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Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase

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Summary

Transgenic alfalfa plants expressing *Bacillus licheniformis* alpha-amylase and manganese-dependent lignin peroxidase (Mn-P) from *Phanerochaete chrysosporium* were produced using the *Agrobacterium tumefaciens* transformation system. In each case, there was a range of expression of the introduced gene among independent transgenic plants. Plants producing alpha-amylase showed no alteration of phenotype. Production of Mn-P in alfalfa, however, in most cases adversely affected plant growth and development. Affected plants were stunted with yellowing foliage, but survived and produced seed. Results from field trials showed that Mn-P production in transgenic alfalfa reduced dry matter yield and plant height. The extent of these symptoms and yield reduction was, for the most part, related to the level of foreign protein production as estimated by Western analysis. Field data from transgenic plants expressing alpha-amylase showed that there was no effect of foreign protein production on plant performance. Expression of Mn-P was shown to segregate in sexual progeny derived from transgenic plants.

Abbreviations: Mn-P – manganese-dependent lignin peroxidase

Introduction

Currently, most industrial enzymes are produced from micro-organisms by large-scale fermentation. An alternative approach would be to express and recover these enzymes from transgenic plants. The production of commercially-interesting heterologous proteins and peptides in plants has been reported by several researchers (DeZoeten et al., 1989; Hiatt et al., 1989; Vandekerckhove et al., 1989; During et al., 1990; Krebbers & Vandekerckhove, 1990; Sijmons et al., 1990). There is also interest in using transgenic crop plants for the production of industrial bulk enzymes (Pen et al., 1992). We are currently conducting a multidisciplinary feasibility study on the production of industrial enzymes in transgenic alfalfa. The overall goal of the research is to develop genetically-engineered alfalfa that produces high levels of industrially-important enzymes, to develop rapid methods for extracting and purifying these enzymes and thus provide a high value product which takes advantage of existing agricultural productivity. Alfalfa has certain advantages for this purpose. It is a perennial, widely-grown crop capable of three or more harvests a year. It is beneficial environmentally since it does not require annual tilling and planting, and needs fewer applications of fertilizer and pesticides than conventional row crops. Furthermore, technology for extracting protein from alfalfa while leaving a valuable residue for animal feed is well-developed.

As part of our feasibility study for the production of industrial enzymes in transgenic alfalfa, we have recently demonstrated the production of manganese-dependent lignin peroxidase (Mn-P) from the fungus *Phanerochaete chrysosporium* in transgenic plants (Mathews et al., 1993; Austin et al., 1994). This

enzyme has potential for large-scale industrial usage for lignin degradation and/or as a bleaching agent in biopulping processes. Expression of this enzyme in alfalfa may also have the potential for increasing the fibre digestibility of forages for ruminants. In addition we have expressed alpha-amylase from *Bacillus licheniformis* in alfalfa. This enzyme is involved in starch degradation and has several current and potential large-scale industrial uses in starch processing (Schwardt, 1990). The production of this enzyme has already been demonstrated in tobacco (Pen et al., 1992). It was chosen as a model enzyme for the overall feasibility study to test recovery protocols since it is a robust enzyme that is active over a wide pH and temperature range.

This report outlines the production and characterization of transgenic alfalfa expressing alpha-amylase or Mn-P. Data from the first year of a field test of transgenic plants are also presented.

Materials and methods

Plant materials. A preliminary survey to screen for regenerative ability and response to kanamycin was performed using 18 alfalfa genotypes derived from Regen-SY (Bingham, 1991). Based on data obtained on basic tissue culture responses, five genotypes were selected for use in transformation studies using leaflets as explant sources. One genotype, RSY#27, Regen-SY-27), was identified as the most amenable for transformation and was used in the experiments described in this report. Plants were maintained in growth rooms with a 16 h photoperiod of 300 μE $m^{-2}s^{-1}$ and day and night temperatures of 21° C and 19° C, respectively.

Vectors. A detailed description of the construction of the plant transformation vectors will be presented elsewhere. Briefly, pDM321 and pDM322 consist of the binary plant transformation vector pCGN1578 (McBride & Summerfelt, 1990) into which was inserted a plant expression cassette containing the coding sequence of a Mn-P gene from *Phanerochaete chrysosporium*. The expression cassette contains the 'Mac' promoter (Comai et al., 1990) and the mannopine synthase transcription terminator (McBride & Summerfelt, 1990). The Mn-P coding sequence was obtained from a cDNA clone (kindly supplied by D. Cullen, USDA, Forest Products Laboratory, Madison, WI) encoding an allelic variant of MP-1 (Pease et al., 1989). pDM321 contains the complete Mn-P coding sequence, whereas in pDM322, the sequence encoding the amino terminal 24 amino-acids was replaced with sequence encoding the amino-terminal 21 amino acids of the soybean vegetative storage protein, VSPβ (Mason et al., 1988). In effect, the fungal amino-terminal endoplasmic reticulum (ER) signal sequence was replaced with a plant ER signal sequence. pDM408 is identical to pDM322 but contains the coding sequence for *Bacillus licheniformis* alpha-amylase mature protein (Yuuki et al., 1985) instead of Mn-P. Thus, the sequence encoding the 29 amino acid bacterial secretion signal was replaced with the sequence encoding the 21 amino acid amino-terminal ER signal sequence of VSPβ. Plant transformation constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404.

