Published online February 1, 2006

Polyphenol Oxidase and *o*-Diphenols Inhibit Postharvest Proteolysis in Red Clover and Alfalfa

Michael L. Sullivan* and Ronald D. Hatfield

ABSTRACT

Many forages experience significant proteolytic losses when preserved by ensiling. Such losses in alfalfa (*Medicago sativa* L.) are especially high, with degradation of 44 to 87% of the forage protein to nonprotein N (NPN). In contrast, red clover (*Trifolium pratense* L.) has up to 90% less proteolysis during ensiling. Here we demonstrate that the combination of polyphenol oxidase (PPO) and *o*-diphenol PPO substrates, both abundantly present in red clover, is responsible for postharvest proteolytic inhibition in this forage crop. Proteolysis in red clover leaf extracts increased nearly fivefold when endogenous *o*-diphenols were removed by gel filtration but returned to starting levels by adding back an exogenous *o*-diphenol. Proteolysis in leaf extracts of red clover plants silenced for PPO expression was dramatically increased compared to control plants. Leaf extracts of transgenic alfalfa expressing a red clover PPO gene showed a nearly fivefold *o*-diphenol–dependent decrease in proteolysis compared to those of control alfalfa. We also demonstrate that PPO levels 10- to 20-fold lower than those typically found in red clover are sufficient for proteolytic inhibition, that as little as 0.25 μmol *o*-diphenol mg^{-1} protein has a substantial impact on proteolysis, that a wide variety of *o*-diphenols are functional substrates in proteolytic inhibition, and that proteolysis is reduced for PPO-expressing alfalfa in small-scale ensiling experiments. Together, these results indicate that PPO and *o*-diphenols can be an effective treatment to prevent protein loss in ensiled forage crops.

Ensiling is a popular method of preserving crops, especially in regions with humid climates. During harvest and the early stages of ensiling, disruption of plant tissues releases cellular proteases resulting in degradation of forage protein to small peptides and amino acids. As fermentation by lactic acid bacteria progresses, silage pH drops and the rate of proteolysis decreases. Unfortunately, substantial conversion of protein to NPN can take place before silage pH becomes sufficiently acidic to inhibit proteolysis. Because dairy cows and other ruminant animals poorly utilize excess NPN, loss of true protein during ensiling results in economic losses to farmers. Such losses for alfalfa alone approach $100 million annually in the United States based on an estimated loss of $70 per hectare (Rotz et al., 1993) and USDA reports of acreage devoted to alfalfa haylage production in 2004 (NASS, 2005). Further, ruminants excrete much of this NPN as urea resulting in increasing N burdens on the environment. For alfalfa, proteolytic losses during ensiling are especially high with degradation of 44 to 87% of the forage protein (Albrecht and Muck, 1991; Jones et al., 1995c; Muck, 1987; Papadopoulos and McKersie, 1983). Red clover, a legume forage with protein content similar to alfalfa, experiences up to 90% less proteolysis than alfalfa when ensiled. Jones et al. (1995a, 1995c) found that red clover tissues do not differ substantially from those of alfalfa in terms of proteolytic activities present and postharvest proteolytic inhibition in red clover is O_2–dependent and involves a heat labile factor. These, along with the observations that PPO *o*-diphenol substrates disappear concomitantly with proteolytic inhibition (Hatfield and Muck, 1999) and that alfalfa has little if any foliar PPO or *o*-diphenols (Jones et al., 1995b; Sullivan et al., 2004a) suggested that the proteolytic inhibition seen in ensiled red clover is due to the action of PPO on endogenous *o*-diphenols.

Recently, we cloned several red clover PPO genes, including the major PPO gene expressed in red clover leaves and showed that active red clover PPO enzyme could be expressed in transgenic alfalfa (Sullivan et al., 2004a). We have recently generated red clover lacking foliar PPO by expression of double-stranded PPO RNA in transgenic plants (RNAi) (Wang and Waterhouse, 2002). Using transgenic PPO-expressing alfalfa, as well as red clover plants silenced for foliar PPO expression, we have now been able to demonstrate that postharvest proteolytic inhibition in red clover is due to the action of PPO on *o*-diphenols and have begun to define the critical parameters of this natural system of protein protection.

MATERIALS AND METHODS

Plant Materials and Transformation

A red clover genotype (lab designation "PPO") (Sullivan et al., 2004a) selected from a population of WI-2 (lot C136) germplasm (Smith and Maxwell, 1980) was used in several experiments as indicated. For experiments with transgenic red clover, two highly regenerable genotypes (designated NEWRC27 and NEWRC30) derived from a population of NEWRC germplasm (Smith and Quesenberry, 1995) were used. These red clover genotypes were transformed via *Agrobacterium*-mediated transformation using a modification (Sullivan and Quesenberry, 2006) of the method of Quesenberry et al. (1996). Control and red clover PPO1-expressing transgenic alfalfa, generated by *Agrobacterium*-mediated transformation of a highly regenerable clone of Regen-SY (Bingham, 1991), have been previously described (Sullivan et al., 2004a). All experiments utilizing transgenic plants were performed with primary

M.L. Sullivan and R.D. Hatfield, U.S. Dairy Forage Research Center, ARS-USDA, 1925 Linden Drive West, Madison, WI 53705. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Received 15 June 2005. *Corresponding author (mlsulliv@wisc.edu).

Published in Crop Sci. 46:662–670 (2006).
Crop Physiology & Metabolism
doi:10.2135/cropsci2005.06-0132

677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: bp, base pair; DM, dry matter; FW, fresh weight; nkat, nanokatal; NPN, nonprotein N; PPO, polyphenol oxidase; RNAi, RNA interference; TCA, trichloroacetic acid.

transformants (T_0). Transgenic alfalfa plants were propagated from stem cuttings in vermiculite. NEWRC red clover plants (untransformed and transformed) were maintained in a growth chamber at 26°C with 16 h d^{-1} of approximately 40 μmol m^{-2} sec^{-1} illumination from fluorescent lamps and propagated by splitting the crown. All other plants were maintained in a greenhouse year-round at 20 to 30°C with light intensities between 400 and 1000 μmol m^{-2} sec^{-1}. Supplemental lighting (13 h d^{-1}) was used during all but summer months. All plants were fertilized weekly with Peter's soluble 20–20–20 (Scott's, Marysville, OH).

Preparation of a Gene Silencing (RNAi) Construct for Red Clover PPO

An approximately 1100-bp fragment from the coding region of the red clover PPO1 gene (Genbank AY017302) was generated using well-established methodologies from a cloned cDNA (Sullivan et al., 2004a) template by PCR using the primers 5′-GGGGGATCCCTCGAGGCCCTTGAACTCA-TGAGAG-3′ and 5′-GGGATCGATGGTACCTATCATCT-TCATCATTCAC-3′. The resulting fragment was digested with either *Xho*I and *Kpn*I or *Bam*HI and *Cla*I and cloned into the sense and antisense arms, respectively, of the intron-containing gene silencing vector pHANNIBAL (Wesley et al., 2001). The silencing cassette of this pHANNIBAL construct was subcloned as a *Not*I fragment into the *Not*I site of the binary vector pART27 (Gleave, 1992) such that the promoters for the hairpin silencing RNA and the npt II selectable marker of pART27 were divergently transcribed. The resulting plasmid was transferred to *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1993) by triparental mating (Rogers et al., 1986) and used to transform red clover as described above.

