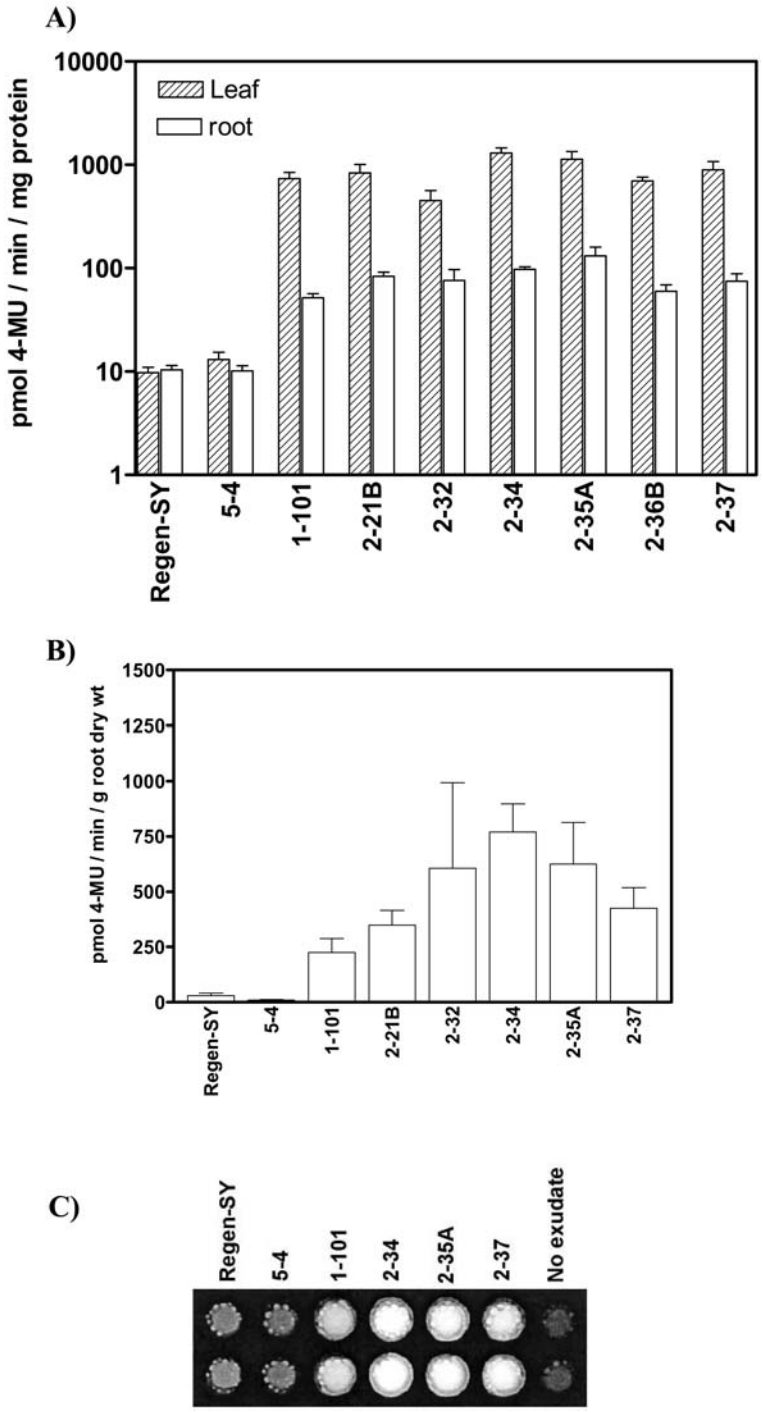


*Figure 3.* Chitinase activity in alfalfa: (A) Leaf and root tissue protein extracts, B) concentrated root exudates and C) visual demonstration of chitinase activity in concentrated root exudates of alfalfa. Chitinase activity bars = means + SE (N = 8 in A and B; and N = 5 in C). Chitinase activity was determined using 4-methylumbelliferyl $\beta$-D-N, N′, N″ triacetylchitotriside in a 96-well black-sided microtiter plate. The high intensity of the fluorescent 4-methylumbelliferyl substrate in plate in C under a 365 nM UV light indicates increased chitinase activity. Samples were Regen-SY (untransformed control), 5-4 (transformed with vector only), 1-101 (transgenic alfalfa containing *ech42*-FSP construct) and other plants contain the *ech42*-APSP construct.

