Planta (2008) 227:943–956
DOI 10.1007/s00425-007-0669-x

ORIGINAL PAPER

Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1

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Received: 8 August 2007 / Accepted: 8 November 2007 / Published online: 29 November 2007

Abstract Cinnamoyl-CoA reductase 1 (*CCR1*, gene At1g15950) is the main CCR isoform implied in the constitutive lignification of *Arabidopsis thaliana*. In this work, we have identified and characterized two new knockout mutants for *CCR1*. Both have a dwarf phenotype and a delayed senescence. At complete maturity, their inflorescence stems display a 25–35% decreased lignin level, some alterations in lignin structure with a higher frequency of resistant interunit bonds and a higher content in cell wall-bound ferulic esters. Ferulic acid-coniferyl alcohol ether dimers were found for the first time in dicot cell walls and in similar levels in wild-type and mutant plants. The expression of *CCR2*, a *CCR* gene usually involved in plant defense, was increased in the mutants and could account for the biosynthesis of lignins in the *CCR1*-knockout plants. Mutant plantlets have three to four-times less sinapoyl malate (SM) than controls and accumulate some feruloyl malate. The same compositional changes occurred in the rosette leaves of greenhouse-grown plants. By contrast and relative to the control, their stems accumulated unusually high levels of both SM and feruloyl malate as well as more kaempferol glycosides. These findings suggest that, in their hypolignified stems, the mutant plants would avoid the feruloyl-CoA accumulation by its redirection to cell wall-bound ferulate esters, to feruloyl malate and to SM. The formation of feruloyl malate to an extent far exceeding the levels reported so far indicates that ferulic acid is a potential substrate for the enzymes involved in SM biosynthesis and emphasizes the remarkable plasticity of *Arabidopsis* phenylpropanoid metabolism.

Keywords *Arabidopsis* · Cinnamoyl-CoA reductase · Feruloyl malate · Lignin · Sinapoyl malate

Abbreviations

Col 0	Columbia
CCR	Cinnamoyl-CoA reductase
FeG	Feruloyl glucose
FeM	Feruloyl malate
FST	Flanking sequence tag
F5H	Ferulate -5-hydroxylase
GC-MS	Gas chromatography-mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
SE	Standard error
SM	Sinapoyl malate
TMS	Trimethylsilylated
WS	Wassilewskija
WT	Wild-type

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Introduction

Cinnamoyl-CoA reductase (CCR) is the first enzyme committed in the lignin-specific branch of the phenylpropanoid pathway (Lacombe et al. 1997; Boerjan et al. 2003). Plants with down-regulated CCR activities display various degrees of reduction in lignin content (Piquemal et al.

1998; Jones et al. 2001; O'Connell et al. 2002; Goujon et al. 2003a; van der Rest et al. 2006; Wadenbäck et al. 2007). However, few studies report the impact of this down-regulation on the other branches of the phenylpropanoid pathway. CCR-deficient Solanaceous species (transgenic tobacco and tomato lines) were reported to have a decreased lignin level associated with an increased pool of chlorogenic acid and its isomers (Chabannes et al. 2001; van der Rest et al. 2006). *Arabidopsis thaliana* and other members of the Brassicaceae accumulate sinapic acid esters, sinapoylmalate in leaves and sinapoylcholine in seeds (Chapple et al. 1992). Flavonoids and sinapoyl malate (SM) are considered as UV protectants (Landry et al. 1995). The modification of the phenylpropanoid metabolic flux in CCR-repressed *Arabidopsis* could also have an impact on these UV-protecting compounds. *CCR1* is the main CCR isoform implied in the constitutive lignification of *A. thaliana* (Lauvergeat et al. 2001; Goujon et al. 2003b). In this work, we have identified and characterized two new *Arabidopsis* knockout mutants for *CCR1*. We have evaluated the consequences of this mutation not only on the lignins of inflorescence stems at complete maturity, but also on the pool of soluble phenolics in vegetative tissues at various developmental stages. Our findings reveal that the reduced flux to lignin biosynthesis in the two *CCR1*-deficient mutants is associated with an unusually increased flux to feruloyl malate.

Materials and methods

Plant material and culture conditions

Most experiments were performed on *A. thaliana* ecotype Columbia (Col 0). The transformed and wild-type (WT) plants were grown together in the same greenhouse to ensure the same environmental conditions. The different growth conditions used according to the experiments are indicated in the text.

Two T-DNA mutants were selected in *Arabidopsis* mutant collections. One mutant (*ccr1s*) was identified in the Salk Institute Genomic Analysis laboratory (Alonso et al. 2003) and the second (*ccr1g*) in the GABI collection (Rosso et al. 2003). The mutants were identified using the systematic border sequencing program of the Salk Institute (San Diego, CA, USA) on the TAIR site (http://www.Arabidopsis.org) and the GABI site (http://www.gabi-kat.de/), respectively. The homozygous mutants were characterized using gene specific primers and flanking sequences of the T-DNA insertion site.

Some analyses of soluble phenolics were carried out on a knockout mutant for ferulate-5-hydroxylase 1 *F5H1* (gene At4g36220). This homozygous mutant (*f5h1*) obtained in the Wassilewskija (WS) background, was identified in the Versailles collection (Samson et al. 2004).

Analysis of RNA

Total RNA was extracted and reverse transcriptase and PCR reactions were performed as described in Eudes et al. (2006). The expression of the *CCR1* gene was checked by RT-PCR using specific primers (forward 5′-GTC GGT GGT GGA TAC ATC GCT TC-3′ and reverse 5′-TCC ATG TAG ACG GCA CCA AT-3′). The expression level of *CCR2* gene (At1g80820) was determined using the primers forward 5′-GAA CCC AAC TGA TCC CAA-3′ and reverse 5′-GAA CTT GGC TCC GTT CCA CC-3′ which amplify a fragment of 202 bp. The amplification of the beta-tubulin 4 gene (*βTUB*, At5g44340) was performed as a control for the cDNA quantity. The primers *βTUB* forward 5′-GTC CAG TGT CTG TGA TAT TGC ACC-3′ and *βTUB* reverse 5′-GCT TAC GAA TCC GAG GGT GCC-3′ were used to amplify a fragment of 318 bp.

Lignin staining

Stem cross sections (60 µm thick) obtained with a vibratome (Leica VT1000S) were stained for 3 min with phloroglucinol-HCl reagent (Prolabo, VWR International, Strasbourg, France) according to Jones et al. (2001) and observed using a microscope (Zeiss, Axioplan 2).

Fourier-transformed infrared spectroscopy

For Fourier-transformed infrared spectroscopy (FTIR) experiments, we used 3-week-old plants grown on compost in a growth chamber under continuous light (20°C, 60% relative humidity). In these controlled conditions, growth and bolting were accelerated. Three biological replicates of WT and mutant stems were embedded in 8% agarose then sectioned with a vibratome (Leica VT1000S). Five different sections per replicate were subjected to FTIR analysis. Areas of 50 µm × 50 µm in the xylem bundles or in the interfascicular fibers were selected for spectra collection with a spectrometer (ThermoNicolet, Nexus, Madison, WI, USA) with a continuum microscope accessory. Fifty interferograms were collected in transmission mode with an 8 cm^{-1} resolution and co-added to improve the signal-to-noise ratio of the spectrum. Spectra were then baselined and normalized as described in Robin et al. (2003). Student's *t*-tests were applied as described in Mouille et al. (2003).

Lignin analyses

For the analysis of lignins and cell wall-bound phenolics, plants were grown in compost in the greenhouse (20°C,

60% relative humidity) under long-day (16 h light, first culture) or short-day (8 h light, second culture) regime.

