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Phytochemistry 68 (2007) 1521–1529
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

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

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Received 21 September 2006; received in revised form 27 February 2007
Available online 26 April 2007

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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doi:10.1016/j.phytochem.2007.03.022

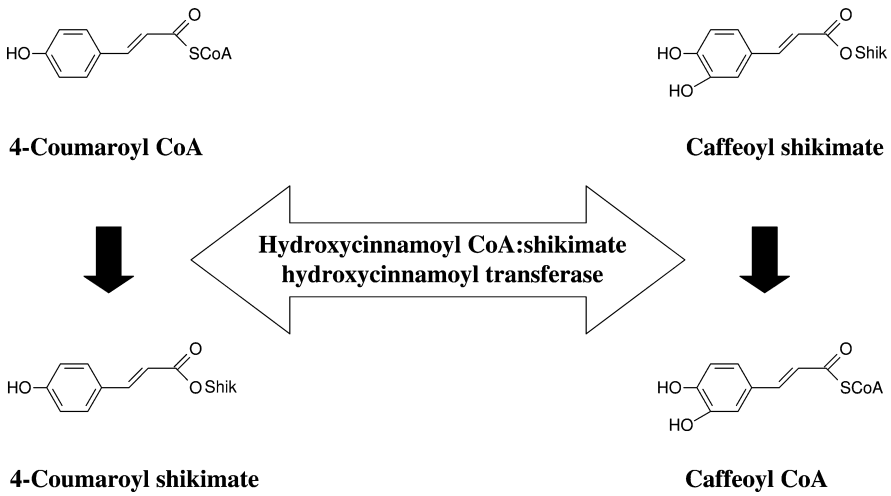

Fig. 1. The position of the reactions catalyzed by HCT in monolignol biosynthesis. HCT converts 4-coumaroyl CoA to 4-coumaroyl shikimate and then converts the resulting caffeoyl shikimate, formed by hydroxylation of 4-coumaroyl shikimate by "C3H", to caffeoyl CoA.

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.

