The Growth Reduction Associated with Repressed Lignin Biosynthesis in Arabidopsis thaliana Is Independent of Flavonoids

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Defects in phenylpropanoid biosynthesis arising from deficiency in hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT) or p-coumaroyl shikimate 3'-hydroxylase (C3' H) lead to reduced lignin, hyperaccumulation of flavonoids, and growth inhibition in Arabidopsis thaliana. It was previously reported that flavonoid-mediated inhibition of auxin transport is responsible for growth reduction in HCT-RNA interference (RNAi) plants. This conclusion was based on the observation that simultaneous RNAi silencing of HCT and chalcone synthase (CHS), an enzyme essential for flavonoid biosynthesis, resulted in less severe dwarfing than silencing of HCT alone. In an attempt to extend these results using a C3' H mutant (ref8) and a CHS null mutant (tt4-2), we found that the growth phenotype of the ref8 tt4-2 double mutant, which lacks flavonoids, is indistinguishable from that of ref8. Moreover, using RNAi, we found that the relationship between HCT silencing and growth inhibition is identical in both the wild type and tt4-2. We conclude from these results that the growth inhibition observed in HCT-RNAi plants and the ref8 mutant is independent of flavonoids. Finally, we show that expression of a newly characterized gene bypassing HCT and C3' H partially restores both lignin biosynthesis and growth in HCT-RNAi plants, demonstrating that a biochemical pathway downstream of coniferaldehyde, probably lignification, is essential for normal plant growth.

INTRODUCTION

In vascular plants, the phenylpropanoid pathway is responsible for the biosynthesis of a variety of metabolites, including lignin, a major structural component of secondary cell wall, and flavonoids, a diverse collection of compounds involved in pigmentation, herbivory defense, and plant–microbe interactions (Figure 1). The presence of lignin protects cell wall polysaccharides from enzymatic and chemical degradation, with substantial negative impacts on forage quality, pulping efficiency, and cellulosic biofuel production (Li et al., 2008). Accordingly, a number of approaches have been employed to identify or engineer plants with low lignin content. Although such plants have been obtained in several species, including tobacco (Nicotiana tabacum), popular (Populus spp), alfalfa (Medicago sativa), and Arabidopsis thaliana, and do indeed exhibit increased cell wall degradability, they typically also show reduced growth and decreased biomass, making them unsuitable for practical applications (Piquemal et al., 1998; Pincon et al., 2001; Franke et al., 2002a; O’Connell et al., 2002; Hoffmann et al., 2004; Reddy et al., 2005; Leple et al., 2007; Shadle et al., 2007).

The growth defects of lignin-deficient plants have previously been attributed to impaired water transport resulting from collapse of the xylem (Piquemal et al., 1998; Jones et al., 2001; Franke et al., 2002a), which in wild-type plants is highly lignified and is subjected to strong negative pressures during water transport. By contrast, the authors of a recent report argued that the growth phenotype resulting from downregulation of one lignin biosynthetic gene is instead due to accumulation of flavonoids and inhibition of auxin transport (Besseau et al., 2007). In this report, the authors used RNA interference (RNAi) to knock down the expression of the gene encoding hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT), which acts at a branch point in the phenylpropanoid pathway where p-coumaroyl CoA can be committed either to lignin production if acted upon by HCT (Hoffmann et al., 2003, 2004), or to flavonoid biosynthesis if acted upon by the competing enzyme chalcone synthase (CHS; Burbulis et al., 1996). It had been previously shown that plants expressing an HCT-RNAi transgene accumulate high levels of flavonoids (Hoffmann et al., 2004), compounds known to have an inhibitory effect on auxin polar transport (Jacobs and Rubery, 1988; Brown et al., 2001). To test whether the hyperaccumulation of flavonoids in HCT-RNAi plants could be the cause for the growth phenotype, Besseau et al. downregulated the flavonoid biosynthesis in these plants by RNAi silencing of CHS and were able to show that the dwarf phenotype could be significantly alleviated. The recovery in growth was also associated with the restoration of auxin transport, which was greatly inhibited in the HCT-RNAi plants. The
Figure 1. The Arabidopsis Phenylpropanoid Pathway.