Inflorescence stems were collected at complete maturity (dry stems from completely senescent and dead plants) after 10 (first culture with the long-day regime) or 15 weeks (second culture with the short-day regime) of cultivation in the greenhouse. The stem samples were obtained from 20 plants grown at the same time and in the same conditions, with one biological replicate for the first culture and three biological replicates for the second culture. With two exceptions (Klason determination on the mutant samples from the first culture), each biological replicate was subjected to two or three analytical replicates of lignin analysis and the comparison of the WT and of the mutant analytical parameters were carried out by the Student's *t*-test using the mean of the analytical replicates. All lignin analyses were performed on extract-free samples, ground to pass a 0.5-mm sieve before solvent extraction (toluene-EtOH, 2:1 (v/v) EtOH, and H_2O). The lignin content was determined on the extract-free samples using the standard procedure of Klason and acid-soluble lignins (ASL, Dence 1992). It was also measured using the acetyl bromide method without any addition of perchloric acid (Fukushima and Hatfield 2001). The determination of lignin composition was performed on extract-free material using the thioacidolysis procedure as previously described (Lapierre et al. 1995; Abdulrazzak et al. 2006). The quantitative analysis of the main *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin-derived monomers, analyzed as their trimethylsilylated (TMS) derivatives, was carried out from specific ion chromatograms reconstructed at m/z 239 for the H monomers, 269 for G monomers and 299 for S monomers. Beside the lignin-derived conventional H, G and S monomers, we evaluated thioacidolysis-released ferulic acid (Fe) as well as another minor monomer, G-CHSET-CH$(SEt)_2$ originating from lignin substructures derived from ferulic acid (Ralph et al. 2007). In addition to conventional thioacidolysis experiments, additional thioacidolyses were run from exhaustively permethylated samples in order to evaluate the frequency of free methylatable phenolic groups within the H, G and S lignin units only involved in β-O-4 bonds and according to a previously described procedure (Lapierre and Rolando 1988).

Alkaline hydrolysis and GC-MS analysis of cell wall-bound phenolics

The extraction procedure and the Gas chromatography-mass spectrometry (GC-MS) analyses of the low molecular weight phenolics as their TMS derivatives were performed according to Jacquet et al. (1995) and Chabannes et al. (2001). The GC-MS determinations were performed on ion chromatograms reconstructed at the most specific ions of the TMS derivatives (molecular ion and base peak ion) of the various compounds and relative to the internal standard (ethylvanillin for the TMS monomers and dieugenol for the TMS dimer) with a response factor arbitrarily set at 1. We nevertheless checked that all the response factors of the various phenolic monomers were close to 1 (in the 0.8–0.9 range).

Analysis of soluble phenolics

For the analysis of soluble phenolics at early developmental stages (3-day-old and 16-day-old plantlets), seedlings were grown in vitro on Estelle and Somerville medium (Estelle and Somerville 1987) at 20°C, 60% relative humidity with a 16 h photoperiod in a growth chamber. Rosette leaves and stem fragments were collected on plants cultivated at 20°C, 60% relative humidity, with a 16-h photoperiod in a greenhouse. The extraction and the Liquid chromatography-mass spectrometry (LC-MS) analyses of soluble phenolics from in vitro-grown plantlets and from the stems and leaves of greenhouse-grown plants were carried out as previously described (Goujon et al. 2003c; Abdulrazzak et al. 2006). The main soluble phenolics quantitatively evaluated by LC-MS (electrospray, negative mode) were as follows: K1, kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside ($(M-H)^-$ at m/z 577); K2, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside [$(M-H)^-$ at m/z 593); K3, kaempferol 3-*O*-[6″-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside ($(M-H)^-$ at m/z 739); Q1, quercetin 3-*O*-glucoside 7-*O*-rhamnoside. ($(M-H)^-$ at m/z 609); I1, isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside ($(M-H)^-$ at m/z 623). In addition, SM, sinapoyl glucose (SG), feruloyl malate (FeM) and feruloyl-glucose (FeG) were evaluated from ion chromatograms reconstructed at their specific (M-H)− ions as follows: 339 for SM, 385 for SG, 309 for FeM and 355 for FeG. All analyses were run as triplicates.

A few analyses of soluble phenolics were carried out on the plantlets from a knockout mutant (*f5h1*) for *F5H1* grown in vitro as previously described.

UV treatment

Seeds were sown in vitro on Estelle and Somerville medium (Estelle and Somerville 1987), placed 2 days at 4°C, then transferred to the growth chamber (25°C, 16 h light 8 h dark, RH 60%) for 2 weeks. The UV treatment was performed under a UV lamp (Transilluminator UV 312 nm, 180 W, model TF-20-M, Fischer Bioblock Scientific, Illkirch, France) for 1 h. The plantlets were then transferred to the growth chamber for 6 days before observation.

Results

Identification of two new *CCR1 Arabidopsis* mutants

Cinnamoyl-CoA reductase (EC 1.2.1.44) is the first enzyme specific to the monolignol biosynthetic pathway. In *Arabidopsis,* at least two genes (*CCR1*, At1g15950 and *CCR2*, At1g80820) encode this enzyme (Goujon et al. 2003b). *CCR1* is highly expressed in lignified tissues and is considered as primordial for lignin biosynthesis (Lauvergeat et al. 2001). Two new T-DNA mutants for *CCR1* were selected in the SALK (FST 123689) and the GABIKat (FST 622C01) collections. The T-DNA insertions were located in the fourth intron and second intron, respectively (Fig. 1). Homozygous lines (*ccr1s* and *ccr1g*) for the insertions were identified using PCR and growth on selective media. They were null mutants as shown by RT-PCR (Fig. 2a). By contrast, the *CCR2* gene, which was not expressed in WT stems, was found to be expressed in the stems of both mutants (Fig. 2b for *ccr1g*, and data not shown for *ccr1s*).

Relative to the WT sample, both *ccr1* knockout mutants displayed an altered developmental program and a dwarf phenotype (Fig. 3a–c). Their floral stem height was decreased by about 50% and their rosettes and siliques were smaller. The seed number was reduced. The senescence of the *ccr1* mutants was delayed and the mutants were still green when the WT inflorescence stems were completely dry (Fig. 3d). Cross-sections of stems, which have a smaller diameter than WT, displayed a collapsed xylem phenotype. Wiesner staining suggested a lignin content reduced in the interfascicular fibers and in the xylem (Fig. 4).