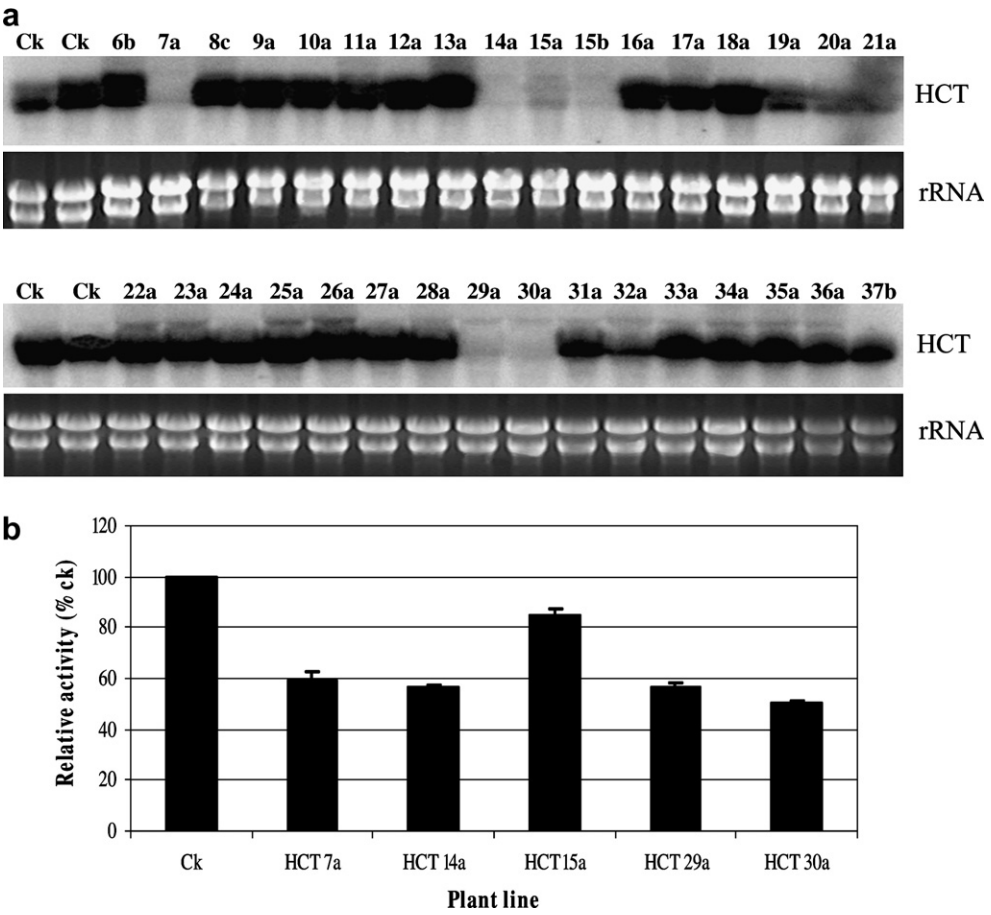

Fig. 2. HCT expression in control alfalfa plants and plants expressing an HCT antisense construct. (a) Transcript levels determined by RNA gel blot analysis. HCT, autoradiogram showing HCT signal; rRNA, ethidium bromide-staining of the original gel showing ribosomal RNA bands as loading controls. (b) Extractable HCT enzyme activities. Numbers refer to independent control (Ck, empty vector transformed or non-transformed) or antisense transgenic lines. Error bars indicate mean ± SD.

2.2. Phenotypic consequences of HCT down-regulation

It was obvious while growing the control and transgenic plants in the greenhouse for molecular analysis that several of the HCT down-regulated lines displayed phenotypic abnormalities. Most of the nine lines with intermediate to strong down-regulation of HCT transcripts and enzymatic activity were dwarf (Fig. 3a), varying in size from around 25% to 50% the height of control lines when grown in parallel to early bud stage.

Although all the HCT down-regulated lines flowered normally, the time from cut-back to flowering was often delayed, by as much as 20 days, when compared to control lines which flowered from around 40–50 days after cut-back (Fig. 4a), and the biomass at early bud stage was likewise significantly reduced (Fig. 4b).

Transverse cross-sections of stems (fifth internodes) from control and HCT-down-regulated lines were prepared and stained with Maule reagent (Fig. 3b), which produces a red coloration specifically with S lignin units. Lignin lacking S units stains a dull brown color. All control lines, and HCT transgenics in which transcript levels were not significantly down-regulated (e.g. 4b), retained strong red/pink staining in the vascular tissues, primarily in the cell walls of xylem elements, pith rays, and pith parenchyma cells. Lines 3a and 15a, which retain low levels of HCT transcripts, showed red to red-brown staining, indicative of the presence of mainly H or G lignin with residual S lignin. Lines 1a, 7a, 14a, 29a and 30a, which almost completely lack HCT transcripts, showed an intense dark brown staining indicative of a severe reduction of S lignin. In several of these lines, best exemplified by line 7a (Fig. 3b and c), the xylem elements appeared distorted, suggesting structural abnormalities affecting the physical properties of the cell walls.

2.3. Effects of HCT down-regulation on lignin content and composition

Lignin content of pooled internodes 1–6 was initially analyzed by the acetyl bromide method (Fukushima and Hatfield, 2004). This revealed reductions in total lignin content of around 50% in some of the antisense lines (Fig. 5a). Subsequent analysis of total thioacidolysis yields from control and HCT down-regulated transgenic lines revealed a striking reduction in extractable monomer units,