Transformation and regeneration of alfalfa. The transformation method was defined after testing several modifications of the basic explant co-cultivation system (Horsch et al., 1985) as a way of optimizing the transformation procedure. These included pretreatment of the tissue, longer co-cultivation times and the use of different levels of the antibiotic kanamycin monosulphate to select for transformed tissue. The optimized procedure is as follows. New-growth trifoliates were taken from RSY27 plants maintained in a growth room (conditions as described above) and sterilized using alcohol and bleach washes (30s in 70% alcohol, 90s in 20% hypochlorite + 0.1% SDS, followed by three rinses in sterile distilled water). Leaf edges were cut on moist filter paper and tissue dropped into liquid SH-II medium (Bingham et al., 1975). When sufficient explants had been taken, they were moved to a suspension of *Agrobacterium* cells (containing the engineered plasmid) from an overnight culture grown in liquid YEP selection medium. Cell density was adjusted to fall between 0.6–0.8 at A_{660}. After 30 minutes inoculation, the explants were gently blotted on filter paper and placed on B5H medium (Brown & Atanassov, 1985) for 4 days. They were then rinsed twice in sterile water and cultured on B5H for a further 4 days. At the end of this period, they were rinsed three times and transferred to B5H containing 25 mg l^{-1} kanamycin and 250 mg l^{-1} carbenicillin. Plates were maintained at 24° C, 16 h photoperiod and light intensity of 60–80μE $m^{-2}s^{-1}$. Explant-derived calli (and occasionally embryoids) which formed within 3 weeks on this medium were moved to B5H with antibiotics but without hormones to allow for further embry-

oid production and development of existing embryoids. After 3–4 weeks, embryos were transferred to MS medium (Murashige & Skoog, 1962) plus the two antibiotics to allow for development into plantlets. Callus did form on untreated explants in the presence of 25 mg l^{-1} kanamycin but embryos were never produced. Each explant piece can give rise to multiple (up to 40) embryos. Plantlets were rooted on MS medium lacking antibiotics. For evaluation purposes only, one rooted plant per explant piece was assessed, thus assuring that each was the result of an independent transformation event. Plants were maintained in a growth room under conditions as described above.

Expression assays. 1. Alpha-amylase assays. Sample preparation and starch plate assays were carried out basically as described by Pen et al. (1992). Briefly, leaf tissue (200 mg) was homogenized by hand in 500 μl 0.5 M glycine buffer (pH 9.0) containing 10 mM $CaCl_2$. The homogenate was centrifuged for 10 min at 14,000 rpm (16,000 g) in a table top centrifuge and the supernatant collected. Soluble protein content was determined following the Bradford procedure (Bradford, 1976), using BSA as standard. The supernatant was then divided into two samples and half was heated to 60° C for 15 min. This was to discriminate between endogenous plant alpha-amylase activity and the heat-stable activity of the *Bacillus* protein. Samples (2 μl) of heated and non-heated extract were placed on 1% starch plates solidified with 0.9% agar and the extent of starch degradation determined after 16 h at room temperature by iodine staining. Halo size was compared with that of standard dilutions of *B. licheniformis* alpha-amylase (Sigma). Plant samples were taken at various times. For the initial testing of putative transgenics, several trifoliates were pooled from 2 to 3-week old, rooted *in vitro* plants. The third fully-expanded trifoliate from several stems of mature plants just prior to flowering was taken for assay of both growth room and field-grown plants. Attempts to quantify alpha-amylase activity in crude alfalfa extracts using the spectrophotometric assay of Saito, (1973) gave inconsistent results. We are currently working on modifying the method of sample preparation and assay conditions such that we can accurately quantify the enzyme.

2. Analysis for Mn-P production. Samples from *in vitro* and mature plants were taken and prepared as described for alpha-amylase, except that they were ground in 50 mM L-tartrate buffer, (pH 4.5). Representatives of all 16 plant lines in the field (generally, leaves from 2–3 clones of any individual) were sampled at the end of the first and second harvest growth period. Eight of the 16 lines were also sampled at weekly intervals during the whole of the second harvest growth period. In addition, older leaf, stem and root samples were taken from the group of 8 plants just prior to the second cutting. Denatured proteins were separated on 10% SDS-PAGE gels, blotted onto nitrocellulose and immunoblots obtained following established protocols as described by the manufacturer of the 'Stable Blue' developer used to visualize the protein (Promega Corporation, Madison, WI). The primary anti-Mn-P antibody was kindly supplied by P. Kirsten, USDA, Forest Products Laboratory, Madison, WI.

Several attempts were made to assay for Mn-P activity in crude extracts following established methods used to assay fungal culture filtrate. In one such attempt, extracts prepared as described above were diluted 10-fold with 50 mM sodium L-tartrate (pH 4.5). Assays were based on the procedure of Glenn & Gold (1985) and used pinacyanol chloride (Sigma) as the substrate. The reaction mixture consisted of 50 mM sodium acetate (pH 4.5), 50 mM sodium L-lactate (pH 4.5), 100 μM $MnSO_4$, 3 mg ml^{-1} gelatin, 10% (v/v) DMSO, and 5 $\mu g\ ml^{-1}$ pinacyanol chloride. After adding 2–10 μl diluted extract, H_2O_2 was added to 100 μM from a freshly prepared 10 mM stock to start the reaction. The reactions were carried out at 37° C and the decrease in absorbance at 603 nm was continuously monitored with time points recorded every 15–30 seconds. The assays were not linear so the slope at the start of the reaction was used to calculate the activity. In cases where there was an initial lag in the reaction, the assay slope at the intersection of the lines for the assay and the lag was used to calculate the activity. A molar extinction coefficient of 120,000 $cm^{-1}M^{-1}$ was used to calculate the International Units (IU) of enzyme activity. Each extract was also assayed by the above procedure in the absence of $MnSO_4$.