Lignin modifications in the *ccr1* mutants

The lignin content of the inflorescence stems at complete maturity of each line was determined from the extract-free material collected from two cultures by the Klason and by the acetyl bromide methods as there is no single method that can accurately measure the lignin content of extract-free samples (for a review, see Hatfield and Fukushima 2005). In agreement with previous results (Goujon et al. 2003a), the culture conditions were found to affect the lignin level of the inflorescence stems. A long-day regime led to a faster development and a lower lignin content (culture 1 in Table 1) than a short-day regime (culture 2 in Table 1). Relative to the WT samples, the mutants displayed a Klason lignin level substantially decreased (by 25–35%). As an acid-soluble and syringyl-rich lignin fraction can be lost when the Klason method is applied to wood samples (Musha and Goring 1974), we determined the ASL content of the extract-free stems by the standard procedure (Dence 1992). The lower lignin level was not restored when the ASL fraction was considered as shown by the similar ASL levels observed in WT and *ccr1g* samples (Table 1). To further confirm the reduction in total lignin content, the acetyl bromide procedure was applied to the *ccr1g* or *ccr1s* sam-

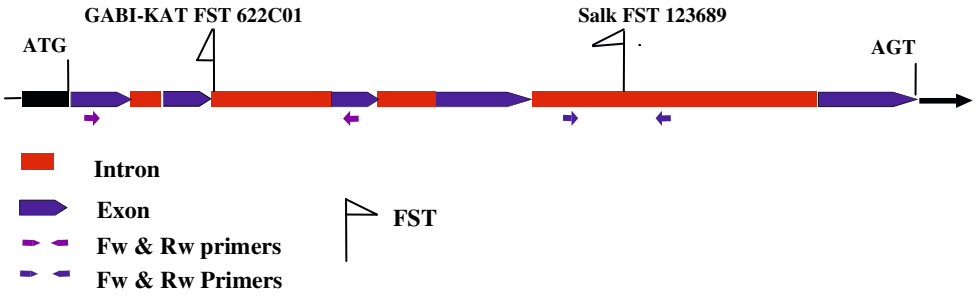

Fig. 1 Schematic representation of the *AtCCR*1 gene and localization of the T-DNA insertions for each *ccr1* line (*Flag*). The exons and introns are represented in *blue* and *red*, respectively. The position of primers (*arrows*) used to verify the absence of mRNA expression are indicated. FST flanking sequence tag

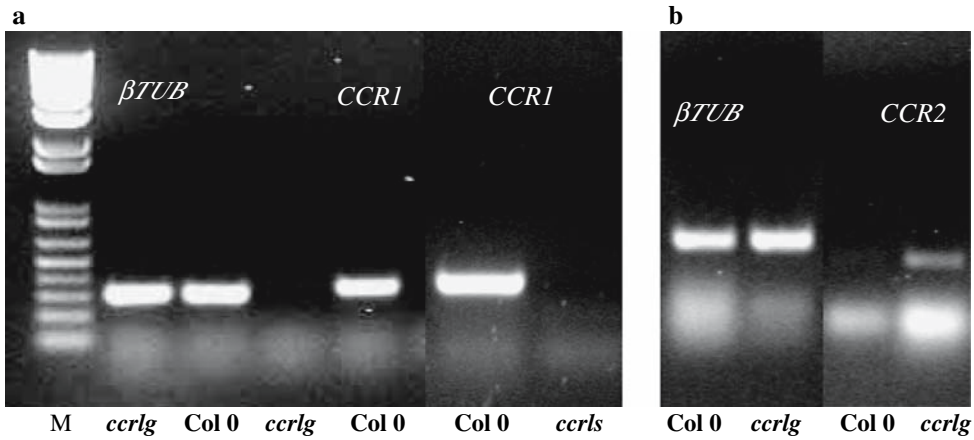

Fig. 2 RT-PCR performed in total RNA extracted for stems of Col 0 (WT), *ccr1s* and *ccr1g*. **a** No transcripts are detected in the mutants when using *CCR1* primers demonstrating that they were null mutants. **b** *CCR2* is expressed in the *ccr1g* mutant whereas not expressed in the WT (Col 0). In each case, expression of the beta-tubulin 4 gene (*β*TUB) used as a control is detected. Lane M**,** 1 kb+ ladder (Invitrogen)

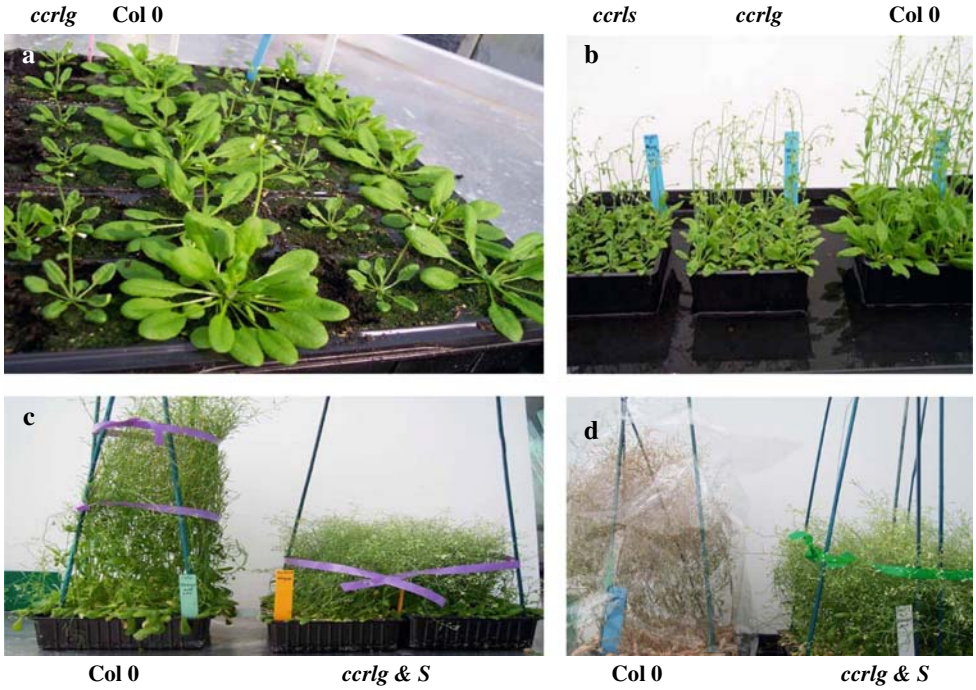

Fig. 3 Comparison of the phenotype of WT and *ccr1* mutants at different stages of growth in greenhouse. **a** Rosette stage: the rosettes of the *ccr1* mutants are smaller than those of Col 0 (WT). **b, c** Inflorescence stage: the size of the inflorescence stem is reduced in the *ccr1* mutants. **d** Senescence stage: the Col 0 plants are dry whereas the *ccr1* mutants are still green

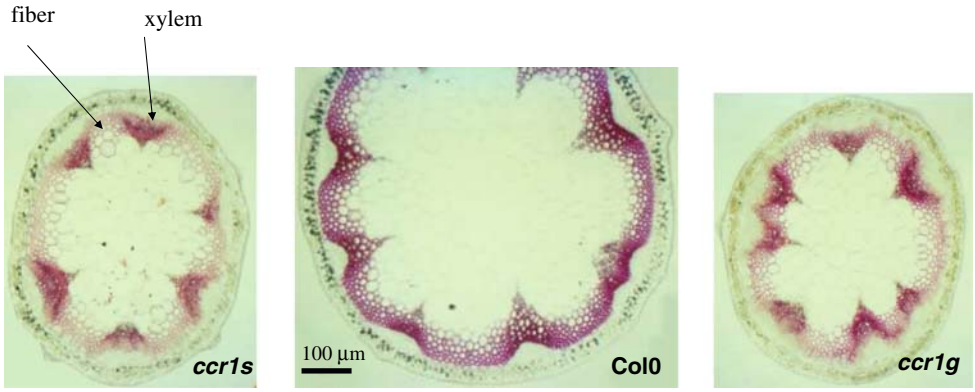

Fig. 4 Wiesner staining of cross sections of stem of *ccr1s*, WT and *ccr1g* plants. The diameters of the stems of the mutants are reduced (all cross sections were performed at the base of the main stem and the scale was the same for all photographs). The xylem is collapsed and the lignin staining is reduced in the *ccr1* mutants

Table 1 Impact of CCR1 absence on the lignin content (percent by weight) of extract-free inflorescence stems collected at complete maturity (10- to 15-week-old samples)

Sample	First culture		Second culture		
	KL	ABL	KL	ASL	ABL
Col 0	14.42 (0.22)	16.38 (0.19)	18.26 (0.18)	2.03 (0.00)	14.53 (0.99)
*ccr1*g	10.60	–	11.73 (0.13)	2.31 (0.26)	9.53 (0.53)
*ccr1*s	10.90	10.25 (0.85)	–	–	–

Two series of cultures were carried out (first culture: long-day regime and second culture: short-day regime) and analyzed for their content in Klason Lignin (KL), acid-soluble lignin (ASL) and acetyl bromide lignin (ABL). Except for the first culture of the mutant samples (KL run as single analyses), the data represent mean values (standard errors in parentheses) of duplicate or triplicate analyses (biological replicates)

ple and the corresponding controls. We employed the protocol without any perchloric acid as it has been reported that this acid favors the degradation of xylans to compounds displaying an absorbance in the 270–280 nm zone that is used to quantify lignin (Hatfield et al. 1999). This spectrometric procedure confirmed the reduced lignin level (decreased by about 35%) of the *ccr1* samples.