The biosynthetic pathways for flavonoids and lignin are interconnected at p-coumaroyl CoA. Mutations in CHS (tt4) and C3'H (ref8) affect the flavonoids and lignin biosynthesis, respectively. Sinapate esters and syringyl lignin share a common precursor, sinapaldehyde. The F5H gene from S. moellendorfii (Sm-F5H) has the unique ability to catalyze the hydroxylation of p-coumaraldehyde and p-coumaryl alcohol to generate caffealdehyde.
authors thus suggested that the growth phenotype resulting from downregulation of HCT is due to accumulation of flavonoids and inhibition of auxin transport, rather than perturbation of lignin content and composition.

After its synthesis by HCT, p-coumaroyl shikimate is subsequently hydroxylated at the 3-position of the hydroxycinnamoyl moiety by p-coumaroyl shikimate 3'-hydroxylase (C3'H; the product of the REF8 gene in Arabidopsis) to yield caffeoyl shikimate (Figure 1; Schoch et al., 2001; Franke et al., 2002b; Nair et al., 2002). Like HCT-deficient plants, C3'H-deficient ref8 mutants are also dwarf and accumulate high levels of flavonoids. However, as we show here, blocking flavonoid biosynthesis with a CHS null mutation (tt4-2; Burbulis et al., 1996) has no effect on the ref8 growth phenotype. Unexpectedly, we also found that the phenotype of HCT-RNAi plants is similarly unaffected by tt4-2. By contrast, we found that rerouting lignin biosynthesis with a heterologously expressed gene to bypass HCT and C3'H in HCT-RNAi plants substantially rescues their growth phenotype, consistent with the previously held notion that lignin deficiency is the cause of growth reduction and dwarfing in phenylpropanoid mutants.

RESULTS

Elimination of Flavonoid Biosynthesis by Disruption of CHS Does Not Rescue the Growth Phenotype of ref8 Mutants

Arabidopsis ref8 mutants exhibit growth reduction and increased accumulation of flavonoids when compared with wild-type controls (Figure 2; Franke et al., 2002a; Abdulrazzak et al., 2006). Based on the previous report that flavonoid accumulation is the cause of the reduced growth of HCT-RNAi plants (Besseau et al., 2007), we sought to test if the growth inhibition observed in the ref8 mutant could also be due to the hyperaccumulation of flavonoids. To this end, we crossed the ref8 mutant with the CHS null mutant tt4-2, which is devoid of flavonoids (Burbulis et al., 1996). Initial genotyping of the F2 progeny from this cross identified a few homozygous ref8 tt4-2 double mutants, all of which were small and sterile. Further detailed analysis was performed using F3 progeny of an F2 plant with a REF8/ref8 tt4-2/ tt4-2 genotype. Of 27 F3 progeny analyzed, 21 plants showed wild-type growth, whereas six plants exhibited reduced rosette size and dwarfing indistinguishable from that seen in the ref8 mutant (Figure 2). Genotyping confirmed that the six smaller plants were all homozygous for both the ref8 and tt4-2 mutations, whereas none of the F3 plants exhibiting wild-type growth were homozygous for the mutant ref8 allele. HPLC analysis of methanolic leaf extracts confirmed that all F3 progeny were devoid of flavonoids (Figure 2), as expected from their lack of a functional CHS. Thus, C3'H deficiency leads to severe growth reduction and dwarfing even in the absence of flavonoids.

Silencing of HCT Has Identical Growth Effects in Both Wild-Type and tt4-2 Mutant Backgrounds

Given the positions of HCT and C3'H in the phenylpropanoid pathway and the similar phenotypes of ref8 mutants and HCT-RNAi plants, we were initially surprised that blocking flavonoid biosynthesis failed to rescue the growth phenotype of the ref8 mutant. This observation led us to further investigate the relationship between flavonoid hyperaccumulation and the dwarf phenotype in HCT-deficient plants. If the reduced-growth phenotype exhibited by HCT-RNAi plants is dependent on the hyperaccumulation of flavonoids as reported, then silencing of HCT in the flavonoid-lacking mutant tt4-2 should have no effect on growth. To test this, we generated an intron-containing hairpin RNAi construct targeting Arabidopsis HCT and subsequently introduced it into both wild-type and tt4-2 mutant plants. Consistent with previous reports (Hoffmann et al., 2004; Besseau et al., 2007), RNAi downregulation of HCT in wild-type plants led to reduced growth and increased flavonoid accumulation, as revealed by analysis of independent lines exhibiting phenotypes of varying severity (Figures 3A and 3G). The suppression of HCT in these lines was evident at both the mRNA level and the level of enzyme activity (Figures 3C and 3D). Surprisingly, however, HCT-RNAi lines generated in the tt4-2 mutant background and analyzed in parallel also exhibited the reduced growth phenotype (Figures 3B, 3E, and 3F), despite the fact that they lacked flavonoids (Figure 3H). It is noteworthy that in moderately dwarfed HCT-RNAi plants, levels of HCT activity only 2% of that found in wild-type plants are sufficient to prevent the extreme dwarfing seen in more severely affected plants grown under the same conditions.

