Cloning of HCT and C3H Genes from Red Clover and Characterization of the Encoded Enzymes

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Many forages, including alfalfa (*Medicago sativa*), experience significant proteolytic losses when preserved by ensiling. An exception is red clover (*Trifolium pratens*e), which experiences up to 90% less proteolysis than alfalfa when ensiled. My laboratory has recently demonstrated that the reduced level of post-harvest proteolysis seen in red clover is due to the oxidation of endogenous *o*-diphenols by a polyphenol oxidase (PPO, see Fig. 1A) (Sullivan and Hatfield, 2006, *Crop. Sci.* 46:662). Proteolytic inhibition is presumably the result of the PPO-generated *o*-quinones reacting with nucleophilic sites on proteases, protease substrates, or both. We have also shown that post-harvest proteolysis can be reduced in alfalfa (which normally lacks significant levels of PPO and *o*-diphenol PPO substrates) by transformation with a red clover PPO gene and treatment with exogenous *o*-diphenols. The lack of endogenous *o*-diphenol PPO substrates in alfalfa leaves prevents practical use of this natural system of protein protection in this important forage crop. As a first step towards overcoming this limitation, we are trying to understand the biosynthesis and accumulation of relatively large amounts of the caffeic acid derivatives phasic acid and clovamide in red clover (Fig. 1B). Work has begun to identify key enzymes involved in the biosynthesis of *o*-diphenols and related phenylpropanoid compounds in *Arabidopsis*, basil, and other plant species (Fig. 1C). We have isolated cDNA clones from a red clover leaf cDNA library corresponding to some of these key enzymes including phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), *p*-coumaroyl CoA ligase (4CL), hydroxycinnomoyl transferase (HCT), and *p*-coumaric acid 3-hydroxylase (C3H). We are focusing on HCT and C3H, hypothesizing that their substrate specificities could be related to the accumulation of specific *o*-diphenols such as phasic acid (i.e., R=malic acid in Fig. 1C). To analyze the substrate specificities of the red clover HCT and C3H, we are expressing them in *Escherichia coli* and *Saccharomyces cerevisiae*, respectively. Concurrently, we are using RNAi to down-regulate the genes in red clover, and will assess the impact of down-regulation on accumulation of phasic acid and related phenylpropanoid compounds.

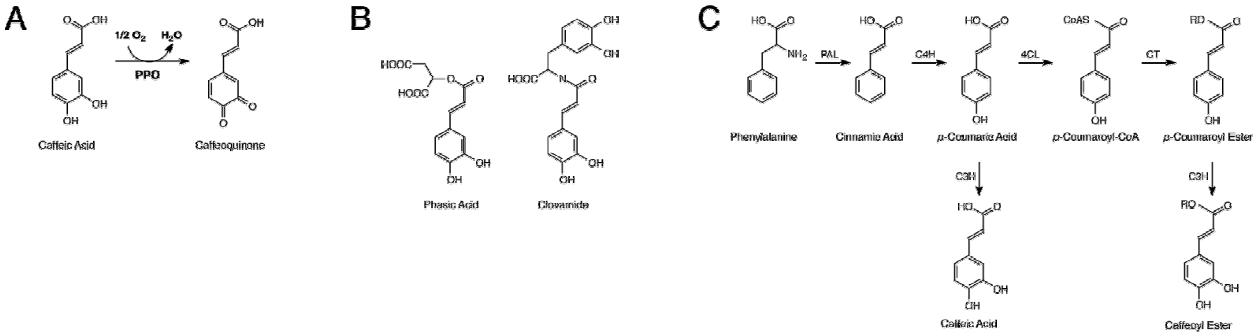

Figure 1. (A) *PPO catalyzes the oxidation of* o*-diphenols to their corresponding* o*-quinones.* (B) *Chemical structures of phasic acid and clovamide,* o*-diphenols abundant in red clover leaves.* (C) *Possible pathway for the biosynthesis of caffeic acid derivatives such as phasic acid. Direct hydroxylation of* p*-coumaric acid by C3H is not thought to occur* in vivo.