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Phenylalanine ammonia lyase genes in red clover: expression in whole plants and in response to yeast fungal elicitor

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Abstract

In red clover (*Trifolium pratense* L.) four unique cDNAs encoding phenylalanine ammonia lyase (PAL, EC 4.3.1.5) were identified (PAL1-4). PAL2-4 encode nearly identical proteins (> 97 %) that are only 89 % identical to that encoded by PAL1. Under normal growing conditions, in young leaves and flowers PAL1 mRNA accumulates to higher levels than that of PAL2-4 whereas in mature leaves and stems, mRNA levels are similar for PAL1 and PAL2-4. Treatment of red clover seedlings with yeast elicitor preparation results in an approximately six-fold induction of PAL2-4 transcripts within 1 h of treatment but only a modest induction of PAL1 transcripts. These results suggest that while both classes of enzymes play a role in biosynthesis of phenylpropanoid compounds under normal growing conditions, PAL2-4 enzymes are also involved in pathogen defense responses.

Additional key words: *o*-diphenols, quantitative PCR, *Trifolium pratense*.

Introduction

In contrast to many other forage legumes, red clover (*Trifolium pratense* L.) accumulates relatively high levels of two phenylpropanoid *o*-diphenols in its leaves, phaselic acid and clovamide (Hatfield and Muck 1999, Winters *et al.* 2008). Phaselic acid, a malic acid ester of caffeic acid, is consistently found in red clover leaves whereas the presence of clovamide, a 3,4-dihydroxy-L-phenylalanine amide of caffeic acid, is more variable (Winters *et al.* 2008) and may be inducible by jasmonic acid (Tebayashi *et al.* 2000). In red clover, upon cellular disruption these *o*-diphenols are readily oxidized by a soluble polyphenol oxidase to produce their corresponding *o*-quinones (Hatfield and Muck 1999, Steffens *et al.* 1994). For forages preserved by ensiling, oxidation of *o*-diphenols by polyphenol oxidase prevents degradation of protein during storage (Sullivan *et al.* 2004, Sullivan and Hatfield 2006) and appears to have positive effects on the lipid profile of products derived from animals fed polyphenol oxidase- and *o*-diphenol-containing forages (Lee *et al.* 2004, Lee *et al.* 2006). Because alfalfa and many other forages lack the required *o*-diphenols for this natural system of protein protection (Jones *et al.* 1995, Sullivan and Bringe 2005), understanding how red clover is able to accumulate these compounds will be a key step to adapt the polyphenol oxidase/*o*-diphenol system to a wide range of economically important forages.

The biosynthetic pathways of *o*-diphenols in red clover have not been defined. However, based on work carried out on monolignol biosynthesis in several species (Dixon *et al.* 2001), as well as characterization of phenylpropanoid pathways in basil (Gang *et al.* 2002) and other plants (Matsuno *et al.* 2002, Niggeweg *et al.* 2004), pathways for the biosynthesis of phaselic acid and clovamide likely involve the deamination of L-phenylalanine to cinnamic acid by L-phenylalanine ammonia lyase (PAL, EC 4.3.1.5) as one of the first steps. Subsequent reactions mediated by cinnamic acid 4-hydroxylase (C4H, EC 1.14.13.11), specific hydroxycinnamoyl transferases, and *p*-coumarate 3-hydroxylases would result in the formation of specific *o*-diphenols. In tobacco, production of cinnamic acid by PAL is the controlling step in the production of the phenylpropanoid

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Abbreviations: C4H - cinnamic acid 4-hydroxylase; CHS - chalcone synthase; Ct - threshold cycle; PAL - L-phenylalanine ammonia lyase; PCR - polymerase chain reaction; q-rtPCR - quantitative real time polymerase chain reaction; YE - yeast elicitor.
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o-diphenol chlorogenic acid (Howles *et al.* 1996). In many plants, substituted and hydroxylated phenylpropanoids presumably act as antioxidants and in plant defense (Gang *et al.* 2001, 2005, Niggeweg *et al.* 2004 and references therein). Consequently, enzymes responsible for biosynthesis of many of these compounds, including PAL, are often induced in response to pathogen attack and wounding. To understand the roles of PAL enzymes in biosynthesis of *o*-diphenols in red clover, we have isolated and characterized several PAL genes.