To investigate the relationship between HCT silencing and plant growth reduction in the above two genetic backgrounds and to eliminate the remote possibility that T-DNA insertion of the above described HCT-RNAi construct had disrupted a growth-regulating gene, the inactivation of which could not be rescued by flavonoid depletion, we analyzed HCT expression and growth in multiple T2 transgenic lines derived from independent T1 transformants. We measured plant height at maturity, after inflorescence stems had ceased elongating, to eliminate any possible confounding effects due to differences in rates of development. When the HCT mRNA levels in these plants were compared with their heights, it was apparent that HCT silencing was strongly correlated with plant growth reduction in both wild-type and tt4-2 backgrounds (Figure 4). Multiple regression analysis showed that HCT expression has a significant effect on plant height (P < 0.001), and this effect is not affected by these two different genetic backgrounds (test for interaction of HCT expression and genetic background, P = 0.6).
The Ability to Synthesize Flavonoids Has No Effect on the Growth Phenotype of HCT-RNAi Plants Containing Identical Transgene Insertions

While the above data strongly suggest that flavonoid accumulation is not the mechanism of growth inhibition in HCT-RNAi plants, a direct comparison of the growth phenotype of the HCT-RNAi lines generated in different genetic backgrounds is unavoidably confounded with potential position effects associated with the T-DNA insertions in different transgenic plants. To generate wild-type and tt4-2 mutants with identical HCT-RNAi transgene insertions, we crossed a homozygous T2 HCT-RNAi plant containing wild-type TT4 with a tt4-2 mutant (scheme shown in Figure 5A).

The resulting F1 plants, which are heterozygous for the tt4-2 allele and hemizygous for the HCT-RNAi transgene, were again crossed with the tt4-2 mutant. Thirty-eight F1 plants from this second cross were genotyped and their heights at maturity were measured. As expected, four genotypes were found at approximately equal frequency in the F1 population: tt4-2/TT4 (n = 11), tt4-2/tt4-2 (n = 10), tt4-2/TT4 with HCT-RNAi (n = 9), and tt4-2/tt4-2 with HCT-RNAi (n = 8). The F1 plants with the former two genotypes lacking the HCT-RNAi transgene have a typical height of ~45 cm (Figure 5B). By contrast, plants possessing the HCT-RNAi transgene exhibit a maximum height of ~25 cm. Critically, no statistically significant difference in height (Student’s t test, P = 0.57) was detected between the plants with the latter two genotypes.
Figure 3. Silencing of HCT in Either the Wild-Type or tt4-2 Background Results in Growth Inhibition.

HCT-RNAi plants showing a moderate (M) or severe (S) growth phenotype with either wild-type TT4 (A) or mutant tt4-2 background (B) were compared with their cognate controls at 3 weeks after planting. Bars = 1 cm. HCT mRNA levels (C and E) and enzyme activity (D and F) as well as HPLC profiles of soluble phenylpropanoids (G and H) were determined for the above plants with either wild-type TT4 (C, D, and G) or mutant tt4-2 (E, F, and H) background. The values represent the average of three biological replications and the error bars represent 1SD. nd, not determined; K1, kaempferol 3-O-(6'-O-rhamnosyl) glucoside] 7-O-rhamnoside; K2, kaempferol 3-O-glucoside 7-O-rhamnoside; K3, kaempferol 3-O-rhamnoside 7-O-rhamnoside; SM, sinapoylmalate; mAU, milli absorbance unit; WT, wild type.

[See online article for color version of this figure.]
which carry the same HCT-RNAi transgene insertions but differ in their ability to produce flavonoids.

