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Gene Expression Patterns, Localization, and Substrates of Polyphenol Oxidase in Red Clover (*Trifolium pratense* L.)

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ABSTRACT: Polyphenol oxidase (PPO) genes and their corresponding enzyme activities occur in many plants; natural PPO substrates and enzyme/substrate localization are less well characterized. Leaf and root PPO activities in *Arabidopsis* and five legumes were compared with those of high-PPO red clover (*Trifolium pratense* L.). Red clover PPO enzyme activity decreased leaves > stem > nodules > peduncle = petiole > embryo; PPO1 and PPO4 genes were expressed early in leaf emergence, whereas PPO4 and PPO5 predominated in mature leaves. PPO1 was expressed in embryos and nodules. PPO substrates, phaselic acid and clovamide, were detected in leaves, and clovamide was detected in nodules. Phaselic acid and clovamide, along with caffeic and chlorogenic acids, were suitable substrates for PPO1, PPO4, and PPO5 genes expressed in alfalfa (*Medicago sativa* L.) leaves. PPO enzyme presence and activity were colocalized in leaves and nodules by cytochemistry. Substrates and PPO activity were localized in developing squashed cell layer of nodules, suggesting PPO may have a developmental role in nodules.

KEYWORDS: *Trifolium pratense L., red clover, leaf, nodule, polyphenol oxidase, PPO, gene expression, enzyme localization, o-diphenol substrate, Raman microspectrometry*

INTRODUCTION

Polyphenol oxidases (PPO) are copper-containing enzymes that catalyze two different reactions: hydroxylation of monophenols to *o*-diphenols and oxidation of *p*- and *o*-diphenols to *p*- and *o*-quinones. *p*-Diphenol oxidases and *o*-diphenol oxidases differ in their cellular localization and in their roles and are easily distinguishable from their gene and amino acid sequences. *p*-Diphenol oxidases include laccase (EC 1.10.3.2), an extracellular enzyme involved in cell wall thickening and lignification. Laccases utilize a wide range of substrates, including *o*- and *p*-diphenol substrates, such as catechol and hydroquinone.

o-Diphenol oxidase (catechol oxidase; EC 1.10.3.1) and monophenolase (tyrosinase; EC 1.14.18.1) are the focus of this paper and will be referred to as polyphenol oxidases or PPOs. These enzymes work on *o*-diphenol substrates, such as the caffeic acid derivatives phaselic acid and clovamide.

PPOs are implicated in protection against both abiotic and biotic stresses and have been described in an increasing number of plant species,[1,2] with differential expression of large PPO gene families dissected.[3−5] Less attention has been paid to enzyme and substrate localization and the substrate preferences of these enzymes.

PPO gene classes EC 1.10.3.1 and EC 1.14.18.1 are absent from some species. For example, in *Arabidopsis* there is only a laccase gene, the product of which is found in the seed testa.[6] PPO enzyme activity also varies among different species:[1] low leaf PPO activity was described in potato[4] and high leaf activity was observed in tomato,[3,7] perennial peanut,[8] and red clover.[9]

PPO enzymes are encoded by nuclear genes and in almost all cases targeted to plastids, such as thylakoid lumen of chloroplasts.[10,11] All six or seven PPO genes in the gene family of red clover[12] have plastid-targeting sequences. The large families of six or more PPO genes in tomato,[3] potato,[4] and aubergine[5] show differential gene expression, suggesting functional diversity of these genes.

PPO enzyme activity is high in leaf tissues of red clover,[9,13] but its level in other tissues has not been investigated. In healthy leaves oxidation of PPO substrates by PPO enzymes is prevented by cellular compartmentalization; the mature enzyme is targeted to the intrathylakoid region of the chloroplast, and the natural substrates identified to date are exclusively located elsewhere in the cell, largely reported within the vacuole.[14] PPO enzyme is the focus of many studies, but substrate availability may also limit PPO activity. Potential natural *o*-diphenol substrates for PPO enzymes include caffeic acid derivatives (e.g., chlorogenic acid, phaselic acid, clovamide), flavonoids (e.g., epicatechin, catechin), and L-1,3,4-dihydroxyphenylalanine (L-DOPA). The natural *o*-diphenols and concentrations may vary with species, tissues, and cells, as well as with genotype and environment.[9,15] Recent work has focused on identifying natural substrates of PPO in important PPO-expressing forage crops.[16]

In red clover leaves, the main endogenous *o*-diphenol substrates reported are phaselic acid and clovamide.[9,17] The latter has been shown to be induced by jasmonic acid in treated roots of red clover seedlings,[18] although relatively high levels can be found in glasshouse- or field-grown red clover. Red clover PPO1 utilizes both phaselic acid and clovamide effectively.[15] By contrast, although alfalfa has at least two PPO genes, it has little endogenous PPO activity or *o*-diphenol substrate in its leaves;[19]

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this fact has been successfully exploited to gain valuable information about the physical and chemical characteristics of three isolated red clover PPO enzymes and their substrates.[13,20,21]

PPO enzyme activity is found in roots of tomato,[22] pearl millet,[23] poplar,[24] beetroot,[25] and aubergine[5] and in roots of legumes, such as soybean,[26] black lentil,[27] peanut,[28] and pea.[29] In nonphotosynthetic roots, PPO is nearly always targeted to plastids, such as starch-packed amyloplasts of potato tubers.[30] The precise location of PPO and its substrates within legume roots, and particularly within N_2-fixing nodules, is unclear.

Here we extend these studies to the recently identified red clover PPO4 and PPO5 genes,[12] analyzing the expression of PPO1, PPO4, and PPO5 genes in red clover tissues and determining how effectively PPO4 and PPO5 gene products utilize various substrates compared with PPO1. We localized potential PPO substrates in nodules using Raman microspectrometry and colocalized PPO enzymes and their activity within cells of leaves and nodules using immunogold labeling and cytochemistry with transmission electron microscopy (TEM).

MATERIALS AND METHODS

Reagents were of molecular biology grade or higher. Phaselic acid (2-*O*-*E*-caffeoyl-L-malic acid) was prepared and characterized as described.[15] Clovamide (*N*-*E*-caffeoyl-3-hydroxy-L-tyrosine) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Growth of Nontransformed Plants. *Arabidopsis* was grown in peat under 8 h of light, 25/15 °C day/night, 100 μmol m^{-2} s^{-1}. Legume seedlings, *Lotus japonicus*, *Lotus corniculatus* (bird's-foot trefoil), *Medicago truncatula* (barrel medic), *Medicago sativa* (alfalfa), *Trifolium repens* (white clover), and *Trifolium pratense* (red clover), were grown in medium grade vermiculite under 16 h of light, 25/15 °C day/night temperatures, 300 μmol m^{-2} s^{-1}, inoculated with rhizobium (Table 1) after 3 days, and watered daily with N-free nutrients.[31] The presence of PPO genes in seedlings was detected using degenerate PPO primers described elsewhere.[12]

Production and Growth of Transgenic Alfalfa Expressing PPO4 and PPO5. Red clover PPO4 and PPO5 overexpression constructs were prepared as previously described for PPO1,[13] but using primer pairs 5′-GACTAGTAAACAATGGCATCTATCTCACCCT-CACC-3′ and 5′-GGGTACCTCAAGATTCAAACTCTATCTTGA-TGCC-3′ or 5′-GTCTAGAAAACAATGATATCTATTTCATCCTC-ACCTCTC-3′ and 5′-GGGTACCTCAATTTGCAAATTCTATCT-TGATGCCAC-3′ to generate PPO4 and PPO5 coding region fragments, respectively, from the cloned genes present on BAC clones.[12] These primers incorporated either *Spe*I or *Xba*I restriction sites (underlined) to facilitate cloning behind the CsVMV promoter of pILTAB357 plant transformation vector. PPO4 and PPO5 constructs were introduced into a Regen-SY alfalfa clone via *Agrobacterium*-mediated transformation.[32] Regenerants were PCR-screened for *npt*II and PPO4 or PPO5 transgenes, and transformants were vegetatively propagated. Alfalfa expressing red clover PPO1 and control alfalfa not transformed with a PPO gene were described previously.[15] Alfalfa plants were maintained in a glasshouse with variations in seasonal temperature of 20−30 °C and light intensities of 400−1000 μmol m^{-2} s^{-1}; mature leaves were harvested for comparative studies on substrate utilization by different PPOs, as described below.

