Salicylic acid mediates the reduced growth of lignin down-regulated plants

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Down-regulation of the enzyme hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) in thale cress (*Arabidopsis thaliana*) and alfalfa (*Medicago sativa*) leads to strongly reduced lignin levels, reduced recalcitrance of cell walls to sugar release, but severe stunting of the plants. Levels of the stress hormone salicylic acid (SA) are inversely proportional to lignin levels and growth in a series of transgenic alfalfa plants in which lignin biosynthesis has been perturbed at different biosynthetic steps. Reduction of SA levels by genetically blocking its formation or causing its removal restores growth in HCT–down-regulated *Arabidopsis*, although the plants maintain reduced lignin levels. SA-mediated growth inhibition may occur via interference with gibberellic acid signaling or responsiveness. Our data place SA as a central component in growth signaling pathways that either sense flux into the monolignol pathway or respond to secondary cell-wall integrity, and indicate that it is possible to engineer plants with highly reduced cell-wall recalcitrance without negatively impacting growth.

biofuel crops | defense signaling | lignin modification | *Medicago truncatula*

T he phenylpropanoid-derived polymer lignin cross-links plant secondary cell walls to provide mechanical strength and hydrophobicity to the vascular system as well as contributes to defense against biotic stress (1). Reducing lignin levels by genetic manipulation improves both forage digestibility and processing of lignocellulosic biomass for liquid biofuel production (2). However, transgenic plants down-regulated in the hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) enzyme, which have a strong reduction in lignin levels, show severe defects in growth (3–5). Similar, although less severe, effects are observed in plants down-regulated in some, but not all, of the other enzymes of the monolignol pathway (5–10).

The dwarf phenotype of HCT–down-regulated *Arabidopsis* plants can be alleviated by restoring lignin accumulation through expression of a *Selaginella* enzyme that bypasses the reactions catalyzed by HCT (4), suggesting that structural alterations to the partially lignified secondary cell walls are in some way linked to the growth defects. Such structural alterations include increased extractability of pectic polysaccharides (11). It is not clear whether it is possible to strongly reduce lignin content in plant cell walls without causing structural changes that will lead to deleterious effects.

HCT–down-regulated alfalfa plants contain increased levels of the stress hormone salicylic acid (SA) and SA-inducible pathogenesis-related (PR) protein transcripts (11). *PR* gene expression can be induced by cell-wall pectic fragments via processes involving SA (12, 13), and SA can impact plant growth and development through largely unknown mechanisms (14, 15). A previous report that lignin reduction in HCT–down-regulated plants results from flavonoid-mediated inhibition of auxin transport has recently been refuted (4).

Levels of SA correlate with the extent of lignin reduction in a series of transgenic alfalfa lines expressing antisense or RNAi constructs independently targeting seven enzymes of the monolignol pathway (16). The potential relationship between SA levels and lignin is intriguing because SA is biosynthetically related to lignin (17). Two major routes have been proposed for SA biosynthesis in plants. The initial pathway via isochorismate, derived from the shikimic acid pathway, is supported genetically (18), but enzymes that convert isochorismate to SA have yet to be shown in plants. Similarly, the previously favored pathway via cinnamate is believed to involve a benzoate 2-hydroxylase (19), but no gene encoding this enzyme has been shown in plants. Theoretically, back-up of flux into the lignin pathway could result in increased production of SA via either route. Alternatively, SA accumulation might be a result of activation of endogenous defense responses by elicitor-active polysaccharides released from improperly lignified cell walls (11).

We show here that removal of SA by genetic approaches relieves growth inhibition but maintains low lignin levels in HCT–down-regulated *Arabidopsis thaliana*. Thus, there is no a priori reason why plants cannot be engineered to contain highly processable cell walls yet also produce abundant biomass. The effects of SA are associated with alterations in gibberellin (GA) responsiveness and signaling.

