Cloning of an alfalfa polyphenol oxidase gene and evaluation of its potential in preventing postharvest protein degradation

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Abstract

BACKGROUND: Ensiling forages often leads to degradation of protein to non-protein nitrogen (NPN), which is poorly utilized by ruminants. Postharvest protein degradation is especially high in alfalfa (*Medicago sativa* L.). In contrast, red clover (*Trifolium pratense* L.) has up to 90% less protein loss during ensiling due to polyphenol oxidase (PPO) forming *o*-quinones from endogenous *o*-diphenols and subsequent binding of *o*-quinones to cytoplasmic proteins. Here we determined whether an endogenous PPO might be exploited for postharvest protein protection in alfalfa.

RESULTS: We isolated an alfalfa PPO gene (MsPPO1) that shares limited sequence identity (70–72%) with red clover PPO genes. MsPPO1 is expressed primarily in flowers and developing seed pods, but not in leaves or stems. Expression of MsPPO1 from a strong constitutive promoter in transgenic alfalfa results in accumulation of PPO transcripts in leaves, but little enzyme activity is detected using a variety of *o*-diphenol substrates unless assayed in the presence of sodium dodecyl sulfate (SDS). Under this SDS-activated condition, preference of MsPPO1 for tested substrates is catechol ≥ (−)-epicatechin > caffeic acid. PPO activity in unactivated MsPPO1-alfalfa extracts is sufficient to inhibit proteolysis in the presence of catechol, but not caffeic acid or (−)-epicatechin. Inhibition is less than in extracts of alfalfa expressing the red clover PPO1 gene.

CONCLUSION: Endogenous alfalfa PPO, even if expressed in appropriate target tissues, would be less effective at preventing proteolytic losses in ensiled forages than red clover PPO.
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Keywords: polyphenol oxidase; alfalfa; forage legumes; proteolysis; protein preservation

INTRODUCTION

Polyphenol oxidases (PPOs; EC 1.14.18.1, 1.10.3.1) are capable of catalyzing the oxidation of *o*-diphenols to their corresponding *o*-quinones. Although they are nearly ubiquitous among plants,[1] the exact roles they play in plant growth, development, and physiology are not entirely clear. In at least some cases, PPOs appear to be involved in pathogen defense responses.[2,3] Some PPOs have been implicated in biosynthetic pathways including the biosynthesis of yellow aurone pigments in snapdragon flowers[4] and a specific lignan in creosote bush.[5] PPOs are probably best known for their negative impact on the quality of fresh fruits and vegetables as a result of PPO-mediated oxidation of endogenous *o*-diphenols to *o*−quinones.[6] The resulting *o*-quinones covalently couple to a number of cellular nucleophiles and consequent secondary quinone reactions lead to the formation of brown and black colored polymers (the browning reaction). Despite the usual negative association of PPO with agricultural crops, we have recently demonstrated that oxidation of *o*-diphenols by PPO can be exploited as a natural system of protein protection in forage crops preserved by ensiling.[7]

Postharvest protein degradation in ensiled forage crops results in the conversion of true protein to amino acids and peptides (non-protein nitrogen, NPN). This conversion of true protein to NPN is problematic because dairy cows and other ruminant animals poorly utilize excess non-protein nitrogen, resulting in economic losses to farmers, who must supplement rations with other sources of true protein. Such losses are especially high in alfalfa (*Medicago sativa* L.), approaching $100 million annually in the United States alone.[7,8] Further, loss of true protein in preserved forages has negative environmental impacts, as excess NPN is excreted by ruminants as urea, increasing N burdens to the environment. In contrast to alfalfa, the forage legume red clover (*Trifolium pratense* L.) experiences up to 90% less proteolysis

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when ensiled.[9] We have recently demonstrated that red clover's lower level of postharvest proteolysis is due to the oxidation of endogenous *o*-diphenols by red clover PPO.[7] Using genetically modified alfalfa expressing the red clover PPO1 gene (TpPPO1), we also demonstrated that this natural system of protein protection could be transferred to alfalfa, relatively little PPO activity is required, and a number of *o*-diphenol PPO substrates are effective in the process. Although the mechanism of protein protection by PPO-generated *o*-quinones is not clear, it seems likely the quinones react with nucleophilic sites on cellular proteins resulting in direct inactivation of proteases, modification of proteins such that they become poor substrates for endogenous proteases, or both. PPO-generated *o*-quinones also appear to prevent lipid breakdown in ensiled red clover[10] and have a positive impact on the lipid profile of products derived from animals fed diets high in red clover.[11,12]

Red clover leaves accumulate high levels of both PPO activity (as high as 70 nkat mg^{-1} protein)[7,13] and caffeic acid derived *o*-diphenols phasalic acid and clovamide[14,15] (Winters A, personal communication) that seem to be good substrates for the predominant foliar PPO enzymes.[16,17] In contrast to red clover, alfalfa, including a collection of almost 200 perennial *Medicago* accessions, has little if any PPO activity in its leaves and lacks significant levels of *o*-diphenol PPO substrates.[13,17–19] Here we have identified and characterized an alfalfa PPO gene (MsPPO1) to determine whether alfalfa's endogenous PPO enzyme might be exploited for preserving forage protein.

