

Cloning of Red Clover and Alfalfa Polyphenol Oxidase Genes and Expression of Active Enzymes in Transgenic Alfalfa

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Abstract:

Red clover contains high levels of polyphenol oxidase (PPO) activity and *o*-diphenol substrates. This results in a characteristic post-harvest browning reaction associated with decreased protein degradation during ensiling. To define PPO's role in inhibition of post-harvest proteolysis, we are taking both biochemical and molecular approaches. We have cloned three unique PPO cDNAs from red clover leaves, RC PPO1-3. The cDNAs encode proteins that are predicted to be targeted to the chloroplast thylakoid lumen. RNA blotting and immunoblotting experiments indicate RC PPO1 is expressed predominantly in young leaf tissue, RC PPO2 is expressed most highly in flowers and petioles, and RC PPO3 is expressed in both leaves and flowers. We expressed the red clover cDNAs in alfalfa, which lacks both significant endogenous PPO activity and *o*-diphenol substrates in its leaves, to further characterize the individual proteins encoded by RC PPO1-3. The expressed proteins are active in alfalfa extracts and the individual enzymes show differences in substrate specificity, suggesting different functional roles. Additionally, we have cloned a PPO gene from alfalfa. Preliminary studies indicate alfalfa PPO expression is limited to flowers and seedpods. Expression of the cloned alfalfa PPO gene from a strong constitutive promoter in transgenic alfalfa results in low but measurable enzyme activity, suggesting a low specific activity compared to the red clover enzymes.

1. INTRODUCTION

Ensiling crops is a popular method of preserving forage for animal feed, particularly in the humid northern regions of the USA. Unfortunately, excessive proteolysis of ensiled forages can result in both economic losses to farmers (Rotz et al. 1993) and negative impacts on the environment since in the rumen, non-true protein nitrogen (ammonia, amino acids, and small peptides) is poorly utilized, most being excreted by the animal as urea.

Proteolytic losses in ensiled alfalfa are especially high, with degradation of 44-87% of the forage protein (Papadopoulos and McKersie 1983; Muck 1987). In contrast, red clover, a forage of protein content similar to alfalfa, has been found to have up to 90% less proteolysis than alfalfa during ensiling (Papadopoulos and McKersie 1983). This difference in the extent of post-harvest proteolysis is evident in extracts of clover and alfalfa leaves (Jones et al. 1995a,b,c). Red clover's lower extent of post-harvest proteolysis is not due to differences in its inherent proteolytic activity compared to alfalfa, but seems related to the presence of polyphenol oxidase (PPO) and *o*-diphenols based on several experimental observations (Jones et al. 1995a,b,c). Interestingly, alfalfa leaves and stems have little if any polyphenol oxidase activity or *o*-diphenol PPO substrates (unpublished data). Since PPO catalyzes the oxidation of *o*-diphenols to *o*-quinones (Figure 1), a possible mechanism whereby PPO acts to inhibit proteolysis could be via PPO-generated *o*-quinones binding directly to and inactivating endogenous proteases.

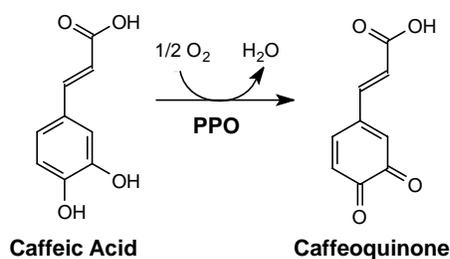


Figure 1. Polyphenol oxidase (PPO) catalyzed oxidation of an *o*-diphenol to an *o*-quinone (caffeic acid to caffeoylquinone in this example).

In an effort to understand the role of PPO in inhibition of post-harvest proteolysis, we have been using both biochemical and molecular approaches. Here we report the isolation of three red clover PPO cDNAs, characterization of their expression pattern, and analysis of their enzymatic activities. Additionally, to understand the lack of PPO activity in alfalfa leaves and stems, we have isolated a genomic PPO clone from alfalfa and have begun to characterize its expression and encoded protein.

