Supporting Information

Gallego-Giraldo et al. 10.1073/pnas.1117873108

SI Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis* hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT)-RNAi lines were obtained from Purdue University and were as previously described (1). Plants used for crossing were the mutant alleles *sid2-2* (2, 3) and *npr1-1* (4) along with plants expressing the NahG transgene (5); all were in the *Arabidopsis thaliana* Columbia-0 ecotype background. Seeds were exposed to a temperature of 4 °C for 2 d and then sown in a seedling mix substrate with controlled fertilizer at 21 °C under a 16-h light/8-h dark photoperiod. Light intensity was 110 $\mu mol \cdot m^{-2} \cdot s^{-1}$, supplied by both incandescent and fluorescence lights. The HCT-RNAi transgene was detected by RT-PCR using the primer pair reported previously (1). Primer pairs for detecting the *sid2-2* mutation and the NahG insertion were as follows: *sid2-2*, 5′-AATTGGCAGGGAGACTTACGAA-3′ and 5′-TCCTCTATCGAATGATTCTATCTCCTT-3′; and NahG, 5′-CACCGGGCGGATTTCAT-3′ and 5′-CCCGAATTGGGCGATACC-3′. Mutants carrying the *npr1-1* mutation were identified with the codominant amplified polymorphic sequences (CAPS) markers described in ref. 4. Plants were genotyped before biochemical or gene-expression analysis. *Arabidopsis cinnamoyl CoA reductase 1* (*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′-TTGTGGAAATATTTCCGGTTG-3′; and LBb1.3, 5′-ATTTTGCCGATTTCGGAAC-3′. Alfalfa antisense lines analyzed were as described previously (6–8). The specific lines used were *4CL* (34a, 13a, and 25a), *C3H* (4a, 9a, and 5A), *HCT* (30a, 3a, and 29a), *CCoAOMT* (CC2-305), *CCR* (48a, 4a, and 41a), *CAD* (17a, 63a, and 56a), *COMT* (C2-310), an empty control vector (CK48), and two WT lines (Ctrl1 and Ctrl49).

Gibberellin (GA) Application. *Arabidopsis* seeds were grown on Murashige and Skoog medium supplemented with 10^{-7}, 10^{-6}, and 10^{-5} M GA_4 (Sigma-Aldrich). Paclobutrazol (10^{-6} M) and Murashige and Skoog medium alone were used as control treatments. Plates were placed in a growth chamber, plant growth was measured, and pictures were taken after 12 d of growth. For plants grown in soil, applications of 10^{-5} M GA_4 were performed by spraying plants and watering soil twice per week for 2 wk. Eight plants were used as biological replicates per treatment. Experiments were repeated three times.

Plant Growth Measurement and Histochemical Analysis. Plant height was measured from the base of the rosette to the tip of the primary inflorescence stem. Both plant height and rosette diameter were measured at mature stage when the primary stem had ceased elongation. Maüle staining for lignin was performed as previously described (9) in cross-sections from the base of the mature stems.

Determination of Salicylic Acid (SA) Levels. SA levels were determined by using the biosensor organism *Acinetobacter* sp. ADPWH_lux as described previously (10, 11). Stems (100 mg fresh weight) were ground in fresh LB liquid medium (2.5 mL of LB per 1 g of stem) by vortexing for 30 s and sonicating for 5 min on ice, and the homogenates were then centrifuged at 12,000 × *g* for 15 min. The supernatants were used for SA measurement, and an equivalent volume of LB medium was used to make an SA standard curve (SA final concentrations of 0, 0.05, 0.25, 0.5, 1.6, 8.3, 20, 40, 83, 166, and 200 μM). An overnight culture of *Acinetobacter* sp. ADPWH_lux was diluted in LB medium (1:20) and grown at 37 °C for ∼2 h to an OD_{600} of 0.4. Then, 60 μL of LB medium, 50 μL of SA biosensor culture, and 20 μL of each crude extract were mixed in a 96-well cell-culture plate. The plate was incubated at 37 °C for 1 h without shaking, and bioluminescence and OD_{600} of negative controls (LB alone or water) were read with a GloMax-Multi Detection System (Promega). SA standards and negative controls were read in parallel with the experimental samples, and every sample was replicated five times. Relative bioluminescence was obtained by subtracting bioluminescence OD_{600} of negative controls, and SA concentration was estimated according to the SA standard curve.