Preparation of Leaf Extracts for Proteolysis Assays

Mature red clover or alfalfa leaves were harvested, powdered in liquid N_2 using a mortar and pestle, and extracted into 50 m*M* MES (2-[N-morpholino]ethanesulfonic acid), pH 6.5 using 3 mL buffer g^{-1} fresh weight. For red clover tissue, 20 m*M* ascorbic acid was included in the extraction buffer to prevent extensive browning by endogenous PPO and *o*-diphenol PPO substrates. Extracts were kept on ice or at 4°C during the entire extraction procedure. Extracts were centrifuged at 10 000 *g* for 5 min, filtered through Miracloth (Calbiochem, San Diego, CA), and centrifuged again at 10 000 *g* for 10 min. For some experiments, low molecular weight compounds were removed by gel filtration. Briefly, a spin column was prepared by plugging the bottom of a 5-mL disposable syringe barrel with glass wool and filling the syringe to the top with Sephadex G-25 (Amersham Biosciences, purchased from Sigma, St. Louis, MO) equilibrated with 50 m*M* MES, pH 6.5. The column was placed in a 15-mL tube and centrifuged at 200 *g* for 1 min at 4°C (final volume of packed matrix was approximately 4.5 mL). Freshly prepared leaf extract (1.5 mL) was applied to the packed column, the centrifugation was repeated, and the eluate collected. Aliquots of the clarified crude or desalted extracts were frozen in liquid N_2 and stored at −80°C. Protein concentrations of extracts were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as a standard. PPO activity of extracts was measured as previously described (Sullivan et al., 2004a) using the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) quinone trap assay method of Esterbauer et al. (1977) with caffeic acid as the substrate. Enzyme activities are expressed in nkat (i.e., nmol sec^{-1}).

In Vitro Proteolysis Assay

Proteolysis assay reactions contained 2 mg mL^{-1} leaf extract protein in 50 m*M* MES, pH 6.5 and either the indicated *o*-diphenol (3% v/v of a 100 m*M* stock in ethanol, 3 m*M* final concentration except as noted) or an equivalent amount of ethanol as a control. Duplicate samples of each reaction were removed after 0, 1, 2, 3, or 4 h of incubation at 37°C in a loosely capped 15-mL test tube. Once removed, the samples were immediately mixed with one-half volume 15% TCA (5% [w/v] final) and placed on ice for at least 30 min to precipitate proteins and peptides. TCA-insoluble material was removed by centrifugation at 16 000 *g* for 5 min. Amino acid concentration of the 5% TCA supernatants was determined using ninhydrin reagent (Nin-Sol AF, Pierce Biotechnology, Rockford, IL) (Moore, 1968) with glycine as the standard, and values for duplicate samples were averaged. Amino acid release, as μmol mg^{-1} of extract protein, was determined by subtracting the amino acid concentration of the initial (unincubated) samples from the amino acid concentration of the samples following incubation. For the experiments in Fig. 5 and 6, amino acid release was determined for the 4-h time point only. For each experiment, the results using three extracts prepared from tissue samples harvested on different days were averaged. Statistical significance of pairwise comparisons was determined using the *t* test with a directional hypothesis, where appropriate.

Analyses of PPO-Silenced Red Clover

Extracts of red clover plants transformed with the PPO-silencing construct (or nontransformed control plants of the same genotype) were prepared as described above except the extraction buffer was 100 m*M* ammonium acetate, 20 m*M* Tris, 50 m*M* ascorbic acid, pH 7.5 (adjusted with NaOH). SDS-PAGE, immunoblotting, and detection with anti–red clover

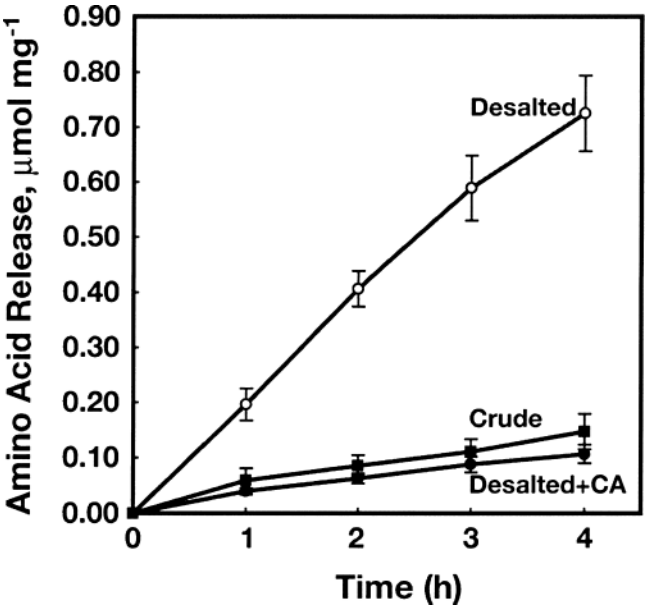

Fig. 1. Proteolytic inhibition in red clover extracts requires *o*-diphenols. Proteolysis, as measured by amino acid release over time, was measured in red clover leaf extracts that were unfractionated (Crude, ■), desalted by gel filtration to remove low molecular weight compounds (Desalted, ○), or desalted and supplemented with the *o*-diphenol caffeic acid at a final concentration of 3 m*M* (Desalted+CA, ●). Results are the average ± SEM of three experiments using independently prepared extracts.

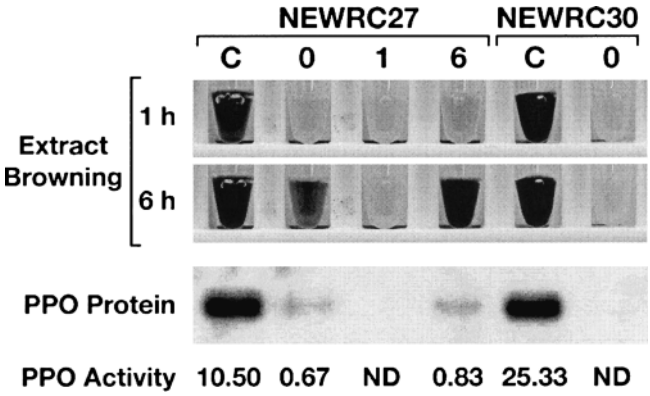

Fig. 2. RNAi reduces PPO expression in red clover leaves. Leaf extracts of nontransformed control (C) or independently derived silenced (numbered) plants from NEWRC27 and NEWRC30 red clover genotypes were analyzed for extract browning, PPO protein by immunoblotting (5 μg protein per lane) with a PPO antibody, or for PPO activity by a quantitative PPO activity assay. PPO activity is expressed as nkat mg^{-1}. For NEWRC27–1 and NEWRC30–0 plants transformed with the silencing construct, PPO activity was not detectable (ND) and no browning was seen, even after 24 h.

