Molecular Plant • Volume 4 • Number 1 • Pages 70–82 • January 2011 RESEARCH ARTICLE

The Simultaneous Repression of CCR and CAD, Two Enzymes of the Lignin Biosynthetic Pathway, Results in Sterility and Dwarfism in *Arabidopsis thaliana*

Johanne Thévenin[a], Brigitte Pollet[a], Bruno Letarnec[a], Luc Saulnier[b], Lionel Gissot[c], Alessandra Maia-Grondard[d], Catherine Lapierre[a] and **Lise Jouanin[a,1]**

a Institut Jean Pierre Bourgin (IJPB), INRA-AgroParisTech, UMR1318, 78026 Versailles Cedex, France
b Unité de Recherche 1268, Biopolymères, Assemblages, Interactions (BIA), INRA, BP71627, 44316 Nantes Cedex 03, France
c Laboratoire Commun de Cytologie et d'Imagerie Végétale, IJPB, INRA-AgroParisTech, UMR1318, 78026 Versailles, France
d Plateau technique de Chimie du végétal, IJPB, INRA-AgroParisTech, UMR1318, 78026 Versailles, France

ABSTRACT Cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) catalyze the last steps of monolignol biosynthesis. In *Arabidopsis*, one *CCR* gene (*CCR1*, At1g15950) and two *CAD* genes (*CAD C* At3g19450 and *CAD D* At4g34230) are involved in this pathway. A triple *cad c cad d ccr1* mutant, named *ccc*, was obtained. This mutant displays a severe dwarf phenotype and male sterility. The lignin content in *ccc* mature stems is reduced to 50% of the wild-type level. In addition, stem lignin structure is severely affected, as shown by the dramatic enrichment in resistant inter-unit bonds and incorporation into the polymer of monolignol precursors such as coniferaldehyde, sinapaldehyde, and ferulic acid. Male sterility is due to the lack of lignification in the anther endothecium, which causes the failure of anther dehiscence and of pollen release. The *ccc* hypolignified stems accumulate higher amounts of flavonol glycosides, sinapoyl malate and feruloyl malate, which suggests a redirection of the phenolic pathway. Therefore, the absence of CAD and CCR, key enzymes of the monolignol pathway, has more severe consequences on the phenotype than the individual absence of each of them. Induction of another CCR (*CCR2*, At1g80820) and another CAD (*CAD1*, At4g39330) does not compensate the absence of the main CCR and CAD activities. This lack of CCR and CAD activities not only impacts lignification, but also severely affects the development of the plants. These consequences must be carefully considered when trying to reduce the lignin content of plants in order to facilitate the lignocellulose-to-bioethanol conversion process.

Key words: *Arabidopsis*; auxin; Cinnamyl Alcohol Dehydrogenase (CAD); Cinnamoyl CoA Reductase (CCR); cell wall; dwarfism; lignins; phenolics; sterility.

INTRODUCTION

Lignins are important components of secondary cell walls. The different steps of the lignin pathway were studied and most genes involved in monolignol biosynthesis, the first part of this pathway, was identified (Boerjan et al., 2003). Many reports have described the manipulation of monolignol biosynthesis obtained by repressing the expression of single genes and the consequences of this gene silencing on lignification. This is the case for *CCR* and *CAD*, the two genes encoding the specific and last steps of the monolignol pathway. Several studies demonstrate the effects of gene silencing by the antisense or RNAi strategies in tobacco and poplar or knockout mutations in *Arabidopsis* (reviewed in Boerjan et al., 2003). In contrast, a smaller number of reports have described the simultaneous suppression of CCR and CAD. The conventional crossing of antisense *CCR* and *CAD* down-regulated tobacco lines provided a heterozygous line with a normal phenotype and development in spite of a dramatic reduction of lignin content (Chabannes et al., 2001). The simultaneous silencing of *CCR* and *CAD* genes by a single chimeric construct (Abbott et al.,

[1] To whom correspondence should be addressed. E-mail lise.jouanin@versailles.inra.fr.

doi: 10.1093/mp/ssq045, Advance Access publication 9 September 2010
Received 14 April 2010; accepted 14 July 2010

2002) provided tobacco plants with a smaller size than the double transformant obtained by crossing and with yellowish foliar areas (Dauwe et al., 2007). The studies of the transcriptome and metabolome of double *CCR*/*CAD* plants obtained by Chabannes et al. (2001) and Abbott et al. (2002) revealed large perturbations of the transcripts and metabolites of the monolignol biosynthesis pathway and also of other metabolic pathways (Dauwe et al., 2007).

Arabidopsis thaliana is of particular interest for studying the consequences of gene silencing due to the availability of null mutants and the possibility of obtaining double mutants by crossing. Two genes encode CCR in *Arabidopsis* (Lauvergeat et al., 2001). One of them, *CCR1* (At1g15950), is highly related to lignification. Knockout mutant lines for this gene were characterized, such as *irx4* (Jones et al., 2001), *ccr1s*, and *ccr1g* (Mir Derikvand et al., 2008). These lines have a dwarf phenotype, a 25–35% reduced lignin content, and a modified pool of soluble phenolics. Among the nine *CAD* genes identified in *Arabidopsis* (Goujon et al., 2003b), two genes (*CAD C*, At3g19450 and *CAD D*, At4g34230) are the primary genes involved in lignin biosynthesis, as demonstrated by the characterization of a double mutant for *CAD C* and *CAD D* (Sibout et al., 2003, 2005). The size of this *cad c cad d* double mutant is almost normal, in spite of a stem lignin content reduced by 40%. In this work, we have crossed the *cad c cad d* and the *ccr1g* lines and selected a homozygous line for the three genes (named *ccc* for *cad c cad d ccr1*). We have evaluated the consequences of silencing the main genes involved in the last two reductive steps of monolignol synthesis on the development and phenotype of the *ccc Arabidopsis* mutant as well as on its lignification and on its soluble phenolic compounds. Our findings reveal that repressing both the CAD and the CCR activities specifically involved in the lignification of *Arabidopsis* has severe consequences, not only on the cell wall composition of the stems, but also on the plant growth and development. The most severe developmental impact is male sterility, which could be related to the lack of appropriate lignification of the anthers.

RESULTS

Identification and Phenotyping of the *ccc* Triple Mutant

The triple mutant was identified by PCR in the progeny of a cross between the double *cad c cad d* mutant (Sibout et al., 2005) and the *ccr1*g mutant (Mir Derikvand et al., 2008). RT–PCR analysis verified the absence of expression of the three target genes (Figure 1). The growth of the triple mutant, the parental lines (*cad c cad d* and *ccr1g*), and of the wild-type (WT) accessions (WS and Col 0) was followed in greenhouse conditions and from the plantlet to the senescence stages.

At 30 days (d) (stage 5.10, Boyes et al., 2001), *ccc* and *ccr1g* plantlets displayed some abnormal leaves (pointed and rolled, Figure 2A). Size of *ccc* rosette leaves was highly reduced, even when compared to *ccr1g*, whereas the rosettes of WT and *cad c cad d* plants had similar diameters (Figure 2B). For example, the rosette leaf number 3 of *ccc* plants was five-fold shorter than the corresponding leaf of the WT (WS and Col 0) or *cad c cad d* lines whereas the corresponding *ccr1g* leaf was three-fold shorter than WT (Supplemental Figure 1). The *ccc* plantlets were dark green and could be identified by their rosette leaf diameter at 21 d old. At full maturity, the size of the floral stem was highly reduced in the *ccc* mutant and its dwarf phenotype was more pronounced than for the *ccr1g* mutant (Figure 2C and 2D). When compared to WT (WS and Col 0), the triple mutant displayed a 1-month-delayed senescence, even if its first inflorescence was prematurely shriveled (Figure 3A and 3B). Moreover, the triple mutant was almost sterile and the observation of flowers and siliques suggests a male sterility. In fact, the flowers prematurely shriveled and the siliques did not develop normally (Figure 3A). A detailed examination of the triple mutant flowers revealed that the pollen was normal, but that it was not released from the anthers (Figure 3B). In consequence, the seed development was very rare and most seeds aborted (Supplemental Figure 2). In addition, less than 50% of them were able to germinate, may be due to embryo lethality.