Assay of HCT Activity. Approximately 0.2 g of ground stem tissue was resuspended in 2.7 mL of extraction buffer [100 mM Tris·Cl (pH 7.5), 10% glycerol, 1 mM PMSF, and 0.5 mM DTT] and 0.1 g of polyvinylpolypyrrolidone was then added. The suspension was kept on ice for 45 min with occasional vortexing. The supernatant was recovered after centrifugation (12,000 × *g* for 5 min) and desalted by passing through a PD-10 column (GE Healthcare) according to the manufacturer's instructions but with collection of only 1.5 mL of sample after the first 1.5 mL had been discarded. The protein concentrations of plant extracts were determined with the Bio-Rad protein assay. For enzyme assay, 3–5 μg of protein extract was incubated at 30 °C for 20 min with 100 mM sodium phosphate buffer (pH 7.5), 5 mM shikimic acid, 1 mM DTT (Roche), and 60 μM 4-coumaroyl CoA in a final volume of 100 μL. The reactions were stopped by adding 10 μL of glacial acetic acid, and products were analyzed by reverse-phase HPLC on a C18 column (Spherisorb 5μ ODS2; Waters) in a step gradient using 1% phosphoric acid in water as solvent A and acetonitrile as solvent B. Chlorogenic acid (caffeoyl quinic acid) was used to construct the calibration curve.

Determination of Lignin and Flavonoid Levels. Lignin content and composition was determined by thioacidolysis as described previously (12). Soluble phenolic compounds were extracted by using the protocol described in ref. 1. Forty microliters of the supernatant was injected onto a ODS2 reverse-phase column (5-μm particle size, 4.6 × 250 mm) and eluted in 1% (vol/vol) phosphoric acid with an increasing gradient of acetonitrile (0–5 min, 5%; 5–10 min, 5–10%; 10–25 min, 10–17%; 25–30 min, 17–23%; 30–65 min, 23–50%; 65–69 min, 50–100%) at a flow rate of 1 $mL \cdot min^{-1}$ for 70 min. The main peaks present in all samples were kaempferol-3-*O*-[rhamnosyl(1>2)glucoside]-7-*O*-rhamnoside (K1), kaempferol-3-*O*-glucoside-7-*O*-rhamnoside (K2), and kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside (K3). Compound identification was confirmed by liquid chromatography/MS. Flavonoid content was quantified by adding the K1, K2, and K3 peak areas at 320 nm (mAU) and using a kaempferol calibration curve as standard.

Determination of Pectic Compounds. Extraction of pectic materials from alcohol-insoluble cell-wall residues was determined as described previously (13). The proportion of pectic material released by cold-water extraction was determined as a proportion of total pectin.

Measurement of Transcript Levels by Quantitative RT-PCR (qRT-PCR). qRT-PCR analysis was performed as described previously (13). 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.

1. Li X, Bonawitz ND, Weng JK, Chapple C (2010) The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. *Plant Cell* 22:1620–1632.
2. Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414:562–565.
3. Dewdney J, et al. (2000) Three unique mutants of *Arabidopsis* identify *eds* loci required for limiting growth of a biotrophic fungal pathogen. *Plant J* 24:205–218.
4. Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88:57–63.
5. Ryals JA, et al. (1996) Systemic acquired resistance. *Plant Cell* 8:1809–1819.
6. Reddy MS, et al. (2005) Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). *Proc Natl Acad Sci USA* 102:16573–16578.
7. Shadle G, et al. (2007) Down-regulation of hydroxycinnamoyl CoA: Shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. *Phytochemistry* 68:1521–1529.
8. Chen F, et al. (2006) Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.). *Plant J* 48:113–124.
9. Nakashima J, Chen F, Jackson L, Shadle G, Dixon RA (2008) Multi-site genetic modification of monolignol biosynthesis in alfalfa (*Medicago sativa*): Effects on lignin composition in specific cell types. *New Phytol* 179:738–750.
10. Huang WE, et al. (2005) Chromosomally located gene fusions constructed in *Acinetobacter* sp. ADP1 for the detection of salicylate. *Environ Microbiol* 7: 1339–1348.
11. Huang WE, et al. (2006) Quantitative *in situ* assay of salicylic acid in tobacco leaves using a genetically modified biosensor strain of *Acinetobacter* sp. ADP1. *Plant J* 46: 1073–1083.
12. Zhao Q, et al. (2010) An NAC transcription factor orchestrates multiple features of cell wall development in *Medicago truncatula*. *Plant J* 63:100–114.
13. Gallego-Giraldo L, Jikumaru Y, Kamiya Y, Tang Y, Dixon RA (2011) Selective lignin downregulation leads to constitutive defense response expression in alfalfa (*Medicago sativa* L.). *New Phytol* 190:627–639.