EXPERIMENTAL

Plant materials

A highly regenerable clone of Regen-SY[20] was used for alfalfa (*Medicago sativa* L.) transformation. Additionally, alfalfa expressing red clover PPO1 and control alfalfa transformed with pILTAB 357 empty vector were the same Regen-SY background.[13] Transformed alfalfa was maintained in a growth chamber at 26 °C with 16 h d^{-1} of approximately 3000 lx illumination. Alfalfa clone P derived from variety 'Blazer XL'[21] used in some experiments was maintained in a greenhouse year-round at 20–30 °C with light intensities between 25 000 and 60 000 lx. Supplemental lighting (13 h d^{-1}) was used during all but summer months. All plants were fertilized weekly with Peter's soluble 20-20-20 (Scott's, Marysville, OH).

DNA and RNA methodologies

Preparation and characterization of nucleic acids

Genomic DNA from young leaves of alfalfa plants, plasmid DNA, and lambda DNA were prepared using the DNeasy Plant Maxi Kit, QIAprep Spin Miniprep Kit, and Lambda Midi Kit, respectively (Qiagen, Valencia, CA, USA). For DNA blotting, DNA digested with restriction endonucleases was fractionated on agarose gels and transferred to nylon membranes essentially as described by Sambrook *et al*.[22] DNA sequence was determined by cycle sequencing using Big Dye v3.1 (Applied Biosystems, Foster City, CA, USA) and run on ABI automated sequencers by the University of Wisconsin Biotechnology Center. Sequence analyses were carried out using the Wisconsin Package, Version 10 (Accelrys, San Diego, CA, USA), ChloroP[23] and SignalP[24] algorithms available online at http://www.cbs.dtu.dk/services, and BLAST using the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Institute for Genome Research (www.tigr.org) web sites.

Total RNA was prepared from plant tissues using an RNeasy kit (Qiagen) or by the method of Chang *et al*.[25] Formaldehyde agarose gels were run and RNA blots prepared as previously described.[26] DNA and RNA hybridizations were carried out as previously described,[13] except that in some experiments ^{32}P-labeled riboprobes, prepared using the Strip-EZ RNA Kit (Ambion, Austin, TX, USA), were used instead of ^{32}P-labeled DNA probes.

Generation of PPO gene fragments by polymerase chain reaction (PCR)

A previously described primer pair corresponding to the conserved region flanking the CuA copper binding site of plant PPOs[13] was used to amplify a PPO gene fragment from an alfalfa DNA preparation. The primers were 5′-GGGGAATTCCAACAAGCTARKRTHCATTG TGCTT-3′ (sense) and 5′-GGGAAGCTTATCC CAATTCCARWAHGG-3′ (antisense). A series of three G deoxyribonucleotides and either an *Eco*RI or *Hin*dIII restriction endonuclease site (underlined) were included in each primer to facilitate subsequent cloning. These primers (50 pmol) and alfalfa clone P genomic DNA (50 ng) were used in a 50 μL PCR reaction using Taq polymerase (Promega Corp., Madison, WI, USA) in the supplied reaction buffer supplemented with 2 mmol L^{-1} $MgCl_2$ for 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C. The resulting DNA fragment was digested with *Eco*RI and *Hin*dIII and cloned into pBluescript SKII(−) (Stratagene, La Jolla, CA, USA) digested with the same enzymes using standard methodologies.[22]

PCR of full-length MsPPO1 from alfalfa cDNA was accomplished using the primers 5′-ATGGCATCTA TCTCACCCCTTG-3′ (sense) and 5′-TCAATCTT CAAGCTCTATC-3′ (antisense), KlenTaq LA (Sigma, St Louis, MO, USA) and cDNA template prepared from total RNA. cDNA was prepared using Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) from DNase I-treated total RNA isolated from MsPPO1-expressing transgenic alfalfa (described in detail below).

Library screening
Approximately 5×10^5 phage from an alfalfa genomic library (derived from cultivar Saranac) in the Lambda DASH II vector[27] were plated and lifted to nylon or nitrocellulose filters using standard protocols,[22] then screened by hybridization with a cloned 196 bp PPO fragment derived by PCR (described above). This screening procedure was repeated once for a total of 10^6 phage screened.

Overexpression of MsPPO1 in transgenic alfalfa

An MsPPO1 overexpression construct was prepared in a manner analogous to that previously described for red clover PPO genes[13] using the primers 5′-GGGGAATTCAAACAATGGCATCTATCTCA-CCCCTTG-3′ (sense) and 5′-GGGGAATTCAGA-TCTTCAATCTTCAAGCTCTATC-3′ (antisense) to generate an MsPPO1 coding region fragment by PCR from the cloned gene. These primers incorporated the proposed dicot consensus sequence AAACA[28] immediately upstream of the initiating Met codon and *Eco*RI restriction sites (underlined) to facilitate cloning behind the cassava vein mosaic virus (CsVMV) promoter of the pILTAB 357 plant transformation vector.[29] The cloned insert was sequenced to ensure that no mutations were introduced that would alter the sequence of the translated protein. The MsPPO1 construct was transferred to *Agrobacterium tumefaciens* strain LBA4404 and transformed into a Regen-SY alfalfa clone as previously described.[13,30,31] Putative transformants were initially screened for the presence of the *npt II* gene by PCR as previously described.[32]