In a reciprocal approach, we selected a single hemizygous T2 HCT-RNAi plant generated in the tt4-2 background and crossed it with both tt4-2 and wild-type plants in parallel (scheme in Figure 6A). The first cross resulted in F1 progeny with the genotypes tt4-2/TT4 (n = 20) and tt4-2/tt4-2 HCT-RNAi (n = 16), whereas the progeny of the second cross had the genotypes tt4-2/TT4 (n = 11) and tt4-2/TT4 HCT-RNAi (n = 14). All plants containing the HCT-RNAi transgene exhibited a strong dwarf phenotype (average height ~10 cm) regardless of whether they possessed a wild-type or mutant allele of TT4 (Figure 6B). Taken together, these data demonstrate that flavonoids have no effect on the reduced-growth phenotype associated with RNAi silencing of HCT.

HCT-RNAi Plants Contain Higher Concentrations of Flavonoids Than Do Wild-Type Plants, but the Total Amount of Flavonoids per Rosette Is Similar in Both Genetic Backgrounds

At 3 weeks of age, HCT-RNAi plants are dwarfed and contain a higher concentration of flavonoids than do wild-type plants. However, the concentration of sinapoylmalate in HCT-RNAi plants is approximately the same as that in wild-type plants, in apparent conflict with the established role of HCT in sinapate ester biosynthesis (Figures 3G and 3H; Hoffmann et al., 2003, 2004). However, we reasoned that both flavonoid and sinapoylmalate measurements could be confounded in HCT-RNAi plants by the fact that their rosette leaves expand substantially less than those of the wild type. To gain more insight into the dynamics of soluble phenylpropanoid accumulation, we quantified sinapoylmalate and flavonoids in whole rosettes of soil-grown wild-type and HCT-RNAi plants at five time points from 14 to 22 d after planting (Figure 7). Wild-type and HCT-RNAi plants begin to diverge from one another phenotypically ~16 d after planting, when rosettes of wild-type, but not HCT-RNAi, plants continue to grow and expand exponentially (Figures 7A and 7B). After this point, the concentration of flavonoids, expressed in nmol per mg fresh weight, was significantly higher (Student’s t test, P < 0.05) in HCT-RNAi plants than in wild-type plants (Figure 7C). However, the total amount of flavonoids in these plants, expressed in nmol

Figure 4. Plant Height Is Negatively Correlated with HCT Silencing in Both Wild-Type and tt4-2 Backgrounds.

Height at maturity and HCT mRNA level in 3-week-old rosettes were measured for HCT-RNAi plants with either wild-type TT4 (A) or mutant tt4-2 (B) background. For each genetic background, two T2 progeny of each of five independent T1 transformants were analyzed. Open boxes indicate untransformed wild-type or tt4-2 plants.

Figure 5. Elimination of Flavonoids from HCT-RNAi Plants Does Not Rescue Their Growth Phenotype.

(A) Schematic illustration of the crosses made to generate the F1 population composed of four genotypes. The number of plants for each genotype is indicated in brackets.

(B) Height at maturity of the four types of F1 plants shown in (A). The averaged values are presented, and the error bars represent 1 SD.
units and trace amount of \( p \)-hydroxyphenyl (H) lignin (Figure 8). Silencing of HCT in either background led to an \( \sim 80\% \) decrease in total lignin and to an increase in the proportion of H lignin monomers, which do not require HCT for their synthesis, relative to the HCT-dependent G and S monomers (Figure 8). The presence or absence of the wild-type \( TT4 \) allele had no effect on total lignin content or composition in plants carrying the HCT-RNAi transgene or in control plants lacking the transgene. These results demonstrate that the HCT RNAi transgene was highly effective in altering lignin and that this effect was independent of flavonoids.

**Flavonoid-Independent Growth Inhibition**

We recently reported the cloning and characterization of a gene encoding a cytochrome P450 monooxygenase from the lycophyte *Selaginella moellendorfii* (Weng et al., 2008). This enzyme, which we have named F5H, cannot only hydroxylate the 5-position of coniferaldehyde and coniferyl alcohol, as does *Arabidopsis* ferulic acid 5-hydroxylase (At-F5H), but also can hydroxylate the 3-position of \( p \)-coumaraldehyde and \( p \)-coumaryl alcohol (Weng et al., 2010). Expression of Sm-F5H in *Arabidopsis* thus provides an alternate biosynthetic route from \( p \)-coumarate to coniferaldehyde and downstream phenylpropanoids that bypasses both HCT and C3’H (Figure 1). We have shown that transformation of HCT- or C3’H-deficient plants with an Sm-F5H expression construct leads to alleviation of their dwarf phenotype as well as to an increase in both total lignin and the fraction of lignin made up by syringyl monomers (Weng et al., 2010). We concluded from these data that the increase in lignin in HCT-RNAi plants was most likely responsible for the alleviation of their dwarf phenotypes; however, given the report by Besseau et al. (2007), we undertook here to test whether alterations in flavonoid accumulation in the HCT-RNAi plants expressing Sm-F5H could play a role in the suppression of their growth phenotype.