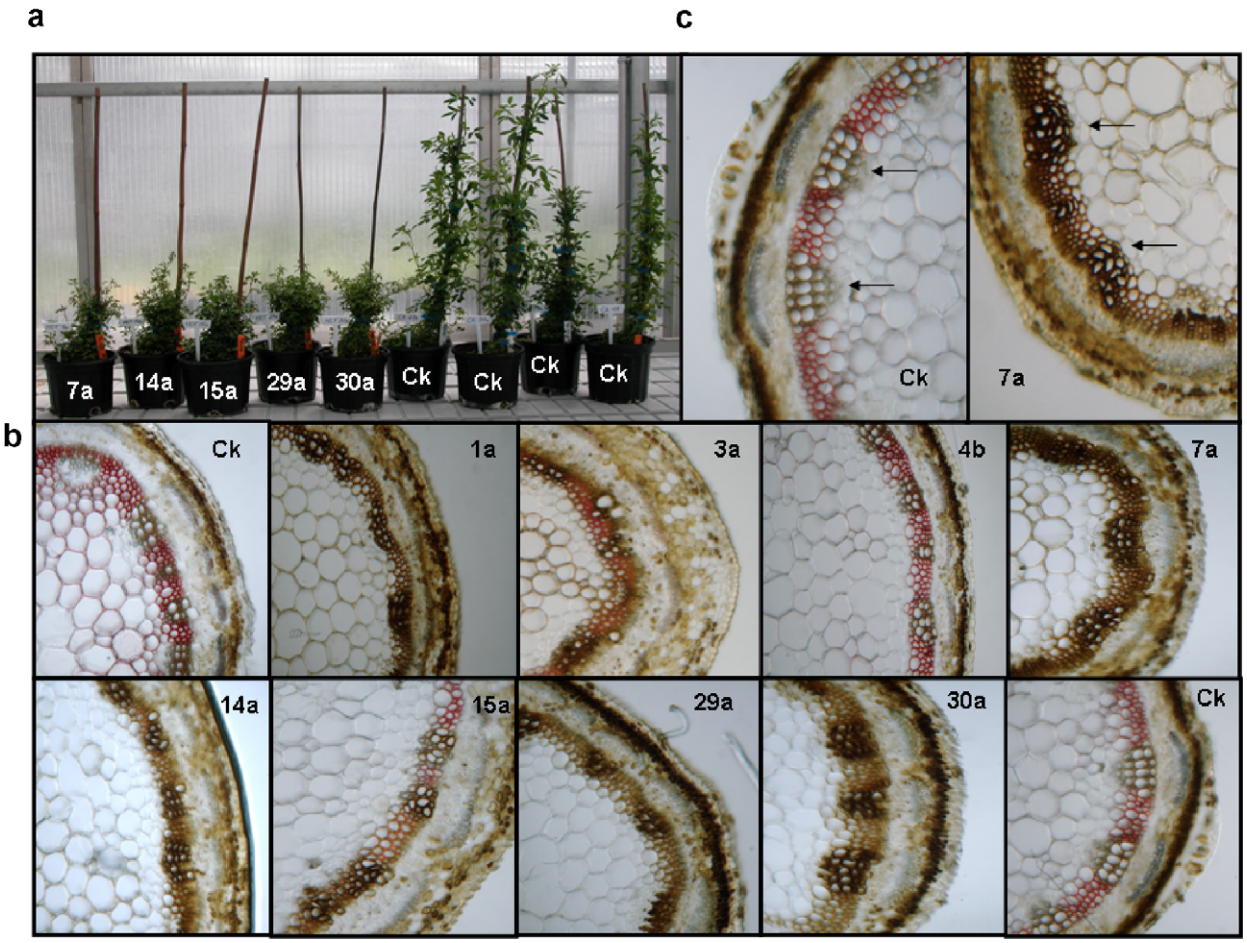

Fig. 3. Phenotypic appearance of HCT down-regulated transgenic alfalfa plants. (a) Whole plants at the early bud stage. (b) Cross-sections of the 5th internodes of stems stained with Maule reagent. Purple/red coloration indicates the presence of S lignin units. (c) Higher magnification of Ck and line 7a, showing distorted xylem vessels in 7a compared to the normal vessels in Ck (arrows). Numbers represent independent transgenic lines and Ck represents untransformed or empty vector transformed alfalfa lines used as controls.

to less than 15% of the control values in the most down-regulated lines (Fig. 5b). Consistent with the results of the Maule staining, the relative levels of S lignin were strongly reduced, although G lignin units were reduced by a similar amount. However, the most striking compositional change in the HCT down-regulated lines was the large increase in H units, comparable to the situation in transgenic alfalfa plants down-regulated in C3H (Reddy et al., 2005; Chen et al., 2006).

2.4. Forage quality analysis

Standard wet chemistry protocols were used to determine acid detergent lignin (ADL), neutral detergent fiber (NDF, the entire fiber component minus pectin), acid detergent fiber (ADF, the lignocellulosic fraction) and in vitro dry matter digestibility (IVDMD) for whole (stem plus leaf) forage samples of control and HCT-down-regulated lines. Consistent with the thioacidolysis yield data, acid detergent lignin levels were strongly reduced in the five lines chosen for forage quality analysis, to 58–65% of the average value of the three control lines (Table 1). HCT down-regulation also led to average reductions in NDF and ADF of 12–32% and 13–35%, respectively, accompanied by striking increases in IVDMD. All the HCT down-regulated lines had similar IVDMD values, representing increases of 15–22% over the digestibility of the control lines.

Analysis of in vitro digestion kinetics indicated that digestibility improvements in the HCT down-regulated lines were apparent as early as 12 h after immersion in rumen fluid (Fig. 6). However, the increase at this early time point was less in lines 29a and 30a than in the other three lines analyzed. By 24 h, forage from the HCT lines attained comparable, near maximum extents of digestion, with the exception of line 29a, which remained slightly less digestible than the other lines at all time points.