Results

Correlations Between SA Levels and Growth in a Population of Lignin-Modified Plants. To investigate whether SA levels were directly related to growth phenotypes, we examined a series of transgenic alfalfa lines in which lignin biosynthesis had been perturbed via antisense or RNAi-mediated down-regulation (2). The collection of plants analyzed consisted of three WT controls and sets of three independent transgenic events down-regulated independently at each of six successive steps of the monolignol pathway (24 plants grown in parallel) (Fig. 1). Lignin levels (thioacidolysis yields) in the first six internodes were from WT levels (130 nmol·g^{-1} dry weight) to as low as 30 nmol·g^{-1} dry weight and inversely correlated with SA levels (R^2 = 0.796) (16). Stem height was inversely correlated with endogenous SA levels across this population of plants (Fig. 1A). In addition, a positive correlation was observed between SA levels and the amount of cell-wall pectic material that was extractable by cold water from alcohol-insoluble cell-wall residues prepared from the aerial portions of the plants (Fig. 1B). Altogether, these data indicate that the previous observations of elevated SA levels and water-soluble pectic elicitors of PR protein induction in HCT–down-regulated alfalfa (11) are not specific to HCT down-regulation but reflect the extent of lignin reduction independent of the lignin pathway enzyme targeted.

Blocking SA Production Restores Growth to HCT–Down-Regulated *A. thaliana* Plants. We used *A. thaliana* to genetically separate reduced lignin from elevated SA levels because mutants of
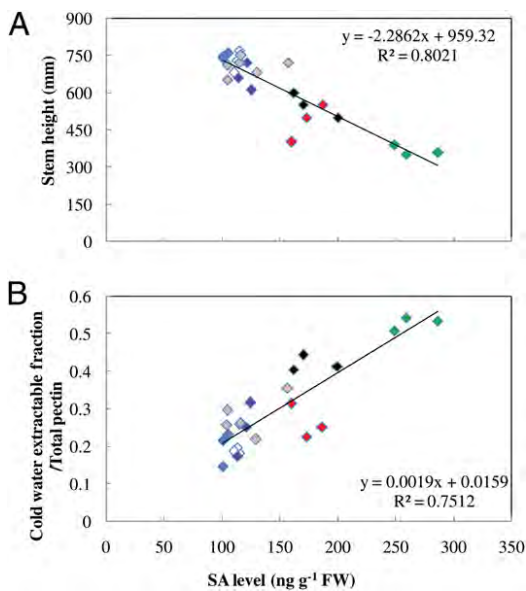
Fig. 1. SA levels, growth, and cold-water–extractable pectin in a population of transgenic alfalfa plants with lignin levels down-regulated at different steps in the monolignol pathway. (*A*) Stem height. (*B*) Pectin extractable from cell walls by cold water. The plant lines are color-coded depending on the gene target: red, 4-coumarate CoA ligase; black, coumaroyl shikimate 3′-hydroxylase; green, *HCT*; white, caffeoyl CoA 3-*O*-methyltransferase; violet, *CCR*; pink, cinnamyl alcohol dehydrogenase; gray, caffeic acid 3-*O*-methyltransferase; blue, WT. *P* < 0.0001. Stems with six internodes were harvested before flowering. For each transgenic line, three biological replicates were taken, each consisting of a pool of three stems from different plants.

Arabidopsis in which SA biosynthesis is blocked are available. As in alfalfa (11), *Arabidopsis* HCT-RNAi lines are severely dwarfed compared with WT plants (Fig. 2*A*) (3) and exhibit increased SA levels and expression of the *PR-1*, *PR-2*, and *PR-5* defense genes (Fig. S1 *A* and *B*). SA and PR transcript levels are also elevated in the *cinnamoyl CoA reductase 1* (*ccr1*) mutant of *Arabidopsis* (Fig. S2), suggesting that, as in alfalfa, elevated SA production is not restricted to plants with lignin modification targeted only to HCT.

