

A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*)

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Abstract

The activity of constitutive promoters was compared in transgenic alfalfa plants using two marker genes. Three promoters, the 35S promoter from cauliflower mosaic virus (CaMV), the cassava vein mosaic virus (CsVMV) promoter, and the sugarcane bacilliform badnavirus (ScBV) promoter were each fused to the β -glucuronidase (*gusA*) gene. The highest GUS enzyme activity was obtained using the CsVMV promoter and all alfalfa cells assayed by *in situ* staining had high levels of enzyme activity. The 35S promoter was expressed in leaves, roots, and stems at moderate levels, but the promoter was not active in stem pith cells, root cortical cells, or in the symbiotic zones of nodules. The ScBV promoter was active primarily in vascular tissues throughout the plant. In leaves, GUS activity driven by the CsVMV promoter was approximately 24-fold greater than the activity from the 35S promoter and 38-fold greater than the activity from the ScBV promoter. Five promoters, the double 35S promoter, figwort mosaic virus (FMV) promoter, CsVMV promoter, ScBV promoter, and alfalfa small subunit Rubisco (RbcS) promoter were used to control expression of a cDNA from *Trichoderma atroviride* encoding an endochitinase (*ech42*). Highest chitinase activity in leaves, roots, and root nodules was obtained in plants containing the CsVMV:*ech42* transgene. Plants expressing the endochitinase were challenged with *Phoma medicaginis* var. *medicaginis*, the causal agent of spring black stem and leaf spot of alfalfa. Although endochitinase activity in leaves of transgenic plants was 50- to 2650-fold greater than activity in control plants, none of the transgenic plants showed a consistent increase in disease resistance compared to controls. The high constitutive levels of both GUS and endochitinase activity obtained demonstrate that the CsVMV promoter is useful for high-level transgene expression in alfalfa.

Introduction

A number of promoters have been identified that confer high levels of constitutive expression of

heterologous genes in transgenic plants. The 35S promoter from the cauliflower mosaic virus (CaMV) in various configurations has been the most widely used constitutive promoter in dicot species. Several other promoters from viruses have been reported to have activity similar to the 35S promoter in some transgenic dicot plants. Such promoters include the full-length transcript promoter or 34S promoter from figwort mosaic virus (FMV) (Sanger et al., 1990; Maiti et al.,

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1997), the sugarcane bacilliform badnavirus (ScBV) promoter (Tzafrir et al., 1998), the cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1996), and the carnation etched ring virus promoter (Holmberg et al., 2002). Constitutive promoters from plants that confer high levels of transgene expression in dicots have also been reported (Holtorf et al., 1995; An et al., 1996; Khoudi et al., 1997; Kelemen et al., 2002; Malik et al., 2002). In addition, chimeric promoters that combine regulatory elements from more than one promoter have been shown to confer high levels of constitutive transgene expression in some dicot plants (Comai et al., 1990; Ni et al., 1995; Ziegelhoffer et al., 1999). A qualitative and quantitative analysis of expression of transgenes from constitutive promoters has not been reported for alfalfa.

Previous studies have shown that the amount of activity and tissue-specific expression of nominally constitutive promoters can differ substantially depending on plant species, organ, and plant developmental stage. For example, Malik et al. (2002) found that β -glucuronidase (GUS) activity from a 35S:*gusA* construct was much greater in leaves of transgenic tobacco compared to GUS activity in leaves of transgenic canola, *Arabidopsis*, or alfalfa. Although expression of a 35S:green fluorescent protein (GFP) construct was observed in almost all parts of cotton plants, stronger GFP expression was seen in vascular tissues of cotyledons and leaves (Sunilkumar et al., 2002). In transgenic tobacco plants, the 35S promoter conferred high levels of GUS expression in leaves and stems, but expression was lower in flowers and seeds (Malik et al., 2002). In contrast, similar levels of GUS expression were observed in all organs of transgenic canola (Malik et al., 2002) and *Arabidopsis* (Holtorf et al., 1995) expressing a 35S:*gusA* transgene. The 35S promoter has also been shown to be developmentally regulated in some dicots. In transgenic cotton, the 35S promoter was not active during early embryo development (Sunilkumar et al., 2002) and developmental regulation of the 35S promoter was observed in transgenic tobacco leaves (Williamson et al., 1989).

In transgenic alfalfa plants the 35S promoter has been used successfully to confer increased disease resistance (Hill et al., 1991; Hipskind and Paiva, 2000), improved winter survival (McKersie

et al., 2000), and enhanced tolerance to salt (Winicov, 2000) and aluminum in acidic conditions (Tesfaye et al., 2001), among other traits. In efforts to utilize transgenic alfalfa as a bioreactor for production of high value proteins, the 35S promoter was used to drive the production of a monoclonal antibody at levels between 0.13 and 1% of total soluble protein (Khoudi et al., 1999). Nonetheless, in contrast to the strong activity of the 35S promoter in other dicot plant species, several reports suggest that this promoter may have less activity in alfalfa (Narváez-Vásquez et al., 1992; Tabe et al., 1995; Khoudi et al., 1997; Malik et al., 2002).

The objectives of this study were to compare the performance of several nominally constitutive promoters in alfalfa using two marker genes, *gusA* and an endochitinase from the biocontrol fungus *Trichoderma atroviride* (*T. harzianum*) (Hayes et al., 1994), to identify promoters with the highest activity, evaluate expression in different tissues, and to test whether high levels of endochitinase activity increased resistance to a fungal pathogen. We compared GUS activity driven by the 35S promoter, CsVMV promoter, and ScBV promoter. Endochitinase activity was assayed in plants with constructs in which the cDNA was controlled by the double or enhanced 35S promoter (E35S), FMV promoter, CsVMV promoter, ScBV promoter, or an alfalfa Rubisco small subunit promoter (Khoudi et al., 1997). Highest activity of both GUS and endochitinase was obtained using the CsVMV promoter. However, expression of the *T. atroviride* endochitinase in transgenic alfalfa did not improve resistance to *Phoma medicaginis* var. *medicaginis*, the causal agent of spring black stem and leaf spot in alfalfa.