2. RESULTS

To clone red clover PPO genes, we took advantage of the conserved sequences of the copper binding motifs of several previously cloned plant PPOs (Figure 2) to design primers for reverse transcription and PCR of red clover leaf mRNA. Several PCR fragments were isolated, cloned, and sequenced. To obtain full-length clones, a 300 bp PCR fragment was used to screen a red clover leaf cDNA library. Three unique PPO cDNAs were identified and sequenced (Table 1).

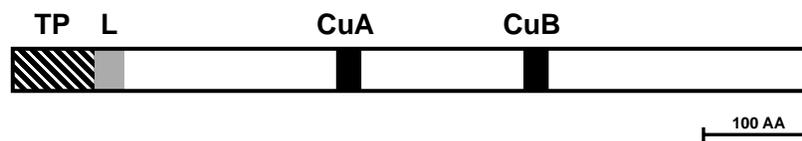


Figure 2. Schematic representation of PPO protein showing predicted transit peptide (TP), luminal targeting sequence (L), and conserved copper binding motifs (CuA and CuB).

Table 1. Characteristics of red clover and alfalfa PPO genes

Gene	Genbank Accession	Protein Length ^a	Expression Pattern
red clover PPO1	AY017302	605/511	leaf
red clover PPO2	AY017303	623/522	flower, petiole
red clover PPO3	AY017304	599/504	leaf, flower
alfalfa PPO	AY283062	607/512	flower, seed pod

^aLength in amino acids, predicted precursor/predicted mature.

Sequence comparison among the three red clover PPO clones indicates they are 84-89% identical at the nucleotide level (76-80% identity at the amino acid level). A BLAST search of Genbank reveals sequence similarity to several previously cloned PPO genes. The red clover PPOs are most similar to that of the legume *Vicia faba* (Genbank accession Z11702, 60-62% amino acid identity). ChloroP and SignalP algorithms (www.cbs.dtu.dk/services) predict an N-terminal chloroplast transit peptide and thylakoid lumen targeting signal (Figure 2) (Peltier et al. 2000; Emanuelsson et al. 1999) which would result in processing to mature proteins of 504-522 amino acids in length. These predictions are consistent with the intracellular localization and processing sites of several other PPO enzymes (Steffens et al. 1994). In the case of RC PPO1, *E. coli*-expressed protein corresponding to the predicted mature form comigrates with a PPO protein present in red clover leaves, indicating that the predicted processing occurs (data not shown).

Expression of the various red clover PPO genes was examined by northern blotting and hybridization using RC PPO1-3 gene-specific probes

(Figure 3 and data not shown). RC PPO1 is most highly expressed in unexpanded and young leaves, RC PPO2 is expressed most highly in flowers and petioles, and RC PPO 3 is expressed in both leaves and flowers. Although leaf expression for RC PPO2 is not apparent in the northern blot, recovery of the cDNA from a leaf library suggests it is expressed in leaves, albeit to a low level. Using antiserum raised against *E. coli*-expressed RC PPO1 on immunoblots of extracts of transgenic alfalfa expressing the individual RC PPO genes (described in more detail below), we found that the individual red clover PPOs could be distinguished by mobility on SDS-PAGE (Figure 3). Immunoblot analysis of red clover leaf and flower extracts show that RC PPO1 and 3 are most highly expressed in leaves whereas RC PPO2 is expressed most highly in flowers. These findings are consistent with the northern analysis.