To test the possible involvement of flavonoids in the rescue of HCT-RNAi by Sm-F5H, we first compared flavonoid levels in wild-type and HCT-RNAi plants either containing or lacking the Sm-F5H expression construct (Figure 9). Consistent with previous experiments, we observed that HCT-RNAi plants lacking Sm-F5H contained higher concentrations of flavonoids than did wild-type plants. Whereas expression of Sm-F5H had no effect on flavonoid accumulation in wild-type plants, HCT-RNAi plants expressing Sm-F5H showed slightly lower flavonoid levels than those lacking Sm-F5H (Student’s \( t \) test, \( P < 0.001 \)). However, these data are confounded by the fact that expression of Sm-F5H also results in increased rosette leaf expansion in HCT-RNAi plants, allowing the possibility that the lower flavonoid concentration in HCT-RNAi plants is the result of alleviation of the growth reduction, rather than the cause.

To conclusively test whether flavonoids are responsible for suppression of the HCT-RNAi phenotype by Sm-F5H, we transformed \( tt4-2 \) HCT-RNAi plants with our Sm-F5H expression construct. These plants lack flavonoids, and thus any phenotypic rescue resulting from expression of Sm-F5H must be independent of flavonoid accumulation. Analysis of a population of 39 T2 progeny from a single T1 transformant revealed a substantial and

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**Figure 6.** Restoration of the Ability to Synthesize Flavonoids Does Not Aggravate the Growth Phenotype of the \( tt4-2 \) HCT-RNAi Plants.

(A) Schematic illustration of the crosses made to generate the two F1 populations. The number of plants for each genotype is indicated in brackets.

(B) Height at maturity of the four types of F1 plants shown in (A). The averaged values are presented, and the error bars represent 1 SD.

**Silencing of HCT in \( tt4-2 \) Plants Decreases Lignin Content and Alters Lignin Composition**

As shown above, RNAi silencing of HCT has identical effects on the growth phenotype of wild-type and \( tt4-2 \) plants, despite the fact that they differ in their ability to synthesize flavonoids. To investigate if this growth defect is associated with common changes in lignin, we analyzed lignin content and composition of HCT-RNAi and \( tt4-2 \) HCT-RNAi plants using the derivatization followed by reductive cleavage (DFRC) method (Lu and Ralph, 1997). The RNAi lines with a moderate growth phenotype were used in this experiment to allow collection of enough inflorescence stem material for lignin analysis. These analyses showed that wild-type and \( tt4-2 \) plants have similar total lignin content and lignin compositions, with a majority of guaiacyl (G) and syringyl (S) lignin per rosette, was similar at all time points (Figure 7D). Sinapoylmalate measurements followed a similar but distinct trend. Whereas sinapoylmalate concentration (nmol per mg) was apparently unaffected by the presence of the HCT-RNAi transgene (Figure 7E), the total amount of sinapoylmalate per rosette was clearly lower in HCT-RNAi plants than in wild-type plants after day 18 (Figure 7F). These data are consistent with a role for HCT in sinapoylmalate biosynthesis and suggest that the high concentration of flavonoids and sinapoylmalate in HCT-RNAi plants may be a result of reduced leaf expansion.
statistically significant difference (Student’s t test, \( P < 0.05 \)) in height between the T2 plants containing the Sm-F5H transgene (13.5 cm, \( \text{SD} = 2.1 \text{ cm}, n = 30 \)) and their siblings lacking the transgene (6.5 cm, \( \text{SD} = 4.7 \text{ cm}, n = 9 \)) (Figure 10). We conclude from these data that the rescue of the HCT-RNAi phenotype by Sm-F5H is independent of flavonoids as previously reported and is due to the restoration of a biosynthetic pathway downstream of coniferaldehyde.