Materials and methods

A red clover (*Trifolium pratense* L.) genotype PPO (Sullivan *et al.* 2004) selected from a population of WI-2 germplasm (Smith and Maxwell 1980) was the source material for isolation of PAL genes and used along with three additional genotypes derived from WI-2 in expression experiments with whole plants. The plants were clonally propagated from crown pieces and grown in a greenhouse with temperatures maintained between 20 and 30 °C and irradiance between 400 and 1000 μmol m^{-2} s^{-1}. Supplemental lighting was used when day length was < 15 h. Plants were fertilized weekly with *Peter's* soluble 20-20-20 (*Scott's*, Marysville, OH, USA). For experiments with seedlings, groups of 50 WI-2 red clover seeds were placed in a 1.5 cm^3 microcentrifuge tube, treated with 1 cm^3 4 % (v/v) *Plant Preservation Mixture* (*Plant Cell Technology*, Washington, DC, USA) in half-strength Murashige and Skoog (1962; MS) basal salts for 6 h with gentle shaking and then transferred aseptically to a piece of filter paper saturated with 1/2 MS in a Petri dish. The Petri dish was wrapped with *Parafilm* (*Pechiney Plastic Packaging*, Chicago, IL, USA), maintained for 36 h at 4 °C in darkness, then incubated in a plant growth room at 25 °C, 14-h photoperiod, irradiance of 40 μmol m^{-2} s^{-1}. After 2 d, 10 germinated seeds were aseptically transferred to 0.75 cm^3 of 1/2 MS in each well of twelve-well tissue culture plates. The plates were wrapped with *Parafilm* and incubated in the plant growth room for 3 additional days.

Yeast elicitor (YE) was prepared from 100 g of fresh compressed baker's yeast (*Red Star*, Milwaukee, WI, USA) essentially as described by Roos *et al.* (1998). The final preparation (270 mg) was resuspended in distilled water at 50 mg cm^{-3} and stored at -20 °C until needed. For YE treatment, the preparation was further diluted to 0.50 mg cm^{-3} in 1/2 MS and 3 cm^3 of solution per well was added to 5-d-old seedlings grown in twelve-well plates as described above. For mock treatment, 3 cm^3 of 1/2 MS without YE was added to seedlings. Additionally, prior to treatment, two wells of seedlings (20 seedlings) were harvested, gently blotted dry, immediately frozen in liquid nitrogen, and stored at -80 °C prior to RNA extraction. This served as an untreated control. Following YE or mock treatment, seedlings from two wells were harvested for RNA extraction as described for the untreated control.

Total RNA was prepared from plant tissues using an *RNeasy* kit (*Qiagen*, Valencia, CA, USA) or by the method of Chang *et al.* (1993). cDNA was prepared using *Superscript III* reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's protocol from DNase I-treated total RNA.

To clone PAL sequences, cDNA from young red clover leaves was used as template with degenerate primers (see Table 1) in a polymerase chain reaction (PCR) with *JumpStart KlenTaq LA* polymerase (*Sigma*, St. Louis, MO, USA) using 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 68 °C. The resulting DNA fragment was purified from an agarose gel using *QIAEX II* resin (*Qiagen*) and cloned into *pGEM-T Easy* (*Promega*, Madison, WI, USA) according to manufactures' instructions. Plasmid DNA was prepared using *QIAprep Spin Miniprep* kit (*Qiagen*). DNA sequence was determined by cycle sequencing using *Big Dye v. 3.1* (*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), and *BLAST* programs using the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and The Gene Index Project (compbio.dfci.Harvard.edu/tgi) web sites.

For library screening approximately 10^6 recombinant phage from a λ *ZAP II* (*Stratagene*, La Jolla, CA, USA) red clover young leaf cDNA library were screened essentially as previously described (Sullivan *et al.* 2004) using a 350 bp PCR-derived red clover PAL gene fragment as the hybridization probe. Phage clones of interest were rescued to *pBluescript II SK(-)* phagemids according to the manufacturer's protocol.