Field test. It was necessary to obtain a field permit from APHIS (Animal and Plant Health Inspection Service of the United States Department of Agriculture) for the environmental release of these plants. The field test of transgenic alfalfa expressing Mn-P was a randomized complete block consisting of 16 entries containing: 1 untransformed control line RSY27, 12 transgenic lines which expressed the protein and three lines recovered from the same experiment which had no detectable protein expression. Plants were clonally propagated and transplanted in 3 replications, with in most cases 5 propagules in each replicate spaced 0.5 m

apart in rows 1 m apart. The plants were cut back at the time of transplanting into the field on June 21, 1993. The plants had a well-developed root system and had been cut back twice prior to planting. The experiment was monitored weekly for any abnormal phenotypes. In order to comply with current APHIS regulations for transgenic alfalfa, foliage was harvested when plants started to have coloured flower buds but no open flowers. The first harvest was taken on August 5 and the second on September 10. At these times, the phenotypes of the plants were visually rated and a number was ascribed to their appearance: 0 = no phenotypic changes, 1 = yellowing of 10% of leaves, 2 = slightly stunted and 25% foliage affected, 3 = stunted and 50% foliage affected, 4 = severely stunted, more than 50% leaves affected and leaves starting to fall, 5 = plant dead with no signs of new growth. The length of the longest stem was measured. The whole plant herbage was collected and dried for 4 days at 70° C. Plants were cut back to a height of 5 cm.

The alpha-amylase trial consisted of 26 entries containing: 2 untransformed control lines of RSY27, 17 transgenic lines which consistently showed measurable levels of alpha-amylase activity and 7 lines recovered from the same experiment which had no detectable enzyme expression. These plants had only been cut back once prior to planting and their root systems were not as well-developed as the plants used in the Mn-P field test. There were 3 replicates of 5 plants each and the plants were monitored and harvested as above. Planting date was June 24, 1993, with the first harvest taken on August 12 and the second on September 17. In each case the plot was bordered by a double row of cultivated alfalfa.

DNA extraction and analysis. DNA was prepared from alfalfa leaf tissue using a modified CTAB protocol (Lee et al., 1993). DNA was cut with restriction enzyme *Eco*R1, separated by electrophoresis in agarose gels, transferred to Biotrace HP filters (Gelman Sciences, Ann Arbor, MI), hybridized to ^{32}P-labeled random-primed probes and analyzed by autoradiography as described by Reid et al. (1988).

Data analysis. Data were analyzed using the procedure GLM of the SAS system employing the method of least squares to fit the models. In the models it was assumed that there was a plant and a block effect on mean plant height and mean plant weight; i.e., mean height and mean dry weight = global mean + block effect + plant effect + error. The error is assumed to be independent and normally distributed with zero mean and constant variance. Residual plots were checked and no violations of the model assumptions were found.

Sexual transfer of Mn-P production. Seed was produced by crossing transgenic plants expressing Mn-P (used as females) with Blazer-XL, a cultivated alfalfa variety. Ten seeds from crosses using 12 of the individuals which were part of the field test were germinated and established under growth room conditions. The progeny were assessed visually 4 weeks after planting and assessed for Mn-P production. Western analyses were done on 50 of the plants as confirmation of the visual scoring.

Results

Plant transformation. Transformation results are summarized in Table 1. Embryos (up to 40 per explant piece) were produced in 3–6 weeks and plants in 8–12 weeks. A large number of putative transgenic plants were produced in the transformation experiment using the alpha-amylase construct. Embryos were produced on 199 of 231 treated explants and in 175 of 199 cases these embryos developed into plants. Plants derived from 75 different explant pieces were selected at random. Sixty-two of these rooted, and 38 of the 62 had detectable alpha-amylase expression *in vitro* as determined by starch plate assays. Results of transformation experiments using the Mn-P constructs were a little unusual in that the conversion of embryos to plants was lower than expected. Plants were recovered from experiments using both Mn-P constructs and approximately two thirds of the putative transgenics tested *in vitro* had detectable levels of Mn-P as determined by Western analysis. It should be noted that Southern analysis was not performed on the whole set of these plants, so we cannot determine if the plants with no detectable amylase or Mn-P activity were escapes (i.e. untransformed), or if they had the foreign gene, but it was not expressed or was expressed at a very low level which we could not detect.

Characterization of transgenic plants

Plants expressing alpha-amylase. Alfalfa plants expressing *B. licheniformis* alpha-amylase appeared phenotypically normal under all growth conditions and all of the plants recovered were fertile. There was considerable endogenous alpha-amylase activity

Table 1. Summary of transformation experiments

Construct	Explants	Explants giving callus	Explants giving embryos	Explants giving shoots	Rooted individuals tested	Plants with detectable protein expression
amylase pDM408	231	208	199	175	62	38
controls	40	40	38	32	–	–
controls + kanamycin + carbenicillin	19	19	0	0	–	–
Mn-P pDM321	160	153	96	52	33	22
Mn-P pDM322	160	159	139	79	36	21