PPO1 antiserum was performed as previously described (Sullivan et al., 2004a). PPO activity was determined as described above. A qualitative extract browning assay was performed by incubating 100-μL aliquots of extract at room temperature and recording browning over time by photography with a digital camera.

Small-Scale Ensiling

Two ensiling experiments were performed on different days. Greenhouse grown alfalfa was harvested by hand with scissors and dried on a screen to approximately 67% its initial fresh weight (approximately 40% DM). A 200 m*M* solution of catechol in ethanol was applied to the tissue at a rate of 82.5 μL g^{-1} (approximately 14 μmol g^{-1} tissue) with an atomizer and the tissue was macerated using a meat grinder with 5-mm holes. For each treatment, duplicate 5-g samples were removed, frozen in liquid N_2, and stored at −20°C for later analyses of initial pH and free amino acid content. A 10-g sample was removed for analyses of initial DM and total N. The remaining tissue was treated with Pioneer 1174 silage inoculant (Pioneer Hi-Bred International, Johnston, IA) at a rate of 10^5 bacteria g^{-1} tissue and ensiled in triplicate minisilos (30 g per silo) as previously described (Muck, 1987). Following incubation for 14 to 21 d at 30°C, the silos were opened. Two 10-g samples from each silo, as well as the previously collected initial samples, were analyzed for DM by lyophilzation. The lyophilized samples were ground together and analyzed for total N by combustion using a FP-2000A N analyzer (Leco Corporation, St. Joseph, MI). Water extracts, prepared from additional 10-g samples from each silo as well as the previously collected 5-g initial samples, were used for pH determination and preparation of 5% TCA-soluble fractions as described elsewhere (Muck, 1987). Free amino acid content of the TCA-soluble fractions were analyzed by ninhydrin assay essentially as described above. For simplicity, amino acid N was calculated assuming only one N atom per amino acid. Although this assumption underestimates amino acid N, it has no impact on the proportionate effects of the treatments examined. Results of the duplicate initial samples, and final samples from triplicate silos were averaged. Statistical significance of pairwise comparisons was determined using the *t* test with a directional hypothesis, where appropriate.

RESULTS

Proteolytic Inhibition in Red Clover Extracts Requires an *o*-Diphenol

Previous work suggested that postharvest proteolytic inhibition in red clover was due to the action of polyphenol oxidase on the abundant *o*-diphenols present in red clover leaves (Jones et al., 1995c). To test the role of *o*-diphenols in inhibition of postharvest proteolysis in red clover, leaf extracts were desalted by passing through spin columns of Sephadex G-25 to remove *o*-diphenols and other low molecular weight molecules. The desalted extracts did not brown like crude extracts, although 70 to 100% of the starting PPO activity remained in the extracts. We analyzed the extent of proteolysis in the resulting desalted and control crude extracts by measuring the release of free amino acids over time. As shown in Fig. 1, removal of low molecular weight compounds resulted in a significant increase in proteolysis compared to the control crude extracts ($P < 0.01$ at all time points). At the end of the 4-h assay, desalted red clover extracts had nearly fivefold more proteolysis than crude extracts (0.73 vs. 0.15 μmol amino acids mg^{-1} protein, respectively). To rule out the possibility that the gel filtration procedure removed some other low molecular weight protease inhibitor from the red clover extract, we added an *o*-diphenol, caffeic acid, back to the desalted extract at a concentration of 3 m*M*, an amount we estimate was similar to that present in the unfractionated red clover extract based on extent of browning (see Discussion). The *o*-diphenol addition resulted in extract browning, indicating the presence of active PPO, as well as inhibition of proteolysis to an extent indistinguishable from that seen for the crude red clover extract (Fig. 1).

Gene Silencing Effectively Reduces PPO Expression and Enhances Postharvest Proteolysis in Red Clover

To test the role of PPO in inhibition of postharvest proteolysis, we decided to use RNAi to create red clover plants with substantial reductions in PPO activity. Plants generated using this methodology would have matching controls of the same genotype, differing only by the insertion of the silencing transgene and selectable marker. We used a fragment of the coding region of PPO1, the major expressed PPO gene in red clover leaves (Sullivan et al., 2004a), to make a hairpin RNA-producing gene-silencing construct with a kanamycin resistance selectable marker that was subsequently used to transform red clover.

We analyzed four kanamycin-resistant primary transformants derived from two genotypes (NEWRC27 and NEWRC30) and their corresponding nontransformed controls for extract browning. Leaf extracts of plants transformed with the PPO-silencing construct showed delayed browning compared to control plants of the same genotype. Extracts of control plants were beginning to show signs of browning before the extraction procedure was completed (approximately 20–25 min)

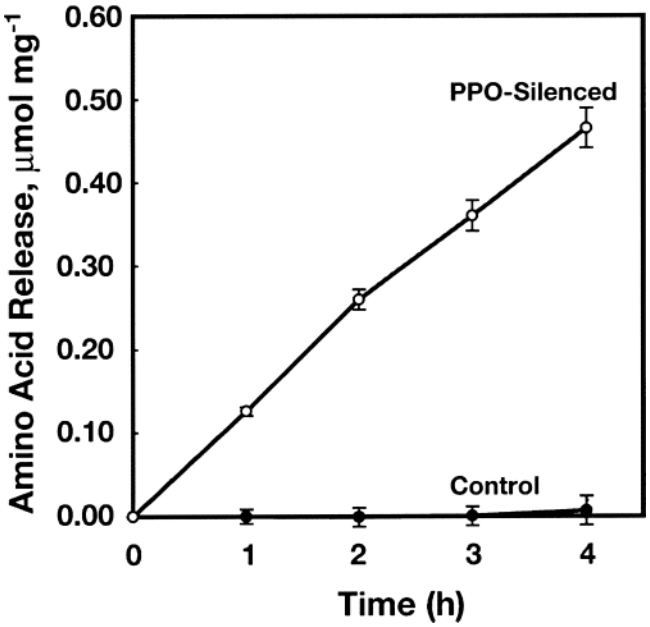

Fig. 3. Proteolytic inhibition in red clover extracts requires PPO. Proteolysis, as measured by amino acid release over time, was measured in leaf extracts from untransformed control (●) or PPO-Silenced (○) red clover. Results are the average ± SEM of three experiments using independently prepared extracts from NEWRC27–1 (2 replicates) and NEWRC30–0 and their corresponding controls.

despite being kept on ice and inclusion of an antioxidant (50 m*M* ascorbate) in the extraction buffer. Subsequent incubation at room temperature resulted in extensive browning of control extracts within less than 1 h (Fig. 2). We observed some browning for extracts of two of the transgenic plants after longer incubation times (Fig. 2, NEWRC27 derived plants 0 and 6). Extracts of two additional transgenic plants, NEWRC27 plant 1 and NEWRC30 plant 0, showed little if any browning even after extended room temperature incubations of up to 24 h (Fig. 2 and data not shown). We also used samples of the control and transgenic extracts for immunoblotting with anti-PPO antiserum and for a quantitative assay for PPO activity using caffeic acid as the substrate (Fig. 2). Consistent with our extract browning observations, foliar levels of PPO protein and activity as detected by immunoblotting and activity assay, respectively, were dramatically reduced in plants whose extracts showed delayed browning (NEWRC27 plants 0 and 6) and not detected at all in plants whose extracts failed to brown (NEWRC27 plant 1 and NEWRC30 plant 0). In the case of the quantitative PPO activity assay, a dilution experiment of the NEWRC27 control indicated we should be able to detect PPO activity that is approximately 1% of that seen in this control.