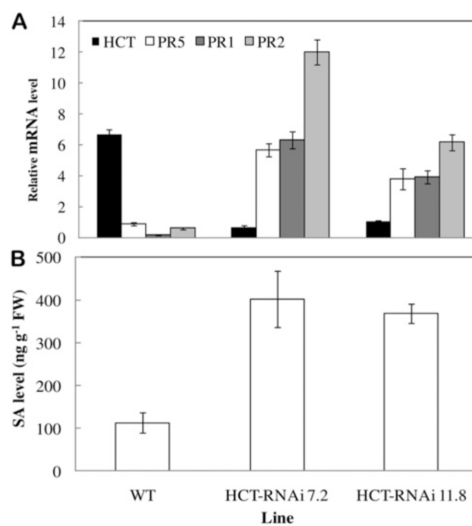

Fig. S1. Silencing HCT induces *PR* gene expression and SA accumulation in *Arabidopsis*. (*A*) Expression level of *HCT*, *PR5*, *PR1*, and *PR2* transcripts determined by qRT-PCR in WT and two HCT-RNAi lines. The relative expression of mRNA was normalized to that of the *Arabidopsis* serine/threonine phosphatase (*PP2A*) and β-tubulin (*BT*) genes. Results are the means ± SD of three biological replicates, and each PCR was run in triplicate. (*B*) SA levels in WT and two HCT-RNAi lines. Results are means of six biological replicates.

PNAS PNAS PNAS PNAS

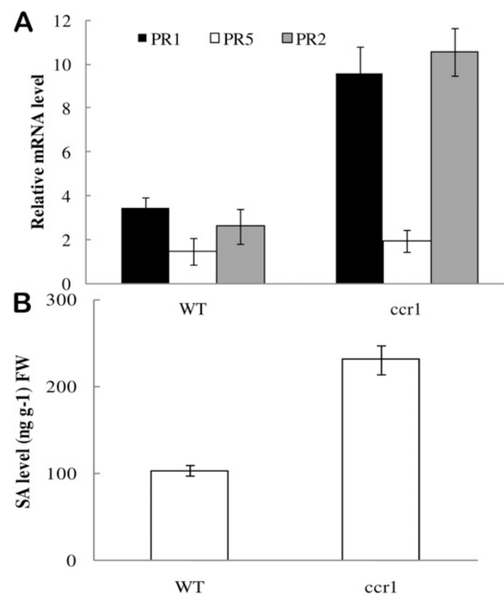

Fig. S2. Loss-of-function of the *Arabidopsis CCR1* gene of monolignol biosynthesis induces SA accumulation and *PR* gene expression. (*A*) Expression level of *PR1*, *PR5* and *PR2* transcripts determined by qRT-PCR in WT and *ccr1* mutant. The relative expression of mRNA was normalized to that of the *Arabidopsis* serine/threonine phosphatase (*PP2A*) and β-tubulin (*BT*) genes. Results are the means ± SD of three biological replicates, and each PCR was run in triplicate. (*B*) SA levels in WT and *ccr1* mutant. Results are means of six biological replicates.