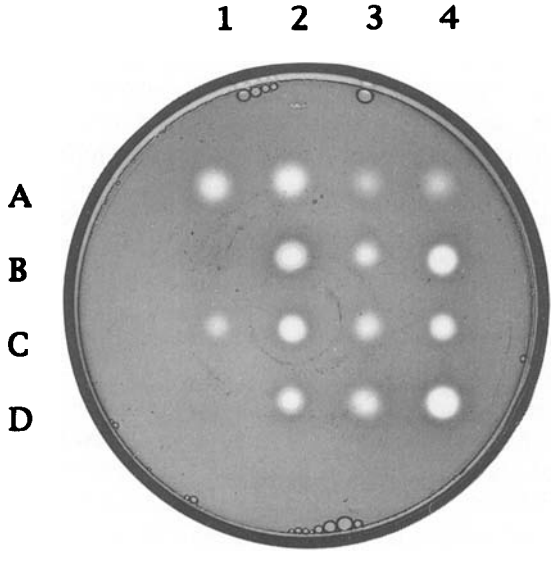

Fig. 1. Starch plate assay showing the expression of active *B. licheniformis* alpha-amylase in transgenic alfalfa. Columns 1 and 3 are heat-treated (60° C) duplicate samples of columns 2 and 4. Row A is *B. licheniformis* alpha-amylase standard, diluted 1:50,000 (A1 and A2) and 1:100,000 × (A3 and A4). Rows B, C and D represent extracts of six alfalfa plants, two tests (heat-treated, i.e. row 1 or 3, or untreated row 2 or 4) for each plant. B1 and B2 represent a control plant, the rest are transformants from the field test with at least one copy of the introduced gene. Note that alfalfa has a relatively high endogenous activity (B2) but this activity is destroyed on heating (B1). Four of the five tested plants are expressing active enzyme.

in untransformed RSY27. This was not heat stable, however, and was not detected in samples treated at 60° C. Thus it was possible to show expression of active *B. licheniformis* alpha-amylase in transformed plants. An example of a starch plate assay showing the activity of heated and non-heated samples is shown in Fig. 1. Crude estimates of *B. licheniformis* alpha-amylase enzyme activity, based on the size of the halo on starch plates compared to native enzyme, were in the range of 0.001–0.01% total soluble protein. Soluble protein values in extracts were typically in the range 3–6 mg ml^{-1} depending on tissue type and plant age. Estimates of expression levels are based on protein values in unheated extracts since there is a significant loss in protein upon heating at 60° C. We are in the process of purifying the introduced alpha-amylase from transgenic plants.

Plants expressing Mn-P. Western analysis of *in vitro* putative transgenic plants clearly showed that some plants were expressing the foreign protein (Fig. 2). Crude estimates of Mn-P levels were in the range 0.01–0.5% total soluble protein. It should be noted that the extraction buffer used (pH 4.5) typically gives lower levels of total soluble protein (1–2 mg ml^{-1}) in plant extracts than when tissue is extracted in a more neutral or basic buffer. Two to three weeks after plants had been placed in rooting medium and roots were forming, there were no obvious phenotypic differences between plants expressing the protein and controls. Two to three

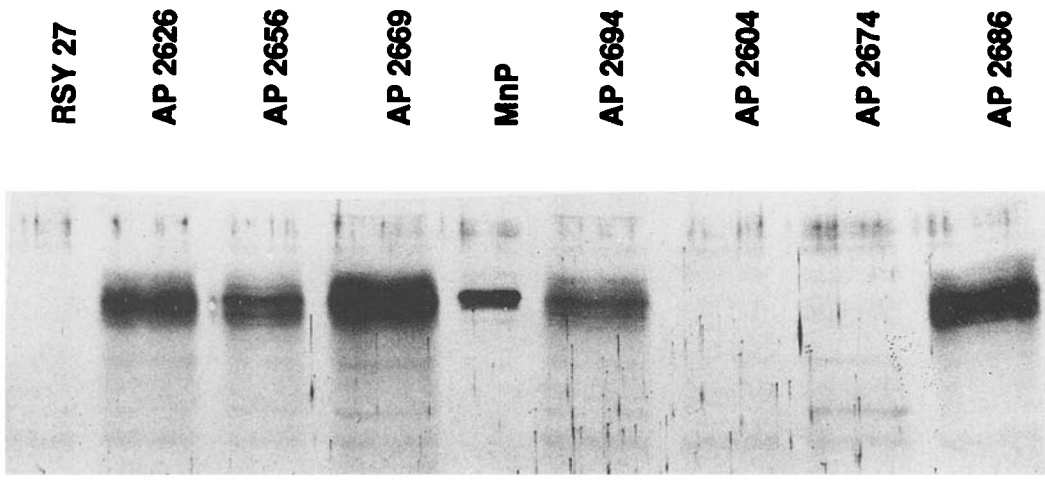

Fig. 2. Western blot of alfalfa extracts showing expression of Mn-P in 2–3 week old *in vitro* plants. Approximately 4 μg of total protein was loaded for each sample; standard is equivalent to 1.86 ng *P. chrysosporium* Mn-P. RSY27 is the untransformed control. Seven transformants are shown; five are positive.

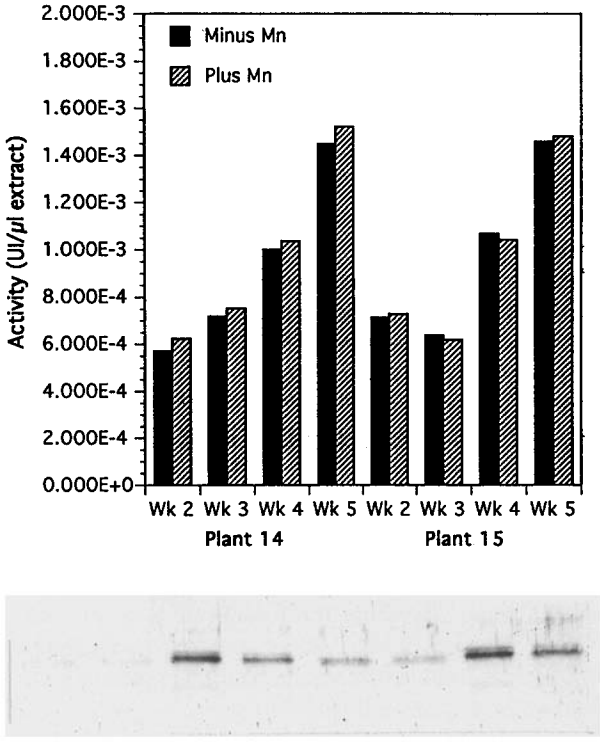

Fig. 3. Time course of peroxidase activity in field-grown alfalfa expressing Mn-P. The graph shows enzyme activity in samples from plants 14 and 15 taken at weekly intervals (2 through 5) during the second harvest period. A Western blot of the same sample (2 μg total protein loaded onto gel) is shown below the graph.