3. Discussion

3.1. Generation of HCT down-regulated alfalfa lines

In a previous study (Chen et al., 2006) we reported the characterization of two alfalfa lines with highly reduced expression of HCT as a result of transformation with an

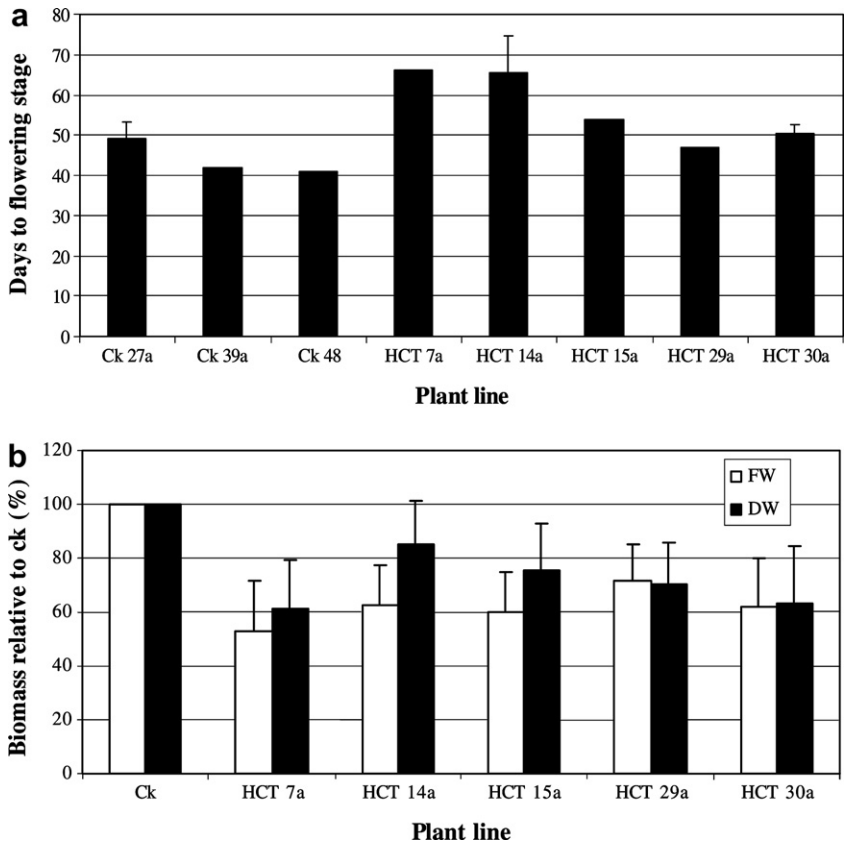

Fig. 4. Growth parameters of control alfalfa plants and plants expressing an HCT antisense construct. (a) Time-to-flowering (following cutback) of control and HCT down-regulated transgenic alfalfa plants. (b) Biomass of HCT down-regulated transgenic alfalfa lines relative to controls. Fresh weight (FW), dry weight (DW). $n = 32$ (Ck), 12 (HCT 7a), 14 (HCT 14a), 13 (HCT 15a), 14 (HCT 29a), 13 (HCT 30a). Error bars indicate mean ± SD.

HCT antisense transgene. Although it was noted that these plants exhibited an overall reduction in plant height, the previous study did not consider detailed phenotypic effects beyond lignin content and composition. The present series of six additional lines, five of which have been analyzed in detail, provided our basis for assessing the phenotypic consequences and biotechnological potential of HCT down-regulation.

Expression of the HCT antisense construct led to a very large decrease in HCT transcript levels in five of the six lines, and a somewhat smaller decrease in line 15a. Although line 15a also exhibited a smaller reduction in HCT enzymatic activity, it was nevertheless surprising that the largest reduction in enzymatic activity, in line 30a, was only to approximately 50% of the wild-type level. A better correlation was seen in previous studies between reductions in transcript levels and extractable lignin pathway enzyme activities (e.g. for phenylalanine ammonia-lyase [PAL], cinnamate 4-hydroxylase [C4H], caffeic acid 3-*O*-methyltransferase [COMT] and caffeoyl CoA 3-*O*-methyltransferase [CCoAOMT]) in transgenic alfalfa (Guo et al., 2001a; Reddy et al., 2005; Chen et al., 2006). It is possible that the level of translatable HCT transcripts is only a small fraction of the steady state HCT transcript level in the total RNA fraction, as analyzed on RNA gel blots, and is less susceptible than the bulk pool to antisense down-regulation. More likely, the enzymatic assay for HCT, which determines formation of caffeoyl shikimate in terms of loss of absorption of caffeoyl CoA, overestimates HCT activity by failing to discriminate it from that of other acyltransferases, not targeted by the antisense RNA, which can catalyze the same reaction in vitro (Niggeweg et al., 2004), or from thioesterase activity. Whatever the interpretation, the phenotypic consequences of HCT down-regulation with regards to lignin content and composition are fully consistent with strong down-regulation of flux through the HCT reaction in vivo.