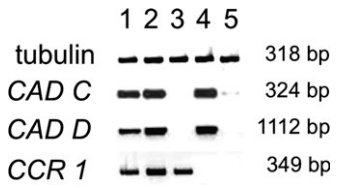

Figure 1. Expression of the Target Genes in the *ccc* Triple Mutant.
RT–PCR amplifications were performed for different genes (tubulin, *CAD C*, *CAD D*, and *CCR1*) on total RNA extracted for stems of WS, Col 0, *cad c cad d*, *ccr1g*, and *ccc* plants.
Lane 1, WS; 2, Col 0; 3, *cad c cad d*; 4, *ccr1g*; 5, *ccc.*
Expression of the beta-tubulin 4 gene was used as a control to equilibrate the cDNA quantity of each line. No transcripts were detected in the *ccc* triple mutant when using *CAD C*, *CAD D*, or *CCR1* primers, demonstrating that it was a null mutant for these three genes.

The *ccc* Triple Mutant Possesses Non-Dehiscent Anthers

In order to determine why the mutant displayed small and empty siliques, the development of its floral and reproductive tissues was monitored. At the first stages of flowering, flower development and the elongation of the stamen filaments were similar in the *ccc* and WT lines (Figure 3A). The *ccc* anthers actually contained pollen and normally developed, but they failed to dehisce (Figure 3B). The size of pollen grains of *ccc* mutant and WT lines were the same. In addition, Alexander and aniline blue stainings revealed that the pollen of the mutant was as viable and as able to germinate as the WT pollen (results not shown). When WT anthers were stained with ethidium bromide and visualized by confocal microscopy, a lignified secondary thickening could be observed in the endothecium (Figure 4). This thickening was still observed in *cad c cad d* mutant. By contrast, it was found to be severely

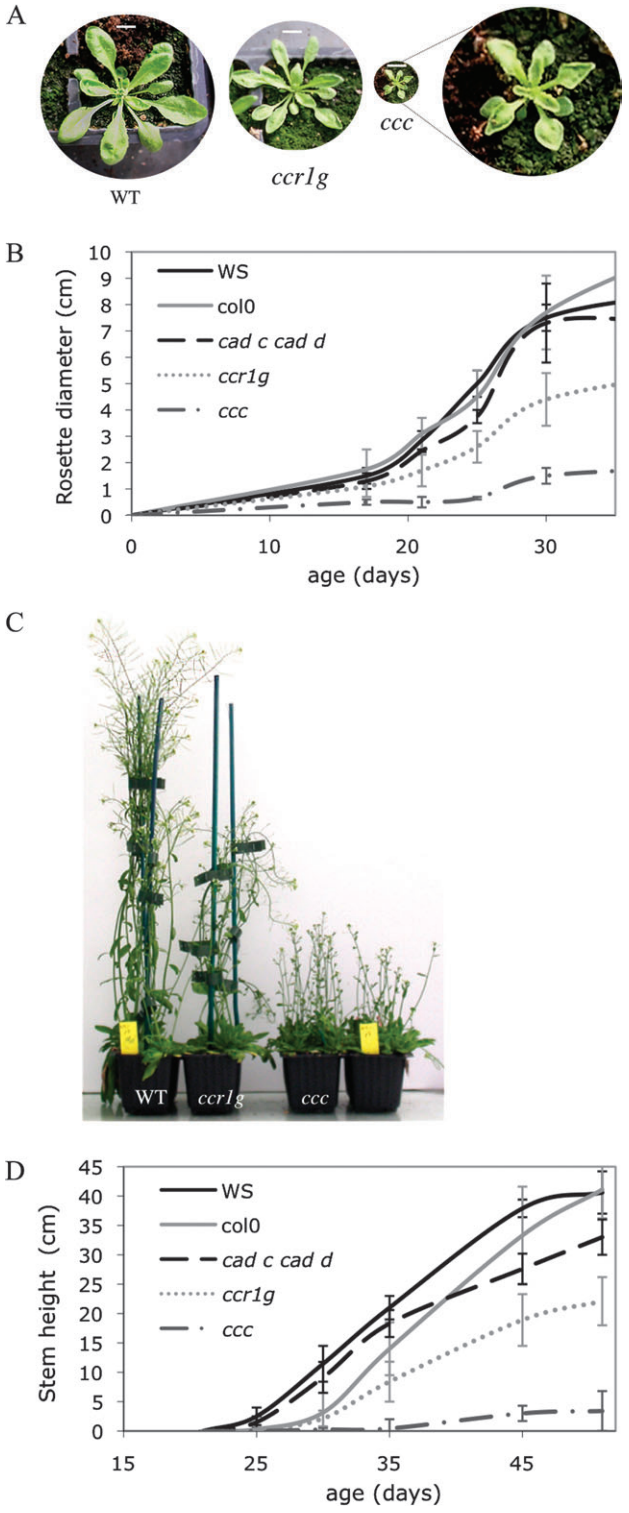

Figure 2. Phenotype and Development of Greenhouse-Grown Wild-Type (WT) and Mutant Lines.

The phenotype of *cad c cad d* was not shown since it was almost the same than those of WS for all the measured criteria. However, the two WT (WS and Col0) and *cad c cad d* are included in the growth curve.

reduced in *ccr1g* (Figure 4D and 4J) and absent in the triple mutant (Figure 4F and 4L). It is noteworthy that the UV absorbance of pollen grains of *ccr1g* and *ccc* mutants was higher than that of WT and *cad c cad d* pollen grains. The cause of

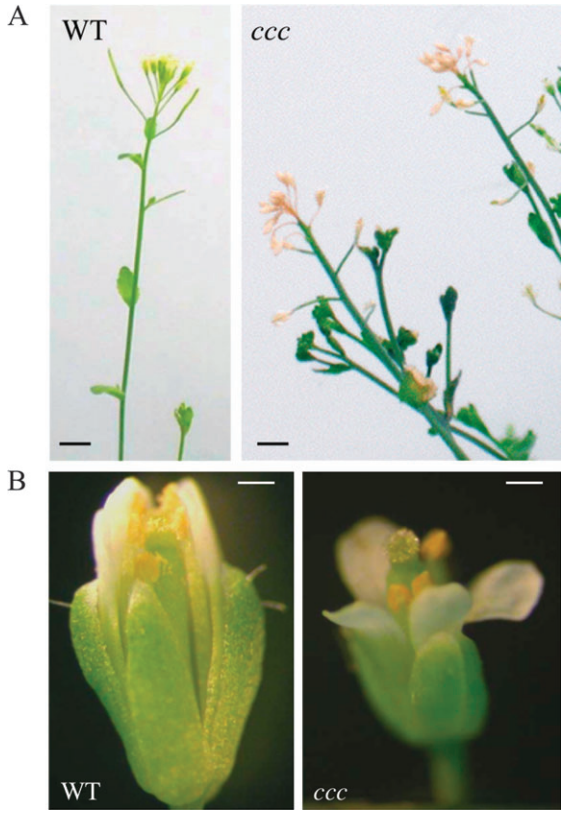

Figure 3. Phenotypes of WT and *ccc* Flowers.

(A) First inflorescence of the WT (WS) and *ccc* plants. WT produces normal flowers and siliques whereas *ccc* shows prematurely shriveled flowers over-blooming or produce siliques that quickly shrivel up before seed maturation. Bar = 1 cm.

(B) Observation of open flowers at anthesis on WT (WS) and *ccc* plants. The sepals partly enclosed the white petals, the stamens (including anthers in yellow), and the central gynoecium with the stigma on top. In WT flowers, the anther dehiscence releases wild-type pollen on the pistil whereas in the *ccc* triple mutant, the flowers are smaller and the shriveled anthers fail to open. Bar = 0.5 mm.

(A) Rosettes of 30-day-old WT (WS), *ccr1g*, and *ccc* observed at the same magnification (bar = 1 cm); the tiny *ccc* rosette was also shown at a four-fold higher magnification.

(B) Growth of WT (WS, black solid line, and Col0, gray solid line), *cad c cad d* (black dashed line), *ccr1g* (dash dotted line), and *ccc* (dotted line) rosette diameter with plant age; the rosette diameters were measured as the mean values of two different cultures (six plants per line and per culture).

(C) Phenotype of 64-day-old WT (WS), *ccr1g*, and *ccc* plants (bar = 4 cm).

(D) Growth of WT (WS, solid line, and Col0, gray solid line), *cad c cad d* (black dashed line), *ccr1g* (dash dotted line), and *ccc* (dotted line) stem height with plant age; the stem heights were calculated as the mean values of three different cultures (six plants per line).