SA biosynthesis may occur through either the isochorismate synthase (ICS) route or via benzoic acid derived from cinnamate (17). The fast-neutron–generated *sid2-2* mutant of *Arabidopsis* contains an exon deletion in the gene encoding ICS (20) that is implicated in SA biosynthesis (18). Homozygous *sid2-2* plants have growth, basal SA levels, and HCT and PR transcript levels similar to those in WT plants (Fig. 2 *A*–*D*). To determine the impact of blocking SA biosynthesis through loss of function of ICS on the growth of HCT–down-regulated plants, homozygous *sid2-2* mutant plants were crossed with a homozygous T$_5$ HCT-RNAi plant containing a WT SID2-2 allele. A control cross between WT and HCT-RNAi plants was performed in parallel. In the F1 population, heterozygous HCT-RNAi × WT plants exhibited reduced plant height similar to that in the HCT-RNAi dwarf parents, whereas heterozygous *sid2-2* × HCT-RNAi progeny showed a growth phenotype intermediate between that of the parents. The heterozygous *sid2-2* × hemizygous HCT-RNAi plants from the F1 population were crossed again with the *sid2-2* null mutant. Among a population of 40 progeny, four genotypes were found at approximately equal frequency: *sid2-2*/SID2-2, *sid2-2*/*sid2-2*, *sid2-2*/SID2-2 with HCT-RNAi, and *sid2-2*/*sid2-2* with HCT-RNAi. The HCT-RNAi × WT progeny retained their dwarf growth habit (Fig. 2 *A* and *B*), high SA levels (Fig. 2*C*), and elevated PR transcript levels (Fig. 2*D*). In contrast, SA and PR transcript levels were reduced and growth was con-

siderably restored in HCT-RNAi lines either homozygous or heterozygous for the *sid2* mutation (Fig. 2 *A*–*D*). The SA levels of the *sid2-2* heterozygotes were intermediate, between those of the HCT-RNAi and *sid2-2* or WT values (Fig. 2*C*). Heterozygous *sid2* mutants in the WT background do not exhibit reduced SA levels after fungal infection (21); the apparent lack of haploinsufficiency with regard to the SA phenotype in the present crosses may be the result of the different HCT expression levels in infected plants in a WT background (such as in ref. 19) and the present uninfected plants with an HCT-RNAi background.

Extractable HCT enzymatic activity, staining of syringyl lignin with Maüle reagent (1), and lignin monomer thioacidolysis yields were strongly reduced in all lines expressing the HCT-RNAi construct, including those homozygous for the *sid2* mutant with restored growth (Fig. 2 *E*–*G*). The lignin composition of the HCT-RNAi lines in the *sid2-2*/*sid2-2* background was typical of plants with reduced HCT expression, namely elevated hydroxyphenyl lignin units, and strongly reduced levels of guaiacyl and syringyl units (22). Thus, HCT–down-regulated *Arabidopsis* plants in which SA biosynthesis is blocked no longer display a dwarf phenotype, despite their strongly reduced lignin levels and drastically altered lignin composition.

Conversion of SA to Catechol Also Restores Growth to HCT–Down-Regulated *A. thaliana* Plants. Because of the potential of multiple pathways for SA biosynthesis, we sought additional genetic confirmation of the role of SA in the growth phenotype of HCT-RNAi plants. The HCT-RNAi line was therefore crossed with plants expressing the bacterial *NahG* gene, which encodes a salicylate hydroxylase that removes SA by converting it to catechol (23). NahG *Arabidopsis* plants have WT growth habit (Fig. 3 *A* and *D*), and introduction of the NahG transgene into HCT-RNAi plants restored growth to WT levels and reduced SA and PR transcript levels (Fig. 3 *A*–*D*) in a manner similar to the introduction of the *sid2* mutation. The extractable HCT activity of the individual NahG/HCT-RNAi progeny plants analyzed varied considerably, as did the overall lignin level, both of which, however, were always lower than those of the NahG parent line (Fig. S3 *A* and *B*). In addition, all NahG:HCT-RNAi lines showed the hydroxyphenyl lignin signature characteristic of HCT down-regulation (Fig. S3*B*). The elevation of HCT expression in many of the lines from this cross is likely the result of partial silencing of the 35S promoter derived HCT-RNAi by the additional copy of the 35S promoter driving the *NahG* gene. Importantly, however, HCT expression and lignin levels in some progeny lines were as low as in the HCT-RNAi parent line, but growth was normal (Fig. S3 *B* and *C*).