Analysis of PPO-mediated browning, PPO activity, protein accumulation, and proteolytic inhibition

For PPO activity and immunoblots, leaf tissues were extracted with 100 mmol L^{-1} CH_3COONH_4, 20 mmol L^{-1} Tris, pH 7.5 (3 mL g^{-1} fresh weight) and protein content determined as previously described.[13] For immunoblotting, a protease inhibitor cocktail (P9599, Sigma) was included in the extraction buffer according to the supplier's instructions. Flower and seed pod protein samples were prepared by phenol extraction as previously described.[13] Browning assays were carried out on plant leaf extracts by addition of 100 mmol L^{-1} *o*-diphenol solution in ethanol to 3 mmol L^{-1} final concentration or ethanol to 3% (v/v) as a negative control followed by incubation at room temperature. PPO activity assays were carried out essentially as described by Esterbauer *et al*.[33] as previously detailed,[13] except assays were carried out in 0.1× McIlvaine's citrate–phosphate buffer, pH 7.0 (15 mmol L^{-1} Na_2HPO_4, 2.3 mmol L^{-1} citric acid). For comparison of different PPO substrates, data for a given extract were normalized to the activity for catechol. Immunoblotting was carried out using antiserum raised against red clover PPO1 as previously described.[13] Proteolysis in leaf extracts (protein content adjusted to 2 mg mL^{-1} protein with extraction buffer) was determined by measuring the release of tricholoroacetic acid (TCA, 50 g L^{-1}) soluble amino acids and peptides over time using previously described procedures.[7] For quantitative PPO activity and proteolytic inhibition experiments, the results using two extracts prepared from different leaf tissue samples harvested on different days were averaged and error reported as standard error of the mean (SEM). For comparison of two sample means, statistical significance was determined using the *t*-test. For comparison of more than two sample means, data were subjected to single-factor ANOVA using the statistical package of Excel (Microsoft Corp., Redmond, WA, USA) and Tukey's HSD post hoc test.

RESULTS AND DISCUSSION

Cloning and sequencing of a PPO gene from alfalfa

Degenerate primers flanking the conserved CuA copper binding site of several previously cloned plant PPO genes, and previously used to amplify approximately 200 bp PPO gene fragments from red clover cDNA,[13] amplified a DNA fragment of the expected size from alfalfa clone P DNA. The 196 bp fragment was cloned and sequenced (Genbank accession EU168793). The resulting nucleotide and predicted protein sequences (excluding sequence derived from the PCR primers), were used in BLASTn and tBLASTn searches of the NCBI database. Sequences with the highest probability matches corresponded to PPO genes from a wide variety of plant species, including red clover, *Ipomoea batatas* L., *Malus domestica* Borkh., and *Triticum aestivum* L., with most showing 60–70% amino acid identity. These results suggested the gene fragment derived from the PCR reaction corresponds to a polyphenol oxidase gene. Interestingly, no high identity matches (i.e., >80%) with *Medicago truncatula* Geartn. ESTs or genomic sequences were identified.

The 196 bp PCR fragment was used as a hybridization probe to screen an alfalfa genomic library.[27] A single hybridizing clone with an approximately 12 kbp insert was obtained following a screen of approximately 5×10^5 lambda clones. By Southern blotting of restriction enzyme-digested clone DNA followed by hybridization with the PCR-derived probe, an approximately 6 kbp *Eco*RI fragment containing the hybridizing DNA sequence was isolated and subcloned into a plasmid cloning vector. Sequence analysis indicated the 6 kbp *Eco*RI fragment contains an entire PPO coding region as well as 1.2 kbp of upstream sequence (Genbank Accession AY283062). This alfalfa PPO coding region contains no introns, typical of PPO genes isolated from dicotyledonous plants.[34] The gene (designated MsPPO1) is predicted to encode a protein of 607 amino acid residues (68.5 kDa) (Fig. 1). Based on ChloroP[23] and Signal P[24] algorithms, the

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  1 MASISPLAFI NTINVSSNSK ISPSSYPFSQ KQQKYSRHRK LPRRQVITFS 50
 51 GNDTNQNNPK EEQEQQNIVV GNRRNVLIGL GGLCGTFTTN PFALASPISP 100
101 PDLSTCGPPD LPLGATPNNI NCCPPNSTKI INYKIPSSNQ PLRVRQAAHL 150
151 VNDEYLQKYK KALELMKALP SSDPRSFVQQ ANIHCAYCDG AYSQVGFPNL 200
201 DLQVHNSWLF FPFHRWYLYF YERILGSLIN DPTFALPFWN YDSPNGMQLP 250
251 SIYADSTSPL YDKLRTASHQ PPTLIDLNYD DVDVDDDANG RISSNLTTMY 300
301 RQMVSNGKTS SLFLGSSYRA GDEPDPGAGS IENVPHGPVH RWSGDNTQPN 350
351 FENMGVFYAA ARDPIFFSHH SNIDRFWSIW KTLGGKRKDF KDKDWLESEF 400
401 LFYDENKNLV KVKVKDCLDT KKLGYVYQDV DIPWLNAKPK PCRKNIQKNV 450
451 EVAQGNFFGI GEARASEINS RSYVTCPLVL DNVVSTIVKR PKKSRSKKEK 500
501 EEEEEVLVIE GIEFDKSLGV KFDVFINDED DKVIKPINTE FAGSFVNVPH 550
551 SSHDHKKKKT NSCLRVGLTD LLEDLGAEDD DSIVVTLVPR YGKGIVKIRN 600
601 IKIELED* 608
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Figure 1. Predicted amino acid sequence encoded by an alfalfa PPO gene, MsPPO1. Predicted cleavage sites for chloroplast transit and thylakoid lumen signal peptides are indicated by open and filled arrowheads, respectively. The conserved CuA and CuB copper binding sites (starting at amino acids 205 and 363, respectively) are underlined.