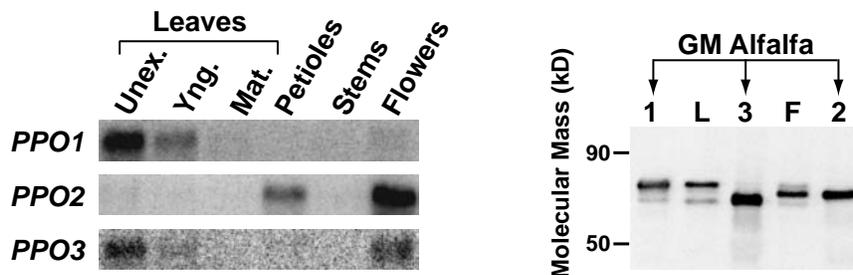


Figure 3. Red clover PPO genes show tissue-specific expression. (Left) Northern blots of RNA isolated from unexpanded (Unex.), young (Yng.) and mature (Mat.) leaves; petioles; stems; and flowers of red clover were hybridized with gene-specific probes as indicated. (Right) An Immunoblot of extracts of red clover leaves (L) and flowers (F) or leaves from transgenic (GM) alfalfa expressing RC PPO1-3 as indicated was probed with anti RC PPO1 antiserum.

Due to the low activity of RC PPO1 enzyme expressed in *E. coli* (data not shown), we decided to express the red clover PPO genes in alfalfa. Alfalfa is easily transformed and alfalfa leaves contain no detectable PPO activity or *o*-diphenol substrates. This lack of endogenous PPO and substrates would facilitate our analyses of the red clover gene products. The entire coding regions from RC PPO1-3 were inserted behind the cassava vein mosaic virus (CVMV) promoter in the pILTAB357 transformation vector (Verdaguer et al. 1996). The resulting constructs were transformed into alfalfa using *Agrobacterium*-mediated transformation (Austin et al. 1995). Several independent transformants were generated for each PPO gene, as well as control plants transformed with the pILTAB357 vector only. When the *o*-diphenol caffeic acid is added to extracts of PPO-expressing alfalfa leaves, a dramatic browning reaction is apparent within minutes, a hallmark of *o*-quinone production by PPO (Steffens et al. 1994). No such browning takes

place in control alfalfa extracts, even after 48 hours. To quantify PPO activity we used a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) quinone trap assay (Esterbauer et al. 1977). For alfalfa expressing PPO1 and PPO3 and using a caffeic acid substrate, we found several independent alfalfa transformants with activities comparable to that seen in red clover leaves (i.e. 1-5 $\mu\text{mol}/\text{min}/\text{mg}$ crude protein). One striking observation using caffeic acid as a substrate is that the activity of RC PPO2 ($\sim 0.1 \mu\text{mol}/\text{min}/\text{mg}$) appears to be substantially lower than that of RC PPO1 and 3. For each of the red clover PPO genes, alfalfa plants showing the highest levels of PPO activity served as the enzyme source for additional analyses of enzyme activity. To determine if there are any differences in substrate utilization among the red clover PPOs, we measured PPO activity for several different *o*-diphenols in extracts of the transgenic alfalfa as well as in red clover leaf extracts (Figure 4).