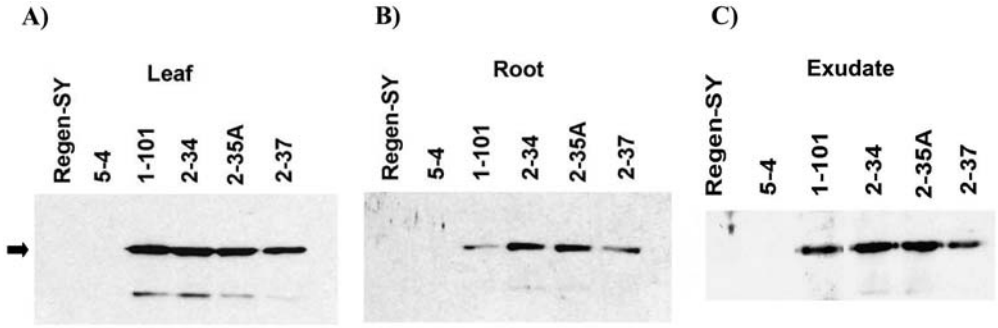


*Figure 4.* Immunoblot of SDS-PAGE. A) Leaf tissue protein extract, B) Root tissue protein extract, and C) Concentrated root exudates. Total soluble protein samples (15 $\mu$g/lane) were resolved on a 10% (w/v) SDS-PAGE, blotted and visualized with anti*ech42* serum and the ECL western-blot detection reagent. Arrowhead indicates 42 kDa endochitinase protein band. Note that the low molecular weight band seen in transgenic alfalfa lanes appear to be a proteolytic degradation product of the endochitinase enzyme that may have occurred during protein extraction. Samples were Regen-SY (untransformed control), 5-4 (transformed with vector only), 1-101 (transgenic alfalfa containing the fungal signal peptide) and other plants contain the *ech42*-APSP construct.

transgenic lines expressing the *ech42*-FSP and *ech42*-APSP constructs (Figure 4C). The endochitinase-specific band was absent in root exudates of untransformed Regen-SY and vector only transformed control plants.

Silver staining of concentrated root exudates separated by SDS-PAGE revealed the presence of a number of secreted protein bands in root exudates of both control and transgenic alfalfa (data not shown). Nevertheless, the endochitinase specific band was only present in transgenic alfalfa expressing the endochitinase gene.

*Bioassay for antifungal activity*

Lytic activity of concentrated root exudates was evaluated after separation on a 10% native PAGE that contained 0.01% (w/v) glycol chitin as substrate. Several lytic zones indicating the presence of chitin-degrading enzymes were detected in both control and transgenic plants as shown in Figure 5A. However, root exudates from transgenic plants showed an additional lytic zone that was not visible in the root exudates of control plants.

The bioassay using the concentrated root exudate from transgenic alfalfa showed significant inhibition of spore germination of two fungal plant pathogens, *P. medicaginis* and *C. trifolii* (Figure 5B). Spore germination in medium with concentrated root exudates from untransformed Regen-SY was 83% and 39% for *P. medicaginis* and *C. trifolii*, respectively. When concentrated root exudates from transgenic plants were added to the medium, spore germination was inhibited to 7% and 1% for *P. medicaginis* and *C. trifolii*, respectively. These results indicate that the secreted endochitinase enzyme retains antifungal activity.

## Discussion

Transgenic plants have been shown to be suitable expression systems for various heterologous proteins. Our results extend the understanding of the use of plants for producing novel proteins by showing: 1) transgenic alfalfa secrets a recombinant protein into the rhizosphere; 2) the exuded recombinant protein retained enzyme activity; and 3) use of the CsVMV promoter in conjunction with the novel signal peptide sequence from a plant protein secreted into the rhizosphere could be very effective strategy to produce relatively pure recombinant protein in root exudates of agronomic crops. Results of this study demonstrate that alfalfa is suitable for the rhizosecretion of heterologous protein that has antifungal activity.