3.2. Visible growth and lignin phenotypes of HCT down-regulated plants

Overall, the lignin phenotypes of the HCT down-regulated alfalfa plants were as observed previously (Chen et al., 2006). However, lignin levels in the present controls were lower than reported previously. In the previous study, internodes 2–8 were analyzed, whereas internodes 1–6 were used in the present work. This explains the lower lignin levels in the control lines in the present study, since lignin levels increase with increasing internode maturity in alfalfa (Chen et al., 2006). HCT down-regulation caused a striking

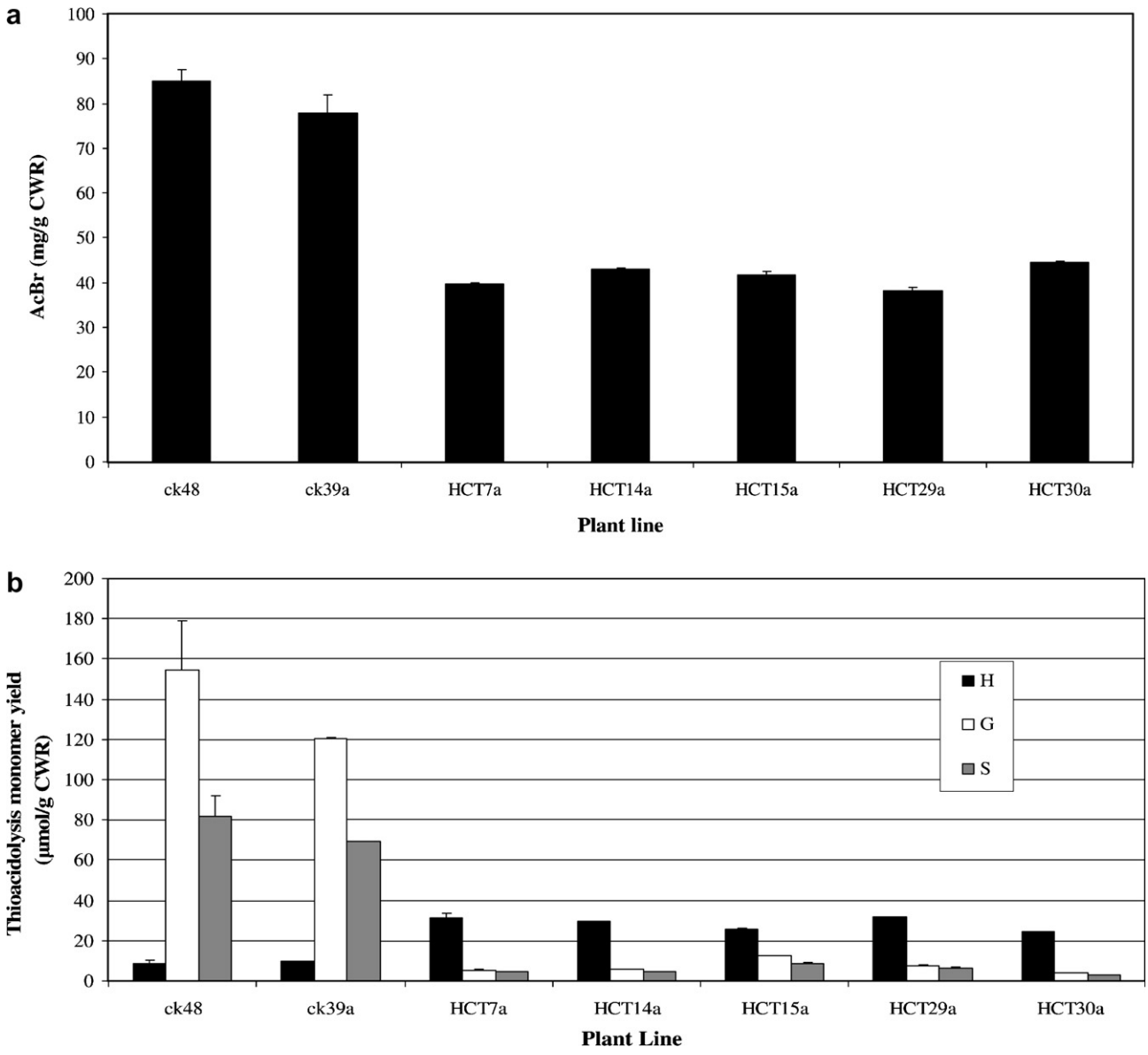

Fig. 5. Lignin and lignin monomer yields in control alfalfa plants and plants expressing an HCT antisense construct. (a) Acetyl bromide lignin levels. (b) Yields of *p*-hydroxyphenyl (H), guiacyl (G), and syringyl (S) monomers. Numbers represent independent transgenic lines and ck represents untransformed or empty vector transformed alfalfa lines used as controls. Error bars indicate mean ± SD.

reduction in lignin content, particularly when measured as thioacidolysis products, but also clear from determination of acetyl bromide or acid detergent lignins. The two latter methods, which are less dependent than thioacidolysis on lignin composition, gave similar values of around 50% reduction in overall lignin in the most strongly affected lines. As observed following down-regulation of C3H (Franke et al., 2002b; Reddy et al., 2005; Chen et al., 2006) HCT down-regulated lines exhibited a striking increase in the proportion of H units in the lignin.