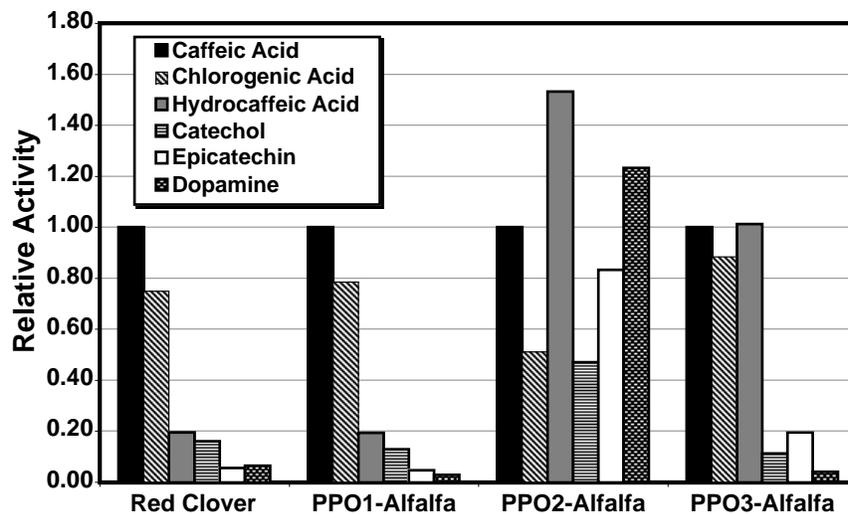


Figure 4. Differential utilization of *o*-diphenols among red clover PPOs. PPO activity towards various *o*-diphenol substrates was measured in extracts of red clover or alfalfa expressing individual RC PPO enzymes using a DTNB quinone trap assay (Esterbauer et al. 1977). To facilitate comparison among the different enzyme sources, activity is expressed relative to that for caffeic acid.

For RC PPO1 we found that the utilization of the *o*-diphenols tested is similar to that of red clover leaf extract, consistent with RC PPO1 being the major PPO of red clover leaves. RC PPO1-3 show substantial differences in their relative preferences for *o*-diphenol substrates. For example, both RC PPO2 and 3 utilize hydrocaffeic acid (3,4-dihydroxyhydrocinnamic acid) as well as or better than caffeic acid, while this is a mediocre substrate for RC PPO1. Also, RC PPO2 utilizes (-)-epicatechin and dopamine, relatively poor

substrates for RC PPO1 and 3, as well as or better than caffeic acid. Additional studies of the red clover PPO enzymes are currently underway.

As noted above, we have detected little if any PPO activity in alfalfa leaves or stems. This lack of PPO activity could be due to lack of a functional gene or lack of PPO expression in the aerial tissues we have examined. Alternatively, PPO enzymes in alfalfa could have substrate specificities different from those of previously characterized PPOs and consequently not be detectable in standard activity assays. To distinguish among these possibilities, we cloned an alfalfa PPO gene by screening a genomic library (Gregerson et al. 1994) with a probe generated by PCR of alfalfa genomic DNA with PPO specific primers. We isolated and sequenced one clone containing an entire intronless alfalfa PPO gene along with 1200 bp of upstream sequence (Table 1). Of cloned PPO genes, alfalfa PPO is most similar to that of *Vicia Faba* (73% amino acid identity). The alfalfa enzyme shares only ~65% amino acid identity with the clover enzymes. Like most other plant PPOs, alfalfa PPO is predicted to be targeted to the chloroplast thylakoid lumen (Peltier et al. 2000; Emanuelsson et al. 1999; Steffens et al. 1994). Expression of alfalfa PPO was examined by northern blotting and hybridization under moderate stringency (to allow detection of transcripts from any related PPO genes). Of the tissues examined, PPO expression appears to be limited to flowers and seed pods (Figure 5).

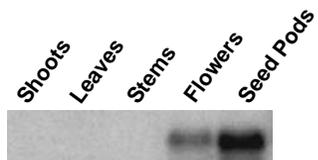


Figure 5. Northern blot analysis reveals alfalfa PPO expression is limited to flowers and seed pods.

We are currently examining the expression pattern of alfalfa PPO using a promoter::GUS fusion in transgenic alfalfa. Initial results indicate PPO is expressed in anthers, sepals, developing seeds and pods. To analyze the alfalfa PPO gene product, we are overexpressing the protein in transgenic alfalfa utilizing a CVMV promoter construct similar to those used for the red clover PPO genes. Extracts of the transgenic plants show significant browning when exogenous caffeic acid is supplied, although the browning reaction is slow compared to that of alfalfa expressing any of the red clover PPO genes (several hours compared to a few minutes). No browning is seen for control plants lacking the PPO transgene, even after several days. Since greater than twenty-five independent transformants have been examined, the slow browning reaction suggests that the specific activity of alfalfa PPO is low (at least for caffeic acid) compared to the red clover PPOs. We are

currently carrying out more detailed studies of the enzymatic properties of alfalfa PPO.

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