weeks later, however, it became evident that expression of the protein had deleterious effects on plant growth and development whether plants were still *in vitro* or in the greenhouse. Trifoliates started to yellow and in severe cases senesced completely and dropped from the plant. Overall, the plants were stunted and flowered later than control plants, also, the highest-expressing plants, usually those with levels estimated to be above 0.3% soluble protein, died. The surviving plants were fertile and seed was recovered. Importantly, viable progeny with high levels have been recovered in crosses. Western analyses were repeated on mature greenhouse plants just starting to show symptoms. Results were essentially the same as for the screening of *in vitro* plants. Levels on a protein basis were 10–20% lower reflecting an increased value in protein levels of greenhouse plants compared to *in vitro* plants. Obtaining enough clonal copies of the transgenic plants (for field studies) by rooting vegetative cuttings was difficult, especially in plants expressing the protein at medium or high levels. Interestingly if the cuttings rooted, the plants grew normally for 2–3 weeks before showing any foliar symptoms. This pattern was seen repeatedly wherever the plants were grown.

Western analyses of field-grown plants, carried out just prior to both harvests, were used to ascribe an expression level to each set of individual transgenic plants. The following levels were ascribed based on crude visual estimations of the intensity of bands as

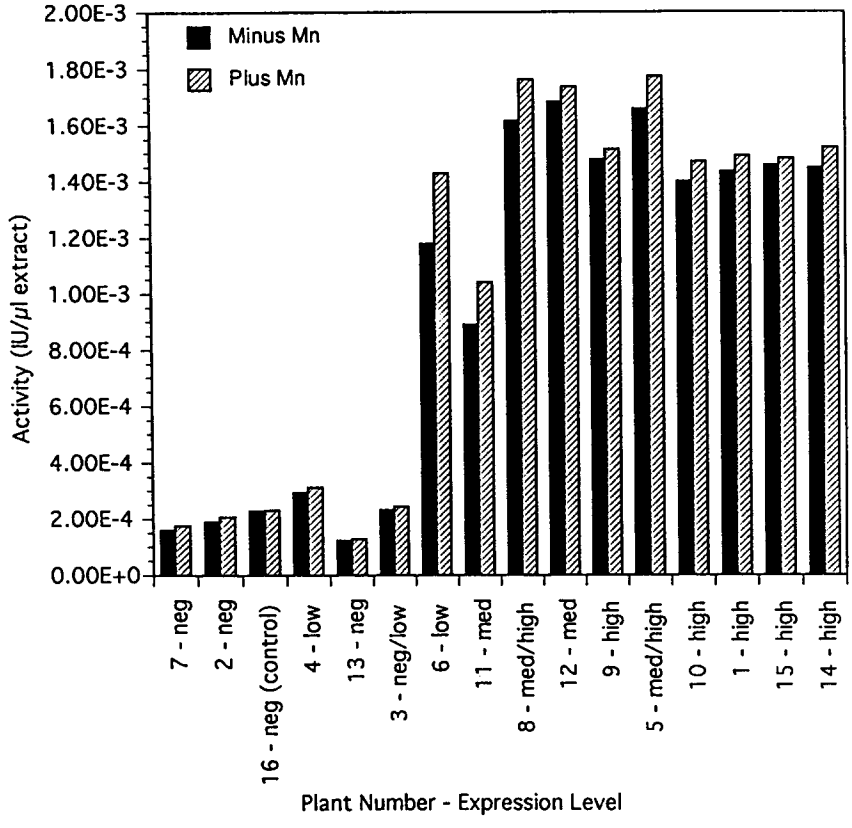

Fig. 4. Peroxidase activity of field-grown transgenic alfalfa expressing Mn-P. Samples were taken immediately prior to the second harvest.

compared with standards; an expression of 0.01–0.05% soluble protein = low, 0.05–0.15% = medium, greater than 0.15% = high. Results from weekly sampling of plants (representative Western analysis data shown in Fig. 3) clearly show that levels of Mn-P increased as plants developed. There were consistently higher levels of the fungal protein in weeks 4 and 5 than in weeks 2 and 3. Thus, increased expression/accumulation of the protein coincided with the appearance of visual symptoms. Results also showed that Mn-P protein was present in all of the plant tissue types tested (data not shown). Attempts to measure Mn-P activity in crude alfalfa extracts were not successful. We could not demonstrate that any of the peroxidase activity was Mn-dependent, i.e., that the amount of peroxidase activity was significantly greater in the presence of Mn than in the absence of Mn (Fig. 3). General peroxidase activity, however, with and without added Mn, was found to correlate with Mn-P levels seen in the Western analyses (Figs 3 and 4). In the weekly time course of Fig. 3, the peroxidase activity was seen to increase as the plants developed just as the Mn-P protein levels did and, therefore, to correlate also with the appearance of the foliar symptoms. This developmental increase in general peroxidase activity was not seen in the control plants or in transgenic plants not expressing Mn-P that were tested in the time course (data not shown). To determine if the correlation between peroxidase activity and Mn-P expression levels was more general, all the field plants were assayed just prior to the second harvest. Figure 4 demonstrates that the increase in general peroxidase activity is only seen in plants that express Mn-P. The higher levels of peroxidase activity in symptomatic plants probably reflect a general wound/stress response of transgenic plants when the levels of Mn-P become deleterious.