The reduction in lignin content following reduction of HCT levels was accompanied by reduced plant height, although the effect was considerably less severe than observed in Arabidopsis plants lacking the ability to convert coumaroyl CoA to caffeoyl CoA (Franke et al., 2002a). Flowering was delayed by as much as 20 days in some individual cuttings from antisense line 14a, although all cuttings from lines 29a and 30a (the line with highest down-regulation of HCT enzyme activity) flowered at the same time as cuttings from control plants grown in parallel. Interestingly, the reduction in plant height was not accompanied by a proportional reduction in overall biomass in all lines. For example, line 14a, which reached only about 30% of the height of wild-type plants at flowering, had about a 20% reduction in dry weight and a 40% reduction in fresh weight. These reductions were associated with a more bushy, branched phenotype. However, the extent of down-regulation of HCT does not seem to correlate well with the extent of change in the biomass and the length of delayed flowering. Overall, the growth phenotype of

Table 1
Forage quality parameters for control alfalfa plants and plants expressing an HCT antisense construct

Line	NDF (%)	ADF (%)	ADL (%)	IVDMD (%)
ck 27a	49.29 ± 0.48	39.01 ± 0.11	8.32 ± 0.41	70.67 ± 0.33
ck 39a	46.10 ± 0.07	36.26 ± 0.41	7.39 ± 0.23	74.23 ± 0.31
ck 48	43.36 ± 0.05	34.75 ± 0.10	7.42 ± 0.04	75.25 ± 0.70
HCT 7a	30.89 ± 0.20	23.88 ± 0.31	4.75 ± 0.27	90.88 ± 0.29
HCT 14a	33.42 ± 0.10	25.10 ± 0.05	4.98 ± 0.12	90.15 ± 0.86
HCT 15a	32.45 ± 0.23	25.15 ± 0.17	4.51 ± 0.12	90.06 ± 0.01
HCT 29a	39.81 ± 0.13	31.94 ± 0.16	4.68 ± 0.24	88.29 ± 0.29
HCT 30a	35.55 ± 0.15	28.24 ± 0.21	5.05 ± 0.00	90.31 ± 0.10

NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; IVDMD, in vitro dry matter digestibility (determined using rumen fluid). Numbers represent independent transgenic lines and ck represents untransformed or empty vector transformed alfalfa lines used as controls. Error bars indicate mean ($n \geqslant 12$) of duplicate pooled samples±SD.

HCT down-regulated alfalfa plants appears to be more severe than observed when C3H is targeted, when a smaller loss of plant height is not accompanied by such a bushy growth habit (Reddy et al., 2005). Since HCT and C3H are believed to act together in the conversion of coumaroyl CoA to caffeoyl CoA, and their down-regulation, driven by the same promoter, results in similar changes in lignin content and composition in alfalfa (Chen et al., 2006) the reasons for these differences in plant phenotype are not clear.

3.3. Applications for forage improvement

Small changes in forage digestibility significantly impact animal performance (Casler and Vogel, 1999), and improving digestibility is therefore an important goal of forage breeding programs (Boerjan et al., 2003). Lignin content and syringyl to guaiacyl ratio increase with stem maturity (Jung and Vogel, 1986; Buxton and Russell, 1988), and correlate negatively with forage digestibility in ruminant animals (Albrecht et al., 1987; Buxton and Russell, 1988; Grabber et al., 1992; Sewalt et al., 1996; Jung et al., 1997). By relating forage digestibility to both lignin content and composition in transgenic alfalfa lines in which these parameters differed widely as a result of down-regulation of the pathway at three different sites, we have recently shown that the actual amount of lignin appears to be more critical for forage digestibility than the lignin composition (Reddy et al., 2005). Based on their large reduction in lignin content, the present HCT transgenic lines would be predicted to have greatly improved digestibility, as confirmed from studies using rumen fluid in vitro. Increases in in vitro digestibility of up to 20% were observed within 24 h of digestion, greater than the increases previously observed in alfalfa plants with reduced C3H or C4H activities (Reddy et al., 2005). These changes in HCT lines were also associated with decreased levels of neutral detergent fiber. NDF represents the total fiber content of the forage, but, in pectin-rich legumes, is often an underestimate as the pectic material is easily solubilized (Jung, 1997). The decrease in NDF is too great to be associated with the decreased lignin component alone, suggesting some loss in nutritive quality in spite of the improved digestibility.