protein is predicted to be localized to the thylakoid lumen following cleavage of a 69 amino acid transit peptide and 26 amino acid signal peptide. Localization of the protein to the thylakoid lumen would be similar to that observed for nearly all characterized PPO proteins. If the alfalfa PPO protein is targeted to the chloroplast thylakoid lumen as predicted, the mature protein would be 512 amino acid residues (58.0 kDa).

The sequence of MsPPO1 isolated from the library had only limited nucleotide sequence identity (70%, excluding primer sequence) with the PCR-derived gene fragment used for the screen. Screening of an additional 5×10^5 genomic clones from the alfalfa genomic library using the 196 bp PCR fragment failed to identify a clone containing its corresponding gene, although three additional clones identical or nearly identical to MsPPO1 were identified. This result suggests that the original PCR product amplified from clone P alfalfa DNA was amplified from contaminating DNA in the original DNA preparation or PCR reactions, represents a PCR artifact, or is derived from a divergent alfalfa gene not present in the genomic library that was screened. That we were unable to identify any potential homologs in searches of *M. truncatula* sequence databases nor detect hybridzing fragments in Southern blotting experiments with clone P DNA (data not shown) suggests the 196 bp PCR fragment does not represent an actual alfalfa PPO sequence.

Using the 1824 bp coding region sequence from the full-length MsPPO1 clone in BLASTn searches of the NCBI Genbank database we identified an *M. truncatula* BAC clone (Genbank accession AC157507, clone mt2-78b21) containing two tandem PPO genes with a high degree of sequence similarity to MsPPO1. The first gene (from position 53 210 to 51 387) and the second gene (from position 59 385 to 57 560) are 95% and 88% identical to MsPPO1, respectively. The two *M. truncatula* genes are 89% identical to each other. We currently have no evidence of tandemly arranged PPO genes in *M. sativa*, although Southern blotting experiments using DNA derived from clone P and Regen SY27 alfalfa genotypes indicate the presence of multiple PPO genes (data not shown). In addition, a BLASTn search of the TIGR *M. truncatula* gene index with the full-length MsPPO1 coding region identified a tentative consensus sequence (TC101697) sharing 91% sequence identity with MsPPO1. Interestingly, TC101697 is not identical to either of the PPO genes contained on mt2-78b21, although sequence comparisons suggest TC101697 is comprised of cDNAs originating from both genes. MsPPO1 shares a high degree of sequence identity (82% over the coding region) with *Vicia faba* PPO (Genbank accession Z11702). MsPPO1 shares lower sequence identity (70%) with PPO cDNAs and genomic clones from red clover (Genbank accessions AY017302, AY017303, AY01704, EF183483, EF183484), suggesting the red clover PPOs may have diverged from the other legume enzymes.

MsPPO1 is expressed in seedpods and flowers

We analyzed MsPPO expression in alfalfa by RNA blotting and hybridization with a probe derived from MsPPO1. Hybridization of a blot containing RNA from young leaves, mature leaves, stems, flower buds, flowers, seed pods 2 weeks post pollination, and 3-day-old seedlings was carried out using an *in vitro*-labeled RNA corresponding to a 1.5 kbp *Xba*I–*Hin*dIII fragment of the MsPPO1 coding region. Hybridization and washes were at moderate stringency (approximately 25 °C below probe-target T_m calculated according to Sambrook *et al.*[22]) to detect MsPPO1 as well as any related PPO sequences. RNA from leaves of transgenic alfalfa expressing MsPPO1 under the control of a constitutive promoter (detailed below) served as a positive control. Under the hybridization and wash conditions used, PPO transcripts of the expected size (approximately 2 kb) were detected in developing seed pods, and to a lesser extent flowers and flower buds (Fig. 2). No transcripts of the approximate expected size of an MsPPO1 transcript were apparent in lanes corresponding to leaves, stems, and seedlings. Although faint higher and lower molecular weight bands are present in several of the lanes, these bands are likely an artifact because their locations on the blot correspond to ribosomal RNA bands that had been visualized by ethidium