The observation that transgenic alfalfa transformed with a chimeric gene composed of a white lupin APase signal peptide fused to a fungal chitinase releases the fungal protein into the rhizosphere demonstrates the potential for directing the exudation of a recombinant protein to the rhizosphere. The exudation of antifungal and antimicrobial recombinant proteins from plant roots is likely to be a useful biocontrol strategy in protecting plants from disease-causing soil organisms thereby maximizing crop yield and reducing cost of cultivation. Rhizosecretion of recombinant proteins and enzymes in transgenic alfalfa also constitutes an effective strategy to influence biological activity near plant roots, to influence microbial symbiosis, and to

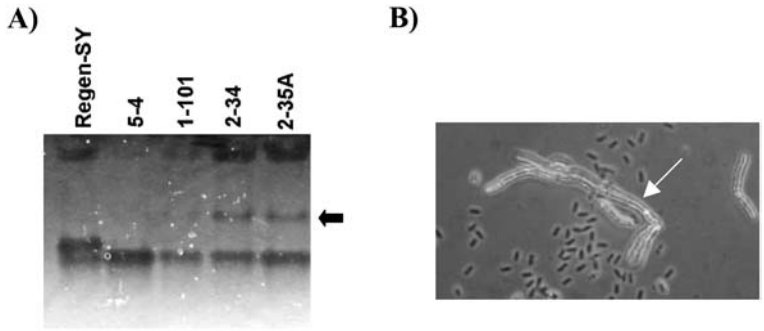


*Figure 5.* Chitionlytic activity and bioassay for antifungal activity. A) Chitinase activity in native PAGE For chitinase activity, concentrated root exudates were resolved on a native 10% (w/v) PAGE that incorporated glycol chitin (0.01% w/v), gels were incubated in 100 mM sodium acetate (pH 5) for 4 h at 37 °C, stained with calcofluor (0.01%, w/v), and then photographed under a 365 nM UV light. Lytic zones appear as dark bands against a brightly fluorescing background and arrowhead indicates lytic zones of the *Trichoderma* endochitinase enzyme. B) Plate shows germinated spores (arrows) and spores without germ tubes (100× magnification).

influence plant growth-promoting soil microbes. It is also useful to modify and improve the acquisition of sparingly soluble plant nutrients in the vicinity of plant roots.

Despite the secretion of proteins in root exudates of all plants tested, the endochitinase-specific band was absent in western blots of root exudates of untransformed Regen-SY plants and transformed alfalfa using the vector only construct. Immunoblot analysis of concentrated root exudates showed the expected endochitinase-specific band only from transgenic alfalfa lines expressing the *ech42* transgene. These results were in agreement with Brants and Earle (2001) who reported higher chitinase activity and an endochitinase-specific protein product in the medium underneath transgenic calli of tobacco and liquid medium of suspension cultures of transgenic tobacco expressing the fungal endochitinase gene with the native signal peptide. Similarly, Lorito et al. (1998) reported extracellular accumulation of the endochitnase protein following immunogold labeling of transgenic tobacco expressing the endochitinase gene containing the native signal peptide. These observations led the authors to conclude that the fungal endochitinase protein was secreted from transgenic tobacco plants, callus and cell culture suspensions. Our results also expand on previous work in that the fungal signal peptide is useful in targeting the endochitinase protein for secretion to the alfalfa rhizosphere. Borisjuk et al. (1999) also reported that transgenic tobacco plants carrying the calreticulin gene signal peptide secrete a heterologous green fluorescent protein (GFP) from jelly fish to the rhizosphere. In contrast, GFP was localized in the cytoplasm of transformed tobacco without a signal peptide sequence (Borisjuk et al., 1999).