The impact of *CCR1* silencing on the composition and structure of the lignins of mature inflorescence stems was studied by thioacidolysis. This analytical degradation

generates H, G and S thioethylated monomers from lignin units only involved in labile ether bonds. Therefore, when calculated on the basis of the lignin content, the total thioacidolysis yield provides an estimate of the frequency of lignin units only involved in β-O-4 bonds. The data of Table 2 reveal that, in addition to reduced lignin levels, *CCR1* silencing induced substantial changes in lignin structure. Upon thioacidolysis and relative to the control, lignins of the two *ccr1* mutants released 20–40% less thioacidolysis main (S + G) monomers. This finding, which was observed independently of the culture conditions, means that the lignins of the *ccr1g* or *ccr1s* mature stems have a higher frequency of resistant interunit bonds. In addition, the relative importance of the H minor thioacidolysis monomers was found to be substantially increased in the mutants. Finally, thioacidolysis-released ferulic acid and G-CHR-CHR_2 (R = SEt) were obtained in five- to ten-times higher amount from the mutant samples. The latter component is the signature of ferulic acid-incorporation in lignins (Ralph et al. 2007).

The frequency of free phenolic groups among the lignin units only involved in β-O-4 bonds was evaluated by thioacidolysis of exhaustively permethylated samples (Lapierre and Rolando 1988). This method confirmed that β-O-4 linked H units were essentially terminal units with free phenolic groups. This can be explained by the high oxidation potential of such units that makes them unreactive in further phenolic coupling after their incorporation into lignins (Brunow et al. 1998). In contrast, S units were primarily etherified at C-4 (data not shown). More importantly, it revealed another significant difference ($P \leq 0.05$) between the control and the *ccr1g* samples : among the G units only involved in β-O-4 linked bonds, the frequency (molar percentage) of G terminal units with free phenolic groups increased from 12.77 ± 0.17 in the control ($n = 4$) to 15.30 ± 0.44 in the mutant ($n = 4$).

To further evaluate the impact of the *ccr1* mutation on lignification, FTIR was performed on cross-sections of the basal part of 3-week-old stems grown under permanent light in a growth chamber. Both mutants displayed similar FTIR difference spectra in the xylem (Fig. 5a, b) and in the fiber (Fig. 5c, d) regions. The highest difference relative to the control was detected in the fibers of the two mutants (Fig. 5c, d). Consistent with the results of chemical analyses, the FTIR difference spectra of the fiber region showed a significant reduction of lignin content (decreased peaks at 1,510 and 1,230 cm^{-1}). In contrast, peaks of the 1,600–1,750 cm^{-1} area were increased which can be assigned to an enrichment in hemicellulose components (Sene et al. 1994) and/or in double bonds conjugated to aromatic rings. The FTIR spectra of the xylem region did not display any significant difference at 1,510 cm^{-1}, but similar trends as the fiber region in the 1,230 and the 1,600–1,700 cm^{-1} areas.

Study of low molecular weight phenolics released by mild alkaline hydrolysis of extract-free stems

The increased incorporation of ferulic acid in the mutant cell walls was further supported by the results from mild alkaline hydrolysis (NaOH 1 M, 20°C, 24 h) of the extract-free stems at complete maturity. The *ccr1g* sample released almost four times more ferulic acid than the control (Table 3). Beside ferulic acid, the extract-free cell walls released a series of low-molecular weight phenolics. Vanillin and syringaldehyde are recovered from the mild alkaline hydrolysis of purified lignin fractions and their recovery yield increases with the severity of the treatment (data not

Table 2 Impact of *CCR1* absence on lignin structure as revealed by thioacidolysis of extract-free inflorescence stems collected at complete maturity (10- to 15-week-old samples)

	Col 0	*ccr1*g	*ccr1*s
First culture (long-day regime)			
Thioacidolysis monomers:			
(H + G + S) in µmol/g KL	1,256 (100)	996* (30)	784* (64)
%H (molar frequency)	0.81 (0.05)	1.31* (0.03)	1.26* (0.03)
%G (molar frequency)	69.79 (0.30)	62.68* (0.34)	66.26* (0.32)
%S (molar frequency)	29.40 (0.30)	36.00* (0.31)	32.38* (0.29)
Thioacidolysis-released ferulic acid (µmol/g KL)	0.66 (0.05)	4.49* (0.09)	4.68* (0.47)
G-CHR-CHR_2 (µmol/g KL)	1.62 (0.27)	8.49* (0.15)	7.54* (0.80)
Second culture (short-day regime)			
(H + G + S) in µmol/g KL	1,208 (29)	773* (63)	–
%H (molar frequency)	0.71 (0.03)	1.05* (0.09)	–
%G (molar frequency)	71.15 (0.19)	71.32 (0.11)	–
%S (molar frequency)	28.14 (0.16)	27.63 (0.17)	–
Thioacidolysis-released ferulic acid (µmol/g KL)	1.10 (0.05)	10.67* (0.50)	–
G-CHR-CHR_2 (µmol/g KL)	1.48 (0.18)	10.66* (0.70)	–

Two series of cultures were carried out. The results are expressed in micromoles per gram of Klason lignin and represent the mean value (standard error in parentheses) of duplicate or triplicate thioacidolysis analyses (R = SEt)

* Indicates a significant difference relative to the control (at $P < 0.05$)

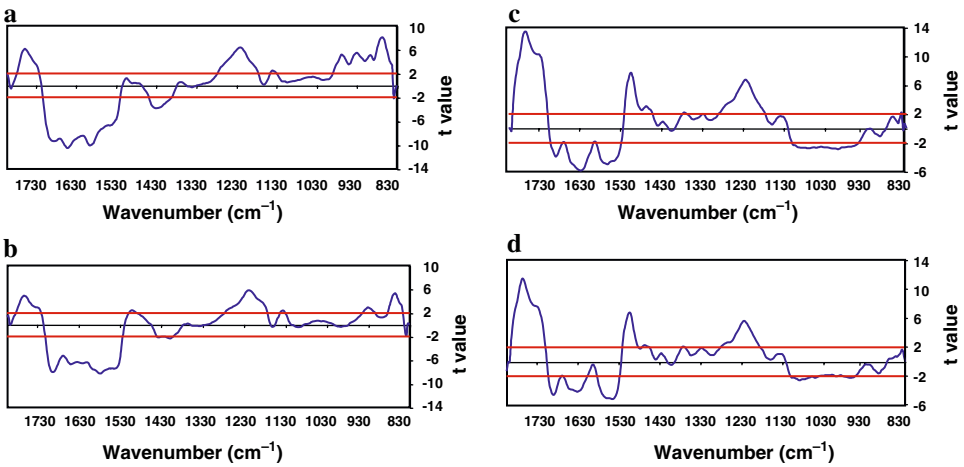

Fig. 5 Fourier-transformed infrared spectroscopy analysis performed on cross sections of inflorescence stems. Student's *t*-test values corresponding to differences between the WT (Col 0) and the *ccr1s* (**a, c**) and *ccr1g* (**b, d**) mutants at the level of xylem (**a, b**) and fibers (**c, d**). Differences are considered as significant if above or under the red lines