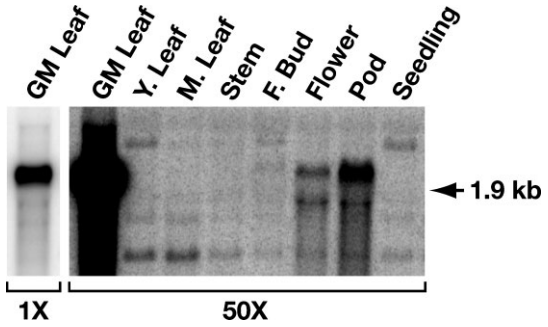

Figure 2. Expression of MsPPO1 as detected by RNA blot hybridization. Total RNA (5 μg) from Regen SY alfalfa young leaves (Y. Leaf), mature leaves (M. Leaf), stems, flower buds (F. Bud), flowers, seed pods (Pod), or 3-day seedlings was used to make an RNA blot. Seed pods (2 weeks post-pollination) and seeds were produced by self-pollination. RNA (1 μg) from leaves of genetically modified MsPPO1-alfalfa (GM Leaf) served as a positive control. Migration position of a 1.9 kb ribosomal RNA (detected on the ethidium stained gel, not shown) is indicated by an arrow. To facilitate comparison of expression levels, a 50-fold shorter exposure of the GM Leaf sample is shown.

bromide staining of the original gel. These results are consistent with a more limited expression analysis that we previously carried out on RNA derived from tissues of clone P alfalfa,[16] in which PPO mRNA was detected in seed pods and to a lesser extent flowers, but not in leaves, stems, or apical shoots. The RNA blotting results are also consistent with *M. truncatula* EST data. Of 14 *M. truncatula* EST sequences with nucleotide identity ≥90% to MsPPO1, seven are from a pod wall library (representing 0.2% of the ESTs from this library). Of the remaining seven ESTs, four are from a drought-stressed plantlet library (representing 0.05% of the ESTs from this library), and one each from developing leaf, insect herbivory, and virus-infected leaf libraries (representing approximately 0.01% of the ESTs from each of these libraries). Together these results suggest that under normal growing conditions PPO genes are expressed at exceedingly low levels, if at all, in alfalfa leaves.

MsPPO1 has low activity in leaves of genetically modified alfalfa

Because alfalfa leaves and stems fail to express MsPPO1 mRNA at levels detectible by RNA blotting and alfalfa leaves have little if any detectible endogenous PPO activity,[13,17–19] we decided to characterize the enzymatic activity of MsPPO1 by ectopically expressing it in alfalfa leaves. Creation of genetically modified alfalfa expressing MsPPO1 in its leaves would also allow us to test whether endogenous alfalfa PPO might be useful in preventing postharvest proteolytic losses if it were expressed in leaves. The entire coding region of MsPPO1 including plastid-targeting signals was inserted behind the CsVMV promoter[29,35] in a plant transformation vector, the resulting construct was transformed into alfalfa, and 16 independent alfalfa transformants containing the *npt II* selectable marker gene were identified. Protein extracts were made from leaves of the alfalfa plants transformed with the MsPPO1 gene and used in quantitative assays of PPO activity. Caffeic acid was used as a substrate in this screening experiment since it had proven to be among the best substrates for red clover PPOs.[16,18] A leaf extract of alfalfa transformed with empty vector (control alfalfa) served as a negative control. For the 16 MsPPO1-transformed alfalfa analyzed in this experiment, measured PPO activities were indistinguishable from that of control alfalfa (0.04 nkat mg^{-1} in this experiment). Because enzyme activities in leaf extracts of MsPPO1-transformed alfalfa were not detectible above background in the quantitative assay, we screened the plants for enzyme activity using a more sensitive qualitative assay. MsPPO1-transformed alfalfa or control alfalfa leaf extracts were prepared and incubated at room temperature in the presence of catechol, caffeic acid, or (−)-epicatechin, and color changes in the extracts characteristic of PPO activity were observed (Fig. 3). These *o*-diphenols were chosen because they are structurally diverse, representing the simplest *o*-diphenol and two distinct classes of naturally occurring *o*-diphenols (caffeic acid derivatives and flavanol *o*-diphenols). Following *o*-diphenol addition and incubation at 25 °C, for four of the MsPPO1-transformed alfalfa plants, color changes were apparent after 1–6 h for all three substrates. Maximum color development was observed at 6–24 h. Extracts of the remaining MsPPO1-transformed and control alfalfa extracts failed to exhibit discernible color changes, even after 2-day incubations. For the four MsPPO1-transformed alfalfa plants that did have PPO activity based on this assay (hereafter referred to as MsPPO1-alfalfa), the length of time for color changes to become apparent were substantially longer (several hours) than that previously observed in leaf extracts of alfalfa expressing TpPPO genes (2–5 min).[13] Interestingly, we observed slight darkening of MsPPO1-alfalfa leaf extracts following a 3-day incubation in the absence of added *o*-diphenol compared to extracts of control alfalfa (no PPO transgene) or alfalfa expressing TpPPO1 (data not shown), although the biological significance of this observation is unclear. A plant consistently showing the most rapid extract color changes in the presence of added *o*-diphenol substrate, and hence the highest levels of PPO activity, was used for the analyses detailed below.