Reduction of SA-Mediated Defense Signaling Does Not Restore Growth to Low-Lignin *Arabidopsis* Plants. To test whether PR expression, distinct from SA production, was causing the growth defects in HCT-RNAi plants, we crossed HCT-RNAi plants with plants harboring a mutation in *NPR1*, a gene involved in signal transduction between SA and PR expression (24). HCT-RNAi lines homozygous for the *npr1* mutation retained elevated SA levels (Fig. 3*B*) and exhibited a partial reduction (∼50%) of *PR5* and *PR2* transcript levels, although *PR1* transcripts were unaltered (Fig. 3*C*). The growth of these plants remained severely stunted (Fig. 3 *A* and *D*). These results are consistent with the fact that PR genes can be regulated by an alternative NPR1-independent pathway (25, 26); for example, constitutive PR gene expression in the *cpr6-1* dominant mutation in *Arabidopsis* requires elevated SA levels but not NPR1 function (25).

Restoring Growth by Removal of SA Is Not Mediated by Altered Flavonoid Levels. *Arabidopsis* HCT-RNAi plants accumulate high levels of flavonoids because of metabolic spillover from the lignin to the flavonoid pathway, which shares 4-coumaroyl CoA, the
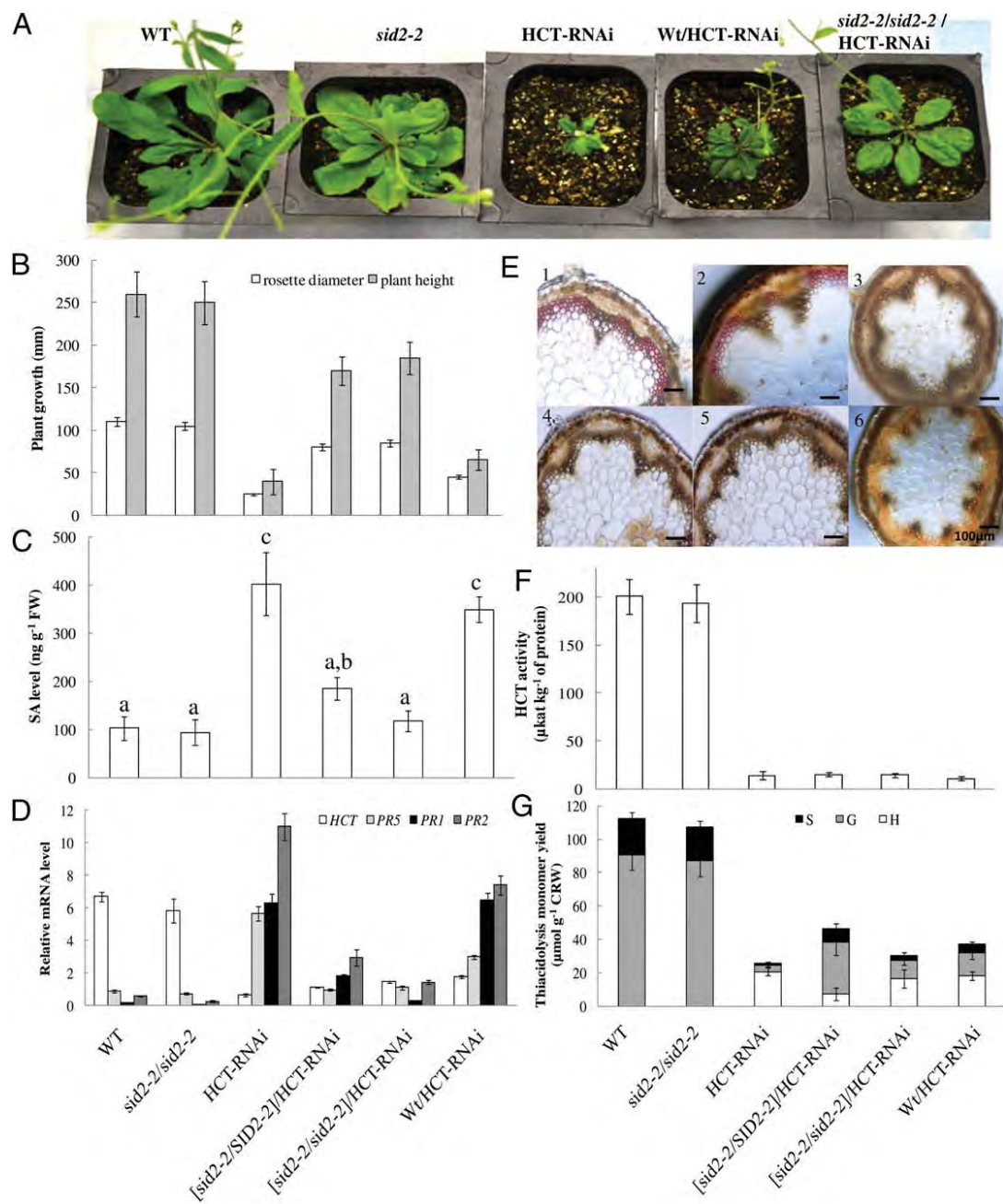
Fig. 2. Blocking SA accumulation restores growth to HCT-RNAi *Arabidopsis*. (*A*) Fifteen-week-old plants grown in soil. (*B*) Rosette diameters and plant heights for the genotypes in *A*. (*C*) SA levels. Values with different letters are significantly different (*P* < 0.05). (*D*) Transcript levels of the *HCT*, *PR5*, *PR1*, and *PR2* genes as determined by qRT-PCR. (*E*) Maüle staining of stem cross-sections. Note loss of red staining in interfascicular fibers in all lines expressing HCT-RNAi. 1, WT; 2, *sid2-2* null mutant; 3, HCT-RNAi; 4, HCT-RNAi with heterozygous *sid2-2*; 5, HCT-RNAi with homozygous *sid2-2*; 6, WT × HCT-RNAi. (*F*) Extractable HCT enzyme activity. (*G*) Lignin content and composition as determined by thioacidolysis. H, hydroxyphenyl unit; G, guaiacyl unit; S, syringyl unit. Results are means ± SD of three biological replicates (each a pool of three plants of the same genotype).