We could not detect any differences in levels of Mn-P in plants produced using constructs pDM321 or pDM322. Thus it would not appear to be necessary to replace the fungal ER signal sequence with a plant signal sequence in this case. Plants derived from both constructs were included in the field test.

Table 2. Summary of data from the second harvest of field grown transgenic alfalfa expressing manganese-dependent lignin peroxidase from *P. chrysosporium*

Plant[a] number	Southern[b] analysis copy number	Protein[c] expression	Field[d] score	LS mean dry weight (g)	Std Error	T grouping[e]	LS mean height (cm)	Std Error	T grouping[e]
7	1	neg	0	59	4.1	A	65	3.76	A
2	1	neg	0	52.8	3.3	AB	59.6	3.03	ABC
16	0	neg (control)	0	46.8	3.3	BC	56.7	3.03	ABCD
4	2	low	0.4	46.8	4.1	BCD	63.5	3.76	AB
13	1	neg	0	44.7	3.3	BCDE	56	3.03	ABCD
3	2	neg/low	0.3	36.6	3.3	DEF	56.3	3.03	ABCD
6	1	low	1.4	35	3.3	EF	56.6	3.03	ABCD
11	1	med	1.5	34.1	3.3	F	58.4	3.03	ABC
8	1	med/high	2.3	30.4	3.3	FG	55.3	3.03	ABCD
12	2, 3	med	2.1	29.1	3.3	FGH	52.3	3.03	CDE
9	3	high	2.9	22.6	4.1	GHI	53.6	3.76	BCD
5	1	med/high	2.3	20.9	3.3	GHI	52.7	3.03	CD
10	1	high	2.7	19.8	3.3	HI	51.2	3.03	CDE
1	nt	high	3.2	16.7	3.3	I	49.4	3.03	DE
15	1	high	3.9	14.4	4.1	I	42.5	3.76	EF
14	1	high	3.8	12.4	3.3	I	40.2	3.03	F

[a] Plants ranked according to LS (least squares) mean dry weights.
[b] nt = not tested.
[c] Levels of Mn-P as estimated by Western analysis.
[d] Visual scoring of plant phenotype at time of harvest.
[e] Values connected by the same letter are not significantly different, P = 0.05.

Southern analysis. All of the plants (except the untransformed control) included in the field test of plants expressing Mn-P had at least one copy of the fungal gene (Table 2). Similarly, all of the plants in the alpha-amylase field study from which data was obtained had one or more copies of the bacterial gene (Table 3).

Field performance

Plants expressing Mn-P. Data from the field tests essentially confirmed earlier growth room and greenhouse observations. Survival rates for transplants were surprisingly very high; only 1 of 224 plants in the test died. All of the test lines had a normal phenotype for the first 2–3 weeks of growth. At this point, plants expressing Mn-P at high levels started to show foliar yellowing and the rate of plant growth and development slowed such that plants became stunted. The appearance of flower buds was typically 7–10 days later in most of the plants expressing the protein than in control lines. Data from the first harvest are shown in Fig. 5 and data from the second are given in Table 2. For easier interpretation of the data in both cases, the plants were ranked according to mean dry weight. The phenotypic scores taken immediately prior to harvest clearly indicate that expression of Mn-P is deleterious to plant growth and development. There is an obvious relationship between expression of the protein and plant performance under field conditions for both harvest periods. Plant dry weight in harvest 2, for example, ranged from 59.0 g in a non-expressing plant to 12.4 g in a plant expressing the protein at a high level. Any group of plants expressing the protein at medium to high levels had a significantly lower mean dry weight than the control plants. The range of plant height was not as great but again there were significant differences between high-expressing and control plants.

Plants expressing alpha-amylase. Expression of *B. licheniformis* alpha-amylase gave no obvious phenotypic effects in field-grown plants. Field survival (92%)

Table 3. Summary of data from the second harvest of field grown transgenic alfalfa expressing *B. licheniformis* alpha-amylase

Plant[a] number	Southern[b] analysis copy number	Enzyme[c] expression	LS mean dry weight (g)	Std Error	T grouping[d]	LS mean height (cm)	Std Error	T grouping[d]
13	1	pos	39.7	4.63	A	61	3.16	ABCDE
11	1	pos	38.1	4.63	AB	60.8	3.16	ABCDE
15	1	pos	35.9	4.63	AB	64.7	3.16	A
9	nt	pos	35.6	4.63	AB	59.6	3.16	ABCDE
20	nt	neg	32.7	4.63	ABC	59.7	3.16	ABCDE
18	1	neg	32.5	4.63	ABC	60.7	3.16	ABCDE
14	1, 2	neg	32.2	4.63	ABC	61.1	3.16	ABCDE
10	0	neg (control)	31.6	4.63	ABC	59.5	3.16	ABCDE
7	nt	pos	31.5	4.63	ABC	60.8	3.16	ABCDE
24	nt	pos	31.4	4.63	ABC	60.2	3.16	ABCDE
6	3	neg	31.4	4.63	ABC	58.1	3.16	ABCDE
3	1	pos	31.1	4.63	ABC	57	3.16	ABCDE
4	3	pos	30.4	4.63	ABC	63.7	3.16	AB
23	3	neg	30.4	4.63	ABC	63.2	3.16	ABC
8	1, 2	neg	30.2	4.63	ABC	61.7	3.16	ABCD
22	4	pos	30.1	4.63	ABC	60.9	3.16	ABCDE
19	2	pos	29.3	4.63	ABC	58.9	3.16	ABCDE
12	nt	pos	28.8	4.63	ABC	55.2	3.16	BCDE
21	nt	pos	28.6	4.63	ABC	60.2	3.16	ABCDE
16	3, 4	pos	26.4	4.63	ABC	59.7	3.16	ABCDE
5	1	pos	25.8	4.63	BC	60.7	3.16	ABCDE
25	0	neg (control)	25.8	4.63	BC	56.3	3.16	ABCDE
26	nt	pos	24.5	5.71	BC	59.3	3.89	ABCDE
2	1	pos	21.9	4.63	C	52.1	3.16	E
17	2, 3	neg	21.7	4.63	C	54.2	3.16	CDE
1	1	pos	20.9	4.63	C	52.7	3.16	DE