**DISCUSSION**

The biosynthetic pathways leading to lignin and flavonoids, two major classes of phenylpropanoids, diverge at the common intermediate \( p \)-coumaroyl CoA. Perturbation of lignin biosynthesis by silencing of HCT or mutation of the gene encoding C3’H leads to both decreased lignin content and increased flavonoids. Either or both of these two biochemical changes could potentially contribute to the growth inhibition observed in these plants, since lignin is important for the water transport function of xylem (Boyce et al., 2004) and some flavonoids are known to be able to modulate the polar transport of auxin (Taylor and Grotewold, 2005; Peer and Murphy, 2007). Previously, Besseau et al. (2007) reported that the reduced-growth phenotype of HCT-RNAi plants can be rescued by RNAi silencing of CHS, suggesting that flavonoid accumulation, not lignin deficiency, is responsible for this growth inhibition. By contrast, we demonstrated here that...
this growth effect is independent of flavonoids by silencing HCT in a CHS null background.

One possible explanation for the discrepancy between our data and that of the previous study is that in Besseau et al. (2007), transcription of both the HCT and CHS RNAi constructs was driven by the same promoter (35S), which in some cases can lead to transcriptional inactivation of one or both transgenes (Fagard and Vaucheret, 2000). Such promoter-homology based suppression could reduce the silencing of HCT in plants carrying both transgenes and consequently alleviate their growth defect. Importantly, both our data and that of Besseau et al. are in agreement that very slight differences in HCT activity (within 1 to 4% wild-type level) lead to dramatic differences in growth phenotype. Such small changes cannot be distinguished at the protein level by the immunoblotting assay that Besseau et al. used to monitor HCT expression in the F1 progeny of the crosses between HCT-RNAi and wild-type or CHS-RNAi plants (see Figures 2 and 9 of Besseau et al., 2007). Thus, it is possible that the alleviation in the growth phenotype of their HCT-RNAi plants was actually due to a suppression of the HCT-RNAi construct that is undetectable at the protein level but that is significant phenotypically. This explanation is consistent with the fact that the HCT-RNAi × CHS-RNAi F1 plants showed more than 14-fold higher lignin content compared with their HCT-RNAi parents, which was not apparent in stem section staining (see Table 2 and Figure 10B of Besseau et al., 2007). This problem was avoided in our analysis by the use of a CHS null mutant instead of RNAi suppression.

There is a well-established role for flavonoids, such as quercetin and kaempferol, in the inhibition of polar auxin transport, and the increased accumulation of flavonoids resulting from suppression of HCT in the wild-type background, as observed by both Besseau et al. et al. and ourselves, might indeed impact auxin transport. This effect, however, is unrelated to the dwarfism seen in these plants. By contrast, the observation that sinapoylmalate levels in HCT-RNAi plants are at least as high as that in wild-type plants appears to contradict the established role of HCT in the core phenylpropanoid pathway. Although this has previously been taken to suggest the existence of an HCT-independent route for sinapoylmalate biosynthesis (Abdulrazzak et al., 2006; Besseau et al., 2007), a simple alternative explanation for the above observations is that the decrease in flavonoids and sinapoylmalate observed in wild-type plant rosettes with time is simply due to a diluting effect resulting from leaf expansion and that the failure of HCT-RNAi leaves to fully expand leads to higher measurements of soluble phenylpropanoids when expressed on a fresh weight basis, as is usually done. It is also possible that the amount of HCT activity present in the leaves of wild-type plants is in great excess of the amount required for normal sinapoylmalate biosynthesis and that the RNAi suppression used here, while sufficient to lead to lignin defects, fails to affect sinapoylmalate biosynthesis.