substrate for HCT, as a common early intermediate (3, 4). Flavonoid levels were reduced somewhat in the *sid2-2* × HCT-RNAi plants, but they still contained significantly higher levels of flavonoids than WT plants did (Fig. S4), confirming the previous conclusion that elevated flavonoid accumulation is not the primary reason for the growth defect in HCT-RNAi plants (4).

SA-Mediated Growth Effects in Low-Lignin Plants May Operate via GA Signaling. HCT-RNAi alfalfa plants show reduced GA levels and perception (11). To test whether the dwarf phenotype of *Arabidopsis* HCT-RNAi lines with elevated levels of SA is mediated

via altered GA sensing or signaling, we first examined the effects of exogenous addition of GA$_4$ to *Arabidopsis* seedlings germinated on Petri plates for 10 d. The GA biosynthesis inhibitor paclobutrazol caused total inhibition of growth of both WT and HCT-RNAi lines (Fig. 4*A*). Both WT and HCT-RNAi seedlings showed similar growth enhancement at GA concentrations of 10^{-6} M and above (Fig. 4*A*). However, at 8 wk postgermination, GA induced similar increases in rosette diameter and inflorescence length in WT, NahG, and *sid2-2* plants but not in HCT-RNAi lines (Fig. 4*B*). GA did, however, increase rosette diameter and petiole length in lines in which the HCT-RNAi was
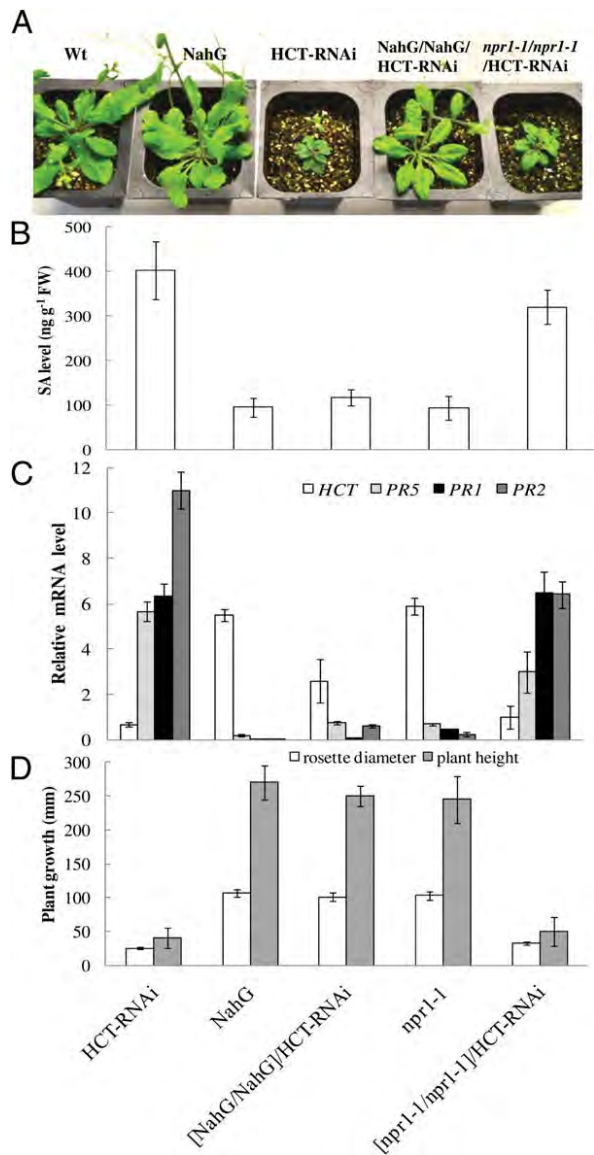
Fig. 3. Removal of SA by expression of NahG restores growth to HCT-RNAi *Arabidopsis*, but interference with SA signaling does not. Plants are F2 progeny of the crosses NahG × HCT-RNAi and *npr1-1* × HCT-RNAi. (*A*) Plant phenotypes. (*B*) SA levels. (*C*) Transcript levels of *HCT*, *PR5*, *PR1*, and *PR2* as determined by qRT-PCR. (*D*) Rosette diameters and plant height. Results are means ± SD of three biological replicates (each a pool of three plants of the same genotype).