Plant Tissues. Plant tissues were harvested, weighed, and flash frozen in liquid N and then stored at −80 °C until required for quantitative analyses of PPO activity, gene expression by qPCR, or natural substrates. Samples from shoots and nodulated roots of all seedlings (5 × 100 mg FW) were combined from 5 and 7 weeks of growth (PPO analysis, Table 1).

Red clover leaves, petioles, stems, and peduncles (3 × 200 mg) were harvested from five genotypes of 12-week-old plants grown in soil in a glasshouse under ambient conditions (12 h day with supplementary light); roots and nodules were harvested (3 × 200 mg) from red clover

Table 1. PPO Activity in Aerial and Root Tissues and Presence of PPO Gene in Seedlings of Three Model Species and Four Agronomically Important Forage Legumes

			rhizobium			PPO activity ($\Delta A_{420\ nm}$/g FW/min)					
						aerial tissues			root tissues		
species	cultivar/ecotype	nodule type	species	strain(s)	PPO gene	active	latent	total	active	latent	total
Arabidopsis thaliana[a]	Landsberg	none	none	none	nd[b]	0.73 ± 0.065	0	0.73 ± 0.065	0.51 ± 0.263	0	0.51 ± 0.263
Lotus japonicus[a]	Gifu	determinate	*Mesorhizobium loti*	NZP2335	nd	1.43 ± 0.084	0	1.43 ± 0.084	1.28 ± 0.101	0	1.28 ± 0.101
Medicago truncatula[a]	Jemalong	indeterminate	*Rhizobium meliloti*	2042	yes	1.37 ± 0.073	0	1.37 ± 0.073	1.01 ± 0.334	0	1.01 ± 0.334
Lotus corniculatus[c]	Leo	determinate	*Mesorhizobium loti*	3011	nd	1.56 ± 0.109	0	1.56 ± 0.109	1.22 ± 0.308	0	1.22 ± 0.308
Medicago sativa[c]	Europe	indeterminate	*Rhizobium meliloti*	2001	yes	1.35 ± 0.092	0	1.35 ± 0.092	1.27 ± 0.327	0	1.27 ± 0.327
Trifolium pratense[c]	Milvus	indeterminate	*Rhizobium leguminosarum*	R5	yes	34.64 ± 14.959	84.88 ± 24.759	119.52 ± 20.913	2.45 ± 0.525	13.89 ± 3.864	16.34 ± 3.879
Trifolium repens[c]	Menna	indeterminate	*Rhizobium leguminosarum*	502, 505, 509, 511, 515	yes[d]	1.71 ± 0.084	5.29 ± 1.472	7.00 ± 1.464	1.53 ± 0.163	0	1.53 ± 0.163

[a]Model species. [b]nd, not detected with degenerate primers for legume PPO genes; minimum 5 replicates from 5−7-week-old plants. [c]Agronomic species. [d]Two sequences submitted to GenBank database, accession nos. KC684556 and KC684557.

Table 2. Red Clover PPO Enzyme Activity in Different Tissues[a]

tissue	PPO activity ($\Delta A_{420\ nm}$/g FW/min) active	latent	total
embryo	2.3 ± 0.31	0	2.3 ± 0.31
glasshouse-grown plants			
leaf			
young, folded	40.5 ± 14.81	211.5 ± 72.74	252.1 ± 86.93
young, expanded	77.0 ± 14.41	215.6 ± 61.91	292.6 ± 85.76
mature	51.8 ± 34.17	135.5 ± 45.39	187.3 ± 64.96
petiole	4.3 ± 1.62	5.7 ± 3.40	10.0 ± 3.38
stem	15.3 ± 5.47	76.0 ± 17.99	91.3 ± 23.20
peduncle	2.2 ± 1.13	9.0 ± 1.27	11.2 ± 1.28
CE[b]-grown plants			
leaf, young, expanded	79.3 ± 18.67	97.7 ± 23.57	177.0 ± 44.09
roots, no visible nodules	4.7 ± 1.56	0	4.7 ± 1.56
nodules	2.7 ± 1.18	45.6 ± 5.92	48.3 ± 6.19

[a] n = 3 replicate samples. [b] CE, controlled environment.

plants grown from seed and inoculated with rhizobium. Embryos were harvested from seeds imbibed in tap water in Petri dishes and germinated in the dark at 25 °C for 24 h for PPO activity (3 × 200 mg FW) and gene expression (400 mg FW) analysis (Tables 2 and 3).

Table 3. Red Clover PPO1, PPO4, and PPO5 Gene Expression Relative to Stem Tissue

tissue	$2^{-\Delta\Delta CT}$[a] PPO1	PPO4	PPO5
embryo	0.89	und	und
leaf			
young, folded	6.37	14.61	0.50
young, expanded	0.87	7.69	0.23
mature	0.13	51.06	8.60
petiole	0.08	0.09	0.00
stem	<u>1.00</u>	13.69	0.54
flower peduncle	0.03	<0.001	0.002
nodules	0.065	<0.001	0.000

[a] Gene expression of foliar tissues was quantified from standard curves (based on quantified DNA for each sequence) and then normalized against PPO1 stem value (underlined).[35] und, undetectable.

Equivalent plant tissues (1 g FW) were harvested for gene expression analysis by qPCR (Table 3) from the genotype used to generate a previously described red clover BAC library.[12] Samples of nodulated roots (3 × 1g) and nodules alone (3 × 70 mg) were also taken for natural substrate analysis by reversed phase HPLC, as described for leaf tissue.[9]

Analysis of PPO Activity. (a) Quantitative analysis to compare PPO activity in various species and tissues using methylcatechol as substrate has been described previously.[9] Methylcatechol was prepared in 2.5 mM HCl (pH 6.0) to prevent oxidation prior to incubation with enzyme. Tissues were ground in liquid N and 1 mL of cold McIlvaine buffer, pH 7, with 60 mM ascorbic acid. Extract was centrifuged at 13000 rpm for 10 min at 4 °C, and supernatant (0.5 mL) desalted by passing through a Bio-Gel P6DG (Bio-Rad) column equilibrated with McIlvaine buffer lacking ascorbate. Active and total (+SDS to give total, i.e., active plus latent forms) PPO activities were measured at 420 nm over 30 s, using a Pharmacia Biotech Ultraspec 4000 UV−visible spectrophotometer coupled to Swift II software. Nonenzymatic oxidation of methylcatechol provided a blank control; it was negligible in incubations including substrate but lacking added PPO enzyme. Average PPO activity ± standard error was expressed on a fresh weight basis due to the low protein content in some tissues.

(b) Comparative studies on substrate utilization by different PPOs were carried out using the 2-nitro-5-thiobenzoic acid (TNB) quinone trap assay.[33] Samples (3 × 1 g FW) of alfalfa leaves expressing red clover PPO genes were extracted in 3 mL of McIlvaine buffer (pH 7.0) supplemented with 50 mM ascorbic acid and desalted as previously detailed.[8,13,20,21] Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as the standard. TNB solution was prepared as previously detailed.[8] Reactions consisted of 950 μL of assay buffer (pH 7.0 McIlvaine's buffer; supplemented with 0.25% SDS for total PPO activity), 20 μL of TNB solution, and 20 μL of 0.1 M caffeic, chlorogenic, or phaselic acid in ethanol. Clovamide (1 and 2 mM) and L-DOPA (2 mM) were dissolved directly in assay buffer due to their limited solubility in ethanol. Reactions were initiated by adding leaf extract (10 μL, diluted in McIlvaine's buffer when appropriate) and then incubated at 25 °C while $A_{412\ nm}$ was monitored over 5 min using a Beckman (Brea, CA, USA) DU50 spectrophotometer and data capture software. Reaction rates were determined by plotting $A_{412\ nm}$ versus time, determining the slope of the linear portion of the reaction by linear regression and converting to nanomoles of substrate using the conversion 91.0 nmol/$A_{412\ nm}$.[33] The specific activity of a given extract was calculated by dividing reaction rate by the amount of extract protein present in the reaction, expressed as nkatal mg^{-1}. Background specific activity measured in control alfalfa extracts (not expressing a red clover PPO transgene) was subtracted from activity in PPO-expressing extracts. For each extract, the relative activity for a given substrate was expressed relative to that of caffeic acid, and results presented as the average ± standard error.