Materials and methods

Construction of transformation vectors and plant transformation

Three plant transformation vectors were obtained to compare promoter activity by measuring activity of GUS in transgenic alfalfa plants containing each of the promoter:*gusA* gene fusions (Figure 1). The vector pBI121 (Jefferson et al., 1987) contains the CaMV 35S promoter fused to

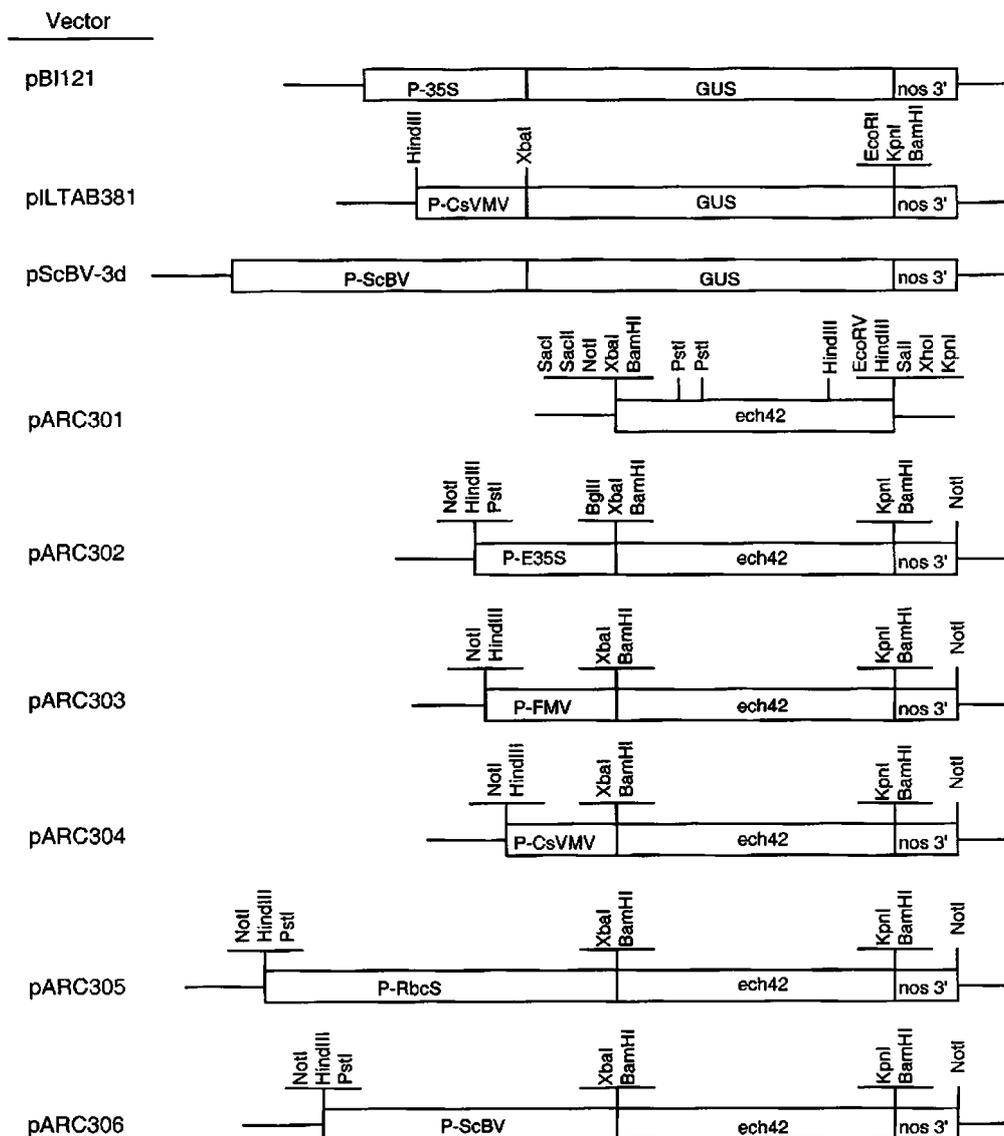


Figure 1. Maps of chimeric marker genes used for plant transformation. For sources and vector construction see Materials and methods. P-35S: cauliflower mosaic virus 35S promoter; P-CsVMV: promoter from the Cassava Vein Mosaic Virus; P-ScBV: promoter from the Sugarcane Bacilliform Badnavirus; P-E35S: enhanced 35S promoter; P-FMV: promoter from the Figwort Mosaic Virus; P-RbcS: Rubisco small subunit promoter from alfalfa; GUS: β -glucuronidase gene; *ech42*: cDNA from *Trichoderma atroviride* encoding an endochitinase.

the *gusA* gene followed by the nopaline synthase (*nos*) 3' terminator. The vector pILTAB381, obtained from C. Fauquet, Donald Danforth Plant Science Center, St. Louis, MO, contains the CsVMV promoter fused to *gusA* and the *nos* 3' terminator (Verdaguer et al., 1996). The vector pScBV-3d, containing the promoter from the ScBV fused to *gusA* and the *nos* 3' terminator (Tzafir et al., 1998), was obtained from

N. Olszewski, University of Minnesota, St. Paul, MN. The promoter:*gusA* gene in all three vectors are transcriptional fusions. The three vectors also contain the neomycin phosphotransferase II (*nptII*) gene controlled by the *nos* promoter and terminator for selection of transformed plants.