Using the red clover plants with no detectible PPO protein or PPO activity in mature leaves, we were able to test the role of PPO in inhibiting postharvest proteolysis. Extracts of mature leaves of NEWRC27–1 and NEWRC30–0 plants and their corresponding nontransformed controls were analyzed for the extent of proteolysis by measuring the release of free amino acids over time as described above. Each extract was also assayed for PPO activity to ensure extracts of the silenced plants had no detectible activity. As shown in Fig. 3, proteolysis in extracts of leaves of PPO-silenced plants was significantly higher than that in extracts of control plants ($P < 0.001$ at all time points). The increased proteolysis was similar to, albeit slightly lower than, that seen for red clover extracts from which *o*-diphenols were depleted (Fig. 1). It is interesting to note that virtually no proteolysis was detected in the extracts of the NEWRC27 and NEWRC30 control plants used in this experiment.

Polyphenol Oxidase Can Inhibit Postharvest Proteolysis in Alfalfa in an *o*-Diphenol–Dependent Manner

We next tested whether PPO and *o*-diphenols are sufficient to inhibit postharvest proteolysis in alfalfa. To establish a model system, we transformed alfalfa with the red clover PPO1 gene (PPO-Alfalfa) and identified a plant with PPO activity comparable to that of red clover (Sullivan et al., 2004a). Alfalfa transformed with the expression vector pILTAB357 served as a control (Control-Alfalfa), and differed from the PPO-Alfalfa only in the presence of the red clover PPO1 transgene. We prepared leaf extracts of PPO- and Control-Alfalfa and analyzed the extent of proteolysis in them by measuring the release of free amino acids over time as described above.

As shown in Fig. 4, substantial proteolysis was seen in extracts of Control-Alfalfa with the extent of proteolysis comparable to that seen in *o*-diphenol–depleted red clover extracts (Fig. 1). Unlike desalted red clover extracts, the addition of caffeic acid had no effect on pro-

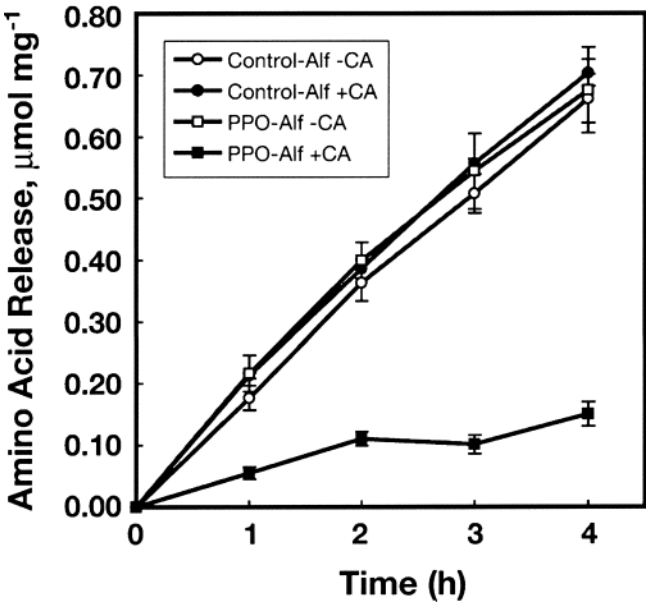

Fig. 4. PPO inhibits proteolysis in alfalfa extracts in an *o*-diphenol-dependent manner. Proteolysis, as amino acid release over time, was measured in leaf extracts from Control- (Control-Alf, ○, ●) or PPO-Alfalfa (PPO-Alf, □, ■) in the absence (○, □) or presence (●, ■) of added caffeic acid (CA, 3 m*M* final concentration). Results are the average ± SEM of three experiments using independently prepared extracts.

teolysis (Fig. 4) indicating that caffeic acid itself is not acting as a protease inhibitor. Proteolysis in extracts of PPO-Alfalfa was indistinguishable from that of Control-Alfalfa (Fig. 4) in the absence of added *o*-diphenol. The addition of caffeic acid, however, resulted in a significant ($P < 0.005$ at all time points) inhibition of proteolysis over the 4-h assay period comparable to that seen in red clover (compare Fig. 1 and 4).

PPO and *o*-Diphenol Dependence of Proteolytic Inhibition

The transgenic alfalfa system described above provided an excellent model with which to assess the PPO and *o*-diphenol requirements for proteolytic inhibition. Using PPO- and Control-Alfalfa plants allowed experimental manipulation of PPO activity levels, and since alfalfa lacks endogenous *o*-diphenol PPO substrates, these were manipulated by simple addition to extracts.

We first examined the PPO dependence of proteolytic inhibition by making a series of dilutions of PPO-Alfalfa extracts of known PPO activity in Control-Alfalfa extract, maintaining a total protein concentration of 2 mg mL^{-1}. Proteolysis, measured as amino acid release following a 4-h incubation at 37°C, was then assessed in the extracts with or without caffeic acid added to a final concentration of 3 m*M*. Proteolysis in the absence of caffeic acid (Fig. 5A) was similar to that seen in the experiment presented in Fig. 4 and did not vary significantly with the amount of PPO activity present in the incubation. In contrast, when the *o*-diphenol caffeic acid was present in the incubation (Fig. 5A), proteolysis was reduced by about twofold (compared to the control lacking *o*-diphenol) at the lowest level of PPO activity tested (0.26 nkat mL^{-1}) to greater then 10-fold at the highest PPO activity tested (8.33 nkat mL^{-1}). The inhibition was significant ($P < 0.022$) at all tested levels of PPO. Substantial proteolytic inhibition, about fivefold, was seen with modest levels (2.08 nkat mL^{-1}) of PPO activity.