Analysis of PPO Gene Expression in Tissues of Red Clover. RNA was extracted and cDNA synthesized from red clover tissues as described elsewhere.[34] Gene expression was measured by qPCR using an Applied Biosystem 7500 Real Time PCR system and SYBR green master mix. Actin expression was monitored with primers specifically designed to detect red clover actin (RC actin F1 5′-TGGGATGAC-ATGGAAAAGATCTGGCA-3′ and RC actin R1 5′-AGATTGGCA-CAGTGTGACTCACACCATC-3′). Abundance of PPO1, PPO4, and PPO5 transcripts was monitored with specifically designed primers: PPO1 F1 5′-AAATGATCTCCAACAGCAAGACCCC-3′, PPO1 R1 5′-CCGGCACCTTTGAAAGGTGTG-3′, PPO4 F1 5′-CTGAGC-AAATGTGTAAAATCAACACCG-3′, PPO4 R1 5′-GCAGCTGGT-CTTTCCCTTAATTTTCC-3′, PPO5 F1 5′-CCAATGTGTAAT-TCCACCAGAAAAATC-3′, PPO5 R1 5′-AAGGGAACTTAAAAT-CGATGATGTCTGC-3′. These primers were designed manually because close homology between genes greatly restricts regions specific to individual genes. Calibration curves were constructed from Ct values obtained following qPCR with known DNA quantities of PPO1, PPO4, and PPO5 fragments incorporating qPCR primer sites. Curves were used to quantify PPO4 and PPO5 transcript abundance from Ct values obtained by qPCR and to convert these values to PPO1 Ct equivalents. Relative transcript abundance was estimated by the $2^{-\Delta\Delta CT}$ method[35] normalized to actin.

Colocalization of PPO Enzyme, Activity, and Substrate Using Transmission Electron Microscopy (TEM) Combined with Cytochemical and Immunogold Labeling and Raman Microspectrometry. Fresh leaf samples and intact mature nodules, with a piece of subtending root, were processed as described elsewhere for leaves[36] with the following general modifications: 0.1 M sodium cacodylate buffer (pH 7.2) was used throughout, and 0.68% imidazole was omitted from the secondary fixative. Nodules were embedded in LR White Resin (hard grade; London Resin Co.) in size 3 gelatin capsules (16 h, 60 °C) to optimize resin penetration; the propylene oxide step was unnecessary with this resin. Thin nodule sections (2 μm) were cut for Raman microspectrometry and mounted on 1 mm thick silicon slides without coverslips. Ultrathin (60−80 nm) sections of leaves and nodules were cut for TEM and collected on Butvar B98 (Agar Scientific) films on GS2x0.5 Nickel slot grids (Gilder Grids, Grantham, UK).

(a) For TEM, samples were initially fixed in 3% glutaraldehyde, which cross-links all cellular proteins into a unified interlocking structure while retaining antigenicity. Soluble proteins and cytochemical and membranous constituents become enmeshed; many enzymes retain their ability to catalyze reactions in this fixed cellular position. Established procedures were followed for cytochemical localization of PPO enzyme activity,[37,38] and fixed tissues were washed once with 0.067 M phosphate buffer (pH 6.9), incubated with 0.5% methylcatechol or L-DOPA in phosphate buffer (16 h, 4 °C), and washed twice with cacodylate buffer before transfer to the secondary fixative, 1% osmium tetroxide in cacodylate buffer (2 h). PPO enzyme reacts with L-DOPA and oxygen, forming an electron-dense dopaquinone−osmium reaction product (DORP) that can be visualized in situ. Sections were randomly selected for colocalization of PPO−DORP and immunogold labeling of PPO by the two-step indirect method using PPO1 antiserum (rabbit)[13] and secondary antibody EMGAR.10 (goat anti-rabbit IgG, 10 nm gold) as described elsewhere.[36] CTM-T blocking buffer lacking PPO1 antiserum served as an antibody-free control to rule out nonspecific binding. The secondary antibody EMGAR.10 was replaced with EMDAS.15 (donkey anti-sheep IgG, 15 nm gold) to demonstrate antigen specificity of PPO immunogold labeling. These control sections had no immunogold labeling, and replacing PPO1 antiserum with preimmune serum resulted in a negligible level of immunogold labeling.

(b) For Raman microspectrometry, multivariate images of nodules were collected from sections (see above) over a range from 1400 to 1800 cm^{-1}. Sections were mounted on 1 mm thick silicon slides without coverslips and spectra acquired using a Renishaw InVia Raman spectrophotometer fitted with a Leica microscope, a Prior H101RNLB mapping stage, and a 514 nm green laser (Modu-Laser, South Centreville, UT, USA). The instrument was optimized for laser and camera slit position and calibrated to the silicon 520 cm^{-1} Raman emission band of silicon prior to image acquisition.

Samples were analyzed initially using Renishaw's Streamline mapping technology, which generated a large mosaic multivariate image giving highly contextual, if low-resolution, chemical data. As the process of Raman imaging caused burning of the section, it was necessary to select regions of interest within the mosaic image for more detailed point mapping analysis. Streamline images were collected using 50× magnification objectives (Leica) and a step size of 1.1 μm. Typically, exposure times for Streamline measurements were 8 s, and measurements were binned by a factor of 3 or more to reduce the number of pixels to be within software limits. Mosaic images of entire nodules could be collected in 8−12 h. Raman spectra for point mapping were collected at each pixel using full laser power and separated from each other by a distance of 1 μm. The exposure time for each spectral measurement was determined empirically by point measurement and was typically 10 s. A typical map consisting of 50 × 50 measurements took approximately 12 h to acquire.

Aromatic compounds in the sections were visualized by their Raman emission at 1601 cm^{-1}. This emission is attributed to a symmetric stretching of the aromatic ring and is the most intense band for many phenolics including lignin.[39]

RESULTS

PPO Enzyme Activity and PPO Genes in Seedlings. Red clover has the highest aerial and root total PPO enzyme activity of all seven species tested, 17- and 11-fold higher, respectively, than in white clover (Table 1). Red clover PPO enzyme was present in both latent and active forms, whereas in root tissues of white clover, PPO enzyme was in the latent form only. *Arabidopsis* had extremely low, barely detectable oxidative activity and no PPO gene sequences (Table 1), thus providing a baseline for nonenzymatic oxidation of methylcatechol in this assay. Both species of *Lotus* and *Medicago* also have low PPO activities, approximately 2-fold higher than that detected in *Arabidopsis*. No PPO genes were identified in *L. corniculatus*, whereas a database search revealed that *L. japonicus* has a PPO-like gene (Genbank accession no. AK339359). This *L. japonicus* gene has low (about 50%) homology with other *Lotus* PPO genes, but the gene product has conserved domains characteristic of PPO. PPO genes were detected in the four remaining legumes, *M. truncatula*, *M. sativa*, *T. pratense* (red clover), and *T. repens* (white clover). These data confirm previous reports of PPO genes in both *M. truncatula* [Genbank accession nos. XM_003593467, XM_003593469 [complete], XM_003605638, XM_003605638 (partial)] and *M. sativa*[19] and for red clover.[12,40] Here, two white clover PPO gene fragments of 527 and 485 bp were detected and sequenced. The sequences had 94% identity with each other and 93% identity with red clover PPO1/4 (FJ587214.1) and 92% with PPO1 (AY017302.1). They were deposited in the GenBank database as TrPPO1 (KC684556) and TrPPO2 (KC684557). The translated proteins from TrPPO1 and TrPPO2 had 84 and 86% identity, respectively, with red clover PPO1/4 (ACM09905.1). These data indicate two different white clover PPO gene sequences.