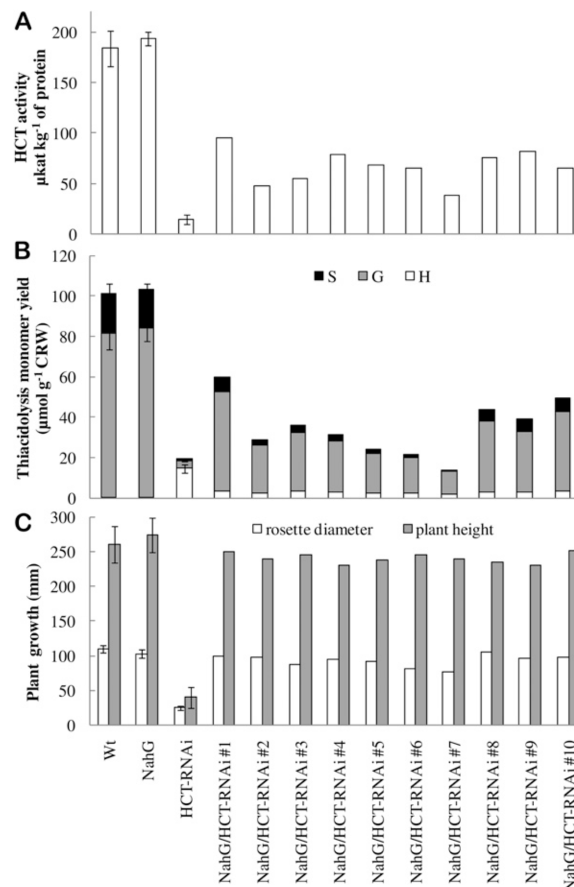

Fig. S3. Effects of expression of NahG in HCT-RNAi *Arabidopsis*. (*A*) Extractable HCT enzyme activity. (*B*) Lignin content and composition as determined by thioacidolysis. H, hydroxyphenyl unit; G, guaiacyl unit; S, syringyl unit. (*C*) Rosette diameters and plant heights. A pool of three plants with the same genotype was considered to be one biological replicate, and results are the means ± SD of three biological replicates in the case of WT, NahG, and HCT-RNAi lines. Ten progeny plants from HCT-RNAi in the NahG background were analyzed independently for each parameter.

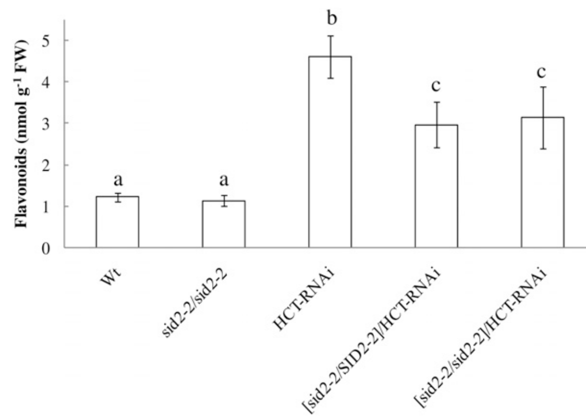

Fig. S4. Abolition of SA accumulation partially restores flavonoid accumulation to HCT-RNAi *Arabidopsis*. Flavonoid levels were determined by HPLC of extracts from stems of WT, *sid2-2* null mutant, HCT-RNAi, and HCT-RNAi heterozygous or homozygous for the *sid2-2* mutation. Values with different letters are significantly different ($P < 0.05$). A pool of three plants with the same genotype was considered to be one biological replicate, and results are the means ± SD of three biological replicates.