To examine *o*-diphenol dependence of proteolytic inhibition, we combined extracts of Control- and PPO-Alfalfa to contain a fixed level (4.17 nkat mL^{-1}) of PPO activity at a total protein concentration of 2 mg mL^{-1}. Control-Alfalfa extract at a protein concentration of 2 mg mL^{-1} served as a negative control. Caffeic acid was added to the extracts at varying levels and the release of amino acids following a 4-h incubation at 37°C was measured. Proteolysis in the absence of PPO (Fig. 5B) was similar to that seen in the previous experiment (Fig. 4) and did not vary significantly with the amount of caffeic acid present in the incubation. In contrast, when PPO was present in the incubation (Fig. 5B), proteolysis was reduced by more than twofold (compared to the no PPO control) at the lowest level of caffeic acid tested (0.5 m*M*) to greater than 10-fold at the highest caffeic acid concentration tested (10 m*M*). Proteolytic inhibition was significant ($P < 0.002$) at all tested levels of caffeic acid. A substantial proteolytic inhibition, about fivefold, was seen with 2 to 3 m*M* levels of caffeic acid.

We also tested the efficacy of several additional *o*-diphenols in PPO-mediated proteolytic inhibition including hydrocaffeic acid (3,4-dihydroxyhydrocinnamic acid), chlorogenic acid, catechol, and (−)-epicatechin. PPO- and Control-Alfalfa extract were combined to contain 4.17 nkat mL^{-1} PPO activity at a final protein concentration of 2 mg mL^{-1}. Control-Alfalfa extract at a protein concentration of 2 mg mL^{-1} served as a negative control. *o*-Diphenols were added to the extracts at a final concentration of 3 m*M* and the release of amino acids following a 4-h incubation at 37°C was measured. In the absence of PPO, none of the tested *o*-diphenols had a significant effect on proteolysis compared to a no *o*-diphenol control (Fig. 6). Also, the presence of PPO in the absence of *o*-diphenol had no significant effect on

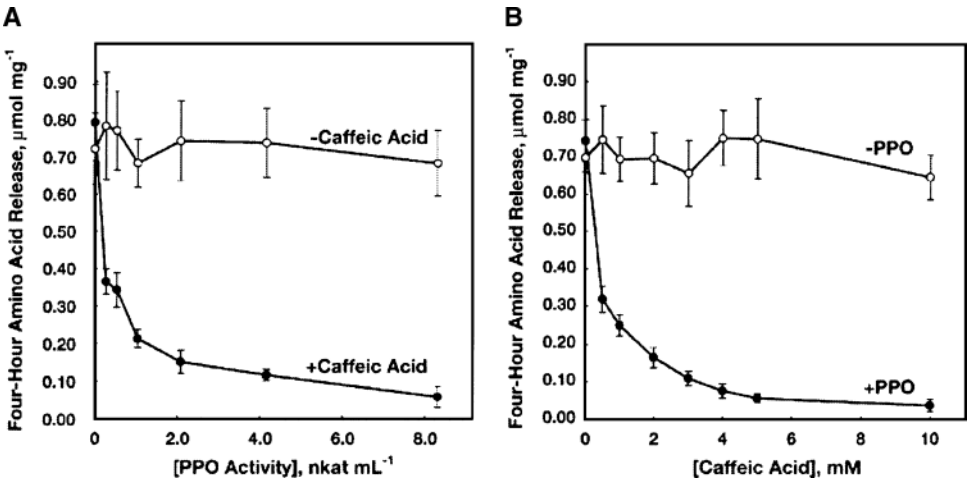

Fig. 5. Proteolytic inhibition in alfalfa extracts requires relatively little PPO or *o*-diphenol. (A) Alfalfa extracts (2 mg mL^{-1} protein) of varying PPO concentration were prepared by mixing PPO- and Control-Alfalfa extracts to give the desired level of PPO activity. The resulting extracts were incubated with (●) or without (○) 3 m*M* caffeic acid as indicated and proteolysis, as amino acid release after 4 h, was measured. PPO activity (caffeic acid substrate) is given as nkat mL^{-1}. (B) Control- (○) or PPO- containing (●, 4.17 nkat mL^{-1}) alfalfa extracts (2 mg mL^{-1} protein) were incubated with various levels of caffeic acid and proteolysis, as amino acid release after 4 h, was measured. Results for both experiments are the average ± SEM of three experiments using independently prepared extracts.

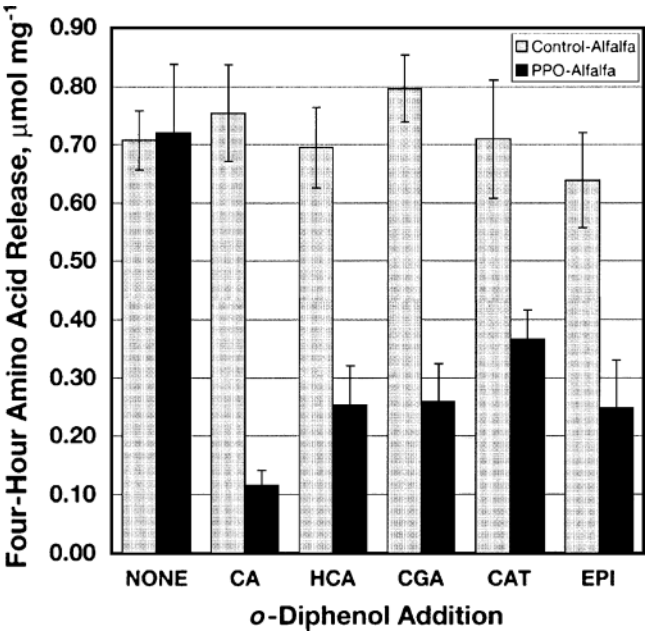

Fig. 6. A variety of *o*-diphenol compounds can function with PPO to inhibit proteolysis. Control- (gray bars) or PPO-containing (black bars, 4.17 nkat mL^{-1}) alfalfa extracts (2 mg mL^{-1} protein) were incubated with various *o*-diphenols (3 m*M* final concentration) and proteolysis, as amino acid release after 4 h, was measured. The tested *o*-diphenols were caffeic acid (CA), hydrocaffeic acid (HCA), chlorogenic acid (CGA), catechol (CAT), and (−)-epicatechin (EPI). Addition of ethanol (the solvent used for the *o*-diphenol stock solutions) served as a negative control (NONE). Results are the average ± SEM of three experiments using independently prepared extracts.

proteolysis. In contrast, the presence of PPO (Fig. 6) and any of the tested *o*-diphenols resulted in significant decreases (two- to sixfold) in proteolysis compared to either the corresponding Control-Alfalfa control ($P < 0.02$) or the no *o*-diphenol control ($P < 0.026$). Caffeic acid was the most effective at reducing proteolysis (sixfold reduction), although the approximately threefold reductions in proteolysis seen with hydrocaffeic acid, chlorogenic acid, and (−)-epicatichin were not significantly different from the caffeic acid result ($P > 0.1$). Catechol was significantly less effective than caffeic acid at reducing proteolysis ($P < 0.02$), although the twofold decrease in proteolysis compared to controls was significant ($P < 0.026$).

