Post-Transcriptional Silencing of Polyphenol Oxidase Gene Expression in Red Clover

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Post-transcriptional gene silencing (PTGS) by transformation of anti-sense, co-suppression, or hairpin RNA-producing constructs is a powerful tool to analyze *in vivo* gene function in plants. We are using this approach to reduce polyphenol oxidase (PPO) gene expression in red clover in order to better understand the role of PPO in inhibition of post-harvest proteolysis as well to elucidate the *in vivo* functions of the red clover PPO gene products. Since we have several tools available to analyze PPO gene expression (gene-specific nucleic acid probes, anti-PPO antibodies, and easy enzyme activity assays), the PPO system also provides an excellent way to evaluate PTGS as a tool for analyzing gene function in red clover.

To silence PPO1 (Genbank AY017302), the major PPO expressed in red clover leaves, we cloned an ~1100 bp fragment from the PPO1 gene into the sense and antisense arms of the intron-containing gene silencing vector pHannible (*Plant J.* 27:581). The silencing cassette was subcloned into the binary vector pART27 and transformed into red clover by *Agrobacterium*-mediated transformation using a procedure modified from Quesenberry *et al*. (*Crop Sci.* 36:1045). We have analyzed the resulting kanamycin resistant transformants from two red clover genotypes by immunoblotting, quantitative enzyme activity assays, and extract browning. Although to date we have assessed silencing in only four transgenic plants, all show marked reductions in PPO expression compared to nontransformed plants of the same genotype. In quantitative assays of PPO activity, all the transformed plants show <10% of the PPO activity of nontransformed controls. Two plants had no detectible PPO protein or enzyme activity based on immunoblotting and quantitative activity assays, respectively. Extract browning, a hallmark of PPO activity, was noticeably delayed for plants with reduced but detectible PPO activity compared to control plants (onset of browning in 1 to 4 hours for the silenced plants versus <5 minutes for control plants). Extracts of the red clover plants with no detectible PPO activity exhibited no browning, even after 48 hours.

These preliminary results strongly suggest that gene silencing by expression of intron-spliced hairpin RNAs in red clover is highly effective, both in terms of percentage of plants displaying silencing (4/4, 100%), and the degree to which the target gene is silenced (two plants with <10% PPO activity and two plants with no detectible PPO activity). Recovery of plants that appear to be completely silenced for foliar PPO1 expression suggests that PPO does not serve any essential function in red clover leaves. We are currently generating additional plants for a more thorough analysis of the efficiency and extent of silencing achievable in red clover. We will also determine whether the PPO1 silencing construct is acting to silence the related flower-expressed PPO2 gene, which may allow us to determine the *in planta* function of PPO2.