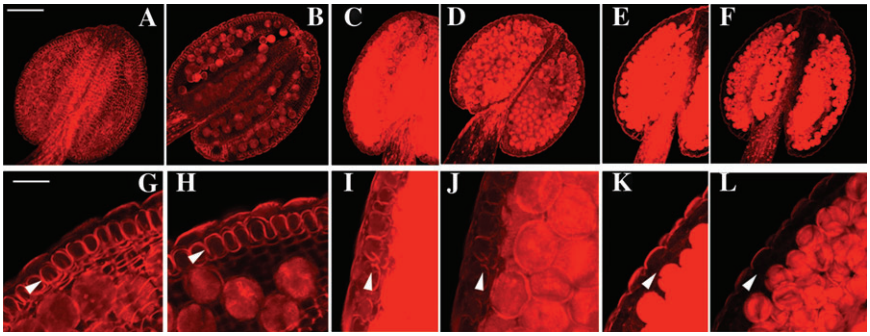

Figure 4. Observation of the Anthers of the *ccc* Triple Mutant and of the Parental Lines.

Anthers were stained by ethidium bromide and visualized by confocal microscopy (λ excitation = 543 nm, λ emission = 610 nm).
(A, G) WT (400 V).
(B, H) *cad c cad d* (400 V).
(C, I) *ccr1g* (400 V).
(D, J) *ccr1g* (330 V).
(E, K) *ccc* (400 V).
(F, L) *ccc* (330 V).
(A–F): bar = 120 μm; (G–L): bar = 25 μm.
Secondary thickening occurs in the endothecium (indicated by an arrow). Thickening is obviously observed in WT (WS) and in *cad c cad d* lines (A, B, G, H). In contrast, this thickening is reduced in *ccr1g* (D, J) and absent in the triple mutant (F, L). It is noteworthy that the UV absorbance of pollen grains of *ccr1g* and *ccc* mutants is higher than the UV absorbance of WT and *cad c cad d* pollen grains. The origin of this high UV absorbance was not determined.

this higher UV absorbance was not further investigated but may be explained by the accumulation of feruloyl malate and feruloyl glucose in the anthers corresponding to the higher quantities of these compounds in flowers (Table 1). Since lines expressing fusions between the β-glucuronidase gene (GUS) and the three target promoters were available (Sibout et al., 2003 for *CAD C* and *CAD D*; Sibout, unpublished work for *CCR1*), their expression profiles in flowers and stamens were determined (Supplemental Figure 3). GUS expression was detected in the stamens and stigmas of these lines. These flower parts are lignified as already shown by Tokunaga et al. (2009).

The *ccc* Mutant Presents Cytological Modifications in the Floral Stems

In the basal area of floral stems, the xylem vessels and the interfascicular fibers of *ccc* and *cad c cad d* mutants displayed a natural reddish coloration whereas no coloration could be observed in the other lines (Figure 5A). In addition, the xylem vessels of *ccr1g* and *ccc* stems were collapsed with a higher collapsing in the triple mutant (Figure 5A). In contrast, xylem of *cad c cad d* was not collapsed in these growth conditions in contrast to other growth conditions such as continuous light (Sibout et al., 2005).

Stem cross-sections (basal area) of the *ccc* mutant were also observed under UV light and compared to the WT. The typical blue autofluorescence of lignins was drastically reduced in its fibers and xylem vessels (data not shown), as already reported for the hypolignified stems of the *cad c cad d* mutant (Sibout et al., 2005). Wiesner staining was also performed on stem cross-sections (Figure 5B). This staining, which is often considered as diagnostic for lignin content, is specific for coniferaldehyde end-groups present in low amounts in native lignins (Nakano and Meshitsuka, 1992). The Wiesner staining was reduced in the fibers of the *ccr1g* mutant, as already observed previously (Mir Derikvand et al., 2008). In contrast, it was found to be increased in the *cad c cad d* mutant, but not to the same extent as in the triple mutant. On the basis of the lower lignin autofluorescence under UV light, the higher Wiesner staining suggests an accumulation of coniferaldehyde end-groups in the lignins of the triple mutant and not an increased lignin content.

Lignin Content and Structure Are Affected to Various Extents in the Different Lines

The lignin content of mature inflorescence stems was determined on the extract-free samples, referred to as cell wall residues (CWR). The amount of CWR recovered from the dried stems of the *cad c cad d* and the *ccr1g* mutants was similar to the WT levels (about 70%). By contrast, it was significantly reduced in the *ccc* triple mutant (Table 2), which suggests that the dry biomass of the *ccc* triple mutant contains fewer cell walls and more soluble components. The Klason lignin (KL) level was also more reduced in the triple mutant than in the *CAD* and *CCR* mutants whereas the so-called acido-soluble lignin (ASL) was increased, which made the total (KL+ASL) amount similar for all the mutant lines (Table 2A).

The impact of the triple mutation on lignin structure was evaluated by thioacidolysis. This analytical degradation generates H, G, and S thioethylated monomers from lignin units only involved in labile β-O-4 bonds. Therefore, when calculated on the basis of the lignin content, the total thioacidolysis yield

Table 1. LC–MS Determination of Flavonol Glycosides, Soluble Sinapoyl Esters (Sinapoyl Malate and Sinapoyl Glucose), and Soluble Feruloyl Esters (Feruloyl Malate and Feruloyl Glucose) Extracted from WT and Mutant Lines.

Compounds (in μg g^{-1} FM)	WT (WS)	WT (Col 0)	*cadc cad d*	*ccr1g*	*ccc*
Three-day-old in vitro-grown plantlets					
Flavonol glycosides FG	1100 (80)	1490 (130)	1010 (100)	990 (60)	570 (60)
Sinapoyl malate + sinapoyl glucose	760 (100)	850 (140)	900 (100)	220 (30)	340 (70)
Feruloyl malate	Nd	Nd	7 (1)	70 (15)	40 (10)
Basal region (1 cm) of 42-day-old inflorescence stems					
Flavonol glycosides FG	290 (40)	–*	140 (40)	–*	580 (20)
Sinapoyl malate SM	70 (5)	–*	450 (10)	–*	920 (40)
Feruloyl malate	Nd	–*	40 (5)	–*	580 (10)
Leaves					
Flavonol glycosides FG	–	1640 (300)	290 (110)	80 (20)	60 (4)
Sinapoyl malate SM	–	880 (200)	1030 (200)	240 (60)	200 (20)
Feruloyl malate	–	Nd	60 (10)	60 (20)	270 (40)
Flowers					
Flavonol glycosides FG	26 340 (200)	27 200 (260)	21 500 (1500)	18 000 (200)	12 900 (400)
Sinapoyl malate + sinapoyl glucose	610 (30)	570 (30)	1150 (60)	260 (20)	430 (40)
Feruloyl malate + feruloyl glucose	Nd	Nd	60 (5)	220 (30)	320 (50)

The data are expressed in μg g^{-1} of fresh material (FM) and correspond to the mean values (and standard errors) of triplicate analyses. Nd, not detected. * Increase of sinapoyl esters in stems of *ccr1g* was reported previously (Mir Derikvand et al., 2008).

provides an estimate of the frequency of lignin units only involved in β-O-4 bonds. The data of Table 2B reveal that, in addition to a reduced lignin level, the stem lignins of the *ccc* mutant release thioacidolysis monomers with a severely reduced yield. This finding means that the lignins of the *ccc* mutant have a dramatically decreased frequency of units only involved in β-O-4 bonds or, conversely, that the *ccc* lignin units are massively involved in resistant inter-unit bonds that affect the recovery of thioacidolysis monomers. Similarly to the *cad c cad d* sample, the frequency of lignin-derived H and G monomers was found to be higher in the *ccc* sample, whereas that of the S monomers was decreased. Beside the conventional H, G, and S lignin-derived monomers, other lignin-derived monomers were recovered from the samples (Table 2C). Relative to the control and *ccr1g* samples, the thioacidolysis monomers released from coniferaldehyde end-groups were recovered in higher relative amounts from the lignins of the *cad c cad d* and *ccc* mutants. This was also the case for the thioacidolysis G and S indene compounds that respectively originate from β-O-4-linked coniferaldehyde and sinapaldehyde units (Kim et al., 2002). This higher frequency of *p*-OH cinnamaldehyde units in lignins is the signature of CAD deficiency. In addition, the *ccr1g* and the *ccc* lines released higher relative amounts of ferulic acid and of a monomer previously reported as the signature of CCR deficiency (Table 2C) (Ralph et al., 2008). Interestingly enough, this marker compound for free ferulic acid incorporation in lignins was also released in higher amounts from the *cad c cad d* mutant (Table 2C and Supplemental Figure 4). The simultaneous absence of CCR and CAD activities has therefore a cumulative effect on the content and composition of lignins of the *ccc* mutant.