PPO Enzyme and Differential Gene Expression in Red Clover. In red clover, all tissues tested had detectable PPO enzyme activity (Table 2). When compared to stem tissues, leaves at all stages of development had 2- to 3-fold higher total PPO activity, with highest total activity measured in young folded and young expanded leaves, although this was not statistically different from mature leaves. Total PPO activity was lowest in embryos germinated for 1 day and in roots stripped of all visible nodules. Root nodules had 10-fold higher total enzyme activity than such roots (Table 2).

Gene expression analyses of PPO1, PPO4, and PPO5 reflect these data (Table 3). Compared to PPO1 expression in stem, PPO1, PPO4, and PPO5 were expressed in leaves, with PPO4 the predominant foliar PPO: PPO1 and PPO4 expression was between 6- and 15-fold higher in young folded leaves, whereas PPO4 and PPO5 expression was 52- and 8-fold higher in mature leaves. PPO1 is the only PPO gene showing significant expression in embryos, which have little or no enzyme activity, after just 1 day of germination. PPO1 is also the most highly expressed PPO gene in nodules, with much lower PPO4 expression.

Identification of the Natural Substrates Present in Nodules and Leaves of Red Clover Plants. The potential natural *o*-diphenol substrates in nodule and leaf tissues differed markedly. We found that nodulated roots of N-fixing plants grown under controlled environment had 1.663 μmol/kg FW clovamide, but nodules alone had 169.967 μmol/kg FW (Figure 1 caption). No phaselic acid was detected in either roots or nodules.

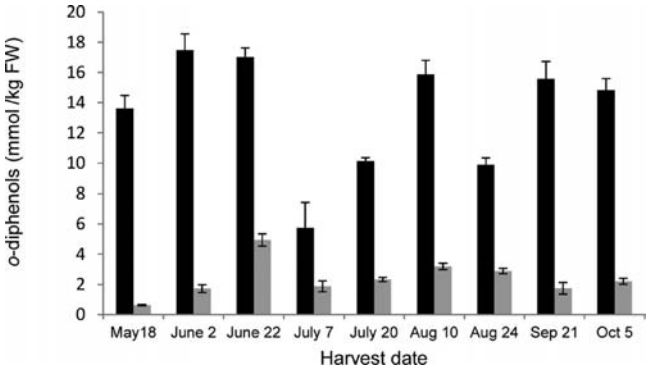

Figure 1. Variation in phenolic substrates in red clover leaves throughout the growing season: phaselic acid (black) and clovamide (gray) content of field-grown red clover leaves. Vertical bars represent standard error (n = 6). Phaselic acid data were previously published.[9] Nodules and nodulated roots of red clover grown under a controlled environment had no detectable phaselic acid, only clovamide at 169.967 and 1.663 μmol/kg FW, respectively.

In contrast, phaselic acid has been reported in field-grown red clover plants at concentrations varying between 5.734 and 17.479 mmol/kg FW over the growing season.[9] Although phaselic acid was the predominant substrate in leaves in this data set, clovamide was also present in the leaves, varying between 5 and 33% of the phaselic acid concentration at 0.633−4.946 mmol/kg FW (Figure 1).

Natural Substrates for Isolated Red Clover PPO4 and PPO5 Genes. Red clover PPO1, PPO4, and PPO5 expressed in leaves of *M. sativa* were assayed for PPO activity with various naturally occurring substrates, using the quinone trap of Esterbauer et al.[33] This method allows easy direct comparison of PPO activity with different substrates. Extracts containing red clover PPO4 and PPO5 were derived from different independent transgenic *M. sativa* plants, resulting in considerable variation in PPO activity in each extract (Table 4, footnote *a*). However, normalization of enzyme activity with each of the substrates relative to caffeic acid utilization gave a data set with relatively little variation between extracts.

Table 4. Utilization of Various Natural Substrates Relative to Caffeic Acid[a] by Activated Red Clover PPOs

	enzyme		
substrate[b]	PPO1	PPO4	PPO5
chlorogenic acid	0.70 ± 0.015	0.33 ± 0.035	0.34 ± 0.013
phaselic acid	0.70 ± 0.037	0.40 ± 0.103	0.70 ± 0.179
clovamide (2 mM)	1.44 ± 0.041	0.67 ± 0.060	0.79 ± 0.032
clovamide (1 mM)	1.05 ± 0.023	0.44 ± 0.047	0.51 ± 0.038
L-DOPA	0.00 ± 0.003	0.00 ± 0.009	0.13 ± 0.039

[a]Mean ± SEM for three independently prepared extracts expressed relative to caffeic acid. Absolute activities with caffeic acid substrate in nkatal/mg protein were PPO1, 108.2, 168.7, 172.7; PPO4, 46.9, 16.9, 880.5; and PPO5, 7.8, 445.5, 6.1. [b]Substrates, including caffeic acid, were tested at 2 mM except as indicated for clovamide.

Caffeic acid and its derivatives, chlorogenic acid, phaselic acid, and clovamide, were suitable substrates for these three PPO genes (Table 4). Although there were some differences between the enzymes for utilization of caffeic acid derivatives, the variation was 2−3-fold. However, L-DOPA was oxidized poorly by red clover PPOs with little or no activity detected for PPO1 or PPO4; L-DOPA oxidation by PPO5 activity was consistently detected, but was only 13% of that seen for caffeic acid. For PPO1, chlorogenic acid and phaselic acid were utilized nearly as well as caffeic acid, and at 2 mM clovamide was oxidized better than caffeic acid. For PPO1 at 1 mM (where oxidizable *o*-diphenol groups would be present at 2 mM), clovamide was utilized about as effectively as caffeic acid at 2 mM.

Less than 10% of alfalfa-expressed red clover PPO was present in the active form for most enzyme substrates tested (data not shown); this is consistent with previous observations for alfalfa expressed red clover PPO1.[41] The one exception was PPO4 incubated with 2 mM phaselic acid, where approximately 35% of the enzyme was active without SDS addition.

Colocalization of PPO Enzyme and Activity in Red Clover Leaves. PPO enzyme and its activity were visualized by PPO immunogold label along with in situ oxidation of L-DOPA, which created electron-dense dopaquinone osmium reaction products (DORP). Similar results were obtained using methylcatechol[37] instead of L-DOPA as a substrate, but the reaction between dopaquinone and osmium formed a more easily visualized reaction product.

The cellular site of both PPO enzyme and substrate oxidation was in chloroplast thylakoids of leaf mesophyll cells (Figure 2). Immunogold labels (arrowed) alone indicated the presence of PPO enzyme within thylakoids (Figure 2A,C). In leaves infused with L-DOPA, electron-dense DORP clearly delineated thylakoid membranes (Figure 2B,D), and chloroplast stroma appeared granular due to numerous small electron-dense lipid inclusions; these inclusions appeared to coalesce into larger lipid plastoglobuli (Figure 2D). There were about 5-fold more plastoglobuli in L-DOPA-infused chloroplasts than in those labeled with immunogold only (Figure 2A,C). Other organelles, such as mitochondria, did not show any positive reaction with L-DOPA.

Colocalization of Potential Phenolic Substrates, PPO Enzyme, and Activity in Nodules. Raman microspectrometry gave an overview of natural phenolic substrates within nodules, whereas TEM studies focused on the developing squashed cell layer (SCL), an area of active cellular differentiation lying between nodule meristem and fully developed, functioning squashed cell layer. Here, terminology for nodule structure, from the outside toward the center, is nodule cortex, squashed cell layer (also called nodule endodermis), nodule parenchyma, and infected or N_2-fixing zone [42] (Figures 3 and 4).

