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Down-regulation of hydroxycinnamoyl CoA: Shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality

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Abstract

The recently discovered enzyme hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) catalyzes the reactions both immediately preceding and following the insertion of the 3-hydroxyl group into monolignol precursors. A number of independent transgenic lines of alfalfa (*Medicago sativa* L.) were generated in which the levels of HCT were reduced through antisense HCT expression under control of the bean PAL2 promoter which is preferentially expressed in vascular tissue. Reduction of enzyme activity in these lines was from at least 15–50%. The most severely down-regulated lines exhibited significant stunting, reduction of biomass and delayed flowering. HCT down-regulation resulted in strongly reduced lignin content and striking changes in lignin monomer composition, with predominant deposition of 4-hydroxyphenyl units in the lignin. Vascular structure was impaired in the most strongly down-regulated lines. Analysis of forage quality parameters showed strong reductions of neutral- and acid-detergent fiber in the down-regulated lines, in parallel with large increases (up to 20%) in dry matter forage digestibility. Although manipulation of lignin biosynthesis can greatly improve forage digestibility, accompanying effects on plant development need to be better understood.

Keywords: *Medicago sativa*; Leguminosae; Forage legume; Digestibility; Lignin biosynthesis; Transgenic plant

1. Introduction

Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) is a recently discovered enzyme of the monolignol pathway, catalyzing the reactions both immediately preceding and following the insertion of the 3-hydroxyl group into monolignol precursors (Hoffmann et al., 2003, 2004). 4-Coumaroyl CoA, a common precursor for both lignin and flavonoid biosynthesis, is converted by HCT to 4-coumaroyl shikimate, the substrate for hydroxylation by 4-coumaroyl shikimate 3-hydroxylase, previously known as coumarate 3-hydroxylase (C3H) (Schoch et al., 2001; Hoffmann et al., 2003) (Fig. 1). The shikimate ester is then converted back to the corresponding CoA ester by HCT reacting in the reverse direction.

An Arabidopsis mutant lacking C3H expression was characterized as a result of its lack of fluorescent sinapate esters, the biosynthesis of which similarly requires hydroxylation of a coumaroyl to a caffeoyl moiety. This mutant, called ref8, was extremely dwarf and plants exhibited poor viability (Franke et al., 2002a). Because HCT is functionally analagous to C3H with regards to its overall role in monolignol biosynthesis (i.e. together, HCT and C3H

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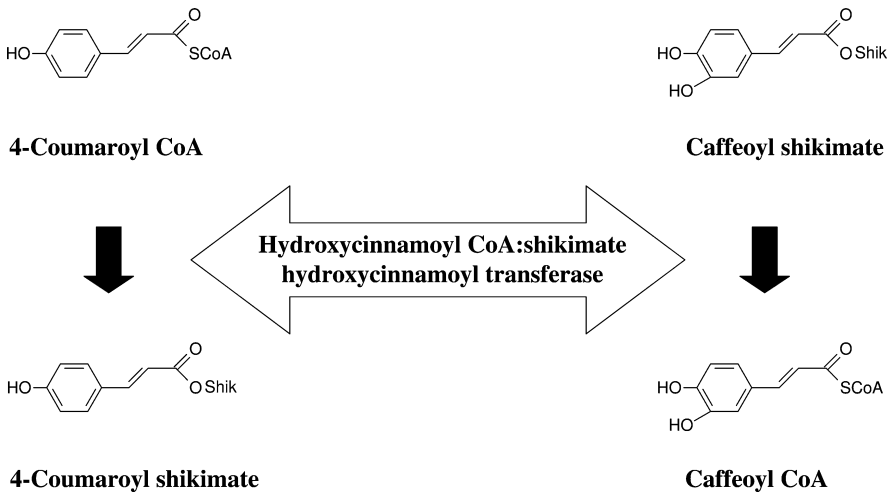

Fig. 1. The position of the reactions catalyzed by HCT in monolignol biosynthesis. HCT converts 4-coumaroyl CoA to 4-coumaroyl shikimate and then converts the resulting caffeoyl shikimate, formed by hydroxylation of 4-coumaroyl shikimate by "C3H", to caffeoyl CoA.

convert coumaroyl CoA to caffeoyl CoA), strong down-regulation of HCT should cause similar phenotypic effects as seen in REF8. To avoid this possibility, initial attempts to down-regulate HCT expression in tobacco (Hoffmann et al., 2004) utilized virus induced gene silencing (VIGS), a technique in which fully developed plants can be subject to down-regulation of the target gene. However, because lignin composition changes significantly during plant development (Lewis and Yamamoto, 1990), such an approach does not address the effects on lignification resulting from down-regulation of the target gene during the early stages of vascular development.

Lignification limits the digestibility of forages in ruminant animals, and several studies have addressed the impacts on forage quality of modifying lignin content or composition through genetic manipulation in the forage legume alfalfa (*Medicago sativa* L.) (Baucher et al., 1999; Guo et al., 2001b; Reddy et al., 2005). As part of a program to investigate the effects of multi-site modulation of monolignol biosynthesis in alfalfa, we recently reported the lignin phenotypes of two transgenic alfalfa lines significantly down-regulated in HCT expression (Chen et al., 2006). The plants were viable, with similar overall growth patterns (shorter compared to wild-type plants) and lignin compositions to alfalfa plants down-regulated in C3H expression (Reddy et al., 2005; Chen et al., 2006). To better understand the phenotypic consequences of HCT down-regulation in alfalfa, and thereby evaluate HCT as a target for forage quality improvement, we have generated additional transgenic lines expressing an HCT antisense transgene under control of the bean phenylalanine ammonia-lyase (PAL2) promoter. Down-regulation of HCT enzymatic activity to between at least 50–85% of wild-type values in these plants was not only associated with striking improvements in forage quality parameters, but also with developmental abnormalities and yield reductions.

2. Results

2.1. Generation of HCT-down-regulated transgenic alfalfa lines

The antisense constructs used a full length *M. truncatula* HCT open reading frame sequence mined from the TIGR *M. truncatula* Gene Index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago) (see Section 4). The antisense transcripts were driven by the bean PAL2 promoter, which exhibits strong expression in vascular tissue of transgenic alfalfa (Guo et al., 2001a). After co-cultivation with *Agrobacterium tumefaciens* harboring the antisense construct, or empty vector for controls, transgenic alfalfa plants were regenerated via somatic embryogenesis.

Thirty nine independent transformants (derived from independent calli) were selected for confirmation of HCT transcript levels by RNA gel blot analysis. A representative blot for 33 of these lines is shown in Fig. 2a. RNA from a number of non-transformed and empty vector control lines was also included on the blot (Fig. 2a). Five lines (7a, 14a, 15b, 29a, 30a) showed reduction of HCT transcripts to virtually undetectable levels, whereas line 15a showed an intermediate level compared to controls.

Crude extracts from stem material from the various down-regulated and control lines were assayed for extractable HCT enzyme activity (Fig. 2b). The enzyme activity determined by spectrophotometric assay was reduced by only 15–50% in the various antisense lines, but generally correlated with the transcript data (e.g. line 15a, in which transcripts were still visible by RNA gel blot, exhibited the smallest reduction in enzymatic activity). The enzyme activity determinations are probably over-estimates due to the potential presence of additional activities in the crude extracts (see Section 3), and therefore under-estimate the degree of enzyme down-regulation in the transgenic lines.