The Sugar Composition Is Modified in the Floral Stem Cell Wall

The quantification of cell wall polysaccharides in the different mutants was performed as the sum of arabinose, xylose, glucose, and uronic acid released by acid hydrolysis of extract-free stems. Rhamnose, fucose, and mannose were detected in low amounts ($<$0.5%) and were not taken into account in the analysis. In spite of its lower lignin amount, the total amount of sugars released by acid hydrolysis of the stem cell walls was found to be lower in *ccc* (decreased by 10% when compared to WT; Table 3). The sugar composition of the *cad c cad d* mutant was slightly modified when compared to WT (WS and Col 0) with a small increase in the proportion of uronic acids (galacturonic acid and glucuronic acid; Table 3). In contrast, a severe reduction in the proportion of glucose was observed in *ccr1g* and *ccc.* This decrease was mainly compensated for by a higher level of xylose and uronic acids in *ccr1g* whereas arabinose and glucuronic acid were mainly increased in *ccc*. Such changes in cell wall composition suggest a modification of the xylan/cellulose ratio in favor of xylan in *ccr1g* as already reported (Ruel et al., 2009). Xyloglucans are present in *Arabidopsis* stems (Gardner et al., 2002) and reduction in the proportion of this polysaccharide might also explain glucose reduction in *ccr1g* and *ccc* mutants. However, the fact that xylose proportion increases in *ccr1g* and remains unchanged in *ccc* does not support a major modification in xyloglucan.

The Hypolignified and Dwarf Stems of the *ccc* Mutant Accumulate Both Soluble Phenolics and Auxin

To further evaluate the impact of the triple mutation on the phenylpropanoid metabolism, the soluble phenolics of

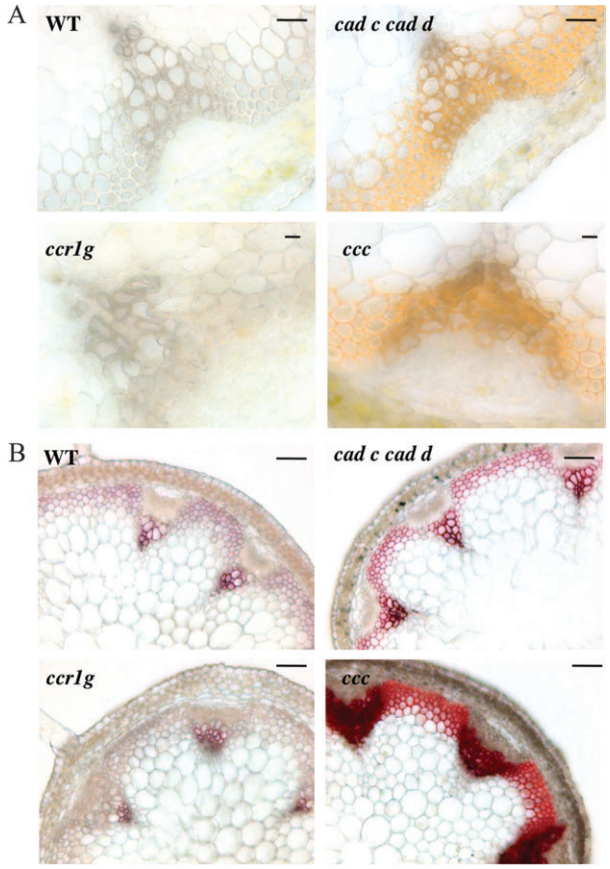

Figure 5. Observation of Cross-Sections of Stems.

(A) Unstained histological stem cross-sections (80 μm) of WT, *cad c cad d*, *ccr1g*, and *ccc* line. Bar = 100 μm. Cross-sections were performed at 1 cm from the base of the primary inflorescence stem (40-day-old). The *cad c cad d* and the *ccc* lines display a natural reddish coloration in xylem vessels and fibers. The *ccr1g* and the *ccc* cross-sections were smaller than the WT and the *cad c cad d*. The *ccr1g* and the *ccc* xylems are strongly collapsed.

(B) Wiesner staining of cross-sections of stems.

Cross-sections (80 μm) were performed at 1 cm from the base of the primary inflorescence stem (height of 10 cm) on Col 0 (WT), *cad c cad d*, *ccr1g*, and *ccc* mutants. Bar = 100 μm. The lignin staining of the *ccr1g* mutant was reduced in xylem vessels and absent in fibers. The lignin staining was slightly increased in *cad c cad d* and highly increased in *ccc* lines. This higher staining is indicative of a higher concentration of coniferaldehyde end-groups.

in vitro-grown plantlets, leaves, flowers, and stems of the different mutants were quantified. High perturbations of the pool of sinapate esters were described in *ccr1* mutants (Mir Derikvand et al., 2008). At the early plantlet stage, the content in flavonol glycosides was similar for all the lines, except for *ccc*, in which it was significantly reduced (Table 1). The *ccr1g* and *ccc* plantlets displayed a similar reduction in sinapoyl esters and the presence of feruloyl malate that could not be detected in the control samples. The same tendency was observed in the rosette leaves and in the flowers of *ccr1g* and *ccc* lines (i.e. a reduced level of sinapoyl esters and the presence of feruloyl malate (Table 1)). By contrast, the content of flavonol glycosides and of sinapoyl malate was dramatically increased in the stems of the triple mutant, as well as the level of feruloyl malate (Table 1). Interestingly enough, feruloyl malate was not only increased in the *ccr1g* plantlets, stems, leaves, and flowers, as previously reported (Mir Derikvand et al., 2008), but was also found in substantial amounts in the stems, leaves, and flowers of the *cad c cad d* mutant.

Table 2A. Cell Wall Content and Lignin Content of Mature Inflorescence Stems from Control (WS and Col 0) and Mutant Lines.

Line	Cell wall residue CWR% of dry matter	Klason lignin KL% of CWR	Acido-soluble lignin ASL% of CWR
WS	72.9 (0.1)	18.72 (0.01)	1.52 (0.22)
Col 0	70.6 (0.1)	18.92 (0.02)	1.60 (0.28)
cad c cad d	69.6 (0.1)	11.87 (0.05)	1.69 (0.33)
ccr1g	71.1 (0.1)	10.92 (0.11)	2.24 (0.20)
Ccc	63.5 (0.1)	8.86 (0.08)	4.83 (0.78)

Data correspond to mean values (and standard errors) of independent duplicate analyses.

Table 2B. Impact of *CAD* and/or *CCR1* Silencing on Lignin Structure as Revealed by Thioacidolysis of Extract-Free Floral Stems.

Line	H+G+S μmol g^{-1} KL	%H	%G	%S
WS	1520 (70)	0.44 (0.00)	70.5 (0.2)	29.0 (0.2)
Col 0	1500 (20)	0.55 (0.04)	68.4 (0.8)	31.0 (0.7)
cad c cad d	55 (0.5)	2.42 (0.16)	90.3 (1.5)	7.3 (1.3)
ccr1g	710 (80)	0.87 (0.03)	66.8 (0.3)	32.4 (0.4)
Ccc	5 (0.5)	1.85 (0.38)	85.2 (0.4)	13.0 (0.5)

The total yield in conventional H, G, and S monomers is expressed in μmoles per gram Klason lignin. Their relative frequency is expressed on a molar basis. Data are mean values (and standard errors) of duplicate analyses.

The relation between flavonoid content and auxin is well documented. Besseau et al. (2007) recently demonstrated that a deregulation of the phenolic pathway induces plant dwarfism due to the disturbance of auxin transport by unusually high flavonoid levels. In this context, the free auxin content of the basal part of floral stem was measured for the various control and mutant lines. Whereas no important modification of the free auxin content was detected in the parental lines, a high accumulation of auxin could be observed in the *ccc* triple mutant (Figure 6).

Expression of Genes Involved in Phenolic Compound Biosynthesis Is Modified in the *ccc* Mutant

In order to determine whether the absence of two primordial steps in the lignin biosynthetic pathway modifies expression of other genes, semi-quantitative RT–PCR experiments were performed. However, the interpretation of these experiments was

Table 2C. Relative Frequencies of Various Thioacidolysis Monomers Released from Extract-Free Floral Stems.

	Thioacidolysis monomers diagnostic for				
Line	Coniferaldehyde end-groups	Coniferaldehyde linked at Cβ	Sinapaldehyde linked at Cβ	Ferulic acid	CCR deficiency
WS	0.3 (0.0)	Tr	Tr	0.1 (0.0)	0.1 (0.0)
Col 0	0.3 (0.0)	Tr	Tr	0.2 (0.08)	0.1 (0.0)
cad c cad d	17.6 (0.3)	72.0 (1.7)	34.8 (0.9)	7.3 (0.4)	9.6 (0.0)
ccr1g	0.6 (0.1)	Tr	Tr	2.5 (0.1)	1.1 (0.0)
ccc	4.0 (0.5)	23.3 (4.3)	13.4 (1.4)	27.2 (1.4)	7.3 (2.5)

The data are expressed as molar percentages of the conventional (H+G+S) monomers. Tr, trace amount. Data are mean values (and standard errors) of duplicate analyses.