[a] Plants ranked according to LS (least squares) mean dry weights.
[b] nt = not tested.
[c] As determined by starch plate assay on heat treated samples.
[d] Values connected by the same letter are not significantly different, P = 0.05.

was less than for the Mn-P field test. This was probably a reflection of the fact that these transplants were smaller than the ones used in the Mn-P field test and their root systems were not as well-developed. This also accounts for the yield data of control plants from this study being considerably less than those seen in the Mn-P study. Plant dry weight and height (Table 3 and Fig. 6) were more uniform for the alpha-amylase set of plants than for those expressing Mn-P. There were significant differences between height and weight for plants at the extremes of the range but the performance of most of the group was not significantly different. The outlying plants probably reflect somaclonal variation commonly seen amongst regenerated plants. The pattern of T grouping for Mn-P and alpha-amylase clearly shows the differences in the two sets of plants. Expression of alpha-amylase has no effect on field performance.

Progeny testing. Only 5 of 120 seeds derived from crosses of transgenic alfalfa producing Mn-P with Blazer-XL failed to germinate. All of the failed seed was derived from crosses of high-expressing plants. Growth and development of the seedlings was normal for the first 17 days, at which time symptoms of foliar yellowing appeared in some of the seedlings. Results

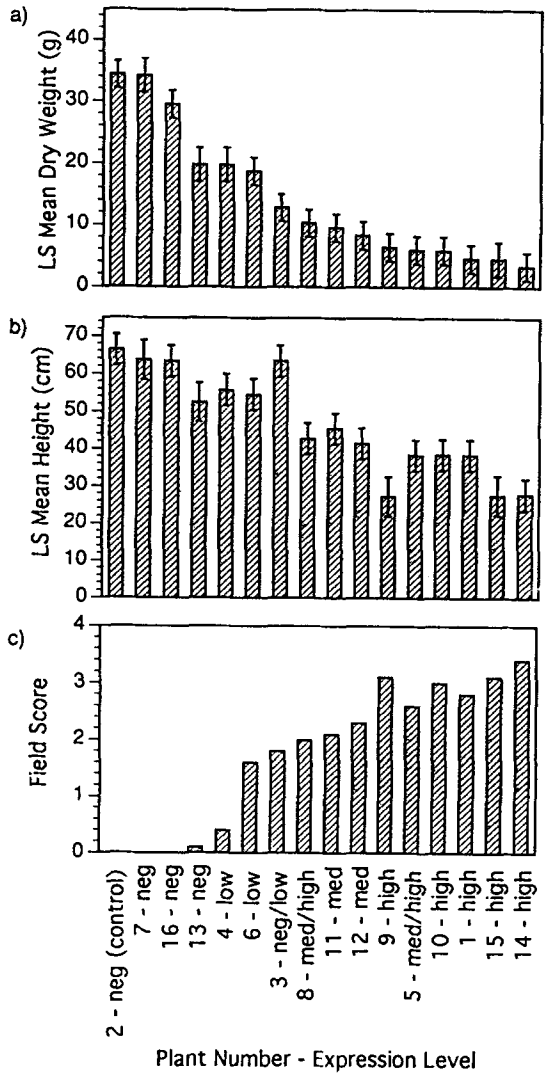

Fig. 5. Dry weight, height and phenotypic score from the first harvest of a field test of transgenic alfalfa expressing *P. chrysosporium* Mn-P.

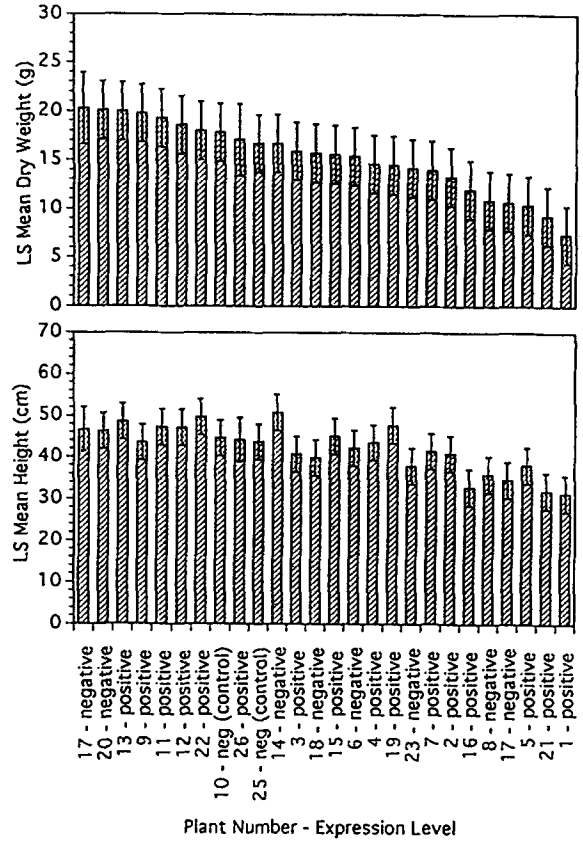

Fig. 6. Dry weight and height data from the first harvest of a field test of transgenic alfalfa expressing *B. licheniformis* alpha-amylase.