The cloning strategy for the five promoter-endochitinase constructs was to assemble the promoter-endochitinase gene-*nos* 3' terminator as a

cassette in a high copy number plasmid, pMON999 (Monsanto Co., St. Louis, MO). The gene cassette is flanked on either side by a *NotI* restriction enzyme site. Once assembled, the gene cassettes were excised by *NotI* digestion and ligated into pMON51850 (Monsanto Co.), which contains T-DNA right and left borders for T-DNA transfer and a *nos* promoter:*nptII* gene for selection of transgenic plants. The cDNA clone, *ech42*, encoding a *T. atroviride* endochitinase (Hayes et al., 1994) was obtained from G. Harman, Cornell University, Geneva, NY. The cDNA was excised from pCR2.1 using *BamHI* and *EcoRV* endonucleases and ligated into the *BamHI-EcoRV* site of pBluescriptSK+ (Stratagene, La Jolla, CA). The resulting plasmid, pARC301 (Figure 1), was digested with *XbaI* and *KpnI* to excise the cDNA sequence. The fragment was ligated into pMON999 digested with *XbaI* and *KpnI*. The resulting vector, pARC302 (Figure 1) contains the cDNA under the control of the double 35S promoter (P-E35S) from pMON999. The vector pARC303 (Figure 1), with the FMV promoter controlling *ech42*, was constructed by replacing the *gusA* gene in pMON10018 (Monsanto Co.) with an *XbaI-KpnI* fragment containing the endochitinase cDNA. The vector containing the CsVMV promoter, pARC304 (Figure 1) was constructed by ligating three fragments, a 0.6-kbp *HindIII-XbaI* fragment containing the CsVMV promoter from pILTAB381, a 1.3-kbp *XbaI-KpnI* fragment containing the *ech42* cDNA from pARC 301 and pMON999 digested with *HindIII* and *KpnI*. The alfalfa Rubisco small subunit promoter, a 1.58-kbp fragment containing the 5' elements of the promoter and 5' untranslated region in pUCBM21 (Khouidi et al., 1997), was obtained from S. Laberge, Agriculture Canada, Québec, Canada. To obtain needed cloning sites pUCBM21 was digested with *SalI* and *XbaI* and the promoter fragment ligated into pUC119. The resulting plasmid was digested with *PstI* and *KpnI* to excise the promoter and this fragment was ligated with a 1.3-kbp *XbaI-KpnI* fragment from pARC301 containing the endochitinase cDNA and pMON999 digested with *HindIII* and *KpnI* to create pARC305 (Figure 1). To obtain restriction sites to construct the gene fusion containing the ScBV promoter, pScBV-3d (Tzafrir et al., 1998) was digested with *EcoRI* and *SacII* to

excise the promoter and the fragment was ligated into the *EcoRI-SacII* site of pBluescriptKS+ (Stratagene). The resulting plasmid was digested with *PstI* and *XbaI* and the promoter fragment ligated with a 1.3-kbp *XbaI-KpnI* fragment from pARC301 containing the endochitinase cDNA and pMON999 digested with *HindIII* and *KpnI* to form pARC306 (Figure 1). Constructs were confirmed by restriction digestion and the promoter-endochitinase cDNA borders were confirmed by DNA sequencing. The gene cassettes were excised by digesting with *NotI* and ligated into the *NotI* site of the plant transformation vector pMON51850. Vectors were mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating.

Agrobacterium-mediated transformation of leaf explants from a highly regenerable clone of Regen-SY (Bingham, 1991) was carried out essentially as described by Austin et al. (1995). Regenerated plants were transferred to potting mix (Metromix 200, The Scotts Co., Marysville, OH) and grown in a growth chamber set at 16 h light/day, 24°C. Transgenic plants were identified by PCR amplification of *nptII* as described previously (Saruul et al., 2002) and amplification of the *Trichoderma* endochitinase using the primers described by Bolar et al. (2000). Due to the obligate out-crossing nature of alfalfa, all experiments were carried out using vegetative cuttings of primary transformants grown in a greenhouse at 24–26°C and 16 h light/day. Plants were watered daily and fertilized monthly with Peters all purpose fertilizer (J. R. Peters Inc., Allentown, PA).

Histochemical localization and fluorometric assays for β -glucuronidase activity

For histochemical analysis, tissue pieces were excised, vacuum infiltrated with a staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl β -D-glucuronic acid sodium salt trihydrate (X-GlcA; Research Products International, Mt. Prospect, IL) as described (Stomp, 1992), and incubated at 37°C for 3 to 16 h. Stems and nodules were sectioned by hand before staining. After staining, samples were rinsed with water and fixed in 70% ethanol.

GUS enzyme activity was quantified using the fluorometric assay described by Jefferson (1987)

with the following modifications. Tissue pieces (approximately 10–20 mg) were excised, placed in 1.5 ml microfuge tubes, frozen in liquid nitrogen, pulverized, and then ground in GUS extraction buffer (Jefferson, 1987) (10 μ l/mg tissue) using a motorized pestle. Insoluble material was pelleted by centrifugation at 14,000 \times g for 10 min. The extracts were diluted 1:10 and 1:100 (v/v) with extraction buffer. Samples of 5 μ l were assayed in triplicate in extraction buffer with 5 μ l of 50 mM 4-methylumbelliferyl β -D-glucuronide trihydrate (MUG; Research Products International) in 250 μ l reactions in 96-well black-sided assay plates (Corning Inc., Corning, NY). Fluorescence of the 4-methylumbelliferone (4-MU) produced was measured over 30 min at 37°C using a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT) equipped with a 360/40 nm excitation filter and 450/50 nm detection filter. Fluorescence units of 4-MU (Sigma Chemical Co., St. Louis, MO) standards in extraction buffer were used to calibrate the plate reader. Protein concentration was determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Each tissue was sampled in duplicate and the experiment was carried out twice.