In addition to C3’H and HCT, perturbation of lignin biosynthesis at other points in the phenylpropanoid pathway also results in growth reduction and dwarfing. For example, transgenic tobacco deficient in Phe ammonia lyase, transgenic alfalfa with downregulated cinnamic acid 4-hydroxylase (C4H), as well as Arabidopsis ref3 and irx4 mutants, which are defective in C4H and cinnamoyl CoA reductase, respectively, all exhibit reduced...
deficiency can readily explain the growth reduction and dwarfing in phenylpropanoid pathway mutants, we cannot exclude the possibility that the accumulation or deficiency of another phenylpropanoid derivative may directly or indirectly influence plant growth and development. For example, dehydrodiconiferyl alcohol glucosides (DCGs), derivatives of coniferyl alcohol, have been shown to have cell division-promoting activities in tobacco tissue cultures (Lynn et al., 1987; Teutonico et al., 1991). It has been reported that, in tobacco, inhibition of phenylpropanoid metabolism by overexpression of MYB308 from *Antirrhinum majus* resulted in decreased content of DCGs and abnormal growth, and feeding the cell culture of these transgenic tobaccos with dehydrodiconiferyl alcohol, the aglycone precursor of DCGs, significantly restored cell expansion (Tamagnone et al., 1998). It would be interesting to explore further whether DCGs and their growth-promoting activities are required for normal development in other plant species and whether their absence could be related to the dwarfing seen in phenylpropanoid pathway-downregulated plants.

Clearly, further investigation will be required to fully understand the mechanism underlying the phenotypic manifestations resulting from disruption of phenylpropanoid metabolism. Given the potential impact of lignin reduction on biofuel production, this knowledge will have a great impact on future genetic engineering strategies for the development of improved biofuel feedstocks.

**METHODS**

**Plant Material and Growth Conditions**

The ref8 mutant was generated previously in our laboratory (Franke et al., 2002a). The *tt4-2* mutant (Burbulis et al., 1996) was kindly provided by Angus Murphy. Plants were grown in Redi-earth Plug and Seedling Mix (Sun Gro Horticulture) supplied with Scotts Osmocote Plus controlled release fertilizer (Hummert International) at 22°C under a 16-h-light/8-h-dark photoperiod. The lighting was supplied at an intensity of 100 μE m⁻² s⁻¹ by both incandescent bulbs and fluorescence tubes.

**Generating Transgenic Plants**

The generation of the HCT-RNAi and the Sm-F5H expression constructs has been described previously (Weng et al., 2008, 2010). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* was performed using a floral dip method (Clough and Bent, 1998). The HCT-RNAi construct was transformed into either wild-type Columbia or *tt4-2* plants, and the transgenic plants were selected by spraying with 1:1000 dilution of Finale Concentrate (glufosinate ammonium; Farnam Companies). The Sm-F5H expression construct was transformed into *tt4-2* HCT-RNAi plants, and the transgenic plants were selected on Murashige and Skoog medium containing 50 μg/mL kanamycin.

**Genotyping**

Tissue samples were ground in a 1.5 mL eppendorf tube, and 0.5mL extraction buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, and 1% SDS) was added. After centrifugation at 16,000g for 5 min, 0.35 mL of supernatant was mixed with 0.35 mL isopropanol in a new tube. The tube was centrifuged at 16,000g for 10 min, the supernatant was discarded, and after the pellet was dry, 0.4 mL TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) was added to redissolve the DNA.
A cleaved amplified polymorphic sequence marker (Konieczny and Ausubel, 1993) that exploits the EcoRV polymorphism caused by the ref8 mutation was used for ref8 genotyping (Franke et al., 2002b). A derived cleaved amplified polymorphic sequence marker (Michaels and Amasino, 1998; Neff et al., 1998) was used for tt4-2 genotyping. The forward primer 5'-AGCTAAACAGATTCTACTTCTCA-3' and reverse primer 5'-TTCGATAGGAGGACA-3' were used to amplify a 372-bp fragment from the CHS locus. The tt4-2 allele can be distinguished from the wild-type TT4 allele from the Ddel digestion pattern of the amplicon. The forward primer 5'-GACCTAAAGACTGCGTGTAAGCA-3', which anneals to the 35S promoter in the HCT-RNAi construct, was used with a HCT-gene-specific reverse primer 5'-TAAAGGTAGGAGCATAATCACCCAAA-3' to detect the HCT-RNAi transgene from the transgenic plants.

HPLC Analysis of Soluble Phenylpropanoid Metabolites

Plant extracts were prepared from fresh tissue with 50% (v/v) methanol (1 mL per 100 mg tissue). Samples were incubated at 65°C for 30 min and centrifuged at 16,000g for 5 min. Ten microliters of the supernatant were injected onto a Shim-pack XR-ODS column (3.0 × 75 mm, 2.2 μm) (Shimadzu) and analyzed using a gradient from 5% acetonitrile in 0.1% formic acid to 25% acetonitrile in 0.1% formic acid at a flow rate of 1 mL min⁻¹. Sinapic acid and kaempferol were used as standard for quantification of sinapoylmalate and kaempferol-derived flavonoids, respectively.