Table 3 GC-MS determination of the main low-molecular weight phenolics released by mild alkaline hydrolysis of *Arabidopsis* control and *ccr1g* extract-free mature stems

TMS derivatives of	Col 0 µg/g	*ccr1g* µg/g
Vanillin	82 (7)	93 (5)
Syringaldehyde	43 (3)	43 (2)
Vanillic acid	43 (3)	43 (3)
Syringic acid	10.3 (0.4)	11.4 (0.8)
p-Coumaric acid (*Z* and *E*)	32 (2)	26 (1)
Ferulic acid (*Z* and *E*)	27 (1)	100 (8)
Arylglycerol-*β*-feruloyl ether dimer (erythro and threo *E* and *Z* isomers)	48 (6)	49 (4)

The data are mean values (and SE) of replicate analyses ($n = 4$) and are expressed in microgram per gram of extract-free samples. The GC-MS determinations were performed on ion chromatograms reconstructed at the most specific ions of the TMS derivatives (molecular ion and base peak ion) of the various compounds and relative to the internal standard (ethylvanillin for the TMS monomers and dieugenol for the TMS dimer) with a response factor arbitrarily set at 1

shown). These aldehydes, which are most likely degradation products from lignins, were recovered in similar amount from the mutant and control samples, in spite of the lower lignin content of the former. This higher susceptibility of the mutant lignins to mild alkaline hydrolysis is another signature of the structural changes induced in lignins by the *ccr1* mutation. The ester-bound *p*-OH benzoic, vanillic, syringic and *p*-coumaric acids were also released in similar amounts (Table 3). Sinapic acid was also detected but in low and poorly reproducible yields (data not shown). In addition to phenolic monomers, extract-free *Arabidopsis* mature stems released ferulic acid-coniferyl alcohol dimers that have been identified for the first time in grass cell walls (Jacquet et al. 1995). As reported for grass cell walls, these dimers were mainly recovered as a mixture of erythro and threo *E* isomers together with lower amounts of their Z analogues (Fig. 6). They could be unambiguously identified from the mass spectra of their TMS derivatives, as compared to analogous compounds released from grass cell walls or to an appropriate authentic compound (Jacquet et al. 1995). To the best of our knowledge, this is the first time that such guaiacylglycerol-*β*-feruloyl ethers are identified in dicot cell walls. As shown in Table 3, their recovery yield from the control is of the same magnitude as the recovery of ferulic acid.

The soluble phenolic pool is altered in the plantlets, stems and leaves of the *ccr1* mutants

To further evaluate the impact of the *ccr1* mutation on the phenylpropanoid metabolism, we studied the soluble phenolics of vegetative tissues from in vitro-grown plantlets (3-day- and 16-day-old) and from greenhouse-grown plants. The rosette leaves of greenhouse-grown plants were collected quite early (18-day-old plants) before any wilting could occur. The stem samples were collected in the basal region of bolting inflorescence stems of 30-day-old plants grown in long day conditions. These phenolics were extracted and analyzed by LC-MS (electrospray ionization in the negative mode) as previously described (Abdulrazzak et al. 2006).

The main soluble phenolics recovered from the control samples were three kaempferol glycosides K1-K3 together with SM (see Sect. "Materials and methods"). Beside these major soluble phenolics and at the early developmental stages (in plantlets), a quercetin glycoside Q1, an isorhamnetin glycoside I1 and SG could be evidenced. In addition to these soluble conventional phenolics, the *ccr1g* and *ccr1s* samples contained feruloyl malate FeM, identified from its mass spectra (Fig. 7a). FeM has been previously described in a ferulate-5-hydroxylase (F5H) null mutant (Hemm et al. 2003). It could be also detected as a minor phenylpropanoid component in a Col 0 extract obtained

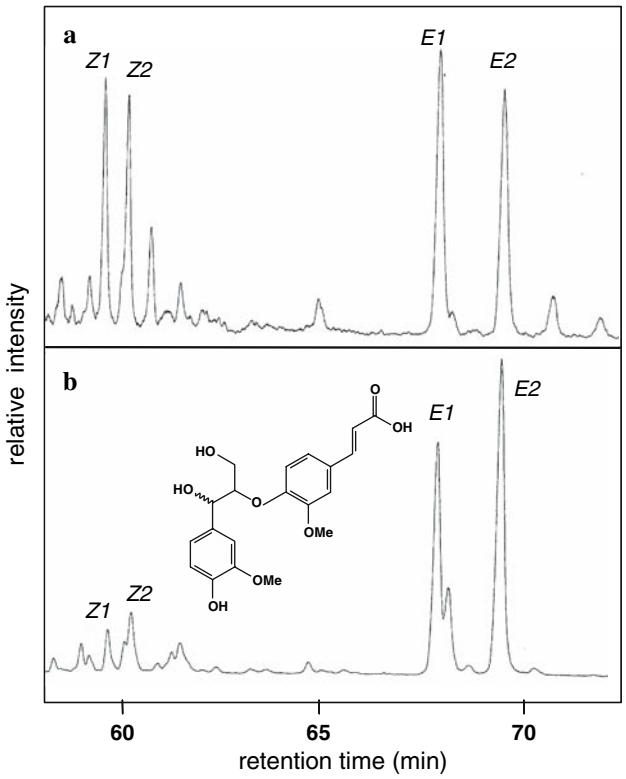

Fig. 6 Gas chromatography-mass spectrometry analysis of the guaiacylglycerol-*β*-feruloyl ethers released by mild alkaline hydrolysis of **a** *Arabidopsis* extract-free and mature stems (*ccr1s*) and **b** a grass cell wall samples (extract-free maize internode). The dimers are analyzed as their TMS derivatives and are recovered as a mixture of erythro/threo *E* (*E1* and *E2*) and *Z* (*Z1* and *Z2*) isomers

from a considerably higher amount of fresh material (Hendrawati et al. 2006). Similar to SM, FeM was obtained as a mixture of *E* and *Z* isomers, the relative importance of which could be changed by UV-induced isomerization (data not shown). Together with FeM which could not be detected in the control samples, 3-day-old mutant plantlets contained small amount of a compound which was speculatively identified as feruloyl glucose FeG on the basis of its MS spectra (Fig. 7a). The data of Tables 4 and 5 reveal that the *ccr1* mutation substantially alters the soluble phenolic pool, whatever the sample.

Relative to the control, the 3-day-old mutant plantlets released more quercetin and isorhamnetin derivatives. In addition and consistently with an altered appearance under UV (data not shown), their sinapoyl ester level was three- to four-times lower than the control level and they accumulated noticeable amounts of FeM and FeG (Table 4). Compared to the control, older plantlets or rosette leaves of the mutants had reduced levels of kaempferol glycosides while their lower content of SM and the substantial occurrence of FeM were still observed (Tables 4, 5).