expressed in the *sid2-2* or NahG background, and inflorescence length also increased in NahG × HCT-RNAi lines (Table S1). Thus, GA responsiveness is lost in HCT-RNAi lines at later stages of vegetative development when lignification is occurring but can be restored by genetic manipulation to block accumulation of SA.

To test whether the reduction in growth of HCT-RNAi lines is associated with SA-mediated impairment of GA biosynthesis/signaling, we designed PCR primers for the *Arabidopsis GA3* and *GA20* oxidases (GA biosynthesis, repressed by exogenous GA application), gibberellic acid-stimulated transcript protein homolog 1 (*GASA1*), and xyloglucan endotransglycosylase/hydrolase (*XTH*) genes *XTH4*, *XTH9*, *XTH24*, and *XTH32* (GA-induced) (Table S2), orthologs of which are down-regulated in HCT–down-regulated alfalfa (11). Quantitative RT-PCR (qRT-PCR) analysis indicated that these genes were repressed in the HCT-RNAi *Arabidopsis* line compared with WT, even after

exogenous GA application (Fig. 4 *C–E*). However, the GA responsiveness of these genes (repression for *GA20* and *GA3* oxidases and induction for *GASA1* and *XTH* genes) was restored in HCT-RNAi lines in the *sid2-2* and NahG backgrounds after exogenous GA application (Fig. S5 *A–C*), with the notable exception of *XTH9* in *sid2* lines (Fig. S5C).

Discussion

Down-regulation of lignin pathway genes in transgenic plants often leads to reduced biomass associated with altered growth and vascular morphology. This phenomenon has been observed in plants with partial knockdown of enzyme activities through expression of antisense or RNAi constructs (5, 7) and is more apparent in genetic knockouts, which are often highly dwarfed (4, 27, 28). If these growth effects are primarily caused by altered vascular morphology and function, it will be difficult to reverse-engineer low-lignin plants for improved growth. This concept was questioned when it was proposed that metabolic spillover from the lignin to the flavonoid pathway was the cause of the reduced growth phenotype in HCT–down-regulated *Arabidopsis* plants via flavonoid-mediated inhibition of auxin transport (3). However, although down-regulation of HCT clearly does result in increased flavonoid levels (3, 11), reduced growth in HCT–down-regulated alfalfa plants is not associated with alterations in auxin transport (11); flavonoids are elevated in some, but not all, reduced lignin lines with altered growth (2). Furthermore, reexamination of the original experiments linking flavonoids to growth effects in *Arabidopsis* (4), as well as the present analyses of flavonoid levels in *Arabidopsis* plants with low lignin but restored growth, strongly support the argument that flavonoids are not causally linked to growth defects in lignin-modified plants but that SA levels are.