Table 3. Chemical Composition of Cell Wall Sugars (g $100g^{-1}$ CWR) in Floral Stems of Wild-Type (WT) and Mutants (*cad c cad d*, *ccr1g*, *ccc*).

Sugar	WT (WS)	WT (Col 0)	*cad c cad d*	*ccr1g*	*ccc*
Arabinose	1.0 (0.1) *1.6*	1.0 (0.1) *1.5*	1.1 (0.1) *1.7*	1.6 (0.1) *2.5*	2.5 (0.1) *4.5*
Xylose	13.4 (0.2) *21.9*	14.1 (0.8) *21.5*	13.7 (0.3) *20.7*	16.7 (0.7) *25.7*	11.6 (0.9) *20.8*
Glucose	36.7 (1.0) *60.0*	41.1 (2.4) *62.6*	38.9 (1.0) *58.8*	34.4 (1.7) *52.8*	29.1 (0.9) *52.2*
Uronic acids	10.1 (0.5) *16.5*	9.5 (0.5) *14.5*	12.5 (0.8) *18.9*	12.4 (0.2) *19.0*	12.5 (0.4) *22.4*
Total	61.2 (1.0)*100*	65.7 (3.8) *100*	66.2 (2.2) *100*	65.1 (2.7) *100*	55.7 (2.3) *100*

Values in italics are relative percentages (% by weight). Standard errors between duplicate analyses are in brackets. Uronic acids correspond to the sum of galacturonic and glucuronic acids.

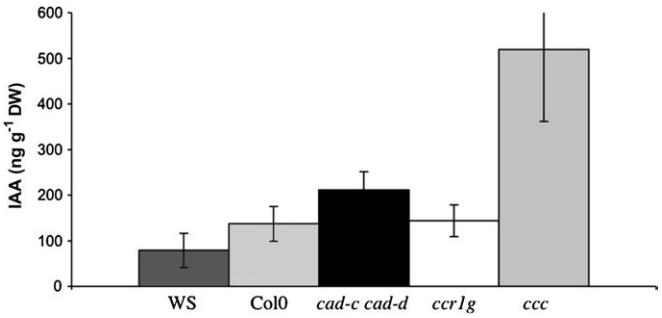

Figure 6. Levels of Free IAA Detected by HPLC/ESI/MS/MS in the Base of the Floral Stem of Greenhouse-Grown 44-Day-Old Plants.

Values represent the average of six biological replicates. The amount of auxin in the WT (WS and Col 0), *cad c cad d*, and *ccr1g* lines is similar whereas a high increase in free auxin is observed in the *ccc* line.

difficult because the delayed development of the *ccc* mutant and its reduced size made difficult the choice of the sampling conditions for RT–PCR experiments (same age or same developmental stage?). It was chosen to sample the stems at the same developmental stage, which was the end of the stem elongation (stage 6.90 according to Boyes et al., 2001). In these conditions and among the genes involved in the biosynthesis of phenolic compounds, no modification of the expression of *PAL1* (At2g37040), *PAL2* (At3g53260), *CHS* (At5g13930), *COMT1* (At5g54160), *CADG* (At1g72680) was observed, whereas *REF1* (At3g24503) and *F5H1* (At4g36220) were down-regulated and *CCR2* (At1g80820) and *CAD1* (At4g39330) were up-regulated and *CADB1* (At4g37980) induced (Figure 7 and data not shown).

DISCUSSION

Simultaneous Silencing of *CCR1*, *CAD C*, and *CAD D* Induces a Severe Dwarf Phenotype in *Arabidopsis* Together with Changes in Gene Expression

The availability of null mutants in the model plant *Arabidopsis thaliana* makes it particularly useful to study the consequences of silencing one or several genes on plant growth and development. In this work, a triple mutant corresponding to the absence of expression of three genes involved in two steps of the lignin pathway was obtained and characterized. The silenced genes correspond to *CCR1* (At1g15950), which is involved in the reduction of hydroxycinnamoyl CoA esters, and to *CAD D* (At3g19450) and *CAD C* (At4g34230), which both contribute to the reduction of hydroxycinnamaldehydes in *Arabidopsis*. These genes belong to multigene families (Goujon et al., 2003b; Raes et al., 2003) but the characterization of mutants knockout for *CCR1*, *CAD C*, and *CAD D* has demonstrated their importance in lignification. Several *ccr1* mutants were previously identified and analyzed (Jones et al., 2001; Mir Derikvand et al., 2008). One double mutant silenced for both *CAD D* and *CAD C* has been obtained and comprehensively studied (Sibout et al., 2005). The lignification was altered to various extents in these CCR1- or CAD-deficient mutants. In order to determine the impact of the simultaneous repression of the lignin-specific *CCR* and *CAD* genes in *Arabidopsis*, a cross was performed

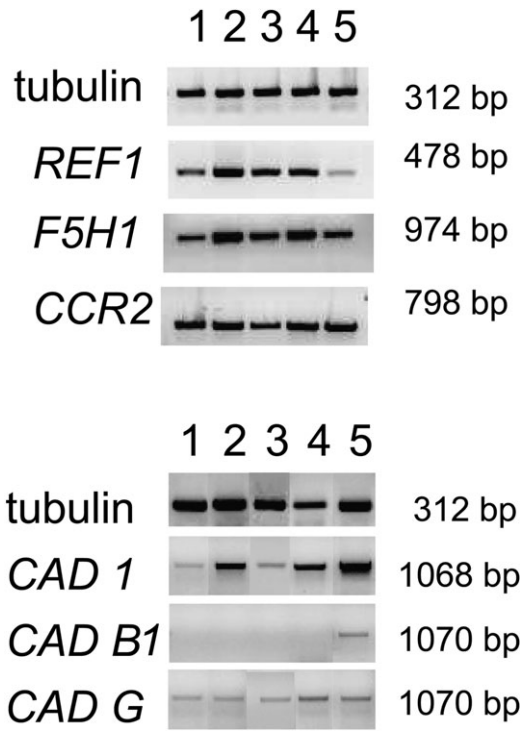

Figure 7. Determination by Semi-Quantitative RT–PCR of the Level of Expression of Different Genes (*CCR2*, *REF1*, *F5H1*, *CAD1*, *CADB1*, *CADG*).

The experiments were performed on total RNA extracted from the bottom of stems (44-day-old) of WS (1), Col 0 (2), *cad c cad d* (3), *ccr1g* (4), and *ccc* (5) lines.

Beta-tubulin 4 gene amplification was used as a control to equilibrate the cDNA quantity for each line.

between the *cad c cad d* and the *ccr1g* lines. The resulting homozygous mutant line, named *ccc* for *cad c cad d ccr1*, was identified and studied.

The first characteristic of the *ccc* line was its dramatically stunted size when compared to the mutant parental lines and to the WTs. This dwarf phenotype affected both the rosette and the inflorescence stem. The senescence of the *ccc* mutant was delayed, a characteristic already observed in the *ccr1* mutants (Mir Derikvand et al., 2008). In addition, the xylem of the *ccc* floral stem was collapsed. Such collapsed xylem vessels were observed in *ccr1g* and *ccr1s* mutants (Ruel et al., 2009) as well as in other irregular xylem (*irx*) mutants (Turner and Somerville, 1997; Brown et al., 2005). The genes mutated in these *irx* lines are involved in the biosynthesis of cellulose, lignins, or xylans, and some corresponding mutants are dwarf. However, among the various *Arabidopsis* mutants null for different steps of the monolignol pathway, only a few ones are dwarf. The size reduction of *CCR1* mutants (Jones et al., 2001; Mir Derikvand et al., 2008) is less severe than the reduction observed herein for the *ccc* mutant. A dwarf phenotype has been also observed in mutants partially down-regulated either for *p*-coumaroyl ester 3′-hydroxylase (C3′H) (Franke et al., 2002; Abdulrazzak et al., 2006) or for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) (Besseau et al., 2007). In contrast, a normal or almost normal size was observed in knockout mutants for COMT1 (Goujon et al., 2003a), CCoAOMT1 (Do et al., 2007), CADC, and CADD (Sibout et al., 2003), and even double CAD C/CAD D (Sibout et al., 2005).