MsPPO1 protein accumulates as a truncated form in genetically modified alfalfa leaves

The relatively low PPO activity detected in leaves of MsPPO1-alfalfa could be due to low-level expression of the transgene, poor accumulation of the active gene product, or poor activity against the tested substrates or under the conditions of the assay. To avoid the first possibility, MsPPO1 was expressed from the CsVMV promoter, which has been shown to direct high-level expression of transgenes in alfalfa.[29,35] An appropriately sized approximately 2 kb transcript corresponding to the MsPPO1 transgene was easily detected

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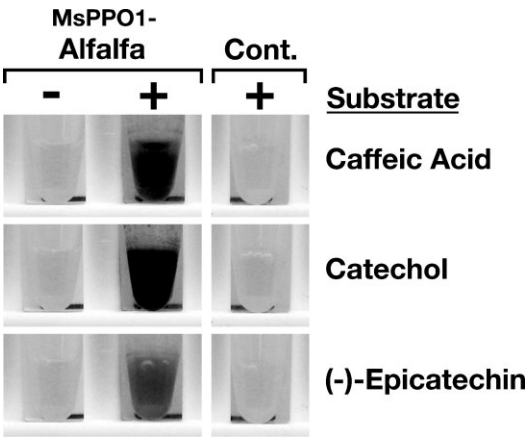

Figure 3. Qualitative assay for PPO activity. Extracts of leaves of MsPPO1- or control alfalfa (Cont.) were supplemented (+) with the indicated *o*-diphenols at 3 mmol L^{-1}. Extracts are shown following a 24 h incubation at 25 °C. A non-supplemented MsPPO1-alfalfa extract (–) incubated for 24 h is shown for comparison.

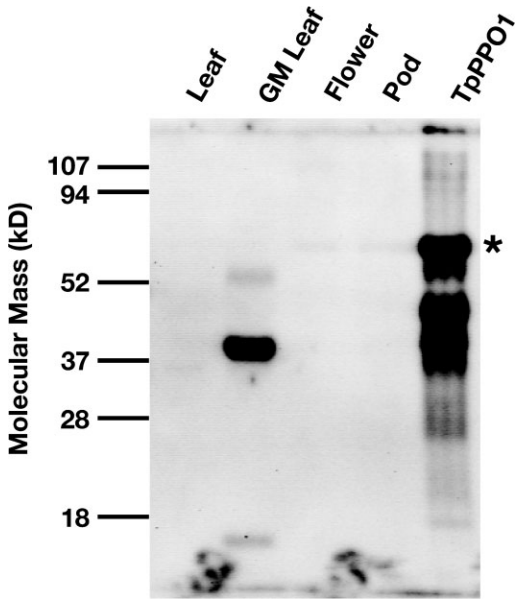

Figure 4. Immunological detection of MsPPO1 protein. An immunoblot of 10 μg protein from untransformed Regen SY alfalfa leaves, flowers, or seed pods; or leaves of MsPPO1-alfalfa (GM Leaf) was developed with anti-TpPPO1 antiserum. Red clover PPO1 expressed in *Escherichia coli* (TpPPO1, approximately 5 ng) served as a positive control.[13] An asterisk indicates the full-length mature (lacking chloroplast targeting signals) TpPPO1 used to produce the antiserum. Lower molecular weight bands in the TpPPO1 lane correspond to truncation products that accumulate when the protein is overexpressed in *E. coli* (Sullivan M, unpublished result).

in leaves of MsPPO1-alfalfa (Fig. 2), indicating the MsPPO1 transgene is well expressed at the mRNA level, accumulating to approximately 250-fold higher levels than the endogenous transcript in seed pods. To make sure that the MsPPO1 protein was accumulating in transgenic plants, leaf extracts were fractionated on SDS-PAGE and analyzed by immunoblotting using anti-TpPPO1 antiserum,[13] which cross-reacts with PPOs from several dicot (Sullivan M, unpublished data) and monocot (Anderson JV and Marita J, personal communication) species. As shown in Fig. 4, a faint band running at approximately 54 kDa is associated with expression of the MsPPO1 transgene and is close to the expected size (58 kDa) of the mature, plastid targeted MsPPO1 gene product. However, a much more intense band associated with expression of the MsPPO1 transgene corresponds to an approximately 42 kDa protein. It is unlikely that this 42 kDa protein represents post-isolation cleavage of the expected mature protein product since a protease inhibitor cocktail was included in the buffer used to prepare this protein extract. A faint band <18 kDa in size is also present in the MsPPO1-alfalfa leaf extract and could be the other cleavage product derived from the expected full-length MsPPO1 protein. To rule out the possibility that the MsPPO1 expression construct contained a premature stop codon, we resequenced the MsPPO1 coding region in the binary vector used for the alfalfa transformation. We also sequenced a PCR fragment of the entire MsPPO1 coding region derived from MsPPO1-alfalfa leaf cDNA and found no coding region mutations had been introduced during the transformation process. These results suggest the 42 kDa form of MsPPO1 in the transgenic plants is a truncated form produced *in vivo* from the full-length protein. This 42 kDa form could represent the mature form of the protein since similar truncation products have been purified as active PPO from other plant species.[36] We have recently demonstrated that for red clover PPOs expressed in alfalfa post-isolation proteolytic cleavage in alfalfa leaf extracts to a 45 kDa form may be partly responsible for enzyme activation.[17] Alternatively, the 42 kDa form of the alfalfa protein could be the result of aberrant processing due to high-level ectopic expression in leaves. If the protein is being aberrantly processed, this might explain the relatively low activity levels observed despite high levels of MsPPO1 mRNA and protein detected in the genetically modified alfalfa.