Raman microspectrometry showed a generally low phenolic signal with localized higher signals, suggesting that natural phenolic compounds were generally low in nodules but accumulated in specific regions, particularly in epidermal cells and nodule cortex outside the clearly defined SCL and within the infected zone (Figure 3A). At the point of SCL origin near the nodule meristem, the phenolic signal can be seen to coalesce ahead of the developing SCL (Figure 3B), which itself tends to have a low Raman signal, indicating low phenolics. These images suggest that phenolics are not present in the nodule parenchyma itself.

Low-resolution images of whole nodules infused with L-DOPA, then cut open, clearly showed a darker staining area comprising the nodule meristem and nodule cortex with reduced staining of nodule parenchyma with N-fixing bacteroids (Figure 4A). This dark staining indicated high enzymatic oxidation in these areas. TEM observations showed starch accumulated in cells near the developing SCL in nodule cortex and parenchyma, but starch predominated in nodule parenchyma in fully

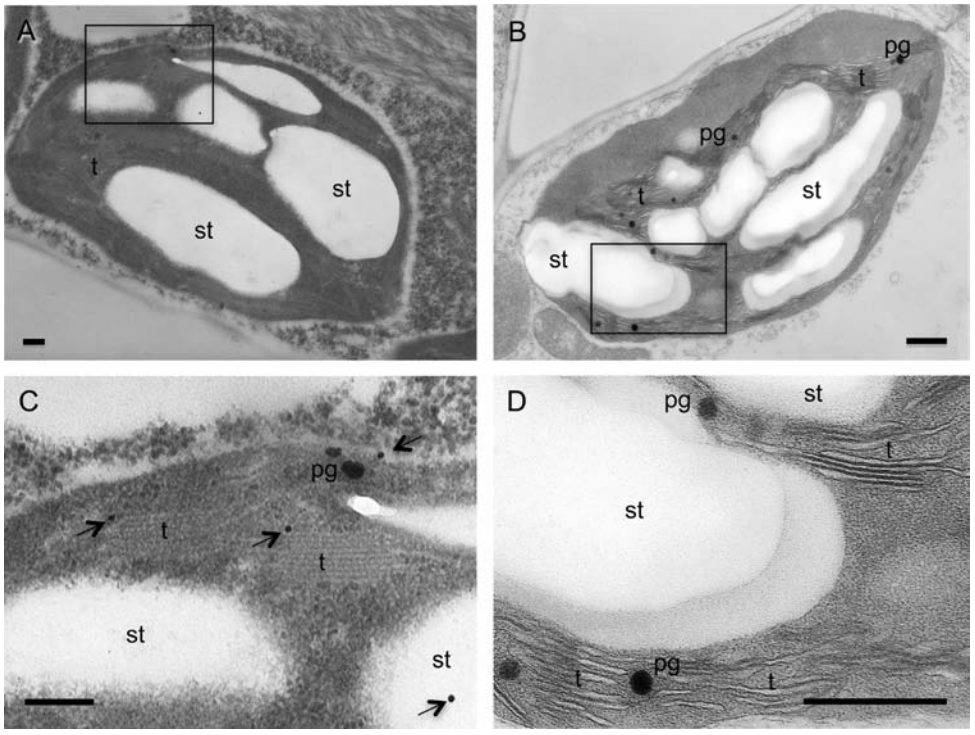

Figure 2. Transmission electron microscope images visualizing active PPO enzyme within chloroplasts of leaf cells. Transmission electron microscope images visualizing active PPO enzyme within chloroplasts of leaf cells by (A, C) immunogold-labeled PPO enzyme and (B, D) incubation with PPO substrate, L-DOPA. (A, C) Immunogold-labeled PPO (arrowed) was detected within thylakoids (t) of the starch-containing (st) chloroplasts. Note the lack of electron-dense reaction deposits within thylakoids and low number of plastoglobuli (pg). (B, D) Incubation with L-DOPA resulted in electron-dense dopaquinone osmium reaction products outlining thylakoid membranes, many small granular lipid inclusions, and plastoglobuli in the chloroplast stroma. Bars = 200 nm.

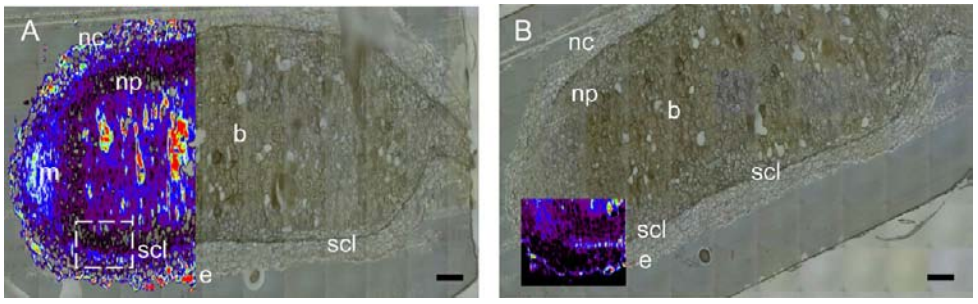

Figure 3. Distribution of phenolics in two red clover nodules as visualized by Raman microspectrometry overlaying an image generated by white reflected light. Raman images were collected using a 514 nm laser from 1400 to 1800 cm^{-1} and overlaid the same image collected by reflected white light in unstained ultrathin sections of two nodules in longitudinal section (A, B). The nodule meristem (m), epidermis (e), nodule cortex (nc), nodule parenchyma (np), and N_2-fixing bacteroids (b) are clearly visible, along with the fully developed squashed cell layer (scl). The white, dashed rectangle indicates the region where the squashed cell layer (scl) begins to differentiate; this region in another nodule is viewed again (3B) and analyzed further by TEM in Figure 4. The colors represent concentration of phenolics (as visualized by Raman emission at 1601 cm^{-1}), from transparent equating with low levels of phenolics through black−purple−blue−white−green−yellow−orange−red equating with high levels of phenolics. Bars = 400 μm.

developed SCL. PPO immunogold label accumulated in various locations, but was particularly high where the SCL was developing and reaction products of L-DOPA were accumulating (Figure 4A,B; dashed rectangles indicate region). This developing SCL region was the focus for TEM studies.

In control sections (not infused with L-DOPA), PPO immunogold label was detected in nodule parenchyma within the developing SCL near amyloplasts and ferritin, along with a few electron-dense globules (Figure 4B,C). Infusion of sections with L-DOPA resulted in more apparent and more abundant PPO immunogold label and electron-dense globules (Figure 4D−H); PPO enzyme clearly accumulated in and around these electron-dense globules (Figure 4B−H), which were also often found close to starch grains in amyloplasts (Figure 4D−G). This colocation of PPO immunogold label with both electron-dense globules and starch-filled amyloplasts strongly suggests that PPO (specifically catechol oxidase) enzyme, which is normally targeted to plastids, was active as well as present in this region of the developing nodule.