Fluorometric assay for endochitinase activity

Chitinase enzyme activity was measured using a modification of the method described by Bolar et al. (2000). Plant samples (approximately 20 mg⁻¹ g) were harvested and held on ice. Samples were ground in extraction buffer (Bolar et al., 2000) (1 ml/20 mg tissue) with a mortar and pestle. Insoluble material was pelleted by centrifugation at 4,000 \times g for 10 min at 4°C. Extracts were diluted 1:10 and 1:100 with extraction buffer. Assays were carried out in duplicate using 100 μ l tissue extract and 50 μ l substrate (0.2 mM 4-methylumbelliferyl β -D-N, N', N'' triacetylchitotriside; Sigma Chemical Co.) in 96-well black-sided assay plates at 37°C. After 30 min, the reaction was stopped by adding 100 μ l 0.2 M Na₂CO₃ and fluorescence of the 4-MU produced read by a BioTek FL600 Microplate Fluorescence Reader using a 360/40 nm excitation filter and 450/50 nm detection filter. A standard curve was prepared using 4-MU standards in extraction buffer. Protein

concentration of extracts was determined using the BioRad Protein Assay with bovine serum albumin as the standard. The experiment was carried out two times and in each experiment each tissue was sampled in duplicate.

Evaluation of disease resistance

Cultures of *Phoma medicaginis* var. *medicaginis* strain 866, obtained from K. Leath (USDA-ARS, University Park, PA), were grown for 14 days on potato dextrose agar plates at room temperature. Fresh spore suspensions were collected in sterile distilled water with 0.01% Tween-20 and spore concentrations were determined using a hemacytometer. Fully expanded leaves were removed from the youngest three nodes and placed abaxial side down on sterile moistened filter paper in a covered plastic 100 mm \times 15 mm petri-plate, five leaves per plate. Plates were spray-inoculated with approximately 1 ml of spores at 1×10^5 , 1×10^4 , or 1×10^3 spores/ml. For each plant line, three plates were inoculated at each concentration; one plate was mock-inoculated. Leaves were incubated at room temperature for 12 days and then scored for leaf spot symptoms on a 0–12 scale where 0 = no symptoms and 12 = 100% of leaf yellow, dead leaf. For whole plant assays, vegetative cuttings were grown for eight weeks in the greenhouse and sprayed until runoff with a fresh spore suspension of *P. medicaginis* strain 866 at 1×10^6 spores/ml in sterile distilled water with 0.01% Tween-20. Plants were maintained in 100% relative humidity in darkness for 48 h then returned to the greenhouse. Defoliation of the primary stem of each plant was evaluated 7 days after inoculation. The study was carried out twice and in each experiment eight plants were sampled from each line tested. Statistical analyses were performed using the general linear model procedure in SAS (SAS Institute, 1989) and mean separations were performed using LSD.

Results

Analysis of GUS activity

Transgenic alfalfa plants were produced using a highly regenerating alfalfa clone from Regen-SY

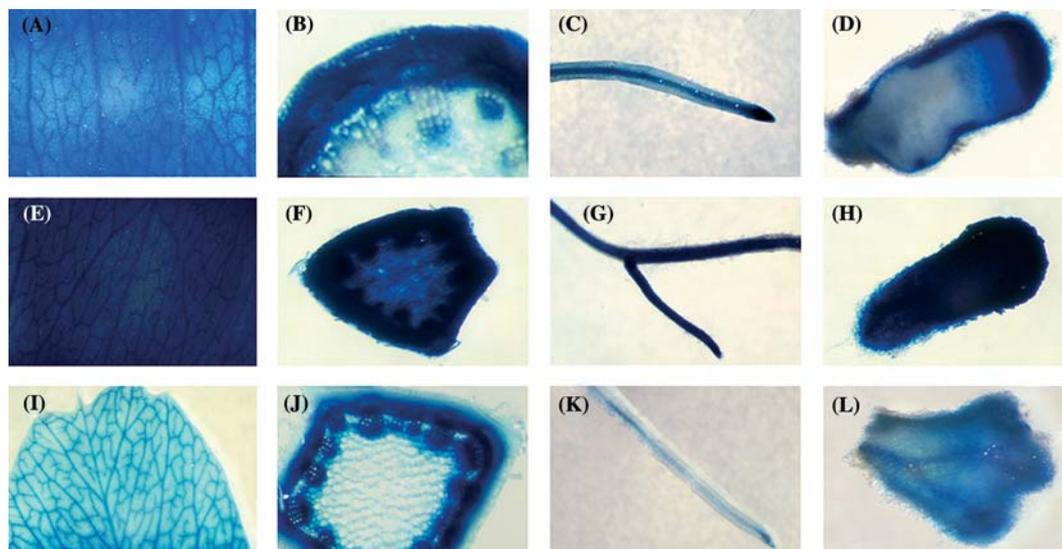


Figure 2. Histochemical assay of GUS activity. (A–D) Staining of plants containing the 35S:*gusA* transgene, (E–H) plants with the CsVMV:*gusA* transgene, and (I–L) plants with the ScBV:*gusA* transgene. Staining patterns in: leaves (A, E, I); stem cross sections (B, F, J); roots (C, G, K); and root nodules (D, H, L). All samples were stained for 3 h at 37°C except those from plants containing the 35S:*gusA* gene (A–D) which were stained for 16 h.

and *A. tumefaciens* LBA4404 containing either pBI121 (35S:*gusA*), pILTAB381 (CsVMV:*gusA*), or pScBV-3d (ScBV:*gusA*). Plants were confirmed to contain the *nptII* gene and *gusA* gene by PCR. A total of 21 plants from independent transformation events were confirmed to have the 35S:*gusA* gene, 47 independent transgenic plants were obtained with the CsVMV:*gusA* gene, and 16 independent transgenic plants containing the ScBV:*gusA* gene were obtained.