In extracts of leaves from control plants not transformed with MsPPO1, a faint band of approximately 36 kDa molecular weight cross-reacting with the TpPPO1 antibody was detected. It is not clear if this represents a breakdown product of an endogenous PPO protein or an unrelated alfalfa protein that fortuitously contains an epitope recognized by the red clover PPO antiserum. Given the lack of MsPPO1 mRNA detected in leaves, the latter explanation seems more likely. We also examined extracts of alfalfa flowers and seed pods, since these were the only tissues in which we identified PPO1 mRNA. Faint bands co-migrating with recombinant TpPPO1 (apparent molecular mass of 65 kDa) and cross-reacting with TpPPO1 antiserum were detected. It is not clear if these bands are the proteins translated from the mRNA observed in Fig. 2, since the immunoblot band intensities for flowers and seed pods are similar but the corresponding mRNA levels are different. We cannot rule out the possibility that the bands seen on the immunoblot for flower and pod proteins are not related to PPO, and that actual

alfalfa PPO protein in these tissues is below the limit of detection in this experiment.

MsPPO1 activity is enhanced by sodium dodecyl sulfate

Many PPO enzymes are activated by small amounts of detergent or other denaturants. These agents presumably cause conformational changes in the enzyme that allow substrates better access to the active site.[37] We assayed leaf extracts from control and MsPPO1-alfalfa for PPO activity with caffeic acid, (−)-epicatechin, and catechol, in standard assay buffer or assay buffer supplemented with 8.7 mmol L^{-1} sodium dodecyl sulfate (SDS) to test for activation (Table 1). As in the screening experiment described above, in standard assay buffer (lacking SDS), PPO activity measurements for extracts of MsPPO1-alfalfa could not be distinguished from those of control alfalfa for any of the tested substrates ($P > 0.05$). This result underscores the limitations of this quantitative assay with extremely low activity levels, since the MsPPO1-alfalfa extracts could mediate the substrate-dependent color changes described above, whereas control alfalfa extracts could not. Addition of SDS to the assay buffer resulted in a slight increase in enzyme activity measurements made for extracts of control alfalfa with catechol ($P < 0.025$), but values for caffeic acid and (−)-epicatechin were unchanged by SDS addition ($P > 0.3$). For control alfalfa extracts it is unclear what any of these background values represent, since extract browning is not seen in the presence of PPO substrate, even with SDS addition (data not shown), and although these background assay values are extract-dependent, addition of more or less extract does not result in the expected corresponding changes in measured activity (data not shown). In contrast to control alfalfa extract, MsPPO1-alfafla extracts showed substantial increases in PPO activity when assayed in the presence of SDS. A high degree of variability was observed in the SDS-activated measurements for MsPPO1-alfalfa leaf extracts prepared from different tissue samples, presumably due to uncontrolled environmental variables that affected enzyme accumulation. This high degree of variability resulted in a relatively high *P*-value for the difference between non- and SDS-treated MsPPO1 alfalfa extracts ($0.07 < P < 0.09$) despite the obvious increase in activity. We have previously observed similar high levels of variation when working with transgenic alfalfa expressing red clover PPO genes from the CsVMV promoter, although normalizing activity data to a given substrate greatly reduces variability between extracts of different tissue samples expressing the same PPO.[7,13,17] Using this approach allowed us to evaluate the relative substrate specificities for MsPPO1 (Table 2). SDS-treated MsPPO1 activity for catechol and (−)-epicatechin was approximately the same, although catechol may be favored ($P = 0.08$), and about 10-fold higher than for caffeic acid ($P < 0.01$). It is unclear what the natural substrates for MsPPO1 might be, but with its preference for a flavanoid *o*-diphenol and seedpod expression pattern, a role in the formation of condensed tannins during seed development is an intriguing possibility (e.g., see Dixon *et al*.[38]). Interestingly, the substrate preference of activated MsPPO1 is different from those of characterized red clover PPO gene products, which seem to prefer caffeic acid over (−)-epicatechin and catechol.[16,17] With the SDS-induced increase in the enzyme activity measured for MsPPO1-alfalfa leaf extracts, PPO activity levels approach those of freshly prepared (and not SDS-activated) leaf extracts of alfalfa expressing TpPPO1 from the CsVMV promoter (up to approximately 5 nkat mg^{-1}), although additional activation of the clover enzyme in alfalfa extracts results in a 5- to 10-fold increase in PPO activity.[13,17] This finding suggests the truncated form of the protein accumulating in MsPPO1-alfalfa leaves represents the active form of the enzyme.

MsPPO1 inhibits proteolysis in alfalfa extracts in the presence of catechol

Previous studies utilizing red clover PPO1 (TpPPO1) ectopically expressed in alfalfa indicated that even relatively low levels of PPO activity can inhibit postharvest proteolysis in alfalfa leaf extracts.[7] To determine whether MsPPO1 is capable of inhibiting

Table 2. Relative preference of SDS-activated MsPPO1 for various substrates

Substrate	Percent maximum activity[a]
Catechol	100a
(−)-Epicatechin	74 ± 9a
Caffeic acid	8 ± 2

[a] Average of two experiments ± SEM. For each experiment, activities for each substrate were corrected for background activity measured in control alfalfa extract and normalized to the catechol value. Difference marked with 'a' is significant at $P = 0.08$. Other differences are significant at $P < 0.01$.