Quantitative RT-PCR

RNA was isolated from plant tissue using the RNeasy plant mini kit (Qiagen). TURBO DNase (Applied Biosystems) treatment was performed to eliminate possible genomic DNA contamination. cDNA synthesis was performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the protocol provided by the manufacturer. SYBR green-based quantitative PCR was performed using the relative quantification method. The forward primer 5'-GAATTCCATACGAGGGTTTGTCTT-3' and reverse primer 5'-GGGGAATGGGCAAGCATA-3' were used to detect the HCT mRNA. We used the At1g13320 gene, which encodes a regulatory subunit of a Ser/Thr protein phosphatase 2A, as a reference gene using the previously published primers since its expression has been shown to be stable over a wide range of conditions (Czechowski et al., 2005).

Plant Height Measurement

To determine whether there were height differences between the wild-type and any of the mutant and transgenic lines, the length from the base of rosettes to the tip of primary inflorescence stems was measured at a mature stage when inflorescence stems had ceased elongation.

Lignin Analysis

Floral stems from 8-week-old Arabidopsis plants were harvested (with siliques removed) and ground to a powder in liquid nitrogen. Cell wall preparations from the above material were performed by an initial extraction with 0.1 M sodium phosphate buffer, pH 7.2, for 30 min at 37°C, three subsequent extractions with 70% ethanol at 80°C, and a final acetone extraction. Fifteen milligrams of the dried cell wall material were used for DFRC analysis, which was performed following the published protocol (Lu and Ralph, 1998). Briefly, cell wall material was dissolved in acetyl bromide/acetic acid solution (20:80, v/v) containing 4.4-ethylidenobenzophenol as an internal standard. After evaporating the solvent with N2 gas, dioxane/acetic acid/water (5:4:1, v/v/v) and zinc dust were added to cleave the solubilized lignin. The reaction products were purified with a C18 SPE column (SUPELCO) and acetylated with pyridine/acetic anhydride solution (2:3, v/v). The resulting lignin monomer derivatives were analyzed by gas chromatography/flame ionization detection. The response factors used for quantification are 1.26, 1.30, and 1.44 for H, G, and S monolignol derivatives, respectively.

HCT Activity Assay

Tissue for each assay was prepared by removing cauline leaves and siliques from a single mature inflorescence stem in the case of wild-type or tt4-2 mutants. In the case of HCT-RNAi or tt4-2 HCT-RNAi plants, two or three individual inflorescence stems were pooled for each tissue sample to compensate for their small size. Tissue was flash frozen in liquid nitrogen, ground with a mortar and pestle, and suspended in 400 mM Tris, pH 7.8, and 2 mM ascorbic acid with an equal amount of wet polyvinyl pyrrolidone. Samples were stirred 5 min on ice, and particulates were removed by filtering through Miracloth (Calbiochem) and centrifugation. The resulting reaction mixture was desalted using a PD-10 column (GE Healthcare) according to the manufacturer’s instructions. Plant extract was preincubated with 200 μM p-coumarate, 220 μM CoA, 5 mM ATP, 5 mM MgCl2, and recombinant 4CL to form p-coumaroyl-CoA. Recombinant 4CL enzyme was obtained by cloning the Arabidopsis 4CL open reading frame (amplified using primers 5'-GGAGAGCATAT-GGCGCCACAAAGACAA-3' and 5'-GATACGCGACATCCATTGTGACTTTTGC-3') into the Ndel and XhoI sites of the pet30a+ (+) (Novagen) bacterial expression vector and transforming this construct into BL21 DE3 cells. The log phase cultures were induced with 0.8 mM isopropyl β-D-thiogalactoside and grown overnight at 18°C, followed by lysing in 400 mM Tris, pH 7.8, and 7.1 mM PMSF by freeze-thaw and desalting the resulting lysate using a PD-10 column (GE Healthcare). Finally, HCT activity was determined by adding shikimate to a final concentration of 10 mM and monitoring p-coumaroyl shikimate formation using HPLC.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: C3'H, At2g40890; HCT, At5g48930; CHS, At5g13930; and Sm-F5H, EU032589.

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