All transgenic plants that were PCR positive for the *gusA* gene were stained for GUS activity using an X-GlcA staining solution. Staining patterns of representative plants are shown in Figure 2. In plants containing the 35S:*gusA* gene, staining for up to 16 h showed that the gene was constitutively expressed in leaves with the heaviest staining occurring in the vascular tissues (Figure 2A). In some plants, staining in mesophyll tissue was light. In stems, staining for GUS activity was observed in the epidermis, all cortical cells, phloem, fascicular and interfascicular cambium, and protoxylem (Figure 2B). In several plants, staining was observed only in stem vascular tissues. In roots, GUS activity was observed in vascular tissues and the root tip (Figure 2C). The heaviest staining for GUS activity in root nodules was seen in vascular tissues with lighter staining in the symbiotic zone in which biological

nitrogen fixation occurs (Figure 2D). For some plants the nodule meristem was positive for GUS activity. Staining patterns of GUS activity were similar 6 months later (data not shown). Control nontransformed plants showed no GUS activity after 16 h in the staining solution.

Thirteen plants containing the 35S:*gusA* transgene with similar patterns of GUS staining were analyzed for GUS enzyme activity after the plants were established in soil for approximately 2 months. GUS activity in leaves ranged from 450 to 7810 pmol 4-MU/min/mg protein with an average activity across all plants of 2728 pmol 4-MU/min/mg protein (Figure 3A). In roots, GUS activity ranged from 41 to 15599 pmol 4-MU/min/mg protein with an average activity across all plants of 4236 pmol 4-MU/min/mg protein. Average GUS activity in stems was 3043 pmol 4-MU/min/mg protein, with values ranging from 510 to 5227 pmol 4-MU/min/mg protein. To assess stability of GUS expression, activity in leaves of eight of the same plants was measured 6 months later. Average GUS enzyme activity of these plants was 3513 and 3405 pmol 4-MU/min/mg protein at 2 months and 8 months after establishment in soil, respectively.

Histochemical staining for GUS activity was carried out on all PCR positive plants containing the CsVMV:*gusA* gene when plants had been

established in soil for approximately 2 months. Staining for 3 h in the X-GlcA solution showed that heavy staining occurred in practically all cells of each organ tested (Figure 2E-H) and all plants had similar patterns of expression. In leaves, vascular tissues stained the most rapidly and had the heaviest staining (Figure 2E). In young stems, all tissues stained for GUS activity with the vascular tissues staining first and showing the heaviest staining (Figure 2F). In older woody stems, staining occurred primarily in the vascular tissues. In roots, all tissues stained for GUS activity, with the root tips and vascular tissues showing the heaviest staining (Figure 2G). In older roots, staining occurred primarily in vascular tissues. All cells within the nodule stained heavily for GUS activity although the nodule meristem, vascular tissues, and inner cortex showed the heaviest staining (Figure 2H). The senescent zone of some nodules showed only light GUS staining. Staining of plants 6 months later revealed no changes in expression patterns (data not shown).

GUS enzyme activity was measured in 15 plants containing the *CsVMV:gusA* transgene when the plants had been established in soil for approximately 2 months. Average GUS enzyme activity in leaves across all plants was 66,490 pmol 4-MU/min/mg protein with a range in activity from 14,710 to 106,300 pmol 4-MU/min/mg protein (Figure 3B). In roots activity ranged from 32,370 to 136,480 pmol 4-MU/min/mg protein with average activity across all plants of 48,910 pmol 4-MU/min/mg protein. In stems

activity ranged from 19,060 to 85,120 pmol 4-MU/min/mg protein and the average activity across all plants was 37,240 pmol 4-MU/min/mg protein. GUS enzyme activity was measured in 11 of the same plants 6 months later to assess stability of expression. The average GUS activity in leaves of these plants at 2 months and 8 months after establishment was 67,182 and 79,455 pmol 4-MU/min/mg protein, respectively.

The 16 PCR-positive alfalfa plants containing the *ScBV:gusA* transgene were stained for GUS activity approximately 2 months after establishing plants in soil. Staining for 3 h in the X-GlcA solution showed activity in leaves, stems, roots and nodules, however, staining occurred primarily in vascular tissues (Figure 2 I-L). Cross sections of stems showed GUS activity in xylem and phloem, fascicular and interfascicular cambium, and cortical parenchyma. All plants had a similar pattern of activity but individual plants varied in the intensity of staining. Longer incubation in the staining solution led to diffusion of the stain but did not reveal activity in additional tissues. A similar pattern of GUS activity was observed in plants stained 6 months later (data not shown).

GUS enzyme activity was measured in 11 plants containing the *ScBV:gusA* transgene showing the strongest expression when plants had been established in soil for approximately 6 months. Average activity in leaves across all plants was 1866 pmol 4-MU/min/mg protein, with activity in individual plants ranging from 956 to 2697 pmol 4-MU/min/mg protein (Figure 3C). In roots, GUS activity ranged from

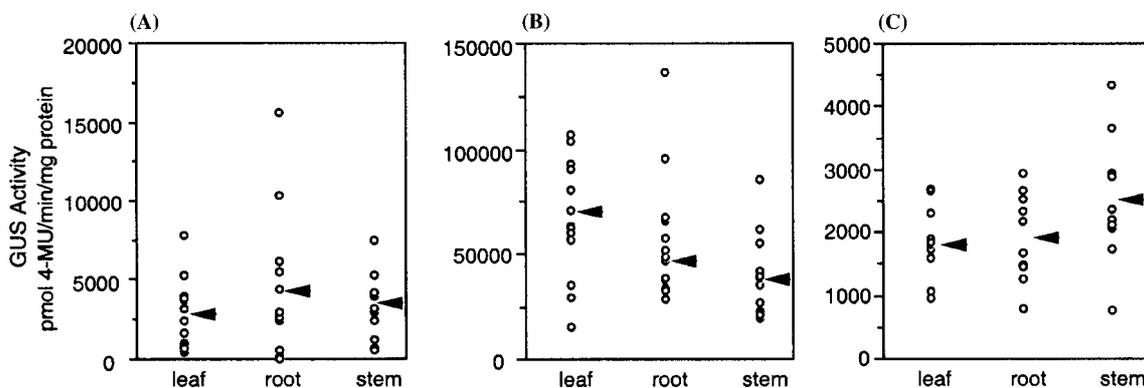


Figure 3. GUS enzyme activity in alfalfa plants. Each circle represents the mean GUS activity (pmol 4-MU/min/mg protein) in a single plant with average GUS activity indicated by the arrow head. (A) Plants containing the *35S:gusA* transgene. (B) Plants containing the *CsVMV:gusA* transgene. (C) Plants containing the *ScBV:gusA* transgene. Note that some data points are covered by overlapping symbols and the Y-axis scale is different in each graph.