Primers for quantitative real time PCR (q-rtPCR) were designed using *Primer Express* software (*Applied Biosystems*) using the factory default parameters (melting temperature of 59 ± 1 ºC, 30 - 80 % GC content, length between 9 and 40 nucleotides with an optimal length of 20 nucleotides) for *Taqman* primer design (Table 1). q-rtPCR was carried out using *SYBR Green PCR Master Mix* (*Applied Biosystems*) in 25 mm^3 reaction mixture containing cDNA equivalent to 0.025 ng total RNA or 2×10^3 to 2×10^6 copies of cloned target DNA (to generate standard curves) and 50 nM each primer, with triplicate reactions run for each sample for a given primer pair. Reactions were run in an *ABI Prism 7000 Sequence Detection System* (*Applied Biosystems*). Initial denaturation was 95 °C for 10 min followed by 40 cycles of 95 °C 15 s and 58 °C 1.5 min. Data were analyzed with auto Ct (threshold cycle) and auto baseline functions of the system and Ct values of the triplicate reactions were averaged. ΔCt for mock- and elicitor-treated seedlings

was determined as $Ct_{treated}$ - $Ct_{untreated}$ for each gene in each of nine biological replicates. ΔΔCt for each gene was determined as $\Delta Ct_{elicitor}$ - ΔCt_{mock}, which is equivalent to $Ct_{elicitor}$ - Ct_{mock}, in each biological replicate. In all cases, values were averaged for the biological replicates and are reported with standard error of the mean. Statistical significance of paired comparisons was determined using Student's *t* test (Samuels 1989).

Table 1. Oligonucleotide primer sequences. [A] - *M. truncatula* Gene Index (TC106669) or GenBank (all other) sequence upon which primer design is based; [B] - forward (F) or reverse (R) orientation of each primer is indicated.

Gene	Use	Accession[A]	Sequence[B]
PAL	Cloning	AY303128	(F) 5´ TYTCCTYAACMWCAACRTCACYCCAT 3´
		TC106669 and others	(R) 5´ TGRTCRGTRAAYTCAGGYTT 3´
PAL1	q-rtPCR		(F) 5´ CATATAATGGAGGGAATTACCAATGG 3´
		DQ073809	(R) 5´ ACGCTTCACCTCATCCAAATG 3´
PAL2-4	q-rtPCR	DQ073808	(F) 5´ CTTAACTCATGCTAATGCTAATAACATGAA 3´
		DQ073810, DQ073811	(R) 5´ TTCCGGTACTCCTCCACCATAC 3´
Actin	q-rtPCR	AY372368	(F) 5´ GTGTGAGTCACACTGTGCCAATC 3´
			(R) 5´ ACGGCCAGCAAGATCCAA 3´
CHS	q-rtPCR	BB919594	(F) 5´ AGAACATTGATAAAGCATTGGTTGAG 3´
		BB914952, BB919590	(R) 5´ TCAGGCTTCAAGGCTAACTTTTG 3´
C4H	q-rtPCR	EU573999	(F) 5´ CCAGATCTTCAGAAGCTACCTTACCT 3´
		EU574000, EU574001	(R) 5´ GGGACGAGAAGTGGAATTGC 3´

Results and discussion

To obtain a red clover PAL sequence, degenerate oligonucleotide PCR primers were designed based on conserved sequences between the *Arabidopsis thaliana* PAL1 gene (GenBank AY303128) and a PAL sequence from the model legume *Medicago truncatula* (Gene Index TC106669) (Table 1). These primers were used in a PCR reaction with red clover cDNA derived from young leaves, a tissue with high contents of a variety of phenylpropanoid compounds, including *o*-diphenols. The resulting PCR product was of the expected size (approximately 350 bp) and sequencing showed it homologous to PAL genes from several plant species (data not shown). To obtain full-length cDNA clones, the red clover PAL gene fragment was used as a hybridization probe to screen a red clover leaf cDNA library. The screen was carried out under low stringency conditions (0.4 M NaCl, 55 °C) to allow efficient detection of PAL sequences with > 75 - 80 % sequence identity to the hybridization probe (Sambrook *et al.* 1989).