For comparison, we studied the soluble phenolic pool of a knockout mutant *f5h1* for ferulate-5-hydroxylase 1 that was identified in the Versailles collection (Samson et al. 2004). In agreement with literature data (Meyer et al. 1998), *F5H1* silencing led to the disappearance of both SM (Table 6) and of the S units in stem lignins (data not shown). In addition and as already reported for another *F5H1* knockout mutant (named *fah1-2*, Hemm et al. 2003; Nair et al. 2004), this *f5h1* mutant was found to accumulate FeM, but to a lower extent than the *ccr1* mutants studied herein (Table 6).

Sinapate esters and flavonol glycosides are considered as protective against UV light (Landry et al. 1995). Since the amount of these phenolic sunscreens was found to be decreased in the mutant plantlets, their resistance to UV was tested. Two-week-old in vitro-grown plantlets were exposed to UV light for one hour, then transferred to the growth chamber to recover for 6 days. After this delay and in agreement with their reduced pool of soluble phenolics, the mutant plantlets displayed more severe alterations than the control samples (Fig. 8 for WT and *ccr1g*, data not shown for *ccr1s*).

By contrast to weakly lignified samples (plantlets and leaves), the basal region of 30-day-old stems displayed a substantial enrichment in SM (Table 5). Their FeM content was also dramatically increased. This outstandingly high level of FeM was confirmed by subjecting the extracts to a mild alkaline hydrolysis. Ferulic and sinapic acids were recovered in similarly high amounts (Fig. 7b). After the analyses of soluble phenolics, we subjected the same stem samples to thioacidolysis in order to characterize their lignins. While the lignin-derived thioacidolysis monomers could be clearly evidenced from the control samples, they could not be detected from the mutant ones (data not shown).

Discussion

Cinnamoyl-CoA reductase 1 silencing induces a delayed development, a dwarf phenotype and a reduced lignin level in the two new *Arabidopsis* mutants

Cinnamoyl CoA reductase, identified as the first enzyme specific to the monolignol branch, was originally cloned and characterized in *Eucalyptus* (Lacombe et al. 1997), then in tobacco (Piquemal et al. 1998). In *Arabidopsis*, the first mutant for *CCR1* was named *irx4* (for irregular xylem 4, Landsberg ecotype) and was identified from its collapsed xylem (Jones et al. 2001). A construct containing the *AtCCR1* gene under the control of the CaMV 35S promoter was introduced in antisense orientation in *Arabidopsis* (Goujon et al. 2003a). Similar to the *irx4* mutant, the ASCCR plants (WS ecotype) displayed a reduced size and a lower lignin content in floral stems collected at maturity. In the present work, two new *Arabidopsis CCR1* knockout

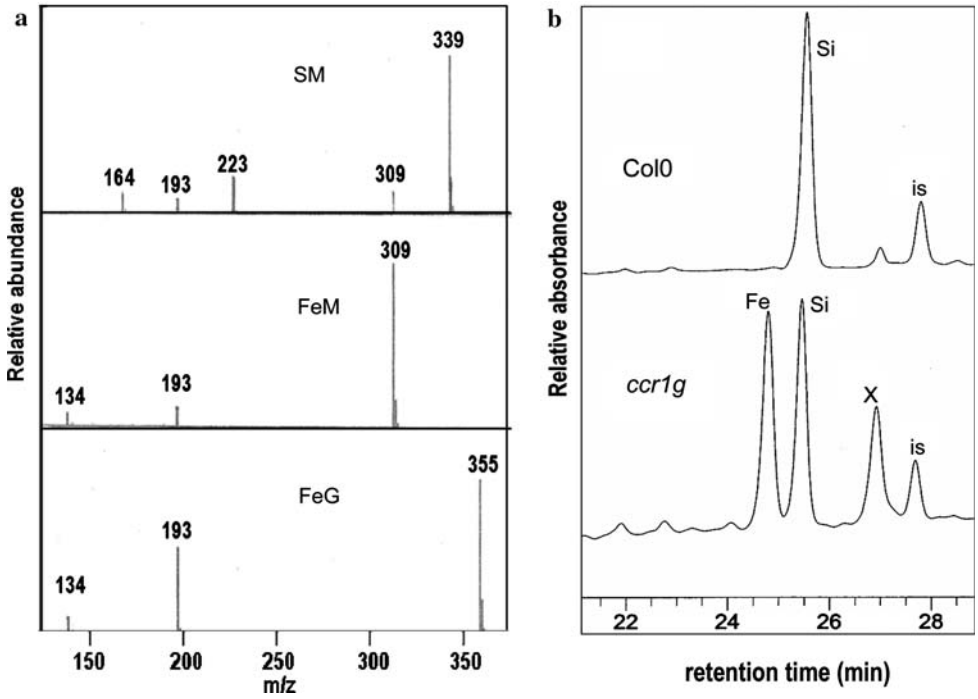

Fig. 7 Liquid chromatography-mass spectrometry analysis of sinapoyl and feruloyl soluble derivatives recovered from Col 0 and *ccr1* samples. **a** Mass spectra of sinapoyl malate SM, feruloyl malate FeM and feruloyl glucose FeG, extracted from 3-day-old *ccr1g* or *ccr1s* plantlets. **b** HPLC analysis of sinapic acid Si and ferulic acid Fe obtained after alkaline hydolysis of the soluble phenolics recovered from Col 0 and from *ccr1g* stems (30-day-old, basal region); *is*, internal standard. Peak *X* is a kaempferol glycoside which is not reproducibly recovered from alkaline hydrolysis

Table 4 LC-MS determination of soluble phenolics extracted from control and *ccr1* mutant plantlets

Compound	$(M-H)^-$	3-day-old plantlets			16-day-old plantlets		
		Col 0	*ccr1g*	*ccr1s*	Col 0	*ccr1g*	*ccr1s*
Flavonol glycosides (in µg/g FM)							
K1	577	164 (30)	55 (5)	139 (21)	216 (9)	48 (8)	61 (8)
K2	593	500 (60)	405 (17)	411 (50)	201 (9)	41 (6)	52 (5)
K3	739	72 (11)	52 (2)	65 (14)	92 (6)	35 (6)	35 (3)
Q1	609	349 (24)	420 (23)	457 (53)	26 (3)	4 (0)	4 (1)
I1	623	70 (6)	97 (5)	85 (5)	Nd	Nd	Nd
Total		1,155 (131)	1,029 (52)	1,157 (143)	535 (27)	128 (20)	152 (17)
p-Hydroxycinnamoyl esters (in µg/g FM)							
SM	339	355 (9)	72 (9)	93 (16)	1123 (97)	427 (38)	404 (48)
SG	385	33 (1)	34 (3)	16 (2)	27 (2)	10 (2)	13 (1)
FeM	309	Nd	55 (7)	59 (5)	Nd	95 (9)	78 (3)
FeG	355	Nd	80 (1)	39 (4)	Nd	8 (1)	8 (1)

The main flavonol glycosides (FG) are representatives of kampferol (K1–K3), quercetin Q1 and isorhamnetin I1 derivatives (see Sect. "Materials and methods"). The main p-hydroxycinnamoyl esters are representatives of sinapoyl malate (SM, *E* and *Z* isomers), sinapoyl glucose (SG, *E* and *Z* isomers), feruloyl malate (FeM, *E* and *Z* isomers) and feruloyl glucose (FeG, one major isomer). The data are mean values (and SE) of triplicate analyses and are expressed in micrograms per gram of fresh material (FM). Three series of 20 and 6 plantlets were collected when 3-day and 16-day old, respectively. LC-MS determination was performed on ion chromatograms reconstructed at *m/z* $(M-H)^-$ for each compound and relative to the internal standard (morin) with a response factor arbitrarily set at 1