Although the fungal and APase signal peptides were recognized by alfalfa plants for secretion of the endochitinase protein into the rhizosphere, there were indications that the plant-derived signal peptide may have greater potential for targeting recombinant proteins to the alfalfa rhzosphere. The first indication was that PSORT predicted the secretion of endochitinase protein with much higher certainty using the APase signal peptide sequence compared to using the fungal signal peptide sequence. Secondly, transgenic alfalfa containing the APase signal peptide showed considerably higher chitinase activity in leaf and root tissues as well as in concentrated root exudates than transgenic alfalfa containing the fungal signal peptide. Promoter elements also appear to influence the level of recombinant protein accumulation in transgenic plants. For example, the amount of human placental secreted alkaline phosphatase (SEAP) protein in transgenic tobacco was higher with the mas2′ promoter construct compared to the 35S promoter (Borisjuk et al., 1999). We did not compare different promoters in this report, but the CsVMV promoter used in this study has been shown to confer constitutive expression of a chimeric gene in vegetative organs of rice and tobacco (Verdaguer et al., 1996). Recent work from our laboratory showed that transgenic alfalfa with the CsVMV promoter driving the *ech42* gene displayed highest chitinase activity in leaves, roots, and root nodules compared to alfalfa with the *ech42* cDNA controlled by the CaMV 35S promoter and figwort mosaic virus promoter, among others (Samac et al., 2004).

The biocontrol fungus, *T. harzianum*, produces three different endochitinase enzymes (CHIT33, CHIT37 & CHIT42) (De La Cruz et al., 1992). *In vitro* assays showed that CHIT42 encoded by *ech42* has

the most significant lytic activity over the other isoforms, as demonstrated by degradation of purified cell walls and inhibition of spore germination and hyphal elongation of the pathogenic fungus *Botrytis cinerea* (De La Cruz et al., 1992). As a result, other investigators have employed constitutive expression of the fungal *ech42* cDNA in transgenic plants as a strategy to improve plant resistance to fungal diseases (Lorito et al., 1998; Mora and Earle, 2001). Our observations that transgenic alfalfa expressed the fungal endochitinase transgene and displayed significantly increased chitinase activity in leaf and root tissues were in agreement with published reports for other transgenic plants such as broccoli and tobacco among others (Brants and Earle, 2001; Lorito et al., 1998; Mora and Earle, 2001). In all cases the fungal endochitinase transgene contained the native signal peptide and was expressed in transgenic plants, in transgenic calli, and in transgenic cell suspension cultures. Similar to our results in protein immunoblots, transgenic plants as well as transgenic calli and cell suspension cultures expressed a protein recognized by the anti*ech42* serum (Brants and Earle, 2001; Lorito et al., 1998; Mora and Earle, 2001). Lorito et al. (1998) reported higher endochitinase protein accumulation mainly in leaves and stems compared to roots and flowers of transgenic tobacco and potato plants that contained the fungal endochitinase gene with the native signal peptide. The fungal endochitinase protein was absent in protein extracts from control tobacco plants and calli without the transgene (Brant and Earle, 2001; Lorito et al., 1998; Mora and Earle, 2001). The increase in chitinase activity in leaf and root tissues of transgenic alfalfa lines was paralled by an increase in endochitinase transgene transcription in these tissues. Nevertheless, the anti*ech42* serum showed a reaction to a low molecular weight protein band seen in leaf and root tissue protein extracts of transgenic alfalfa. Because this product was absent from untransformed Regen-SY and vector only transformant control alfalfa plants, the most likely explanation is proteolytic degradation that may have occurred during tissue protein extraction steps (Bolar et al., 2000; Farran et al., 2002).

Additional as yet unidentified protein bands were visualized on silver stained SDS-PAGE, indicating that additional proteins were also secreted into the root exudates of control and transgenic alfalfa plants (data not shown). Some of the protein bands in root exudates appear to show lytic activity to a glycol chitin substrate in a native protein gel, suggesting that they could be plant-derived chitinase(s). The mechanism and consequences of secreting plant chitinases in root exudates of alfalfa plants are not clear, but it may represent yet another plant defense mechanism not known previously. In conclusion, the demonstration that transgenic alfalfa plants together with an APase signal peptide sequence from white lupin are suitable for the expression of secreted proteins in alfalfa may be a useful strategy to modify soil microbial activity, or to influence plant health near plant roots, and may provide alternative uses for traditional crop species and would open new markets.

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