From this library screen, 24 clones hybridizing with the red clover PAL probe were isolated. Sequence analysis of the 5´ ends of the clones identified four unique PAL gene sequences: these were designated PAL1, PAL2, PAL3, and PAL4 (Table 2). Seventeen full-length cDNA clones corresponded to PAL1, and one each corresponded to PAL2, PAL3, and PAL4. The remaining clones were not full-length. The longest of the PAL1 clones and the PAL2, PAL3 and PAL4 clones were sequenced in entirety on both strands and the sequences deposited in GenBank. The PCR-derived hybridization probe appears to be derived from PAL1 because their nucleotide sequences are identical. PAL2 and PAL3 encode nearly identical proteins (99 %) that are very similar to that encoded by PAL4 (97 % identity). The protein encoded by PAL1 is truncated at the N-terminus relative to those of PAL2-4 and is only about 89 % identical to them. PAL2-4 have high peptide sequence identity (93 %) to an elicitor-induced PAL gene (GenBank accession X58180) from alfalfa (*Medicago sativa* L.) (Gowri *et al.* 1991). PAL1 has more limited amino acid sequence identity (86 %) to the elicitor-induced alfalfa PAL gene product.

To examine the tissue specific accumulation of mRNA corresponding to the isolated red clover PAL genes, primer pairs for q-rtPCR were developed. Because of the high degree of sequence similarity between PAL2, PAL3, and PAL4, even in their 3´ UTRs two sets of primer pairs were developed for q-rtPCR: one pair recognizing PAL1 and a second pair that recognizes PAL2, PAL3, or PAL4 (Table 1). Specificity of the primer pairs was confirmed and calibration curves were generated by carrying out q-rtPCR on a dilution series of purified plasmid DNAs corresponding to actin, PAL1, PAL2, PAL3, and PAL4. The PAL1-specific primer pair

Table 2. Red clover PAL genes and proteins. [A] - % amino acid identity compared to PAL2.

Gene	cDNAs isolated	GenBank Accession	Peptide data length	identity[A]
PAL1	17	DQ073809	712	89
PAL2	1	DQ073810	725	100
PAL3	1	DQ073808	724	99
PAL4	1	DQ073811	725	97

detected PAL1 signal but failed to detect signal from PAL2, PAL3, and PAL4 whereas the PAL2-4-specific primer pair detected PAL2, PAL3, and PAL4 to a similar degree but failed to detect PAL1. Because this experiment allowed the construction of gene specific calibration curves, relative quantitation of PAL1 versus PAL2-4 in the tissue-specific expression experiment described below was possible.

cDNA prepared from young leaves, mature leaves, stems, and flowers from four red clover genotypes derived from WI-2 germplasm was used in q-rtPCR experiments with the PAL1- and PAL2-4-specific primer pairs. A primer pair for red clover actin served as a control for cDNA synthesis and was used to normalize the data (Table 3). The level of plant-to-plant variability was relatively high in these experiments compared to similar studies analyzing the expression of red clover hydroxycinnamoyl transferase and *p*-coumaroyl 3-hydroxylase genes (unpublished data). Because the same cDNA preparations were used for these other studies and the PAL analysis presented here, this finding suggests that PAL gene expression might vary among different genotypes or be particularly responsive to unidentified environmental stimuli that were not controlled in the experiment. In flowers and young leaves, PAL1 mRNA accumulated to three- to four-fold higher levels than PAL2-4 mRNA. Although this difference was significant only for flowers ($P < 0.04$), the finding for young leaves is consistent with the high number of PAL1 clones recovered in the library screen compared to PAL2-4 clones. In mature leaves and stems PAL1 and PAL2-4 mRNA accumulated to similar levels. PAL1 expression was highest in flowers, young leaves, and stems and lowest in mature leaves. PAL2-4 expression showed little variation among the tissues examined.

Table 3. PAL gene expression in various red clover tissues. Means ± SEM, $n = 4$ (mRNA levels are normalized to actin mRNA and reported relative to PAL1 mRNA level in flowers).