Table 1. PPO activities[a] (nkat mg^{-1}) in MsPPO1- and control alfalfa leaf extracts without and with SDS

	Without SDS		With SDS	
Substrate	MsPPO1	Control	MsPPO1	Control
Catechol	0.09 ± 0.01b	0.13 ± 0.01a	4.22 ± 1.69b	0.22 ± 0.02a
(−)-Epicatechin	0.06 ± 0.03b	0.11 ± 0.01	3.13 ± 1.49b	0.15 ± 0.03
Caffeic Acid	0.05 ± 0.04b	0.07 ± 0.05	0.43 ± 0.17b	0.07 ± 0.05

[a] Average of two experiments ± SEM. Differences within a row marked with the same letter are significant at $P < 0.025$ (a) and $P < 0.10$ (b).

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Table 3. Proteolysis in control, MsPPO1-, and TpPPO1-alfalfa extracts in the presence of various *o*-diphenols

	Amino acid release (4 h), μmol mg^{-1}[a]			Amino acid release (20 h), μmol mg^{-1}[a]		
Extract	Caffeic	(−)-Epi	Catechol	Caffeic	(−)-Epi	Catechol
Control	0.74 ± 0.07a	0.68 ± 0.02a	0.75 ± 0.01	1.50 ± 0.07a	1.47 ± 0.05a	1.43 ± 0.00
MsPPO1	0.69 ± 0.04a	0.57 ± 0.11a	0.45 ± 0.01	1.41 ± 0.08a	1.35 ± 0.22a	1.00 ± 0.05
TpPPO1	0.09 ± 0.02	0.09 ± 0.02	0.13 ± 0.01	0.21 ± 0.03	0.17 ± 0.03	0.32 ± 0.02

[a] Average of two experiments ± SEM. Data marked with the same letter within a column are not significantly different ($P > 0.56$). All other differences within a column are significant ($P < 0.025$).

proteolysis in alfalfa extracts in the presence of PPO substrate, leaf extracts of control and MsPPO1-alfalfa were incubated at 37 °C in the presence of 3 mmol L^{-1} caffeic acid, (−)-epicatechin, or catechol. An extract of alfalfa expressing TpPPO1[7] served as a positive control. These assays were carried out without SDS activation, since during ensiling such extraordinary steps would be impractical. Proteolysis was measured as release of TCA-soluble amino acids after 4 and 20 h (Table 3). Trends at the 4 h and 20 h time points were similar. As previously observed,[7] in the TpPPO1-alfalfa extract proteolysis was reduced by >75% in the presence of any of the tested *o*-diphenols relative to a control extract. While no significant reduction in proteolysis was seen for MsPPO1-alfalfa extracts in the presence of either caffeic acid or (−)-epicatechin ($P > 0.5$), a reduction in amino acid release was observed when catechol was the *o*-diphenol used. Although the reduction in proteolysis seen for MsPPO1 was not as great as that seen for TpPPO1, it was substantial given the relative lack of quantifiable PPO activity of these extracts. This finding underscores our previous observation that even small amounts of PPO activity are capable of inhibiting postharvest proteolysis.[7] Although (−)-epicatechin is almost as good a substrate as catechol for SDS-treated MsPPO1, it is unclear why it failed to significantly reduce proteolysis in MsPPO1-alfalfa extracts. Many factors may influence the efficacy with which a given PPO-generated *o*-quinone results in proteolytic inhibition (e.g., rate of quinone formation, relative reactivity towards various nucleophiles, half-life, cross-linking potential). Until the roles these parameters play in proteolytic inhibition are better understood, it may be difficult to fully predict optimal PPO/quinone combinations. The high levels of enzyme activity available from TpPPO1 without additional activation may be sufficient to allow proteolytic inhibition with a wide variety of *o*-diphenol substrates.[7] Unfortunately, the relative lack of enzymatic activity and/or limited substrate specificity of MsPPO1 make it, overall, less effective at preventing postharvest proteolysis than its red clover counterpart.

CONCLUSIONS

The properties of the MsPPO1 gene (e.g., natural expression pattern limited to flowers and seed pods, poor activity of the encoded enzyme in the absence of activation against naturally occurring substrates most likely to be available for an ensiling process) make it a poor target for development of a PPO/*o*-diphenol-based system of protein protection in alfalfa. These results are consistent with a recent screen of approximately 200 accessions of perennial *Medicago* species for useful levels of foliar PPO activity against caffeic acid, (−)-epicatechin, and catechol.[19] In that screen, no accessions were identified with potential for development of a PPO-based protein preservation system. Together, these results suggest development of a PPO-based protein protection system in alfalfa will likely require the use of transgenic approaches (e.g., use of TpPPO1 as a transgene). Additionally, the results reported here underscore the uniqueness of the red clover PPOs among legume species with respect to sequence, expression pattern, and enzymatic activities.

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