DISCUSSION

PPO activity has been implicated in resistance to stress and pathogens[1] but is clearly not essential for plant growth under optimal conditions. Whereas *Arabidopsis* lacks PPO genes, alfalfa does not appear to express leaf PPO genes and lacks the

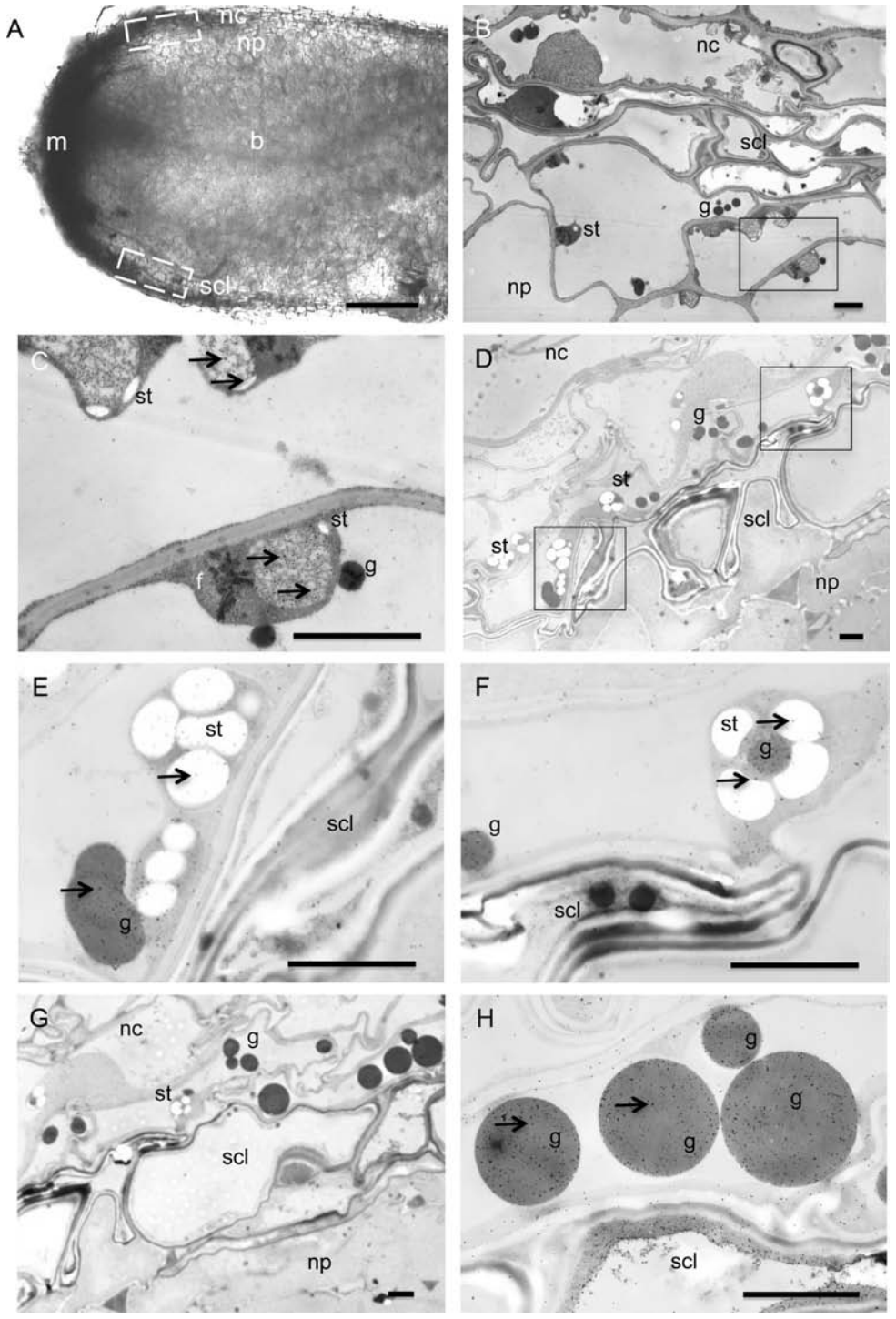

Figure 4. Light and transmission electron microscope images visualizing active PPO enzyme within nodules. (A) Longitudinally cut surface of nodule after infusion with L-DOPA, showing strong staining of meristem (m) and nodule cortex (nc), indicating enzymatic oxidation and reduced staining of nodule parenchyma (np) with N_2-fixing bacteroids (b). Dashed rectangles indicate developing squashed cell layer (scl) selected for ultrathin sections for TEM. Transmission electron microscope images of developing squashed cell layer of nodules: (B–H) immunogold-labeled for PPO enzyme and (D–H) also infused with L-DOPA. Starch-containing amyloplasts (st) and electron-dense globules (g) are located adjacent to the developing squashed cell layer (scl), which has heavily thickened cell walls (B, D, G). Enlarged images (C, E, F, H) show PPO immunogold label (arrowed) located near ferritin (f) in (C) and within or near starch grains (st) and electron-dense globules (g) and thickened cell walls of squashed cells. Bars (A) = 200 μm; (B–H) = 2 μm.

appropriate *o*-diphenol substrates.[19,21] Significant differences also exist between related clovers. Red clover has a large PPO gene family[12] and very high constitutive leaf PPO activity, whereas two PPO genes were detected in white clover and leaf PPO activity was relatively low and mainly present in the latent form. Latent leaf PPO activity was also reported in *Vicia faba*, broad bean,[43] from which three cDNA clones coding for a chloroplast-targetting PPO gene have been identified.[44]

Wounding leaves of pea and chickpea plants resulted in increased localized PPO activity, and not systemic activity.[45] In

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contrast, tomato PPO transcript levels systemically increased in young leaves when mature leaflets were injured.[46] Although PPO may not be considered to play a major role in systemic plant protection,[45] localized PPO activity in tissues and cells may be important. For example, high foliar PPO enzyme activity benefitted a red clover population during multiple successive natural invasions by aphids, mildew, and slugs; a leaf PPO mutant population grown in the same glasshouse[9] succumbed more rapidly.[47]

Most studies on PPO enzyme activity and substrates have focused on leaf PPO, although recent work has reported PPO activity in roots of a range of species, including grain legumes.[26−29] Challenging seedling roots of soybean (*Glycine max*)[26] with L-DOPA enhanced PPO activity by about 30%, whereas nodulated roots of *Pisum sativum*, with a 4-fold elevation of PPO activity as compared to noninoculated roots, was less damaged by broomrape (*Orobanche crenata*).[29]

The physiological function of PPO is not clear, but even the low levels of PPO activity reported here for legume shoots and roots could be significant, if activity is localized to a few cells in specific tissues, or increased under stress. Such cellular localization could provide a mechanism to counter over-reduction in plant tissues in response to stress, for example, in the photosynthetic electron transport chain under high light intensities or in successful development of root colonisation by rhizobium and mycorrhizal symbionts.

Differential PPO gene expression has been reported in aerial tissues of tomato[3] and in aerial and root tissues of both potato[4] and aubergine.[5] In aubergine, two distinct subclasses of PPO genes (*Sme*PPO1−PPO3 and *Sme*PPO4−PPO6) have been identified, on the basis of their differential responses to jasmonic acid and salicylic acid. These molecules are involved in defense against pests and pathogens.[48]

In this study, red clover PPO1, PPO4, and PPO5 genes were expressed in aerial tissues and mainly PPO1 in nodules. Total PPO enzyme activity is stem is 2-fold higher than in nodules, whereas the expression level of the predominant PPO gene (PPO1) is 15-fold higher in the stem tissues. There may be differences in the half-life of transcripts and enzyme in stems and nodules, but there may also be additional, unidentified red clover PPO genes that could be expressed in nodules.

A previous study using RNA and immunoblotting experiments showed PPO1, PPO2, and PPO3 expression in aerial tissues and indicated that PPO1 was the major foliar PPO of red clover,[13] although PPO1 was not expressed in mature leaves. Here PPO4 seemed to be the major foliar PPO gene, with PPO1 showing lower expression in mature leaves. Analysis of a low leaf PPO mutant population derived from a single backcrossed plant[47] indicated that it lacked PPO4 gene expression (Winters, unpublished data). One explanation would be allelic variation in PPO genes: we were not able to detect all PPO genes in the single plant used for both qPCR analysis and production of the red clover BAC library.[12] PPO3 was never detected in that BAC library.

Differential expression of PPO genes supports the possibility that PPO enzymes may have different functional roles, and their activity level could reflect substrate specificities as reported for red clover PPO1, PPO2, and PPO3 previously,[40] or substrate availability, as reported in alfalfa.[20,21] When red clover PPO1, PPO4, and PPO5 genes were expressed in leaves of alfalfa, their gene products showed only slight differences in substrate specificity and natural substrate utilization. Caffeic acid and its derivatives (clovamide, phaselic acid, and chlorogenic acid) were all readily utilizable substrates by the three PPOs, whereas L-DOPA was utilized only by PPO5, which demonstrated a low but consistent activity with this substrate. In this assay, PPO1 more readily oxidized clovamide than the other substrates tested, whereas both PPO4 and PPO5 enzymes utilized clovamide about as effectively as caffeic acid at 2 mM.