792 to 2928 pmol 4-MU/min/mg protein with an average activity across all plants of 1962 pmol 4-MU/min/mg protein. In stems, the average activity was 2546 pmol 4-MU/min/mg protein with activity in individual plants ranging from 770 to 4354 pmol 4-MU/min/mg protein.

Analysis of chitinase activity

Transgenic alfalfa plants were produced with five promoter:*ech42* transgene constructs. Presence of *nptII* and *ech42* was confirmed by PCR in 27 alfalfa plants with the E35S:*ech42* transgene (pARC302), 16 plants with the FMV:*ech42* construct (pARC303), 23 plants containing CsVMV:*ech42* (pARC304), 28 plants with RbcsS:*ech42* (pARC305), and 27 plants with ScBV:*ech42* (pARC306).

Chitinase activity was assayed in all *ech42*-PCR positive plants using a fluorometric chitinase assay to identify plants with the highest chitinase activity. Activity was assayed in the youngest fully expanded leaves from the shoot apex. All assays were conducted using freshly prepared extracts from fresh leaves as chitinase activity was reduced approximately 50% by freezing leaves at -80°C or freezing crude extracts at -20°C (data not shown). The highest amount of chitinase activity was observed in plants with the CsVMV:*ech42* construct (pARC304) (Figure 4B). Average activity across plants with this construct was 11,023 pmol 4-MU/min/mg protein with a range of

116–33505 pmol 4-MU/min/mg protein. In plants containing the E35S:*ech42* construct, chitinase activity in leaves ranged from 45 to 2143 pmol 4-MU/min/mg protein with an average activity of 1457 pmol 4-MU/min/mg protein (Figure 4A). Chitinase activity was less in plants with the FMV:*ech42* transgene, ranging from 22 to 1215 pmol 4-MU/min/mg protein with an average activity across all plants of 780 pmol 4-MU/min/mg protein. In plants with the Rubisco promoter driving the *Trichoderma* endochitinase cDNA (pARC305), mean chitinase activity was 1013 pmol 4-MU/min/mg protein with a range of activity of 23–1930 pmol 4-MU/min/mg/protein (Figure 4A). Chitinase activity in plants with pARC306 (ScBV:*ech42*) was similar to activity in vector control and untransformed plants (data not shown).

To compare chitinase activity in different plant organs, five plants showing high amounts of activity from each promoter were assayed at the same time. Plants were all in bud-stage growth with approximately 5 weeks of regrowth, and bearing 8–11 leaf nodes on the primary stem. Chitinase activity was measured in young leaves (youngest fully expanded leaves from the stem apex), old leaves (leaves from the oldest nodes), root tips (1 cm of actively growing roots), and mature nitrogen-fixing root nodules. Control plants included plants transformed with the vector alone (no promoter:*ech42* insert) and untransformed Regen-SY plants. Chitinase activity in

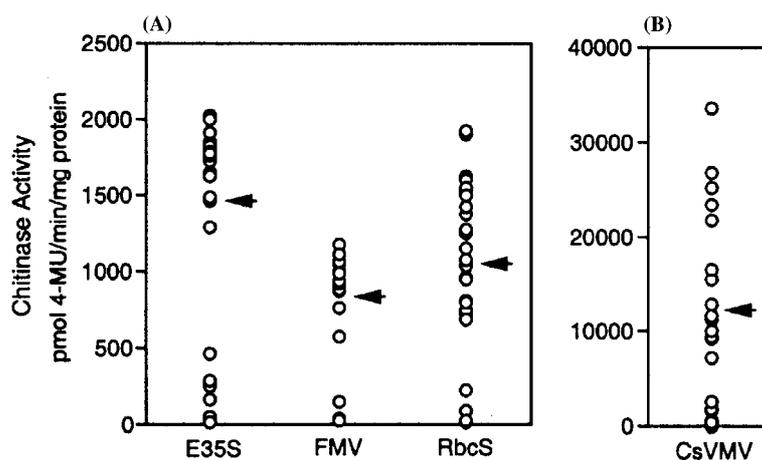


Figure 4. Chitinase enzyme activity in young leaves. Plants contained either the (A) E35S:*ech42* construct (E35S), FMV:*ech42* construct (FMV), RbcsS:*ech42* (RbcS) construct or (B) CsVMV:*ech42* construct (CsVMV). Each circle represents the mean activity (pmol 4-MU/min/mg protein) in a single plant with average activity indicated by the arrow head. Note that some data points are covered by overlapping symbols and the Y-axis scale is different in each graph.

Table 1. Chitinase activity in leaves, roots, and nodules. Values are units of activity with the standard deviation in parentheses

Construct	Young leaves	Old leaves (pmol 4-MU/min/mg protein)	Root tips	Nodules
pARC302 (E35S: <i>ech42</i>)	1883 (147)	1097 (108)	315 (178)	709 (356)
pARC303 (FMV: <i>ech42</i>)	1036 (126)	1172 (139)	411 (232)	1052 (505)
pARC304 (CsVMV: <i>ech42</i>)	7926 (3687)	8690 (939)	884 (516)	1424 (211)
pARC305 (RbcS: <i>ech42</i>)	1199 (214)	767 (166)	18 (8)	48 (44)
Control	3 (1)	11 (4)	22 (5)	18 (2)

both types of controls was similar and is reported as an average in Table 1. Highest chitinase activity in all organs was observed in plants containing the CsVMV:*ech42* construct (Table 1). Average activity in young leaves of plants with the CsVMV:*ech42* construct was 4.2-fold greater than chitinase activity in young leaves of plants containing the 35S:*ech42* construct. Chitinase activity was not significantly different between young and old leaves of plants with the CsVMV:*ech42* construct or between old and young leaves of plants with the other transgene constructs. Chitinase activity in root tips of plants with the RbcS:*ech42* transgene was similar to control plants, although mean activity in root nodules from plants with this transgene was approximately 2.7-fold greater than activity in nodules from controls.