Nd not detected

mutants (Col 0 ecotype) were identified and characterized. All the observed parameters including size, growth and developmental stages, lignin content and structure, were the same for the two independently obtained mutants, which supports the relationship between the phenotype and the *CCR1* mutation. *CCR1* mutation has a strong impact on the growth and development of the two *ccr1* mutants. Both exhibit a delayed senescence when compared to WT plants, which is in agreement with recent studies of the *irx4* mutant (Patten et al. 2005; Laskar et al. 2006). The size of the *ccr1* plants is reduced at all vegetative stages and a longer period of development is necessary to get viable seeds. Dwarf

Table 5 LC-MS determination of soluble phenolics extracted from control and *ccr1* mutant rosette leaves and stems

Compound	$(M-H)^-$	Rosette leaves (18-day-old plants)			Inflorescence stems (30-day old, basal region)		
		Col 0	*ccr1g*	*ccr1s*	Col 0	*ccr1g*	*ccr1s*
Flavonol glycosides (in µg/g FM)							
K1	577	242 (80)	117 (8)	132 (12)	365 (45)	462 (17)	642 (19)
K2	593	287 (47)	54 (7)	46 (8)	169 (27)	234 (38)	331 (1)
K3	739	244 (44)	121 (7)	122 (22)	125 (17)	208 (5)	289 (6)
Total		773 (171)	292 (22)	300 (42)	659 (89)	904 (60)	1262 (26)
p-Hydroxycinnamoyl esters (in µg/g FM)							
SM	339	1495 (176)	452 (38)	453 (16)	138 (8)	292 (41)	458 (30)
FeM	309	Nd	310 (30)	297 (21)	Nd	279 (19)	346 (9)

The main flavonol glycosides (FG) are representatives of kampferol (K1–K3) glycosides (see Sect. "Materials and methods"). The main *p*-hydroxycinnamoyl esters are representatives of sinapoyl malate (SM, *E* and *Z* isomers) and feruloyl malate (FeM, *E* and *Z* isomers). The data are mean values (and SE) of triplicate analyses and are expressed in micrograms per gram of fresh material (FM). LC-MS determination was performed on ion chromatograms reconstructed at *m/z* $(M-H)^-$ for each compound and relative to the internal standard (morin) with a response factor arbitrarily set at 1

Nd not detected

Table 6 Soluble phenolics extracted from 16-day-old mutant plantlets and from the corresponding controls (Col 0 for *ccr1g* and WS for *f5h1*)

Compounds	Col 0	*ccr1g*	WS	*f5h1*
Flavonol glycosides	535 (27)	128 (20)	370 (50)	250 (13)
Sinapoyl malate	1123 (97)	427 (38)	322 (46)	Nd
Feruloyl malate	Nd	95 (9)	Nd	14 (1)

The data are mean values (and SE) of triplicate analyses and are expressed in micrograms per gram of fresh material. LC-MS determination was performed as described (see Sect. "Materials and methods")

Nd not detected

phenotypes following *CCR* down-regulation have already been observed using the antisense strategy in tobacco (Piquemal et al. 1998; Chabannes et al. 2001) and the RNAi strategy in tomato (van der Rest et al. 2006). Another *CCR* gene (*CCR2*, At1g80820) is present in the *Arabidopsis* genome and has been shown to be induced by pathogens (Lauvergeat et al. 2001). We found that *CCR2* expression is increased in the *ccr1* mutants. This increase could account for the lignin level of the mutant mature stems which is 65–75% of the control level in spite of a complete *CCR1* absence. As a support for such a compensatory *CCR2*-dependent mechanism, it has been reported that, when the expression of this gene was induced in *Arabidopsis*, it could lead to an ectopic lignification (Cano-Delgado et al. 2003). However, the unusual *CCR2* expression could not completely compensate for the lack of *CCR1* since the lignin content of the mutant stunted stems is reduced by ca. 25–35%. This substantial reduction was demonstrated by two different methods and for both mutants. Moreover, the FTIR microscopy suggested that the lignification of the mutant was more altered in the fiber region than in the xylem region.

Lignin structure and cell wall-bound ferulic acid are affected in the *ccr1* mutant stems

In addition to a lower lignin level, substantial alterations of lignin structure in stems collected at complete maturity could be evidenced by thioacidolysis and in both mutants. In agreement with a previous study (Goujon et al. 2003a), the frequency of S and G thioacidolysis monomers did not allow a clear discrimination between the mutant and control samples as it seems to be affected by the culture conditions (Table 2). Relative to the control value, the S/G ratio of the mutant is significantly higher when the plants develop rapidly in a long-day regime, but this difference disappears with a short-day culture. By contrast and whatever the culture conditions, lignins from both mutants released substantially less thioacidolysis monomers than WT samples. This finding means that lignins of mutant lines are enriched in resistant interunit bonds (i.e., they have a higher condensation degree). Together with this higher condensation degree, the frequency of free phenolic groups in β-O-4 G units was found to be increased by the *ccr1* mutation. Both parameters consistently increase in the lignins of *ccr1g* mature stems as a higher branching of the polymer (i.e., a higher frequency of terminal units with free phenolic groups) may be expected from a higher condensation degree. Another and more discrete impact of the *ccr1* mutation is the higher incorporation of ferulic acid in the lignin polymers as suggested by the higher recovery of the thioacidolysis monomer G-CHR-CHR_2 (R = SEt) which is the signature of ferulic acid-incorporation in lignins (Ralph et al. 2007).

Consistent with this increased incorporation of ferulic acid in the lignin polymers, the mature and extract-free

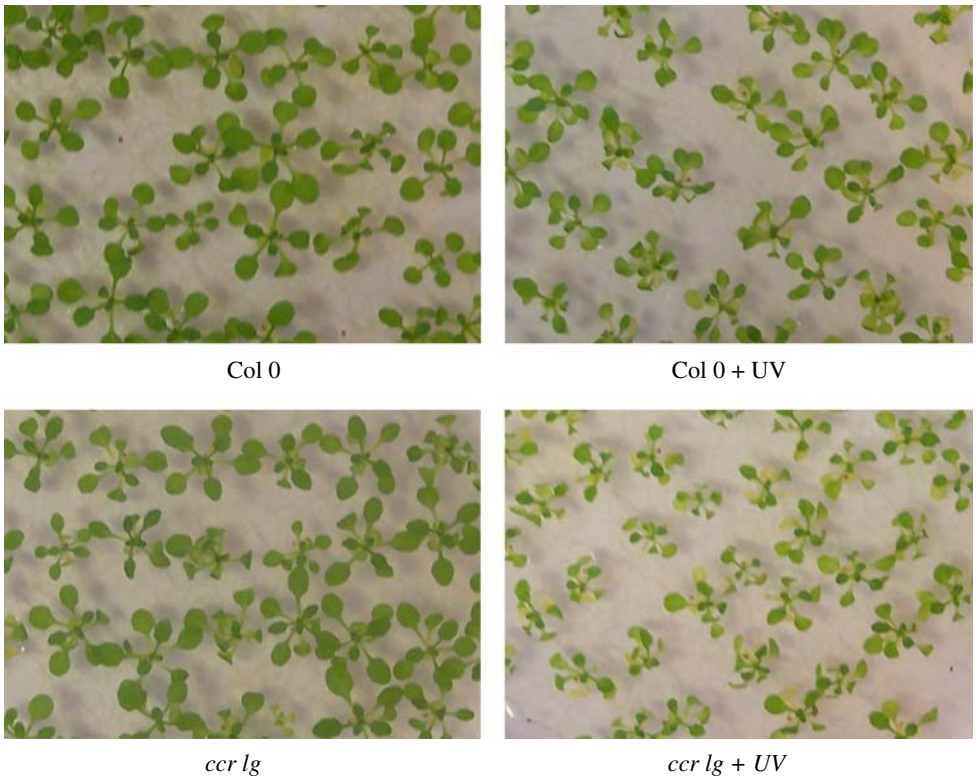

Fig. 8 Phenotype of 20-day-old plantlets without (Col 0 and *ccr1g*) and with (Col 0 + UV and *ccr1g* + UV) a 1-h exposure to UV applied 6 days before. The plantlets of the mutant are smaller than the WT after the treatment. They are pale green and have yellow leaves suggesting a higher sensitivity to UV than WT