High levels of phaselic acid (4.8−17.5 mmol/kg FW) occur in red clover leaves,[9,15] the range reflecting variations in genotype and environment. Here, we presented data from the same leaves of field-grown cv. Milvus showing broadly similar clovamide levels (0.6−4.9 mmol/kg FW) to those found in leaves of glasshouse-grown NewRC germplasm clones of a single plant of cv. Arlington (2.9−5.7 mmol/kg FW);[15] in NewRC, clovamide levels sometimes exceeded those of phaselic acid.[15]

By contrast, only clovamide was detected in red clover nodules. Clovamide in nodules was 400-fold lower than in leaves, but similar to levels reported in another study using non-nodulated red clover seedling roots.[18] In those roots, clovamide was 0.281 mmol/kg FW,[18] equivalent to levels we detected in nodules alone (0.170 mmol/kg FW), but 100-fold higher than in well-established nodulated roots. Substrate levels have also been shown to increase under simulated stresses.[18] In those studies, wounding red clover seedling roots and simulataneously applying 50 μM jasmonic acid increased clovamide levels 10-fold to 2.81 mmol/kg FW.[18]

Studies of PPO activity localization have demonstrated that leaf PPO enzyme activity is targeted to chloroplastic thylakoid membranes, with increased PPO import in response to wounding and methyl jasmonate in tomato,[10,11,49] and in tubers of potato, PPO enzyme was targeted to starch-filled amyloplasts.[30] Our results confirmed the main sites of PPO enzyme activity in red clover were chloroplastic thylakoid membranes in leaves and starch-rich amyloplasts in nodules.

In red clover leaves, cytochemical staining with L-DOPA increased the number of electron-dense plastoglobuli within chloroplasts. Plastoglobuli contain lipid molecules, such as carotenoids, plastoquinone, and tocopherol, and may be in a dynamic equilibrium with lipid quinones located in thylakoid membranes.[50] Thus, plastoglobuli could help protect healthy chloroplasts by ejecting toxic lipid quinones. Alternatively, PPO enzyme may be located within plastoglobuli themselves and transported out of the chloroplast. In red clover nodules, PPO enzyme immunogold label was often associated with starchy amyloplasts and electron-dense globules, which appeared very similar to the electron-dense lipid plastoglobuli seen in chloroplasts and had a high density of PPO immunogold label. Cells containing these amyloplasts and PPO labeled, electron-dense globules were particularly prevalent in cells near the developing SCL in the nodule parenchyma. Raman micro-spectrometry showed that significant amounts of phenolic compounds also accumulated in cells in this developing SCL, which is a key anatomical feature of N_2-fixing nodules.

Thus, red clover PPOs have potential functional diversity due to their differential tissue and cellular localization. The slightly higher specificity of red clover PPO enzymes for clovamide rather than phaselic acid may not be biologically significant. However, PPO activity may form part of the plant's defense. For example, its presence in healthy nodules may offer localized protection against invading symbiotic bacteria, which could become parasitic; it may also have a role in normal cellular differentiation and development of N-fixing nodules. A red clover mutant population lacking the major foliar PPO[9,47] and RNAi knockdowns with little or no PPO expression[19,36] will allow

exploration of subtle roles of PPO both in photosynthesis and in the symbiotic relationship between legumes and rhizobium.

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REFERENCES

(1) Mayer, A. M. Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry* **2006**, *67*, 2318−2331.

(2) Yoruk, R.; Marshall, M. R. Physicochemical properties and function of plant polyphenol oxidase: a review. *J. Food Biochem.* **2003**, *27*, 361−422.

(3) Thipyapong, P.; Joel, D. M.; Steffens, J. C. Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development. *Plant Physiol.* **1997**, *113*, 707−718.

(4) Thygesen, P. W.; Dry, I. B.; Robinson, S. P. Polyphenol oxidase in potato − a multigene family that exhibits differential expression patterns. *Plant Physiol.* **1995**, *109*, 525−531.

(5) Shetty, S. M.; Chandrashekar, A.; Venkatesh, Y. P. Eggplant polyphenol oxidase multigene family: cloning, phylogeny, expression analyses and immunolocalization in response to wounding. *Phytochemistry* **2011**, *72*, 2275−2287.

(6) Pourcel, L.; Routaboul, J. M.; Kerhoas, L.; Caboche, M.; Lepiniec, L.; Debeaujon, I. Transparent TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *Plant Cell* **2005**, *17*, 2966−2980.

(7) Thipyapong, P.; Melkonian, J.; Wolfe, D. W.; Steffens, J. C. Suppression of polyphenol oxidases increases stress tolerance in tomato. *Plant Sci.* **2004**, *167*, 693−703.

(8) Sullivan, M.; Foster, J. Perennial peanut (*Arachis glabrata*) contains polyphenol oxidase (PPO) and PPO substrates that can reduce postharvest proteolysis. *J. Sci. Food Agric.* **2013**, DOI: 10.1002/jsfa.6050.

(9) Winters, A. L.; Minchin, F. R.; Michaelson-Yeates, T. P. T.; Lee, M. R. F.; Morris, P. Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. *J. Agric. Food Chem.* **2008**, *56*, 2817−2824.

(10) Newman, S. M.; Eannetta, N. T.; Yu, H. F.; Prince, J. P.; Devicente, M. C.; Tanksley, S. D.; Steffens, J. C. Organization of the tomato polyphenol oxidase gene family. *Plant Mol. Biol.* **1993**, *21*, 1035−1051.

(11) Sommer, A.; Ne'eman, E.; Steffens, J. C.; Mayer, A. M.; Harel, E. Import, targeting, and processing of a plant polyphenol oxidase. *Plant Physiol.* **1994**, *105*, 1301−1311.

(12) Winters, A.; Heywood, S.; Farrar, K.; Donnison, I.; Thomas, A.; Webb, K. J. Identification of an extensive gene cluster among a family of PPOs in *Trifolium pratense* L. (red clover) using a large insert BAC library. *BMC Plant Biol.* **2009**, *9*, 94.

(13) Sullivan, M. L.; Hatfield, R. D.; Thoma, S. L.; Samac, D. A. Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in *Escherichia coli* and transgenic alfalfa. *Plant Physiol.* **2004**, *136*, 3234−3244.

(14) Lattanzio, V.; Cardinali, A.; Linsalata, V. Plant phenolics: a biochemical and physiological perspective. In *Recent Advances in Polyphenol Research*; Cheynier, V., Sarni-Manchado, P., Quideau, S., Eds.; Wiley-Blackwell: Chichester, UK, 2012, Vol. 3, pp 1−39.

(15) Sullivan, M. L.; Zeller, W. E. Efficacy of various naturally occurring caffeic acid derivatives in preventing post-harvest protein losses in forages. *J. Sci. Food Agric.* **2013**, *93*, 219−226.

(16) Parveen, I.; Threadgill, M. D.; Moorby, J. M.; Winters, A. Oxidative phenols in forage crops containing polyphenol oxidase enzymes. *J. Agric. Food Chem.* **2010**, *58*, 1371−1382.

(17) Sullivan, M. L. A novel red clover hydroxycinnamoyl transferase has enzymatic activities consistent with a role in phaselic acid biosynthesis. *Plant Physiol.* **2009**, *150*, 1866−1879.

(18) Tebayashi, S.; Ishihara, A.; Tsuda, M.; Iwamura, H. Induction of clovamide by jasmonic acid in red clover. *Phytochemistry* **2000**, *54*, 387−392.

(19) Sullivan, M. L.; Hatfield, R. D.; Samac, D. A. Cloning of an alfalfa polyphenol oxidase gene and evaluation of its potential in preventing postharvest protein degradation. *J. Sci. Food Agric.* **2008**, *88*, 1406−1414.

(20) Sullivan, M. L.; Hatfield, R. D. Polyphenol oxidase and *o*-diphenols inhibit postharvest proteolysis in red clover and alfalfa. *Crop Sci.* **2006**, *46*, 662−670.