Together with their reduced size, *Arabidopsis* lines down-regulated for *C3′H* displayed massive changes in gene expression, as shown by microarray analysis (Abdulrazzak et al., 2006). By contrast, and in spite of a dramatic perturbation of the expression of many genes, the size of a *pal1 pal2* mutant was normal (Rohde et al., 2004). In addition to a smaller size, large modifications of gene expression were observed in double *CCR/CAD* down-regulated tobacco plants (Dauwe et al., 2007). The large-scale study of differentially expressed genes in *ccc* using microarray could not be performed, since the genetic background of this mutant is a mix between WS and Col 0. However, semi-quantitative RT–PCR performed on a few target genes revealed some changes in gene expression. Similarly to the parent *ccr1g* mutant line (Mir Derikvand et al., 2008), the *ccc* mutant displayed a higher expression of the *CCR2* gene that is induced in stress conditions (Lauvergeat et al., 2001). Its induction could therefore partially compensate for the absence of CCR1. Among the *CAD* gene family, the *CAD1* gene was overexpressed in *ccc* and the *CADB1* gene induced whereas the expression of *CADG* did not change. In addition, the *REF1* gene and, to a lower extent, the *F5H1* gene were found to be down-regulated in the *ccc* mutant. Whereas the first one has been reported to be specifically involved in sinapate biosynthesis, the second one is involved both in the biosynthesis of sinapate esters and of sinapyl alcohol, the precursor of S lignin units.

The Absence of Lignified Thickening in the Endothecium of the *ccc* Mutant Induces Male Sterility

The second characteristic of *ccc* is its male sterility mainly due to the non-dehiscence of the anthers. Male sterility has been reported in *Arabidopsis* lines co-suppressed for C3′H (Abdulrazzak et al., 2006) as well as in the *pal1 pal2* double mutant (Rohde et al., 2004). The origin of this sterility in these cases was not well documented. In the *ccc* mutant and similarly to these *Arabidopsis* lines, the lignin level was severely reduced, which could be responsible for male sterility through non-dehiscent anthers. The cellulosic and lignified thickening of the endothecium is actually thought to be critical for anther dehiscence (Dawson et al., 1999). A few *Arabidopsis* mutants, which have such reduced or missing endothecium thickenings, were found to be indehiscent. The identified mutated genes responsible for such a phenotype are involved in jasmonic acid signaling (Sanders et al., 2000) or regulate secondary thickening (Mitsuda et al., 2005; Steiner-Lange et al., 2003; Yang et al., 2007). In the latter case are the NAC or MYB transcription factors, such as the SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST2 (Mitsuda et al., 2005) and the MYB26/MALE STERILE35 (MS35) gene (Steiner-Lange et al.,

2003; Yang et al., 2007). In each case, a lack of lignification of the anther endothecium has been detected, which blocks their dehiscence and therefore the release of pollen. The lignin-specific *CCR1*, *CAD C*, and *CAD D* genes were found to be expressed in the reproductive tissues of WT samples, which further demonstrates that lignification is important not only for stem rigidity, but for fertility (pollen release) and seed dispersal (silique dehiscence).

The Cell Wall Composition of *ccc* Is Highly Modified

The third characteristic of the *ccc* mutant is related to its stem cell walls. Modifications of cell wall sugars were observed, suggesting changes in polysaccharide proportions. Glucose content is reduced in *ccr1g* and in *ccc* that is likely due to a cellulose default. However, the proportion of xylan polysaccharides is enhanced in *ccr1g* while the increase in arabinose and uronic acids suggests that *ccc* cell walls contain higher levels of pectins and/or arabinogalactan proteins. Therefore, the absence of expression of two lignin-specific genes induces noticeable changes in the composition of cell wall polysaccharides.

The lignin content of *cad c cad d* and *ccr1g* mutants was reduced in mature inflorescence stems. Not unexpectedly, a similar impact was observed in *ccc* stems displaying a Klason lignin content reduced by 50%. By contrast, their content in the so-called acido-soluble lignin was substantially increased, which might reflect some important changes in lignin structure.

A higher frequency of *p*-OH cinnamaldehydes could explain the natural red coloration of the xylem vessels and fibers in *ccc*. Such a red coloration is typical of *CAD*-silenced tobacco (Halpin et al., 1994; Chabannes et al., 2001), poplar (Baucher et al., 1996), or *Arabidopsis* (Sibout et al., 2005) lines. An orange-brown coloration has been reported for *CCR*-silenced tobacco (Piquemal et al., 1998) and poplar (Leplé et al., 2007), but not for *ccr1* and *irx4 Arabidopsis* lines (Jones et al., 2001; Mir Derikvand et al., 2008). The Wiesner staining, which is generally considered as representative of lignin content, is specific for coniferaldehyde end-groups in lignins. The intense red Wiesner staining of the hypolignified stem cross-sections of the *ccc* mutant suggests accumulation of coniferaldehyde in this line.

When analyzed by thioacidolysis, the lignins of the *ccc* stems released very little amounts of monomers. Such dramatically low yields suggest that more than 95% of lignin units in *ccc* stems are involved in resistant inter-unit bonds. In addition, the marker compounds for CAD (Kim et al., 2002) and CCR (Ralph et al., 2008) deficiencies, already identified in transgenic plants down-regulated for these enzymes, were identified, demonstrating the additive impact of the two missing lignin biosynthesis steps. The *cad c cad d* stems also provided the thioacidolysis marker compound for CCR deficiency. This result suggests that *CAD* silencing has a negative impact on *CCR1* expression, which is consistent with previous transcriptomic studies reporting a two-fold lower *CCR1* expression in *cad c cad d* stems (Sibout et al., 2005). The incorporation of coniferaldehyde, sinapaldehyde, and ferulic acid in the lignins of the *ccc* mutant did not restore its lignin content to the wild-type level. The markedly altered lignin content and structure probably account for its collapsed vessels and altered growth.

The *ccc* Mutant Accumulates Soluble Phenolics in the Stem with Impact on Auxin Transport

Another characteristic of this triple mutant line is the accumulation of soluble phenolics (sinapoyl malate, feruloyl malate, and flavonol glycosides) in its hypolignified stems. By contrast, *ccc* plantlet flowers or leaves contained lower amounts of sinapoyl malate and flavonol glycosides than the controls. Taken together, these results suggest that the perturbed lignification in *ccc* stems is associated with a redirection of the phenylpropanoid pathway. Such a redirection was reported in different lignin mutants, including the *ccr1g* parental line (Mir Devikvand et al., 2008). The accumulation of flavonoids, but not of sinapate esters, was also observed in co-suppressed C3'H (Abdulrazzak et al., 2006) and HCT (Besseau et al., 2007) *Arabidopsis* lines. In the last study, the authors proposed that the reduced size of the HCT-silenced plant was mainly due to the accumulation of flavonoids, which disturbs auxin transportation. The same hypothesis could be proposed for the *ccc* mutant. High amounts of flavonoids may perturbate auxin transportation and explain the auxin accumulation at the base of the *ccc* stems. However, a recent publication (Li et al., 2010) disproves this hypothesis. Interestingly enough, and in spite of a reduced expression of the *REF* and *F5H1* genes, the stems, leaves, and flowers of the *ccc* mutant accumulate feruloyl malate.

Conclusion

Altering both two *CAD* and one *CCR* genes specifically involved in the lignification of *Arabidopsis* has severe consequences, not only on the cell wall composition of the stems, but also on the plant growth and development. The results obtained herein are different from those reported for down-regulated CCR/CAD hybrid tobaccos, which displayed no significant alteration of development in spite of a reduced lignin content (Chabannes et al., 2001). However, in the case of these tobacco plants, the double transformants were issued from the crossing of transgenic plants altered for CCR or CAD activities by the antisense strategy. Therefore, these plants exhibit only one allele of each antisense transgene and their residual CCR and CAD activities were moderately reduced (32% of the control level for CCR and 12% for CAD) (Chabannes et al., 2001). By contrast, the *ccc Arabidopsis* line studied herein is null both for *CCR1* and for two redundant *CAD* (*CAD C* and *CAD D*). Even if another *CCR* (CCR2, At1g80820) and another *CAD* (CAD1, Ag4g39330) involved in *Arabidopsis* lignification (Mir Derikvand et al., 2008; Eudes et al., 2006) are overexpressed in *ccc*, the remaining CCR and CAD activities may be lower than in the case of the transgenic tobaccos and therefore could not restore enzymatic activities to produce enough normal monomers for lignin biosynthesis.

The induction of *CADB1* (At4g37980) may be related to the stress state of the *ccc* line, since the overexpression of *CADB1* in *cad c cad d* was not able to restore the CAD activity (Eudes et al., 2006).