Disease assays

Resistance to infection by *Phoma medicaginis* var. *medicaginis*, the causal agent of spring black stem and leaf spot of alfalfa, was tested using two different methods, a whole plant assay, similar to that used for standardized testing of alfalfa cultivars for spring black stem and leaf spot resistance (Salter and Leath, 1991), and a detached leaf assay. Five plant lines containing the E35S:*ech42* transgene and the five lines containing the CsVMV:*ech42* transgene, two vector control lines, and the untransformed control were tested in the whole plant disease assay. The transgenic lines expressing the chitinase cDNA were selected from preliminary detached leaf

assays in which each selected line showed a significant increase in disease resistance compared to the controls. Disease resistance was assessed by percent defoliation of the primary stem. No significant differences in percent defoliation were detected among transgenic or control lines in the two experiments performed. All inoculated plants were heavily infected. Defoliation of 40 – 70% was observed for inoculated plants while defoliation of mock-inoculated plants was 19% (data not shown).

A detached leaf assay was used in order to control inoculum dose. For this assay, one line containing the E35S:*ech42* transgene (pARC 302M23) and three lines containing the CsVMV:*ech42* transgene (pARC 304M3, pARC 304M6, pARC304 1-100B) were selected from preliminary assays using a single concentration of inoculum (1×10^6 spores/ml). In these preliminary assays, each of the selected transgenic lines had shown a significant increase in disease resistance compared to control lines, which correlated in most cases with a high level of endochitinase activity. As shown in Figure 5A, decreasing the inoculum dose decreased the severity of disease symptoms observed 12 days post inoculation (DPI). Mock-inoculated leaves did not show disease symptoms. However, no significant differences among lines expressing the chitinase cDNA and control lines were observed. In addition, no differences among controls and lines expressing the chitinase transgene were observed when disease ratings were made at 7 DPI (data not shown). To determine whether chitinase activity remained constant in detached leaves, inoculated detached leaves were assayed

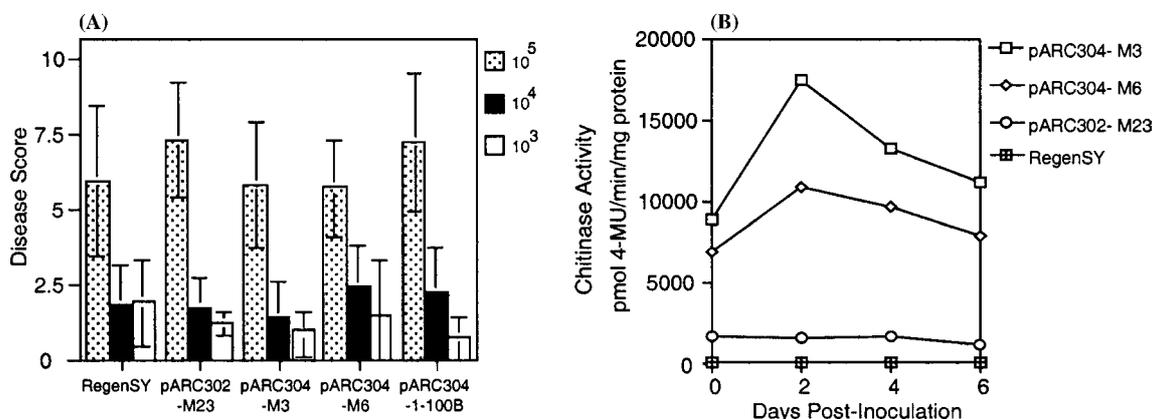


Figure 5. Mean disease scores of alfalfa leaves inoculated with *Phoma medicaginis* var. *medicaginis* and chitinase activity in inoculated leaves. (A) Young alfalfa leaves were placed in moist chambers at room temperature, spray-inoculated with spores at the concentrations indicated, and disease rated on a 0-12 scale 12 days after inoculation. Each bar represents the mean disease score of 15 leaves with the standard deviation. (B) Chitinase activity at 0 days, 2, 4, and 6 days after inoculation. Each point represents the mean activity in 8 leaves. Regen-SY is the untransformed parent, pARC203-M23 contains the 35S:*ech42* transgene, and pARC304-M3, -M6 and -1-100B contain the CsVMV:*ech42* transgene.

for chitinase activity through 6 DPI, when disease symptoms began to be observed. In plant line pARC302-M23 with the E35S:*ech42* construct, chitinase enzyme activity remained constant through 6 DPI (Figure 5B). Chitinase enzyme activity increased from 0 to 2 DPI in detached leaves from lines pARC304-M3 and pARC304-M6 containing the CsVMV:*ech42* transgene then decreased by 6 DPI to levels similar to those at 0 DPI. Chitinase activity in the untransformed control (Regen-SY) leaves remained low and constant through 6 DPI.