Two pathways have been postulated for SA biosynthesis in plants: from cinnamate via benzoate (19) or from shikimate via isochorismate (18). The fact that the elevated SA levels present in HCT-RNAi plants were reduced to WT levels in HCT-RNAi plants homozygous for the *sid2* mutation suggests that lignin down-regulation induces SA formation primarily through the isochorismate pathway, which operates in *Arabidopsis* during responses to pathogens (18). The correlation between SA levels and the proportion of cold-water–extractable pectin to total pectin (Fig. 1*B*) in multiple transgenic reduced lignin alfalfa lines is consistent with the hypothesis that release of pectic elicitors from underlignified secondary cell walls in HCT-RNAi lines induces SA, and thereby defense responses, in the same manner as would occur when pathogens degrade cell walls during ingress (11, 12) rather than that the SA accumulates simply as a result of metabolic spillover from the monolignol pathway. A program of secondary wall formation with limited lignification can result in pectin that is more extractable (11), and pectic oligosaccharides are well known as elicitors of plant defense responses (12, 29, 30).

HCT-RNAi alfalfa (11) and *Arabidopsis* (the present work) are nonresponsive to GA as a growth enhancer when beyond the early seedling stage. GA-induced growth is restored upon reduction of the SA pool in *Arabidopsis*, suggesting that SA may mediate its growth-reducing effects through GA signaling. Our data do not support an alternative model in which reduced growth is largely a result of the diversion of energy into defense responses (e.g., PR protein expression). Although SA is increasingly being shown to impact plant growth (15), there has been little study of its potential involvement in elongation growth. Our work therefore provides a unique system for studying the poorly understood cross-talk between plant defense and growth control mechanisms (31, 32). In conclusion, we have shown that SA-mediated events are central to the orchestration of the reduced growth response of HCT-RNAi plants. This finding has clear implications for the engineering of improved bioenergy crops with optimal agronomic performance.
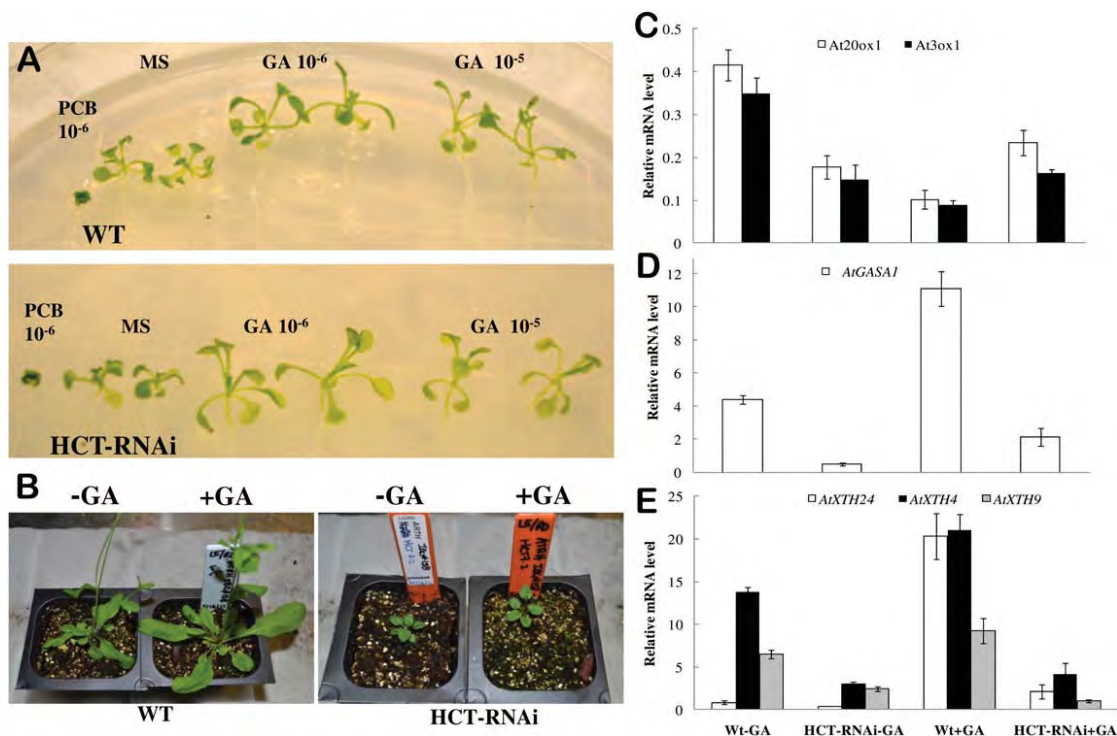
Fig. 4. Mature HCT-RNAi *Arabidopsis* plants have reduced responsiveness to GA. (*A*) Response of 12-d-old WT and HCT-RNAi seedlings to supplementation of the medium with the GA biosynthesis inhibitor paclobutrazol (PCB; 10^{-6} M) or GA_4 at 10^{-6} or 10^{-5} M. (*B*) Response of 8-wk-old plants to 10^{-5} M GA_4. Pictures were taken after 2 wk of twice-per-week GA treatment. (*C–E*) Transcript levels (by qRT-PCR) of GA biosynthesis and response genes in 8-wk-old *Arabidopsis* plants with and without GA application (8 d postapplication). Means and SDs from three biological replicates are given relative to the *Arabidopsis* serine/threonine phosphatase (*AtPP2A*) transcript levels. (*C*) GA20 and GA3 oxidase. (*D*) GASA1. (*E*) XTH4, XTH9, and XTH24.