Intensive and worldwide efforts are currently carried out to understand the factors that govern the efficiency of the lignocellulose-to-bioethanol conversion processes. As lignification primarily affects this process, the main challenge is to modify lignification to an extent that is not detrimental to the plant development and growth, but that makes easier the saccharification processes (Chen and Dixon, 2007). As revealed by the present study, the relationships of lignin modification to plant performances still remain ill-defined. The simultaneous silencing of both *CAD* and *CCR* genes induces a similarly important reduction in lignin content in tobacco (Chabannes et al., 2001) and in the *ccc Arabidopsis* line studied herein. In the tobacco hybrid transgenic line, this dramatic reduction in cell wall lignin does not seem to be associated with any significant change in plant development. By contrast, in the *ccc* hypolignified *Arabidopsis* line, the simultaneous repression of lignin-specific *CAD* and *CCR* genes further induces a very severe alteration of plant development, with dwarfism and male sterility.

METHODS

Plant Materials and Growth Conditions

Experiments were performed on the *Arabidopsis thaliana* accession Wassilewskija (WS), the *cad c cad d* double mutant obtained in the WS background (Sibout et al., 2005), the accession Columbia (Col 0) wild-type, and the *ccr1g* mutant obtained in the Col 0 background (Mir Derikvand et al., 2008).

The *cad c cad d ccr1* (*ccc*) triple mutant line was isolated from the F4 population of a *cad c cad d* double mutant (WS) and a *ccr1g* (Col 0) cross. The putative *ccc* triple mutant line was identified using genomic PCR analyses.

For the screening of the putative *ccc* triple mutant, F2–F4 plants were grown *in vitro* on medium containing kanamycin (50 mg L^{-1}) and sulfadiazine (5.25 mg L^{-1}) in a growth chamber (20°C, 16 h light per day, 70% relative humidity RH) for 10 d. The plants were then transferred to the greenhouse. The transformed and wild-type plants were grown together in the same greenhouse to ensure uniform environmental conditions for 12 weeks at 22°C with 16 h of light per day and 55% RH.

For growth parameter determination, plants of the F4 putative *ccc* mutant line were measured over 60 d in the greenhouse.

RT–PCR

The base of the stems (first 1 cm) was taken from three 44-day-old plants pooled for analysis. Once collected from WS, *cad c cad d*, Col 0, *ccr1g*, or *ccc* lines, the samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

Total RNAs were extracted using the RNeasy extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations, with an extra DNase I treatment (Sigma-Aldrich, St Louis, MO, USA). RNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (LabTech, East Sussex, UK).

Reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA) reactions were performed using 1 μg of total RNA. The reverse-transcribed cDNAs were diluted four-fold before PCR analysis. The amplification of the *beta-TUBULIN 4* gene (βTUB, At5g44340) was performed as a control for the cDNA quantity. Band intensities were analyzed using the Quantity One software (Bio-Rad, Richmond, CA, USA). The expression of βTUB (beta tubulin), *PAL1* (phenylammonia lyase 1), *PAL2*, *CAD C*, *CAD D*, *CAD 1*, *CAD G*, *CAD B1*, *CCR1*, *CCR2*, *REF1* (reduced epidermal fluorescence or hydroxy-cinnamaldehyde dehydrogenase), *F5H1* (ferulate 5 hydroxylase), *COMT 1* (caffeic acid/5 hydroxyferulic acid *O*-methyltransferase 1), and *CHS* (chalcone synthase) was checked by RT–PCR using the specific primers indicated in Supplemental Table 1.

Plant Measurements and Histological Observations

Leaves, stems, and apical floral buds of plants were observed with a digital camera Olympus E410. The foliar length was measured with the Image J software. Anthers and young siliques were observed with binoculars (Nikon SMZ 10A). For confocal microscopy (SP2 AOBS, Leica, Germany), ethidium bromide was used to stain lignified cells (red fluorescence, excitation 543 nm). Fresh tissues were washed in 1X PBS and 2% (v/v) Tween 20 for 10 min, then in 1X PBS for 10 min, stained with 0.05% (w/v) ethidium bromide (1 h, room temperature), and washed (1X PBS, 10 min). This protocol is derived from the protocol described in Yang et al. (2007). Observations were performed at 330 and 400 V.

Fresh stem cross-sections (80-μm thickness) included in agarose 8% (w/v) were obtained with a vibratome (Leica VT1000S, Germany) and stored in PBS 1X. To observe stem cross-sections without staining or under UV (lignin autofluorescence), 40-day-old plants were used.

For Wiesner lignin staining, cross-sections from 10-cm-high stems were stained for 3 min in phloroglucinol-HCl reagent (Prolabo, VWR International, Strasbourg, France) and then observed in ethanol 100%: HCl 37% (9/1, v/v) using a light microscope (Zeiss, Axioplan 2).

Analyses of Cell Wall Sugars, Lignins, and Soluble Phenolic Compounds

For the analyses of cell wall sugars and of lignins, 65-day-old mature stems were collected after removal of leaves and siliques and ground to pass a 0.5-mm sieve before solvent extraction. Extract-free samples, referred to as cell wall residues (CWR), were prepared using a Soxhlet apparatus, by sequentially extracting the ground material with toluene:ethanol (2:1, v/v), ethanol, and then water.

The analyses of cell wall sugars (neutral sugars and uronic acids) were performed in duplicate for each CWR sample as described by Saulnier et al. (1995).

The determinations of lignin content were carried out on the CWR samples using the Klason procedure and the acido-soluble lignin method (Dence, 1992). The evaluation of lignin structure was carried out by thioacidolysis (Lapierre et al., 1995, 1999). The lignin-derived monomers were identified by gas chromatography–mass spectrometry (GC–MS) of their trimethylsilylated derivatives. All the lignin analyses were performed in duplicate.

The analyses of soluble phenolic compounds were carried out from fresh material (plantlets, leaves, stems, and flowers) and as triplicate experiments. The plantlet analyses were conducted from 3-day-old plantlets (20 plantlets gathered per assay). The stem analyses were run from 1-cm-long stem fragments collected in the basal part of 42-day-old plants (three plants per assay). The phenolic compounds (flavonol glycosides, sinapoyl malate, and feruloyl malate) were analyzed by liquid chromatography–mass spectrometry (LC–MS) as previously described (Mir Derikvand et al., 2008).

Determination of Auxin Content in the Basal Area of Floral Stems

The indole-3-acetic acid (IAA) content of stem (3-cm fragments of three plants) was measured on two plant series. The procedure used lyophilized frozen tissue. Samples were weighted and 2.5 ng of a standard $^{13}C_6$-IAA (Euriso-Top SA, France) were initially added as internal tracers for recovery and analytical purposes. Stems were extracted in a potter with 2 mL acetone/water/acetic acid (80/19/1, v/v), then centrifuged at 3000 *g*, 4°C, 15 min. The supernatants were collected and the pellets were re-extracted with 1.5 mL of the same extraction solution then sonicated for 15 min (25 Hz). Following centrifugation, the two supernatants were pooled and concentrated under a nitrogen flow. The dry extract was dissolved in 150 μl acetonitrile/water (50/50, v/v), filtered, and submitted to analysis by HPLC–electrospray–MSMS (HPLC–ES–MSMS).

The compounds were introduced in the ESI source using a Waters 2695 separation module (Alliance) (Waters, Milford, MA, USA) equipped with a Waters 2487 dual UV detector. Separation was achieved on a reverse-phase column (Uptisphere C18 5 μm, 150 × 2 mm i.d., Interchrom) using a flow rate of 0.15 mL min^{-1} and a binary gradient: acetonitrile 0.5 % acetic acid (v/v) (A) and acetic acid 0.5 % (v/v) (B). Typically, the solvent gradient was programmed as follows: 0–5 min 20% A, 5–15 min 65% A, 15–20 min 100% A, before returning to the initial composition at 30 min.

The analyses were performed on a Waters Quattro LC triple quadrupole mass spectrometer (Waters, Milford, MA, USA) operating in a Multiple Reaction Monitoring (MRM) scanning mode. Relevant instrumental parameters were set as follows: capillary 2.75 kV (negative mode), extractor 2 V, source block and desolvation gas temperatures 120 and 350°C, respectively. Nitrogen was used to assist the nebulization and the desolvation (250 and 450 L h^{-1}, respectively), argon was used as collision gas at 3.5×10^{-3} mbar. The parameters used for MRM quantitation of $^{13}C_6$-IAA and IAA in negative mode were: cone potential 17 V, collision energy 10 eV for two transitions used *m/z* 180 > 136 and 174 > 130, respectively.