PPO and *o*-Diphenols Inhibit Proteolysis in Ensiled Alfalfa

We also tested whether the combination of PPO and *o*-diphenols is effective at inhibiting proteolysis in ensiled alfalfa. Because alfalfa lacks significant amounts of endogenous *o*-diphenol PPO substrates, an ethanol solution of *o*-diphenol was applied to the tissue before maceration. Since exogenously applied *o*-diphenols might have difficulty diffusing throughout the macerated plant material, we decided to use an *o*-diphenol that is less readily utilized by the red clover PPO present in our transgenic alfalfa, reasoning that the slower oxidation rate by PPO might allow better substrate diffusion before formation of reactive *o*-quinones. Consequently, catechol was selected as the *o*-diphenol in these experiments because its reaction rate with the PPO present in the transgenic alfalfa is 10- to 20-fold lower than that of caffeic acid (Sullivan et al., 2004b), yet it still is able to substantially reduce proteolysis as demonstrated in extract experiments (Fig. 6).

Two separate ensiling experiments were performed with PPO- and Control-Alfalfa treated before maceration with catechol at a level comparable to that used in the extract experiments (14 μmol g^{-1} tissue, which should correspond to approximately 2 μmol mg^{-1} protein). Because we had limited amounts of plant material and the extract experiments clearly showed that *o*-diphenols alone have no effect on proteolysis, we did not carry out control ensilings without *o*-diphenol addition. Samples of the macerated material before and after ensiling were analyzed for DM, pH, total N, and amino acid N (5% TCA-soluble, ninhydrin-reactive N). Similar results were seen in both experiments. Final silage pH values (Table 1) indicate there were not major differences in fermentation between Control- and PPO-Alfalfa, although PPO-Alfalfa silage had a slightly higher final pH than Control-Alfalfa in Experiment 2 that was statistically significant ($P < 0.003$). In both experiments, the amount of total N present as 5% TCA-soluble amino acids before ensiling did not differ

Table 1. Ensiling data for catechol-treated PPO- and Control-Alfalfa.†

	Experiment 1		Experiment 2	
	Control-Alfalfa	PPO-Alfalfa	Control-Alfalfa	PPO-Alfalfa
Initial conditions				
DM, %	42.0	38.3	42.0	39.7
pH‡	6.30 ± 0.01	6.27 ± 0.01	6.42 ± 0.03	6.27 ± 0.02
Total N, mg g^{-1} DM	34.4	35.3	30.7	32.0
AA N‡§, % total N	9.4 ± 0.3	9.1 ± 0.0	7.4 ± 0.1	7.6 ± 0.2
Final conditions				
DM‡, %	41.0 ± 0.5	38.2 ± 0.1	40.9 ± 0.2	39.3 ± .2
pH‡	4.75 ± 0.05	4.86 ± 0.01	4.81 ± 0.01	4.86 ± 0.01**
Total N‡§, mg g^{-1} DM	41.5 ± 0.1	39.9 ± 0.5	33.9 ± 0.5	32.5 ± 0.5
AA N‡§, % total N	32.0 ± 0.8	27.1 ± 0.1**	37.7 ± 0.2	33.6 ± 0.6**
Change AA N‡§, % total N	22.6 ± 0.8	18.1 ± 0.1**	30.3 ± 0.2	26.0 ± 0.8**

** Significant at <0.01 probability level.
† Catechol added at a rate of 14 μmol g^{-1} tissue.
‡ Average value ± SEM.
§ TCA-soluble amino acid N assuming one N per amino acid.

significantly between Control- and PPO-Alfalfa ($P > 0.3$). Analysis of samples following ensiling, however, indicate less amino acid N was released in PPO-Alfalfa compared to Control-Alfalfa (18.1 vs. 22.6% and 26.0 vs. 30.3% of total N for Experiment 1 and Experiment 2, respectively). These reductions are significant ($P < 0.003$) and represent a 14 to 20% decrease in in-silo proteolysis for PPO-Alfalfa.

DISCUSSION

Studies have consistently shown that when ensiled, red clover experiences significantly less degradation of protein to NPN compared to many other forages, especially alfalfa (Albrecht and Muck, 1991; Jones et al., 1995c; Papadopoulos and McKersie, 1983). Several lines of evidence suggested that the postharvest proteolytic inhibition seen in red clover is due to the action of PPO on endogenous *o*-diphenols. It was, however, difficult to rule out other possible mechanisms, such as the presence of specific protease inhibitors in red clover. Using biochemical and transgenic approaches, we have now been able to rigorously assess the role of PPO and *o*-diphenols in postharvest proteolytic inhibition in forage legumes and begin to define some of the critical parameters of the system.

For many of our experiments, we assessed the extent of proteolysis in leaf extracts by measuring the release of TCA-soluble amino acids over time. The highest levels of proteolysis measured (e.g., in control alfalfa or *o*-diphenol–depleted red clover extracts) corresponded to the release of approximately 0.7 μmol amino acids mg^{-1} protein during the course of the 4-h assay and represent degradation of a significant amount of the protein present in the extract. Assuming an average amino acid residue molecular weight of 110 daltons, this would correspond to the release of nearly 8% of the amino acids originally present as protein. This may be an underestimation of the extent of proteolysis taking place since our assay will only measure release of free amino acids and short peptides. Proteolytic events that result in larger peptides (i.e., endoproteolytic cleavages) would not be detected. Our measured amino acid release values for alfalfa and *o*-diphenol– or PPO-depleted red clover extracts compare well with those of similar studies utilizing red clover and/or alfalfa (Jones et al., 1995c; Lee et al., 2004). Although our values are slightly higher, this may be due to the slightly higher temperature and lower pH conditions we employed in the assays.

We tested the requirement of *o*-diphenols in proteolytic inhibition by removing low molecular weight compounds from red clover extracts using gel filtration. *o*-Diphenol depletion resulted in substantial increases in proteolysis in the extracts to a level comparable to those seen in alfalfa extracts (compare Fig. 1 and 4). The addition of an *o*-diphenol, caffeic acid, to depleted extracts restored proteolytic inhibition to levels indistinguishable from those of the non-depleted extracts. Although this does not rule out the possibility that caffeic acid acts on its own as a protease inhibitor, subsequent experiments with alfalfa extracts show that caffeic acid addition alone has no significant effect on proteolysis (Fig. 4, 5, 6) even at concentrations as high as 10 m*M*.

Using a RNAi approach to generate red clover plants with undetectable levels of foliar PPO, we tested the requirement of PPO for proteolytic inhibition. Extracts of these plants had significantly enhanced proteolysis compared to those of control plants that differed only by the expression of PPO (Fig. 3). Although the absolute level of amino acid release in extracts of the silenced plants was slightly lower than that seen for *o*-diphenol–depleted extracts (Fig. 1), the differences in amino acid release compared to the corresponding controls in each experiment were indistinguishable ($P > 0.2$). Modest proteolysis (0.15 μmol mg^{-1} protein over 4 h) was seen in the control plants in the depletion experiment (Fig. 1), but virtually none was seen in the control plants in the PPO-silencing experiment (Fig. 3). This slight difference in extent of proteolysis during the 4-h incubation could be due to genotype differences between the plants used for the depletion and the PPO-silencing experiments.