Modification of lignin content in alfalfa by targeting cinnamyl alcohol dehydrogenase (CAD), COMT or CCoAOMT improves digestibility less than by targeting the earlier enzymes in the pathway, but has fewer negative phenotypic effects (Baucher et al., 1999; Guo et al.,

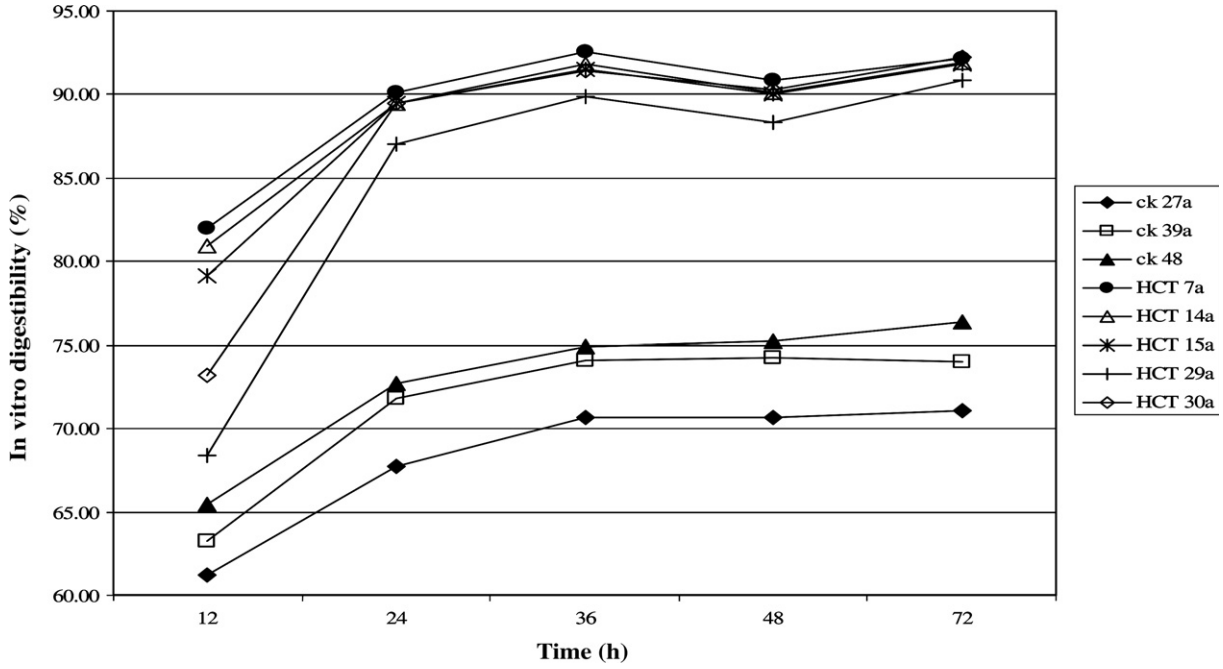

Fig. 6. In vitro digestion kinetics of forage material from control alfalfa plants and plants expressing an HCT antisense construct. Numbers represent independent transgenic lines and ck represents untransformed or empty vector transformed alfalfa lines used as controls.

2001a,b). Work is in progress to determine whether it will be possible to target HCT down-regulation to fewer cell types by using different gene promoters, and thereby capture the benefits of lignin reduction in the absence of adverse agronomic phenotypes.

4. Experimental

4.1. Plant materials and genetic transformation

Alfalfa (*M. sativa* cv Regen SY) plants were grown in the greenhouse under standard conditions with a 16-hour day from 6:00 h to 22:00 h facilitated by supplementary lighting. Genetic transformations were performed with clonally propagated material of one selected highly regenerable line of Regen SY. The entire coding region of *M. truncatula HCT* (TC106825), an ortholog of tobacco HCT (GenBank Accession No. AJ507825) was amplified using forward (ATGATCATAAACGTTAGAGATTCGACAATG) and reverse (CACCTCAAATATCATACAAGAAGTCCTTGAACAC) primers. pCAMBIA-2200-MtHCTas constructs were then made for antisense mediated down-regulation of HCT following the procedure described previously (Reddy et al., 2005). The generation of transgenic alfalfa lines was as reported previously (Guo et al., 2001a; Reddy et al., 2005).