(21) Schmitz, G. E.; Sullivan, M. L.; Hatfield, R. D. Three polyphenol oxidases from red clover (*Trifolium pratense*) differ in enzymatic activities and activation properties. *J. Agric. Food Chem.* **2008**, *56*, 272−280.

(22) Ramamoorthy, V.; Raguchander, T.; Samiyappan, R. Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp *lycopersici*. *Plant Soil* **2002**, *239*, 55−68.

(23) Raj, S. N.; Sarosh, B. R.; Shetty, H. S. Induction and accumulation of polyphenol oxidase activities as implicated in development of resistance against pearl millet downy mildew disease. *Funct. Plant Biol.* **2006**, *33*, 563−571.

(24) Wang, J. H.; Constabel, C. P. Three polyphenol oxidases from hybrid poplar are differentially expressed during development and after wounding and elicitor treatment. *Physiol. Plant.* **2004**, *122*, 344−353.

(25) Gandia-Herrero, F.; Garcia-Carmona, F.; Escribano, J. Purification and characterization of a latent polyphenol oxidase from beet root (*Beta vulgaris* L.). *J. Agric. Food Chem.* **2004**, *52*, 609−615.

(26) Soares, A. R.; Ferrarese, M. D. L.; Siqueira-Soares, R. D.; Marchiosi, R.; Finger-Teixeira, A.; Ferrarese, O. The allelochemical L-DOPA increases melanin production and reduces reactive oxygen species in soybean roots. *J. Chem. Ecol.* **2011**, *37*, 891−898.

(27) Chakrabarti, J.; Chatterjee, S.; Ghosh, S.; Chatterjee, N. C.; Dutta, S. Synergism of VAM and rhizobium on production and metabolism of IAA in roots and root nodules of *Vigna mungo*. *Curr. Microbiol.* **2010**, *61*, 203−209.

(28) Chakraborty, D.; Mandal, S. M. Fractional changes in phenolic acids composition in root nodules of *Arachis hypogaea* L. *Plant Growth Regul.* **2008**, *55*, 159−163.

(29) Mabrouk, Y.; Mejri, S.; Hemissi, I.; Simier, P.; Delavault, P.; Saidi, M.; Belhadj, O. Bioprotection mechanisms of pea plant by *Rhizobium leguminosarum* against *Orobanche crenata*. *Afr. J. Microbiol. Res.* **2010**, *4*, 2570−2575.

(30) Stensballe, A.; Hald, S.; Bauw, G.; Blennow, A.; Welinder, K. G. The amyloplast proteome of potato tuber. *FEBS J.* **2008**, *275*, 1723−1741.

(31) Ryle, G. J. A.; Powell, C. E.; Gordon, A. J. The effect of source of nitrogen on the growth of Fiskeby soyabean: the carbon economy of whole plants. *Ann. Bot.* **1978**, *24*, 637−648.

(32) Samac, D. A.; Austin-Phillips, S. Alfalfa (*Medicago sativa* L.) *Methods Mol. Biol. (N.Y.)* **2006**, *343*, 301−311.

(33) Esterbauer, H.; Schwarzl, E.; Hayn, M. Rapid assay for catechol oixdase and laccase using 2-nitro-5-thiobenzoic acid. *Anal. Biochem.* **1977**, *77*, 486−494.

(34) Webb, K. J.; Jensen, E. F.; Heywood, S.; Morris, S. M.; Linton, P. E.; Hooker, J. E. Gene expression and nitrogen loss in senescing root systems of red clover (*Trifolium pratense*). *J. Agric. Sci.* **2010**, *148*, 579−591.

(35) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. *Methods* **2001**, *25*, 402−408.

(36) Lee, M. R. F.; Tweed, J. K. S.; Cookson, A.; Sullivan, M. L. Immunogold labelling to localize polyphenol oxidase (PPO) during wilting of red clover leaf tissue and the effect of removing cellular matrices on PPO protection of glycerol-based lipid in the rumen. *J. Sci. Food Agric.* **2010**, *90*, 503−510.
(37) Czaninski, Y.; Catesson, A.-M. Polyphenoloxidases (plants) In *Electron Microscopy of Enzymes, Principles and Methods*; Hayat, M. A., Ed.; Van Nostrand Reinhold: New York, 1974; Vol. 2, pp 66−78.
(38) Martyn, R. D.; Samuelson, D. A.; Freeman, T. E. Ultrastructural-localization of polyphenoloxidase activity in leaves of healthy and diseased waterhyacinth. *Phytopathology* **1979**, *69*, 1278−1287.
(39) Sun, L.; Simmons, B. A.; Singh, S. Understanding tissue specific compositions of bioenergy feedstocks through hyperspectral Raman imaging. *Biotechnol. Bioeng.* **2011**, *108*, 286−295.
(40) Sullivan, M.; Thoma, S.; Samac, D.; Hatfield, R. Cloning of red clover and alfalfa polyphenol oxidase genes and expression of active enzymes in transgenic alfalfa. In *Molecular Breeding of Forage and Turf*; Hopkins, A., Wang, Z. Y., Sledge, M., Baker, R., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2004; Vol. 11, pp 189−195.
(41) Lee, M. R. F.; Tweed, J. K. S.; Sullivan, M. L. Oxidation of ortho-diphenols in red clover with and without polyphenol oxidase (PPO) activity and their role in PPO activation and inactivation. *Grass Forage Sci.* **2012**, *68*, 83−92.
(42) Dalton, D. A.; Joyner, S. L.; Becana, M.; Iturbe-Ormaetxe, I.; Chatfield, J. M. Antioxidant defenses in the peripheral cell layers of legume root nodules. *Plant Physiol.* **1998**, *116*, 37−43.
(43) Robinson, S. P.; Dry, I. B. Broad bean leaf polyphenol oxidase is a 60-kilodalton protein susceptible to proteolytic cleavage. *Plant Physiol.* **1992**, *99*, 317−323.
(44) Cary, J. W.; Lax, A. R.; Flurkey, W. H. Cloning and characterization of cDNAs coding for *Vicia faba* polyphenol oxidase. *Plant Mol. Biol.* **1992**, *20*, 245−253.
(45) Dhankher, O. P.; Gatehouse, J. A. Non-systemic induction of polyphenol oxidase in pea and chickpea after wounding. *Physiol. Mol. Biol. Plants* **2003**, *9*, 125−129.
(46) Thipyapong, P.; Steffens, J. C. Tomato polyphenol oxidase − differential response of the polyphenol oxidase F promoter to injuries and wound signals. *Plant Physiol.* **1997**, *115*, 409−418.
(47) Winters, A. L.; Gill, S.; Parveen, I.; Sullivan, M.; Webb, K. J. Role of red clover PPO in pathogen resistance. Polyphenols Communications 2008 (Volume 1). In *Proceedings, XXIVth International Conference on Polyphenols*, Escribano-Bailón, M. T., González-Manzano, S., González-Paramás, A. M., Dueñas-Patón, M., Santos-Buelga, C., Eds.; Globalia Artes Graficas, S.L., Salamanca, Spain; 2008; 433−434.
(48) Shetty, S. M.; Chandrashekar, A.; Venkatesh, Y. P. Promoter analyses and transcriptional profiling of eggplant polyphenol oxidase 1 gene (*Sme*PPO1) reveal differential response to exogenous methyl jasmonate and salicylic acid. *J. Plant Physiol.* **2012**, *169*, 718−730.
(49) Koussevitzky, S.; Ne'eman, E.; Peleg, S.; Harel, E. Polyphenol oxidase can cross thylakoids by both the Tat and the Sec-dependent pathways: a putative role for two stromal processing sites. *Physiol. Plant.* **2008**, *133*, 266−277.
(50) Austin, J. R.; Frost, E.; Vidi, P.-A.; Kessler, F.; Staehelin, L. A. Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. *Plant Cell* **2006**, *18*, 1693−1703.