We extended these finding using transgenic alfalfa expressing red clover PPO as a model system (Sullivan et al., 2004a). Because alfalfa lacks significant levels of endogenous foliar PPO activity and *o*-diphenols, by making transgenic alfalfa that expresses active PPO enzyme, we were able to develop a system in which both PPO activity and *o*-diphenols could be experimentally manipulated. Utilizing this system, we demonstrated that PPO is capable of inhibiting proteolysis in alfalfa extracts in an *o*-diphenol–dependent manner and that the extent of this inhibition is comparable to that seen for red clover. Together, these results demonstrate that PPO and *o*-diphenols are necessary for the postharvest proteolytic inhibition that has been observed in red clover and sufficient to mediate proteolytic inhibition in plant extracts normally lacking these components. Because PPO-silenced transgenic red clover and PPO-expressing transgenic alfalfa had corresponding controls of the same genotype, our experiments allow us to rule out other possible confounding factors such as genetic variation for levels of proteases or protease inhibitors that might interfere with the assays.

Given our success at reconstituting PPO-mediated proteolytic inhibition in alfalfa extracts, we used this system to further characterize the process. With this approach, we were able to determine that modest levels of PPO activity (2.08 nkat mL^{-1}) are capable of inhibiting proteolysis approximately 80% in an alfalfa extract and that even lower levels of PPO can significantly inhibit proteolysis, provided an exogenous *o*-diphenol substrate is supplied. Typical red clover extracts prepared as described in this report have PPO activities in the range of 15 to 120 nkat mL^{-1}(Sullivan et al., 2004a) indicating levels of PPO activity 10- to 20-fold lower than that of red clover are sufficient to inhibit postharvest proteolysis. This finding is consistent with the observation of Lee et al. (2004) that release of free amino acids (and extent of proteolysis) in extracts of a mutant line of red clover with only a fivefold reduction in PPO activity (cv. Milvus) did not differ significantly

from that in extracts of a wild-type line with normal levels of PPO activity. Using caffeic acid as an *o*-diphenol, we were also able to show that proteolytic inhibition is substantial (80–90% inhibition) in the 2 to 4 m*M* range (1–2 μmol mg^{-1} protein) in PPO-containing alfalfa extracts although significant inhibition is seen with as little as 0.5 m*M* (0.25 μmol mg^{-1} protein). We have identified the predominant *o*-diphenols present in red clover as the caffeic acid derivatives phasic acid and clovamide (R. Hatfield, unpublished data, 2000). Unfortunately, we have been unable to quantify the levels present in red clover leaves due to the lack of appropriate standards and efforts to hydrolyze them to caffeic acid moieties have failed. However, based on the extent of browning, we estimate these caffeic acid derivatives to be present at levels about 10 to 15 μmol g^{-1} FW, an amount comparable to those we used in our proteolysis assays (1–2 μmol mg^{-1} protein).

We also examined the effectiveness of various *o*-diphenols in PPO-mediated proteolytic inhibition using a range of *o*-diphenols that varied in chemical properties (structure, size, polarity) and in how well they are utilized by the red clover PPO expressed in the transgenic alfalfa. Although caffeic acid was most effective among the tested *o*-diphenols (proteolysis was inhibited about 80%), all showed substantial proteolytic inhibition. The poorest performer, catechol, still inhibited proteolysis by about 50%. We did not observe any strict correlation between how well an *o*-diphenol is utilized by red clover PPO and how effective that substrate is in proteolytic inhibition. For example, chlorogenic acid, hydrocaffeic acid, and (−)-epicatichin, are utilized 80, 20, and 5% as well as caffeic acid by red clover PPO1 (Sullivan et al., 2004b), respectively, yet all three inhibit proteolysis to about the same extent in the presence of PPO. Even the relatively slow oxidation of a poor PPO substrate like (−)-epicatechin to its corresponding *o*-quinone is capable of inhibiting proteolysis measured over several hours. Thus, it might be expected that even poor substrates for PPO could be quite effective at inhibiting postharvest proteolysis. Still, we did observe a significant difference in the effectiveness at inhibiting proteolysis of catechol versus caffeic acid. It may be that such differences in proteolytic inhibition are due to differences in reactivities of the corresponding PPO-generated quinones. Another possibility is that if quinones ultimately modify proteins by reacting with nucleophilic sites, differing chemical structures of the phenolic moieties might have differential effects on protein structure or enzyme active site function.

Finally, we also tested whether the PPO–*o*-diphenol system is capable of inhibiting proteolysis in ensiled alfalfa. We expected the exogenous application of an *o*-diphenol PPO substrate to plant tissue would be less effective than addition to plant extracts, since there are physical limitations to diffusion of the substrate. For this reason, we macerated PPO- and Control-Alfalfa tissues with a meat grinder to better disrupt them and expose cellular contents. Catechol was used as the *o*-diphenol in these ensiling experiments because we reasoned that its slower oxidation by PPO might allow greater diffusion throughout plant tissue before formation of reactive quinones. Despite the lower efficacy of catechol in inhibiting proteolysis in alfalfa extracts, we saw a 14 to 20% reduction in release of TCA-soluble amino acids in ensiled PPO-Alfalfa compared to Control-Alfalfa in two experiments. The reduction in proteolysis seen for the PPO-Alfalfa cannot be attributed to better fermentation of this material, since the final pH of the PPO-Alfalfa silage was actually slightly higher than that of Control-Alfalfa. It will be interesting to see how the type and amount of *o*-diphenol added and the extent of tissue maceration contribute to PPO-mediate proteolytic inhibition in ensiled forages.

The ensiling results, along with the results of the extract experiments indicate that the PPO–*o*-diphenol system can be used to prevent breakdown of protein to NPN in ensiled forages provided PPO and *o*-diphenol PPO substrates are present or can be supplied. As demonstrated in this report, relatively low levels of PPO are sufficient for proteolytic inhibition and can be easily supplied, if necessary, by introduction of a transgene. Because a wide range of *o*-diphenols can function with PPO to inhibit postharvest proteolysis, these could be supplied exogenously using byproducts from food processing provided *o*-diphenols from such sources were affordable. Additionally, methodologies and equipment for the effective application of *o*-diphenols and maceration of plant material would likely need to be developed. Alternatively, pathways involved in plant secondary metabolism could be modified to produce utilizable *o*-diphenols in forages which lack them (Dixon, 2005). This latter approach would likely not require significant modification of current production practices.