Tissue	Relative mRNA level	
	PAL1	PAL2-4
Young leaves	0.68 ± 0.28	0.23 ± 0.09
Mature leaves	0.16 ± 0.07	0.25 ± 0.10
Stems	0.56 ± 0.14	0.43 ± 0.27
Flowers	1.00 ± 0.09	0.23 ± 0.27

Because PAL2, PAL3, and PAL4 have a high degree of sequence similarity to a PAL gene from alfalfa (Gowri *et al.* 1991) induced in response to a yeast-derived fungal elicitor and a number of PAL genes are rapidly (in 1 to 2 h) induced in *M. truncatula* upon YE treatment of suspension cultured cells (Naoumkina *et al.* 2007), it seemed likely PAL2-4 might be similarly induced. We developed a system whereby intact seedlings were treated with YE since a red clover cell suspension system similar to those used in the *Medicago* YE studies does not exist. For this system, seeds were treated with a *Biocide* and germinated under sterile conditions to prevent growth of contaminating fungi which could potentially affect the results of the study. Newly germinated seedlings were transferred to the wells of twelve-well plates and following a total of 5-d growth, the seedlings were treated directly in the well with YE in MS salts or mock treated with MS salts alone. By treating directly in the tissue culture wells, wounding of seedlings would be minimized, since PAL genes have also been shown to be wound inducible in several systems (see for example Campos-Vargas and Saltveit 2002).

The developed system was used to examine accumulation of PAL1 and PAL2-4 mRNA using q-rtPCR 1 h following elicitor treatment. Accumulation of actin mRNA in response to YE was also evaluated as a potential negative control for YE treatment. There was no significant difference ($P > 0.10$) between the changes in actin mRNA levels relative to untreated seedlings (ΔCt) in mock- and elicitor-treated seedlings 1 h post treatment (Table 4). The magnitude of the measured change from the untreated control was relatively small, corresponding to only 10 and 25 % decreases in transcript abundance for the mock and YE-treated seedlings, respectively (Table 4). In contrast, changes in PAL2-4 mRNA levels relative to untreated seedlings were significantly higher for YE-treated seedlings compared to mock-treated seedlings 1 h post-treatment ($P < 0.01$), although both mock and elicitor treatments increased PAL2-4 mRNA levels (*i.e.* ΔCt is negative). For PAL1, elicitor induced changes in mRNA level were not significantly different from mock treatment at 1 h ($P > 0.10$). Because the mock treatment did seem to affect mRNA levels relative to the untreated control, especially for PAL2-4, data were also expressed as $\Delta Ct_{elicitor} - \Delta Ct_{mock}$ (ΔΔCt) to remove any effects of the method used to deliver the elicitor to the seedling. This approach confirmed any effect of elicitor on actin mRNA levels is small, with ΔΔCt corresponding to only about a 20 % decrease in transcript abundance compared to mock treatment. Because YE treatment had little if any effect on actin transcript abundance, it was used as a negative control. For PAL2-4, ΔΔCt is significantly different than that observed for actin ($P < 0.0001$) and corresponds to an increase in mRNA levels of about six-fold compared to the mock treatment. A smaller induction (ΔΔCt corresponding to a 1.7-fold increase in transcript abundance) was seen for PAL1, although this level of induction is significant compared to that of actin mRNA ($P < 0.02$).

Because PAL2-4 mRNA levels were clearly increased by the YE treatment, two additional genes that have been shown to be rapidly induced by YE in *M. truncatula* suspension cultures, chalcone synthase (CHS, EC 2.3.1.74) and cinnamic acid 4-hydroxylase (C4H) (Suzuki *et al.* 2005, Naoumkina *et al.* 2007) were also tested for induction in red clover seedlings. Primers for q-rtPCR were developed based on red clover EST sequences for CHS available in GenBank and on the sequence of 3 red clover C4H clones isolated in my laboratory (Table 1).

CHS showed a behavior similar to PAL2-4, with ΔΔCt significantly different from that of actin ($P < 0.0001$) and corresponding to a more than four-fold increase in transcript abundance compared to the mock treatment. C4H showed a behavior similar to PAL1, with a small ΔΔCt corresponding to a more modest 1.6-fold increase in transcript abundance that is significantly different than that seen for actin ($P < 0.0001$).