Discussion

In this study, we compared the amount of activity and the expression patterns of three nominally constitutive promoters in alfalfa using the *gusA* gene and measured the performance of four promoters using a *T. atroviride* endochitinase cDNA. The highest expression of GUS in all organs examined was obtained using the CsVMV promoter followed by the 35S promoter and the lowest activity was observed using the ScBV promoter. GUS activity in leaves was stable over multiple forage harvests spanning more than 8 months with the CsVMV and 35S promoters. Average GUS enzyme activity in leaves from the CsVMV promoter was approximately 24-fold greater than expression from the 35S promoter. *In situ* staining

of GUS activity showed that the CsVMV promoter was active in all alfalfa cells examined. Interestingly, activity of this promoter appears to vary by plant species. In tobacco plants containing a CsVMV:*gusA* gene, expression was highest in vascular tissues, leaf mesophyll and root tips with low activity in stem pith and parenchyma and root cortical cells (Verdaguer et al., 1996). In grape plants expression of the CsVMV promoter was very low in roots and most vegetative tissues (Li et al., 2001). Although the 35S promoter has strong constitutive activity in some plant species, we found that this promoter was not strongly expressed in all alfalfa cells. In leaves, stems, roots and nodules the heaviest staining occurred in vascular tissues of all 12 plants examined. Little to no staining was observed in stem pith cells, root cortical cells or root nodule cells in the symbiotic zones. Thus, the 35S promoter may not be the preferred promoter for altering expression of genes involved in biological nitrogen fixation or for altering gene expression in alfalfa roots. The ScBV promoter was previously reported to be strongly expressed in vascular tissues of *Arabidopsis* and tobacco (Tzafrir et al., 1998; Schenk et al., 1999). We found the heaviest staining from the ScBV:*gusA* transgene in vascular tissues of alfalfa. This promoter may have utility in studies aimed at modifying vascular cell wall composition to improve forage quality (Guo et al., 2001).

The highest expression of endochitinase observed was obtained using the CsVMV promoter followed by the 35S promoter, alfalfa Rubisco small subunit promoter, and FMV promoter. Chitinase activity in transgenic plants was 50- to 2650-fold greater compared to control plants. In contrast to results of GUS enzyme assays, chitinase activity in plants was not normally distributed, regardless of transgene construct. Enzyme activities clustered in two groups, one of low activity and the other of higher activity. Plants containing *gusA* gene constructs were preselected by histochemical staining and those with low levels of expression were not assayed for enzyme activity. Preselection of plants with the endochitinase transgene was not done, resulting in inclusion of plants with low expression in enzyme assays. Plants containing the CsVMV:*ech42* transgene showed the largest range in chitinase activity within the group of plants with higher activity. Although 27 plants were obtained with the ScBV:*ech42* construct, none of the plants had chitinase activity greater than that of control plants. It is not clear why plants with this construct did not show increased chitinase activity. Sequence analysis of the complete transgene showed no differences from the published sequence of the promoter and the *T. atroviride ech42* cDNA (data not shown). It is possible that introducing 56 bp of the multiple cloning site from pBluescriptSK between the 3' end of the promoter sequence and the initiating ATG interfered with transcription or translation of this transgene. Activity of the Rubisco promoter in leaves was similar to that of the 35S promoter and FMV promoter. The promoter was not active in roots although some root nodules had significant chitinase activity (Table 1). These nodules may have been harvested from near the surface of the container and received sufficient light for promoter induction, or the promoter may have been induced during harvest. The activity could reflect abnormally high endogenous chitinase activity in some nodules in response to microbes. Khoudi et al (1997) also found that a RbcS:*gusA* transgene was light inducible in alfalfa leaves.

Chitinases catalyze the hydrolysis of the β -1,4-linkages in chitin, a polymer of *N*-acetyl-D-glucosamine, which is found in the primary cell wall of many fungi. Expression of the

T. atroviride ech42 cDNA in transgenic plants was previously shown to increase resistance to several fungal pathogens. Increased resistance to *Alternaria alternata*, *Botrytis cinerea*, and *Rhizoctonia solani* in tobacco and *A. solani* in potato was obtained by expression of the *T. atroviride* chitinase gene, which increased endochitinase activity approximately 400-fold over the activity in controls (Lorito et al., 1998). Reductions in lesion size and sporulation of *Venturia inaequalis* on apple were obtained by expression of the *T. atroviride* chitinase gene (Bolar et al., 2000). In preliminary experiments testing all alfalfa plants expressing *ech42* in a detached leaf assay, we found several lines showed a small significant increase in disease resistance compared to controls. However, these results were not consistent in further tests. In whole plant inoculations, none of the 10 lines tested showed an increase in disease resistance, measured as percent defoliation, from the vector control or non-transformed control lines. Furthermore, in a detached leaf assay with three inoculum doses, no differences in disease symptoms were seen between plants expressing the endochitinase cDNA and control plants (Figure 5A). Although leaves from transgenic alfalfa plants showed no increase in disease resistance, they had high levels of chitinase activity (Figure 5B). The apparent increase in activity over time in leaves containing the CsVMV:*ech42* construct may indicate that this promoter is induced by pathogen infection or some other condition in the moist chamber such as an increased concentration of ethylene. It could also reflect the relative stability of the endochitinase with respect to general proteolytic degradation occurring in the detached leaves.

There are several possible explanations for the high disease scores in alfalfa plants with high amounts of endochitinase activity. Effective disease control by expression of the *T. atroviride* endochitinase in transgenic plants may depend on a number of factors including: contact of the fungus with the enzyme, the accessibility of chitin in the fungal cell wall, and the growth pattern of the fungus, as well as the amount of chitinase activity. In detached leaf assays, *P. medicaginis* infects alfalfa leaves directly through the epidermis and then grows predominantly in the intercellular spaces. Severe disease symptoms of

leaf yellowing and cell collapse occur with relatively little fungal growth (Castell and Samac, unpublished). The limited amount of pathogen growth coincident with leaf spot symptoms suggests that symptoms may be caused in part by phytotoxins produced by *P. medicaginis*. Thus, chitinolytic activity may have little effect on symptom expression of this disease. Successful disease resistance was obtained in transgenic plants expressing *ech42* against *Venturia inaequalis* (Bolar et al., 2000, 2001), *Alternaria alternata*, and *A. solani* (Lorito et al., 1998), which produce abundant mycelial growth *in planta*, although only moderate disease control was observed against *A. brassicicola* (Mora and Earle, 2001). Future experiments will test the resistance of alfalfa plants expressing *ech42* to additional fungal pathogens.

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