Although it is not clear exactly how PPO-generated *o*-quinones limit postharvest proteolysis, it seems likely that PPO-generated quinones react with nucleophilic sites on cellular proteins. Such protein modifications could directly inactivate proteases, make proteins poorer proteolytic substrates, or both. Given how rapidly proteolysis is inhibited (Jones et al., 1995c; Lee et al., 2004), direct inactivation of proteases is an attractive model. Indeed, proteases have been shown to be inhibited by incubation in red clover extracts (Hatfield and Muck, 1999), and data from Lee et al. (2004) suggest that lipolytic enzymes are inhibited by quinone modification as well. Such modifications near enzyme active sites or on enzyme surfaces may be sufficient to inactivate them. We are currently using our red clover and alfalfa systems to elucidate the mechanisms whereby PPO and *o*-diphenols inhibit postharvest proteolysis. Although extensive modification of proteins by PPO-generated quinones might be expected to decrease the nutritional value of forages, animal feeding studies comparing red clover and alfalfa silage indicate that animal performance is not dramatically impacted by diets where red clover is the sole forage and such diets result in decreased excretion of N by animals (Broderick et al., 2000; Broderick et al., 2001). Further studies will be needed, however, to determine whether PPO-generated quinones negatively impact the nutritional value of forages and animal performance.

In the course of the study detailed here, we transformed red clover plants with an intron-spliced, hairpin-RNA gene silencing construct. Although we analyzed a limited number of plants, all (four out of four) showed reduced foliar PPO protein and activity compared to control plants and two lacked detectible PPO protein and activity. This result indicates that RNAi is highly effective in red clover both in terms of number of plants displaying silencing and in extent of silencing. We expect that RNAi will prove useful in analyzing gene function in red clover. Additionally, the PPO-silenced plants generated in this study will be useful for further characterization of PPO with respect to in vivo function, as well as how it impacts proteolysis and other aspects of postharvest quality.

CONCLUSIONS

The combination of PPO and *o*-diphenols is necessary to inhibit postharvest proteolysis in red clover and sufficient to inhibit proteolysis in alfalfa which normally lacks these components. These results indicate that this natural system of protein protection could be utilized to prevent breakdown of protein to NPN in a variety of ensiled forages.

ACKNOWLEDGMENTS

We wish to thank Tyson Wolfe and Sara Rierson for excellent technical assistance, Ken Quesenberry for providing regenerable red clover genotypes and technical advice on red clover transformation, Peter Waterhouse and CSIRO for provision of pHANNIBAL and pART27, Richard Muck and Ursula Hymes-Fecht for assistance in designing and carrying out ensiling experiments, and Jane Marita for helpful comments on the manuscript.

REFERENCES

Albrecht, K.A., and R.E. Muck. 1991. Proteolysis in ensiled forge legumes that vary in tannin concentration. Crop Sci. 31:464–469.

Bingham, E.T. 1991. Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. Crop Sci. 31:1098.

Broderick, G.A., R.P. Walgenbach, and S. Malgnan. 2001. Production of lactating dairy cows fed alfalfa or red clover silage at equal dry matter or crude protein contents in the diet. J. Dairy Sci. 84: 1728–1737.

Broderick, G.A., R.P. Walgenbach, and E. Sterrenburg. 2000. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. J. Dairy Sci. 83:1543–1551.

Dixon, R.A. 2005. Engineering of plant natural product pathways. Curr. Opin. Plant Biol. 8:329–336.

Esterbauer, H., E. Schwarzl, and M. Hayn. 1977. A rapid assay for catechol oxidase and laccase using 2-nitro-5-thio benzoic acid. Anal. Biochem. 77:486–494.

Gleave, A.P. 1992. A versatile binary vector system with a T-DNA organizational-structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. 20:1203–1207.

Hatfield, R.D., and R. Muck. 1999. Characterizing proteolytic inhibition in red clover, p. 147–148. *In* XIIth Int. Silage Conference, Upsala, Sweden. 5–7 July 1999. Swedish Univ. of Agriculture and Sciences, Upsala, Sweden.

Hood, E.E., S.B. Gelvin, L.S. Melchers, and A. Hoekema. 1993. New agrobacterium helper plasmids for gene transfer to plants. Transgenic Res. 2:208–218.

Jones, B.A., R.D. Hatfield, and R.E. Muck. 1995a. Characterization of proteolysis in alfalfa and red clover. Crop Sci. 35:537–541.

Jones, B.A., R.D. Hatfield, and R.E. Muck. 1995b. Screening legume forages for soluble phenols, polyphenol oxidase and extract browning. J. Sci. Food Agric. 67:109–112.

Jones, B.A., R.E. Muck, and R.D. Hatfield. 1995c. Red clover extracts inhibit legume proteolysis. J. Sci. Food Agric. 67:329–333.

Lee, M.R.F., A.L. Winters, N.D. Scollan, R.J. Dewhurst, M.K. Theodorou, and F.R. Minchin. 2004. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. J. Sci. Food Agric. 84:1639–1645.

Moore, S. 1968. Amino acid analysis: Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. 243:6281–6283.

Muck, R.E. 1987. Dry matter level effects on alfalfa silage quality. I. Nitrogen transformation. Trans. ASAE 30:7–14.

NASS. 2005. Crop production 2004 summary. USDA-NASS Cr Pr 2–1 (05) U.S. Gov. Print. Office, Washington, DC.

Papadopoulos, Y.A., and B.D. McKersie. 1983. A comparison of protein degradation during wilting and ensiling of six forage species. Can. J. Plant Sci. 63:903–912.

Quesenberry, K.H., D.S. Wofford, R.L. Smith, P.A. Krottje, and F. Tcacenco. 1996. Production of red clover transgenic for neomycin phosphotransferase II using Agrobacterium. Crop Sci. 36: 1045–1048.

Rogers, S.G., R.B. Horsch, and R.T. Fraley. 1986. Gene transfer in plants—Production of transformed plants using Ti-plasmid vectors. Methods Enzymol. 118:627–640.

Rotz, C.A., R.E. Pitt, R.E. Muck, M.S. Allen, and D.R. Buckmaster. 1993. Direct-cut harvest and storage of alfalfa on the dairy farm. Trans. ASAE 36:621–628.

Smith, R.R., and D.P. Maxwell. 1980. Registration of WI-1 and WI-2 red clover. Crop Sci. 20:831.

Smith, R.R., and K.H. Quesenberry. 1995. Registration of NEWRC red clover germplasm. Crop Sci. 35:295.

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

Sullivan, M.L., and K.H. Quesenberry. 2006. Red Clover. *In* K. Wang (ed.) Agrobacterium Protocols. 2nd ed. Humana Press, Totowa, NJ. (in press).

Sullivan, M., S. Thoma, D. Samac, and R. Hatfield. 2004b. Cloning of red clover and alfalfa polyphenol oxidase genes and expression of active enzymes in transgenic alfalfa. p. 189–195. *In* A. Hopkins et al (ed.) Molecular breeding of forage and turf. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Wang, M.-B., and P.M. Waterhouse. 2002. Application of gene silencing in plants. Curr. Opin. Plant Biol. 5:146–150.

Wesley, S.V., C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, and P.M. Waterhouse. 2001. Contruct design for efficient, effective, and high-throughput gene silencing in plants. Plant J. 27:581–590.