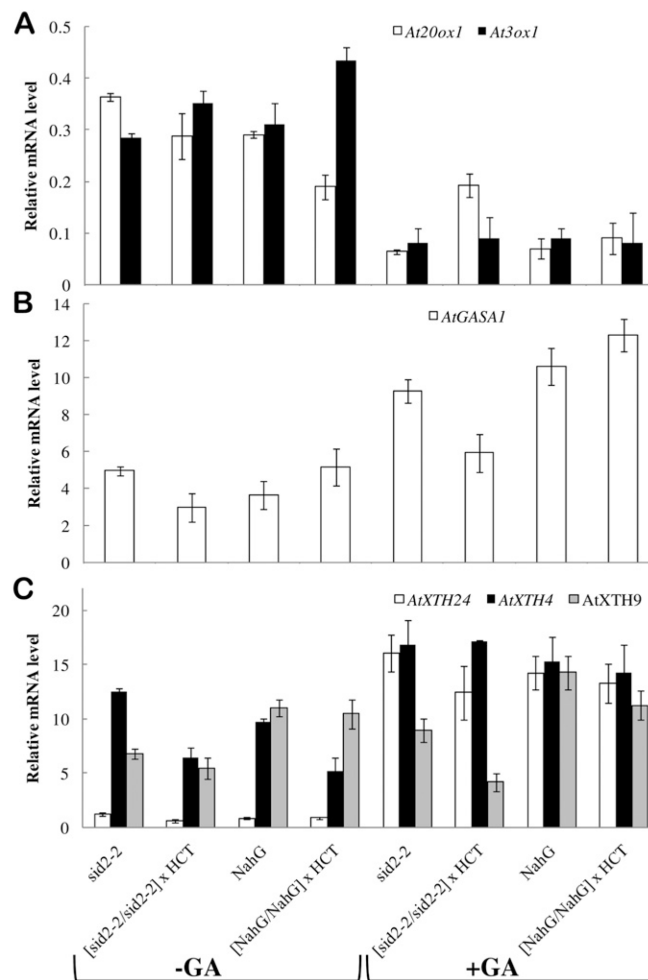

Fig. S5. Transcript levels of GA biosynthesis and response genes in *Arabidopsis* plants with and without GA application (8 d postapplication). Plants are *sid2-2* null mutant, HCT-RNAi with homozygous *sid2-2*, NahG, and HCT-RNAi in the NahG background. Transcripts levels were determined by qRT-PCR, and values (means and SDs from three biological replicates) are given relative to the *Arabidopsis* serine/threonine phosphatase (*AtPP2A*) transcript levels. (*A*) *GA20* and *GA3* oxidase. (*B*) *GASA1*. (*C*) *XTH4*, *XTH9*, and *XTH24*. See Fig. 4 *C–E* for response of WT plants grown in parallel.

Table S1. Responsiveness of *Arabidopsis* lines HCT-RNAi, *sid2-2*, *and NahG* (alone and in the HCT-RNAi background), and control *Arabidopsis* lines to external application of GA_4

Line	GA_4	Rosette diameter (mm)	Inflorescence length (mm)	Petiole length (mm)
WT	–	53.5 ± 4.7*	148.2 ± 9.2*	10.0 ± 1.3*
WT	+	76.5 ± 5.7†	198.2 ± 7.5†	14.0 ± 1.6†
sid2-2	–	42.5 ± 4.7*	139.7 ± 12.8*	9.3 ± 2.0*
sid2-2	+	68.5 ± 4.3†	179.7 ± 20.7†	12.4 ± 2.0*
HCT-RNAi	–	27.7 ± 8.0*	47.3 ± 5.9*	4.0 ± 1.4*
HCT-RNAi	+	29.7 ± 7.1*	51.2 ± 5.5*	3.2 ± 1.1*
NahG	–	46.2 ± 3.7*	142.5 ± 11.5*	8.7 ± 1.5*
NahG	+	70.5 ± 5.1†	183.2 ± 19.3†	12.3 ± 2.0†
sid2-2/*sid2-2*× *HCT*-RNAi	–	48.8 ± 6.1*	116.7 ± 19.2*	5.7 ± 1.2*
sid2-2/*sid2-2*× *HCT*-RNAi	+	58.5 ± 2.1†	130.0 ± 9.9*	7.8 ± 1.0†
NahG/NahG × HCT-RNAi	–	45.5 ± 3.9*	123.4 ± 10.3*	7.0 ± 1.0*
NahG/NahG × HCT-RNAi	+	69.5 ± 2.3†	155.8 ± 16.7†	10.6 ± 2.0†

Rosette diameter, inflorescence length, and petiole length were measured at 15 d after application of 10^{-5} M GA_4 via spraying and watering soil. Plants were 8 wk postgermination at the start of the experiment. Results are means ± SDs of eight replicate plants per treatment. Values for individual genotypes with and without GA treatment are marked with different signs (* or †) if they are significantly different ($P < 0.05$). Statistical analysis was performed independently with each pool of data from each plant genotype.

Table S2. Primers used for qRT-PCR

Gene-specific primer	Forward (5′ → 3′)	Reverse (5′ → 3′)
AtPP2A (serine/threonine phosphatase)	AGATCGCTCGGAACTTGGAAA	ACATCCTCACCAAAACTCAAATCA
AtBT (tubulin)	TGGGAACTCTGCTCATATCT	GAAAGGAATGAGGTTCACTG
AtPR1	GTCTCCGCCGTGAACATGT	TGCACGTGTTCGCAGCGTA
AtPR2	ACGGCCAACATCCATCTAGACT	GAGTACCCTGGATCGTTATCAACA
AtPR5	CGATGGCGGCAAAGATTT	CCCCAGCTTGACATTGTAACC
AtHCT	TCACGCGGCAGATGGTTT	CGAGCCATATCAGACCATGTGT
AtGA20ox1	GATCCATCCTCCACTTTAGA	GTGTATTCATGAGCGTCTGA
AtGA3ox1	GGAGCTCCCGGATTCTTACAA	GAGGAGAAGGAGCAGCGGA
AtGASA1	CTCTTATCGCTTCTCTTCTCATATCTCTT	ACCATTTTTCTTCTGTGAGTTTTCG
AtXTH24	TTCTCGTGGCGGCGTTT	AGCTACGTTGACGTCAGTGTTGA
AtXTH4	GCATGTGGTGGGATCAGAAAG	TTTGAGACGACGCCATTGTTC
AtXTH9	CACTACCGGCGAGCCTTACA	TCTCTGTTTCCAACTCCGTTCAC