mutant stems contain four times more cell wall-bound ferulic esters than the WT samples. This finding suggests that *CCR1* absence favors an increased transfer of ferulic acid to cell wall polysaccharides. This phenomenon would involve an acyltransferase activity catalyzing the transfer of ferulic acid from feruloyl-CoA to polysaccharides, as described in past studies (Meyer et al. 1991). The precise nature of the polysaccharide acceptor, which is well documented in the case of Gramineae and of Chenopodiaceae cell walls (Saulnier and Thibault 1999), remains to be addressed in *Arabidopsis*.

In addition to ferulic acid, the mild alkaline hydrolysis of *Arabidopsis* extract-free stems released guaiacylglycerol-*β*-feruloyl ether dimers. To the best of our knowledge, this is the first time that these dimers are evidenced from *Arabidopsis* cell walls. These structures associating ferulic acid to a lignin unit have been identified in grass cell walls (Jacquet et al. 1995) and have important biochemical implications. They mean that polysaccharide-bound ferulate esters provide nucleation sites for the lignin polymers via ether bonds that anchor lignins to polysaccharides. Our finding indicates that this anchoring, which is well established for grass cell walls, is also present in *Arabidopsis* cell walls and in spite of their considerably lower amount of cell wall-bound ferulate esters. The coupling of feruloyl derivatives to lignin precursors has been recently evidenced in the soluble phenolic pool of *Arabidopsis* and by the identification of a 4-O-8 cross-coupling product of feruloyl malate and coniferyl alcohol (Rohde et al. 2004). In spite of a higher content in cell wall-bound ferulic esters, the *ccr1* mutant cell walls did not release higher amounts of the feruloyl ether dimers than the control. This result is consistent with the reduced carbon flux into the lignin-specific branch of the phenylpropanoid pathway.

The profile of soluble phenolics is dramatically altered in the two *ccr1* mutants with a massive redirection to feruloyl malate in the inflorescence stems

In *Arabidopsis*, the down-regulation of other steps of the monolignol branch has similar consequences on the lignin level of mature stems (reduction by 20–40%), but a lower impact on the phenotype and development. For instance, the stems of a cinnamyl alcohol dehydrogenase mutant are not smaller, but just red and limp (Sibout et al. 2005). The size of a caffeoyl-CoA *O*-methyltransferase mutant is only slightly reduced (Do et al. 2007). In other words, *CCR1* absence has a more severe impact (i.e., dwarfing) on the growth and development of *Arabidopsis*. As suggested in recent studies, dwarfing may result from the perturbation of other branches of the phenylpropanoid pathway (Abdulrazzak et al. 2006; Besseau et al. 2007) or from the perturbation of other metabolisms such as the CoASH metabolism (Patten et al. 2005). van der Rest et al (2006) obtained CCR-down-regulated tomato plants by an RNAi strategy. The most severely affected transgenic lines displayed a

dwarf phenotype together with a reduced lignin level. Moreover, the pool of soluble phenolics (mainly chlorogenic acid and its isomers) was dramatically increased in the hypo-lignified stems. The authors concluded that the pool of precursors left unused by the monolignol biosynthetic pathway had been redirected to the synthesis of other phenolics. In another recent study, Besseau et al (2007) established that the accumulation of flavonoids was primarily responsible for the severely altered phenotype of *Arabidopsis* lines underexpressing the hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (HCT). The soluble phenolic pool of *Arabidopsis* is mainly composed of flavonol glycosides (kaempferol and quercetin derivatives) and sinapate esters. We have therefore studied the consequences of *CCR1* mutation on the pool of soluble phenolics at different developmental stages.

In leaves, whatever the age (3- and 16-day-old in vitro plantlets which are mainly composed of leaves or leaves from 18-day-old plants grown in greenhouse), the mutation induced the appearance of FeM together with a substantial reduction of flavonol glycosides and of SM. While the origin of this reduction remains to be addressed, this reduction induced a lower resistance of the plantlets to UV exposure. Consistently with a previous study (Hemm et al. 2003), this feruloyl derivative, which could not be detected in the control samples, could be also detected in *f5h1* mutant plantlets, but in lower amounts.

By contrast to the plantlets and leaves, the basal stem regions of the mutant stunted plants not only accumulate higher levels of SM (three- to four-times more than the control level) and flavonol glycosides, but also outstandingly high levels of FeM. Taken together, these findings suggested that FeM may be produced byUDP-glucose:sinapic acid glucosyl transferase and sinapoylglucose:malate sinapoyl transferase given the previously identified low substrate specificity of these enzymes (Grawe et al. 1992; Franke et al. 2002). These results suggest that if the metabolic flux toward stem lignins is reduced, there is a redirection of the phenylpropanoid pathway toward other branches, a situation which closely mirrors the one reported in CCR-down-regulated tomato plants (van der Rest et al. 2006) and, more recently, in a CCR-down-regulated tobacco line (Dauwe et al. 2007). The dwarf phenotype of the *ccr1* mutants might be related to the perturbation in the flavonoid pool.

Conclusion

The formation of feruloyl malate to an extent that far exceeds the levels reported so far in the literature indicates that ferulic acid is a potential substrate for the transferases involved in SM biosynthesis. The redirection of the phenolic metabolism in *CCR1*-knockout stunted stems could be accounted for by the following hypothetical scenario. When the flux toward H, G or S lignin units is hampered by *CCR1* silencing, the stunted plants manage to produce a viable lignin level by recruiting the CCR2 activity. However, this enzyme cannot entirely compensate for the lack of CCR1. Accordingly and in lignifying tissues displaying an active phenylpropanoid metabolism, the mutants would avoid the detrimental accumulation of p-coumaroyl-CoA and feruloyl-CoA by a series of redirection mechanisms. The transient excess of *p*-coumaroyl-CoA would be partially processed by its redirection to flavonol glycosides. The accumulation of feruloyl-CoA would be avoided partly by an increased and acyltransferase-mediated transfer of ferulic acid from feruloyl-CoA to cell wall polysaccharides. Another fate of this feruloyl-CoA in preventing excess would be its hydrolysis to ferulic acid and CoASH, by means of a putative thioesterase activity. Such a thioesterase activity could be ensured by HCT (Hoffmann et al. 2003). The resulting ferulic acid would be further processed either to feruloyl malate or to SM. The redirection mechanism could also proceed upstream to the *p*-hydroxycinnamoyl-CoA esters and at the level of the free acids. In agreement with a series of recent studies of the phenolic metabolism in *Arabidopsis* mutants, this study further emphasizes the remarkable plasticity of the phenylpropanoid metabolism.

Acknowledgments We thank Fréderic Legée (AgroParisTech, UMR 206 Chimie Biologique) and Laurent Cézard (INRA, UMR 206 Chimie Biologique) for running the Klason lignin and the thioacidolysis analyses respectively and Christina Gofron (INRA Versailles) for *Arabidopsis* cultivation in the greenhouse.

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