Typically, for a 5-μL injection of sample prepared with 2.5 ng internal standard and reconstituted in 150 μL of 50/50 ACN/H_2O, the limit of detection (LOD) and limit of quantification (LOQ) were calculated from calibration curve and sample using the Quantify module of MassLynx version 4.1 software. The respective LOD and LOQ are 1 and 3 pg mg^{-1} dry mass.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

ACKNOWLEDGMENTS

Authors are grateful to Frédéric Legée for Klason analysis, to Laurent Cézard for thioacidolysis analysis, to Jacqueline Vigouroux for cell-wall polysaccharide analysis, to Richard Sibout for the promoter *CCR1::GUS Arabidopsis* lines, to Daniel Vezon for help in gametogenesis experiments, and to Michael Harrington for critical reading of the manuscript. No conflict of interest declared.

REFERENCES

Abbott, J.C., Barakate, A., Pinçon, G., Legrand, M., Lapierre, C., Mila, I., Schuch, W., and Halpin, C. (2002). Simultaneous suppression of multiple genes by single transgenes: down-regulation of three unrelated lignin biosynthetic genes in tobacco. Plant Physiol. **128,** 844–853.

Abdulrazzak, N., et al. (2006). A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of non redundant meta-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. Plant Physiol. **140,** 30–48.

Baucher, M., et al. (1996). Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiol. **112,** 1479–1490.

Besseau, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., and Legrand, M. (2007). Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell. **19,** 148–162.

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. Annu. Rev. Plant Biol. **54,** 519–546.

Boyes, C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Görlach, J. (2001). Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. Plant Cell. **13,** 1499–1510.

Brown, D.M., Zeef, L.A.H., Ellis, J., Goodacre, R., and Turner, S.R. (2005). Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics. Plant Cell. **17,** 2281–2295.

Chabannes, M., Barakate, A., Lapierre, C., Marita, J.M., Ralph, J., Pean, M., Danoun, S., Halpin, C., Grima-Pettenati, J., and Boudet, A.M. (2001). Strong decrease in lignin content without

significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant J. **28,** 257–270.

Chen, F., and Dixon, R.A. (2007). Lignin modification improves fermentable sugar yields for biofuel production. Nat. Biotech. **25,** 659–761.

Dauwe, R., et al. (2007). Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell-wall metabolism, primary metabolism, stress metabolism and photorespiration. Plant J. **52,** 263–285.

Dawson, J., Sözen, E., Vizir, I., Van Waeyenberge, S., Wilson, Z.A., and Mulligan, B.J. (1999). Characterization and genetic mapping of a mutation (*ms35*) which prevents anther dehiscence in *Arabidopsis thaliana* by affecting secondary wall thickening in the endothelium. New Phytol. **144,** 213–222.

Dence, C.W. (1992). The determination of lignin. In Methods in Lignin Chemistry, Dence, C., Lin, S., eds (Heidelberg: Springer), pp. 33–61.

Do, C.-T., Pollet, B., Thévenin, J., Sibout, R., Denoue, D., Barrière, Y., Lapierre, C., and Jouanin, L. (2007). Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid 0-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in *Arabidopsis*. Planta. **226,** 1117–1129.

Eudes, A., Pollet, B., Sibout, R., Do, C.-T., Séguin, A., Lapierre, C., and Jouanin, L. (2006). Evidence for a role of *AtCAD* 1 in lignification of elongating stem of *Arabidopsis thaliana*. Planta. **225,** 23–33.

Franke, R., Hemm, M.R., Denault, J.W., Ruegger, M.O., Humphreys, J.M., and Chapple, C. (2002). Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of *Arabidopsis*. Plant J. **30,** 47–59.

Gardner, S.L., Burrell, M.M., and Fry, S.C. (2002). Screening of *Arabidopsis thaliana* stems for variation in cell wall polysaccharides. Phytochemistry. **60,** 241–254.

Goujon, T., et al. (2003a). A new *Arabidopsis* mutant deficient in the expression of the *O*-methyltransferase impacts lignins and sinapoyl esters. Plant Mol. Biol. **51,** 973–989.

Goujon, T., Sibout, R., Eudes, A., MacKay, J., and Jouanin, L. (2003b). Genes involved in the biosynthesis of lignin precursors in *Arabidopsis thaliana*. Plant Physiol. Biochem. **41,** 677–687.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M.T., and Schuch, W. (1994). Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. Plant J. **6,** 339–350.

Jones, L., Ennos, A.R., and Turner, S.R. (2001). Cloning and characterization of irregular xylem4 (*irx4*): a severely lignin-deficient mutant of *Arabidopsis*. Plant J. **26,** 205–216.

Kim, H., Ralph, J., Lu, F., Pilate, G., Leplé, J.-C., Pollet, B., and Lapierre, C. (2002). Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. J. Biol. Chem. **277,** 47412–47419.

Lapierre, C., et al. (1999). Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial Kraft pulping. Plant Physiol. **115,** 153–163.

Lapierre, C., Pollet, B., and Rolando, R. (1995). New insights into the molecular architecture of hardwood lignins by chemical degradation methods. Res. Chem. Intermed. **21,** 397–412.

Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D., and Grima-Pettenati, J. (2001). Two cinnamoyl-CoA reductase (*CCR*) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. Phytochemistry. **57,** 1187–1195.

Leplé, J.C., et al. (2007). Downregulation of cinnamoyl-Coenzyme A reductase in poplar: multiple level phenotyping reveals effects on cell wall polymer metabolism and structure. Plant Cell. **19,** 3669–3691.

Li, X., Bonawitz, N.D., Weng, J.-K., and Chapple, C. (2010). The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. Plant Cell. **22,** 1620–1632.

Mir Derikvand, M., Berrio Sierra, J., Ruel, K., Pollet, B., Do, C.-T., Thévenin, J., Buffard, D., Jouanin, L., and Lapierre, C. (2008). Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1. Planta. **227,** 943–956.

Mitsuda, N., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2005). The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell. **17,** 2993–3006.

Nakano, J., and Meshitsuka, G. (1992). The detection of lignin. In Methods in Lignin Chemistry, Dence, C., and Lin, S., eds (Heidelberg: Springer), pp. 23–32.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J., and Boudet, A.M. (1998). Down-regulation in cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. Plant J. **13,** 71–83.

Raes, J., Rohde, A., Christiensen, J.H., Van de Peer, Y., and Boerjan, W. (2003). Genome-wide characterization of lignification toolbox in *Arabidopsis*. Plant Physiol. **133,** 1051–1071.

Ralph, J., Kim, H., Lu, F., Grabber, J.H., Leplé, J.-C., Berrio-Sierra, J., Mir Derikvand, M., Jouanin, L., Boerjan, W., and Lapierre, C. (2008). Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency). Plant J. **53,** 368–379.

Rohde, A., et al. (2004). Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell. **16,** 2749–2771.

Ruel, K., Berrio-Sierra, J., Mir Derikvand, M., Pollet, B., Thévenin, J., Lapierre, C., Jouanin, L., and Joseleau, J.P. (2009). Impact of *CCR1*-silencing on the assembly of lignified secondary walls in *Arabidopsis thaliana*. New Phytol. **184,** 99–113.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000). The *Arabidopsis* DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell. **12,** 1041–1061.

Saulnier, L., Marot, C., Chanliaud, E., and Thibault, J.F. (1995). Cell-wall polysaccharide interactions in maize bran. Carbohydrate Polymers. **26,** 279–287.

Sibout, R., Eudes, A., Mouille, G., Pollet, B., Lapierre, C., Jouanin, L., and Séguin, A. (2005). CINNAMYL ALCOHOL DEHYDROGENASE-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis*. Plant Cell. **17,** 2059–2076.

Sibout, R., Eudes, A., Pollet, B., Goujon, T., Mila, I., Granier, F., Séguin, A., Lapierre, C., and Jouanin, L. (2003). Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in *Arabidopsis*: isolation and characterization of the corresponding mutants. Plant Physiol. **132,** 848–860.

Steiner-Lange, S., Unte, U.S., Eckstein, L., Yang, C., Wilson, Z.A., Schmelzer, E., Dekker, K., and Saedler, H. (2003). Disruption of *Arabidopsis thaliana* MYB26 results in male sterility due to non-dehiscent anthers. Plant J. **34,** 519–528.

Tokunaga, N., Kaneta, T., Sato, S., and Sato, Y. (2009). Analysis of expression profiles of three peroxidase genes associated with lignification in *Arabidopsis thaliana*. Physiol Plant. **136,** 237–249.

Turner, S.R., and Somerville, C.R. (1997). Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell. **9,** 689–701.

Yang, C., Xu, Z., Song, J., Conner, K., Barrena, G.V., and Wilson, Z.A. (2007). *Arabidopsis* MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. Plant Cell. **19,** 534–548.