HCT2, a Novel Hydroxycinnamoyl:Malate Transferase, is Responsible for Phaselic Acid (2-*O*-Caffeoyl-L-Malate) Biosynthesis in Red Clover

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In red clover, post-harvest oxidation of *o*-diphenol caffeic acid derivatives to *o*-quinones by an endogenous polyphenol oxidase (PPO) prevents breakdown of forage protein during storage (1). Agronomically important forages like alfalfa lack both PPO and *o*-diphenols. Consequently, breakdown of their protein upon harvest and storage results in economic losses ($100 million/yr in the US alone) and release of excess nitrogen into the environment. Understanding how red clover is able to synthesize and accumulate *o*-diphenols will help in development of forages that take advantage of this natural system of protein protection. My laboratory has focused on biosynthesis of phaselic acid (caffeoyl-malate), a major *o*-diphenol in red clover leaves that accumulates to approximately 0.5% of dry matter.

Using a bioinformatics approach, we identified and cloned a novel hydroxycinnamoyl-CoA:malate hydroxycinnamoyl transferase, HCT2, from red clover (2). Detailed kinetic analyses indicate the enzyme can transfer *p*-coumaroyl, caffeoyl-, and feruloyl moieties from their corresponding CoA derivatives to malic acid (Fig. 1). HCT2 can carry out the reverse reaction (formation of hydroxycinnamoyl-CoA from hydroxycinnamoyl-malate) for *p*-coumaroyl-malate, but not for phaselic acid. An apparent lack of a 3'-hydroxylating activity capable of converting *p*-coumaroyl-malate to phaselic acid in red clover suggests that in vivo, phaselic acid is formed by transfer of caffeoyl moieties to malic acid by HCT2 (3).

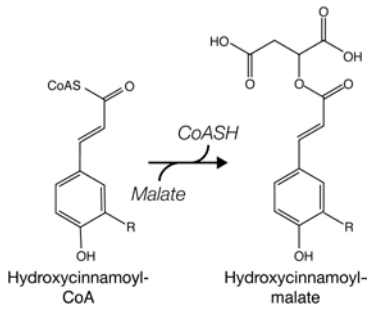

Fig. 1*. Transfer of hydroxy-cinnamoyl moieties to malate by HCT2. R=H, OH, or OCH_3 for* p-*coumaroyl, caffeoyl, and feruloyl derivatives, respectively.*

To demonstrate the in vivo role of HCT2 in phaselic acid biosynthesis, red clover was transformed with a hairpin RNAi gene construct to silence HCT2 expression. Analysis of eleven independent transformants and their three corresponding wild type controls demonstrated a significant and substantial correlation between HCT2 mRNA levels and phaselic acid accumulation ($P<0.001$). In several of the HCT2-silenced plants, phaselic acid and *p*-coumaroyl-malate accumulated to nearly undetectable levels compared to wild type controls. These reductions result in easily observable phenotypes including reduced PPO-mediated browning (Fig. 2) and a reduction in blue epidermal fluorescence under UV-light.We also transformed alfalfa with the red clover HCT2 gene. Leaves of these plants have HCT2 activity approaching that of wild type red clover and accumulate *p*-coumaroyl- and feruloyl-malate, and to a lesser extent phaselic acid. We are currently using these plants to better understand the phaselic acid biosynthetic pathway and as a starting point for optimization of phaselic acid accumulation in alfalfa.

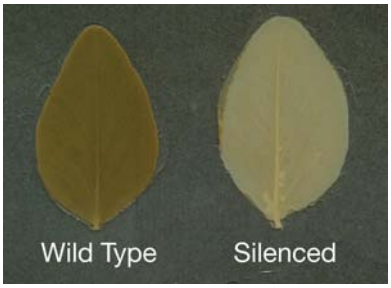

Fig. 2*. PPO-mediated browning of wild type and HCT2-silenced red clover leaves. Leaves were allowed to wilt prior to extraction of chlorophyll with ethanol.*

1. Sullivan, ML and Hatfield, RD (2006) Crop Sci. 46:662.
2. Sullivan, ML (2009) Plant Physiol. 150:1866.
3. Sullivan, ML and Zarnowski, R (2010) Planta 231:319.