Materials and Methods

Plant Material. *Arabidopsis* HCT-RNAi lines were obtained from Purdue University and were as previously described (4). Plants used for crossing were the mutant alleles *sid2-2* (18, 20) and *npr1-1* (24) along with plants expressing the NahG transgene (33); all were in the *A. thaliana* Columbia-0 ecotype background. The HCT-RNAi transgene was detected by RT-PCR using the primer pair reported previously (4). Mutants carrying the *npr1-1* mutation were identified with the codominant amplified polymorphic sequences (CAPS) markers described in ref. 24. *Arabidopsis ccr1* (AT1G15950.1) mutant seeds were obtained from the *Arabidopsis* Biological Resource Center (line SALK_123689). Primer pairs for plant genotyping were as follows: left primer, 5′-GTGTCGTAGAGGCTTTGCTTG-3′; right primer, 5′-TTGTGGAAA-TATTTCCGGTTG-3′; and LBb1.3, 5′-ATTTTGCCGATTTCGGAAC-3′. Alfalfa antisense lines analyzed were as described previously (5, 34, 35). For the specific lines used, see *SI Materials and Methods*.

GA Application. Treatments with GA_4 were as described in *SI Materials and Methods*.

Plant Growth Measurement and Histochemical Analysis. Plant height was measured as described in *SI Materials and Methods*. Maüle staining for lignin was performed as previously described (7) in cross-sections from the base of the mature stems.

Determination of SA Levels. SA levels were determined by using the biosensor organism *Acinetobacter* sp. ADPWH_lux as described previously (36, 37) and in *SI Materials and Methods*.

Assay of HCT Activity and Determination of Lignin and Flavonoid Levels. HCT activity was determined as described in *SI Materials and Methods*. Lignin content and composition was determined by thioacidolysis (38). Soluble phenolic compounds were extracted by using the protocol described in ref. 4 and as further described in *SI Materials and Methods*.

Determination of Pectic Compounds. Extraction of pectic materials from alcohol-insoluble cell-wall residues was determined as described previously (11). The proportion of pectic material released by cold-water extraction was determined as a proportion of total pectin.

Measurement of Transcript Levels by qRT-PCR. qRT-PCR analysis was performed as described previously (11). Gene-specific primers are listed in Table S2.

Statistical Analysis. Statistical treatment of data was performed by ANOVA using Fisher's least significant difference procedure for multiple-comparison tests (Statgraphics Plus, version 5.1 for Windows). Significance of correlations was obtained by using the online calculator for Pearson correlation *P* values at http://www.danielsoper.com/statcalc/calc44.aspx.

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