4.2. RNA gel blot analysis

Total RNA was isolated from 500 mg of ground stem tissue (internodes 2–5) using TRI Reagent solution (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Ten micrograms of total RNA was separated on 1.2% formaldehyde-containing agarose gels and transferred onto a nylon membrane (Hybond-N+, Amersham Biosciences Corp, Piscataway, NJ) by standard procedures (Sambrook et al., 1989) and UV cross-linked using a Stratalinker (Stratagene, La Jolla, CA). The HCT coding sequence was labeled with a α-^{32}P-dCTP labeling kit (Amersham) and purified on Probe Quant G50 micro-columns (Amersham). The purified probe was used for RNA gel blot hybridization as described previously (Reddy et al., 2002).

4.3. Assay of HCT enzyme activity

Alfalfa stems (internodes 1–6) were harvested and ground to powder in liquid nitrogen. One gram of fresh tissue was weighed into 12 ml tubes on ice, and 3 ml of extraction buffer containing 20 mM Tris–HCl (pH 7.5) and 0.1% β-mercaptoethanol were added. The mixture was homogenized with a polytron for 10 s on ice. Phenolic compounds were removed by addition of a spatula tip-full of polyvinylpolypyrrolidone, votexing and incubating on ice for 5 min. The supernatant was separated by centrifugation at 12,000*g* at 4 °C for 15 min, and desalted by passing through a Sephadex G-25 column. HCT activity of the supernatants was determined by measuring the decrease in absorbance at 333 nm using *p*-coumaroyl CoA (1.35 mM) and shikimic acid (10 mM) as acyl donor and acceptor, respectively (Hoffmann et al., 2003). Protein concentrations were determined by the Bradford method (Bradford, 1976). Reactions were linear over the times and protein/substrate concentrations used.

4.4. Determination of lignin content and composition

Lignin content and composition were measured in stem internodes 1–6. Total lignin was determined by the acetyl bromide method (Fukushima and Hatfield, 2004). Thioacidolysis methods (Lapierre et al., 1985, 1995) were used for determination of lignin composition. Thioacidolysis was performed using ~20 mg of extractive-free samples reacted with 3 ml of 0.2 M BF_3 etherate in an 8.75:1 dioxane/ethanethiol mixture. Lignin-derived monomers were identified by gas chromatography mass spectrometry (GC/MS), and quantified by GC as their trimethylsilyl derivatives. GC/MS was performed on a Hewlett–Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60-m × 0.25-mm × 0.25-μm film thickness), and mass spectra were recorded in electron impact mode (70 eV) with 60–650 *m*/*z* scanning range.

4.5. Histochemical staining of lignin

Alfalfa stem cross-sections were made using a vibratome (series 1000; Ted Pella Inc., Redding, CA) and subjected to Maule staining as described previously (Guo et al., 2001a). Photographs were taken using an Olympus SZX stereomicroscope system with a SPOT RT color camera.

4.6. Determination of forage quality

Vegetatively propagated alfalfa cuttings were grown in parallel in one gallon pots in the greenhouse. Aerial portions were harvested at the early bud stage to ensure material was matched developmentally, and dried in a 50 °C oven for at least 72 h. The samples were then ground in a Thomas–Wiley model 4 Laboratory Mill (Lehman Scientific, Wrightsville, PA) with 1 mm sieves. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were estimated by standard protocols (Goering and Van Soest, 1970). For NDF analysis, 0.35 g of ground samples were transferred to a F57 ANKOM filter bag (ANKOM Technology Corporation, Fairport, NY) and heated at 100 °C for 1 h in an ANKOM Fiber Analyzer, according to the manufacturer's instructions. The samples were washed in near boiling water, dried at 105 °C for 6 h, and weighed to determine fiber loss. ADF was estimated sequentially on the material remaining after NDF analysis. The residue was then used for determination of acid detergent lignin by incubation in 72% (v/v) sulfuric acid for 3 h, washing thoroughly and drying at 105 °C for 6 h, prior to weighing.

For determination of in vitro digestibility, ground tissue samples were dried at 105 °C for 6 h prior to determining pre-extraction dry weights. The same procedure was used to obtain post-extraction dry weights. Digestibility analysis (0.5 g samples) was performed using F57 filter bags and the DAISY II incubator (ANKOM Technology Corporation, Fairport, NY) (Vogel et al., 1999), following the manufacturer's instructions.

Acknowledgements

We thank Drs. Elison Blancaflor and Million Tadege for critical reading of the manuscript, and Dennis Walker for forage quality analysis. This work was supported by Forage Genetics International and the Samuel Roberts Noble Foundation.

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