of the visual assessment after 4 weeks of growth (Table 4) showed that symptom expression segregated in the progeny. Western analysis of seedlings from 7 of the 12 sets of progeny confirmed that symptom expression was associated with production of the fungal protein (data not shown). Interestingly, parent plant number 13 had no detectable Mn-P protein production but 2 of the progeny did produce Mn-P. Thus, a silent or very low-expressing copy of the gene in the original transgenic became capable of high expression in some of the progeny. Progeny obtained from high-expressing parents tended to be more severely affected than those from low- or medium-expressing plants. The progeny which are hybrids with an elite cultivar tended to have larger trifoliates and a more vigorous growth habit than RSY27. Crude estimates of Mn-P levels in progeny ranged from 0.09–0.35% total soluble protein in extracted samples. As a general observation, the deleterious effects of relatively high amounts of Mn-P protein were not as severe in the progeny plants. Further crosses are currently in progress.

Discussion

We have presented data showing the expression of a bacterial and fungal protein in transgenic alfalfa. The transformation system used is similar to that of other researchers (e.g. Shahin et al., 1986; Chabaud et al., 1988; D'Halluin et al., 1990; Hill et al., 1991; Schroeder et al., 1991; Bagga et al., 1992). The culture

Table 4. Assessment of progeny derived from transgenic alfalfa expressing manganese-dependent lignin peroxidase

Parent	Expression level in parent	Visual score of progeny 4 weeks after germination									
		a	b	c	d	e	f	g	h	i	j
16	neg (control)	–	–	–	–	–	–	–	–	–	–
13	neg	–	–	–	–	–	++	–	–	+	–
6	low	–	–	+	++	–	–	–	–	–	–
12	med	++	+	–	–	–	–	–	–	–	+
11	med	++	++	–	–	++	+	–	++	–	–
8	med/high	–	–	+	–	–	++	–	+	–	++
5	med/high	–	–	+	++	–	+	–	–	–	–
1	high	–	–	++	+++	+++	–	++	–	–	+
15	high	+++	ng	++	–	–	++	ng	+	+	+
9	high	++	–	ng	++	+++	–	+++	–	+	–
10	high	++	+++	+++	–	++	++	–	+	++	++
14	high	–	ng	–	+++	ng	++	+++	++	+++	+

– No significant phenotypic effects.
+ 10–20% of leaves affected; growth slightly stunted.
++ 20–50% of leaves affected; growth slightly stunted.
+++ > 50% of leaves affected; growth stunted.
ng = Seed did not germinate.

conditions were a little different since they were optimized for an easily-regenerable genotype of alfalfa. The major difference in our system is that kanamycin is selective for inhibition of embryoid formation but not for callus formation. This is probably the reason that our system produces plants in a much shorter time (10–12 weeks) than that reported by others (20–30 weeks). In these and in other experiments using this transformation system, 20–50% of treated explants gave rise to transgenic plants. This high transformation frequency and the speed at which plants can be recovered makes this an ideal system for our studies.

Expression of *B. licheniformis* alpha-amylase had no effect on the yield of field-grown transgenic alfalfa. The expression of active alpha-amylase in transgenic alfalfa was lower than that reported by Pen et al. (1992) for tobacco. It is difficult, however, to compare absolute levels of foreign protein production between plant species. Expression is usually based on the amount of soluble protein in the extract prepared for analysis, for example, protein levels can vary enormously between plant species, between different parts of the same plant and on the conditions used to extract the tissue. We are currently analysing transgenic tobacco to determine whether the relatively low levels of expression are a function of our construct. The field plants will be maintained over two more seasons and used to look at large-scale recovery of *B. licheniformis* alpha-amylase from plant juice.

Levels of Mn-P in transgenic alfalfa were relatively high but expression of the protein had a deleterious effect on plant growth and development. We have not been able to demonstrate that the enzyme is active in the plant. The correlation between the effects on growth and development and protein production strongly suggests that at least a portion of the expressed protein is active enzyme. The delay in the onset of foliar symptoms in the transgenic plants is interesting. It is not clear if it is triggered by a development stage in alfalfa or reflects increased production/accumulation of the protein. If protein expression were deleterious in the very early stages of plant development we would not have recovered any transgenic plants. We have no explanation for the deleterious effects of Mn-P in alfalfa, particularly since these were not seen when we expressed the gene in tobacco using the same constructs (Mathews et al., 1993). The enzyme requires manganese for its activity and converts Mn^{2+} to Mn^{3+}. Foliar symptoms could be due to a deficiency of Mn^{2+} or the result of the damaging action of Mn^{3+}. It is not clear what effect, if any, expression of this enzyme has on lignin content of the transgenic alfalfa. Preliminary data suggest that there are qualitative changes in lignin in transgenic plants expressing Mn-P. This is

being investigated further since it may have potential for increasing the ruminant digestibility of this widely-used forage legume. Mn-P production may be an ideal system to test the use of an inducible promoter. It may be possible to express the protein a few days before harvest and thus be able to recover the enzyme and avoid most of the deleterious effects on growth and development.

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