Table 4. q-rtPCR expression data for various red clover genes. $\Delta Ct = Ct_{treated} - Ct_{untreated}$. Mock versus elicitor treatment differences are significant for PAL2-4, CHS and C4H ($P < 0.02$) but not for Actin ($P = 0.11$) and PAL1 ($P = 0.15$). $\Delta\Delta Ct = \Delta Ct_{elicitor} - \Delta Ct_{mock} = Ct_{elicitor} - Ct_{mock}$. ΔΔCt for PAL1, PAL2-4, CHS and C4H are significantly different from actin at $P < 0.002$.

Gene	ΔCt		ΔΔCt
	mock	elicitor	
Actin	0.14 ± 0.06	0.41 ± 0.14	0.26 ± 0.13
PAL1	0.26 ± 0.43	-0.53 ± 0.31	-0.79 ± 0.24
PAL2-4	-1.18 ± 0.58	-3.84 ± 0.42	-2.65 ± 0.34
CHS	-0.91 ± 0.58	-3.07 ± 0.39	-2.15 ± 0.28
C4H	-0.02 ± 0.21	-0.69 ± 0.15	-0.67 ± 0.12

Although both classes of PAL genes (PAL1 and PAL2-4) appear to play roles in phenylpropanoid biosynthesis under normal growing conditions, one or more genes (the q-rtPCR did not distinguish between them) from the PAL2-4 group were rapidly (< 1 h) induced about six-fold in seedlings in response to a yeast-derived fungal elicitor. This observation suggests that PAL2-4 might be involved in the biosynthesis of phenylpropanoid compounds in response to pathogens. This elicitor induction is similar to that of PAL genes from *M. truncatula* and an *M. sativa* PAL gene with which PAL2-4 share a high degree of sequence identity. Although the magnitude of induction seen with the red clover genes was more modest than that seen for the *Medicago* genes, this could be due to the different tissues used in the induction experiments. Because the seedlings used in the experiment presented here consist of multiple tissue types, in contrast to the more uniform suspension cell cultures used in the *Medicago* experiments, it might be that only certain of the red clover tissues respond to the fungal elicitor, thus making the observed induction averaged over all the tissues appear smaller. Preliminary experiments examining the effect of dose and duration of elicitor treatment suggest the observed induction of PAL2-4 was maximal under the conditions tested (data not shown). PAL1 was also induced by YE treatment, although the magnitude of induction (less than two-fold) was more modest. In contrast, actin transcript accumulation showed little, if any, response to the YE treatment, making it a good negative control in these experiments.

To carry out the YE experiments, we developed a system whereby intact red clover seedlings were treated with the YE preparation and mRNA levels for genes of interest were analyzed by q-rtPCR. For two genes examined using this system, PAL2-4 and CHS, induction by YE was clear by simply comparing the change in Ct for mock- and YE-treated seedlings relative to untreated control seedlings. However, from these data it seemed that for some genes, the mock treatment was causing some changes in expression, presumably due to treatment with the medium, mechanical disruption of the seedlings, *etc*. To more easily assess the effect of YE apart from delivery of YE (*i.e.* mock treatment), data were expressed as the difference between the elicitor and mock treatment. This also had the effect of producing a lower standard error of the mean for ΔΔCt than would be calculated from the errors of the average ΔCt values for the mock and YE treatments, indicating that variation due to uncontrolled variables within the biological replicates affect the mock and YE treated seedlings in similar ways. By analyzing the data in this manner, it became apparent that PAL1 and C4H transcript levels were modestly, but significantly, increased relative to the actin control by YE treatment. With additional optimization, the seedling system should be a useful tool for detailed investigations into changes in red clover gene expression in response to YE as well as other biological signaling agents. For example, it should be possible to adapt this seedling system for jasmonic acid treatment to identify roles for specific genes (*e.g.* those for cinnamoyl transferases and hydroxylases) in the jasmonic acid induced clovamide biosynthesis described by Tebayashi *et al.* (2000). The seedling system described here could also be easily adapted to analyze gene expression in other small seeded species.

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