ORIGINAL ARTICLE

Thomas Goujon · Valérie Ferret · Isabelle Mila
Brigitte Pollet · Katia Ruel · Vincent Burlat
Jean-Paul Joseleau · Yves Barrière
Catherine Lapierre · Lise Jouanin

Down-regulation of the *AtCCR1* gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability

Abstract Cinnamoyl CoA reductase (CCR; EC 1.2.1.44) is the first enzyme specific to the biosynthetic pathway leading to monolignols. *Arabidopsis thaliana* (L.) Heynh. plants transformed with a vector containing a full-length *AtCCR1* cDNA in an antisense orientation were obtained and characterized. The most severely down-regulated homozygous plants showed drastic alterations to their phenotypical features. These plants had a 50% decrease in lignin content accompanied by changes in lignin composition and structure, with incorporation of ferulic acid into the cell wall. Microscopic analyses coupled with immunolabelling revealed a decrease in lignin deposition in normally lignified tissues and a dramatic loosening of the secondary cell wall of interfascicular fibers and vessels. Evaluation of in vitro digestibility demonstrated an increase in the enzymatic degradability of these transgenic lines. In addition, culture conditions were shown to play a substantial role in lignin level and structure in the wild type and in the effects of *At*CCR1 repression efficiency.

T. Goujon · V. Ferret · L. Jouanin (✉)
Biologie Cellulaire,
Institut National de la Recherche Agronomique,
78026 Versailles Cedex, France
E-mail: jouanin@versailles.inra.fr
Fax: +33-1-30833099

I. Mila · B. Pollet · C. Lapierre
Chimie Biologique,
Institut National de la Recherche Agronomique,
Institut National Agronomique Paris-Grignon,
78850 Thiverval-Grignon, France

K. Ruel · V. Burlat · J.-P. Joseleau
Centre de Recherche sur les Macromolécules Végétales,
BP53, 38041, Grenoble Cedex 09, France

Y. Barrière
Unité de Génétique et d'Amélioration des Plantes Fourragères,
INRA, 86600 Lusignan, France

Present address: Y. Barrière
Faculté des Sciences et Techniques,
Laboratoire de Physiologie Végétale,
37200 Tours, France

Introduction

Lignins are the second most abundant biopolymer on earth, just after cellulose, and can represent up to 30% of the dry weight of trees (Sarkanen and Hergert 1971). They result from the dehydrogenative polymerization of cinnamyl alcohols or monolignols. The pathway leading from phenylalanine to monolignols has been determined and revised recently (Humphreys et al. 1999; Osakabe et al. 1999; Li et al. 2000, 2001; Humphreys and Chapple 2002). The monolignol pathway leads to cinnamyl alcohols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, differing by their degree of methoxylation and giving rise respectively to hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units in the polymer. The lignin profile of each plant species displays characteristic features (Baucher et al. 1998). Thus, most lignins of gymnosperms are devoid of S units, whereas those of angiosperms contain both G and S units. The content and composition of lignins depend on the species, the type and stage of maturation of tissues, and the micro-localization within the cell wall. The external conditions such as photoperiod, temperature and hygrometry (Struik 1983) also influence the lignification patterns.

Cinnamoyl CoA reductase (CCR; EC 1.2.1.44) has been identified as the first enzyme specific to the monolignol pathway and was originally cloned and characterized in *Eucalyptus* (Lacombe et al. 1997), and

then in tobacco (Piquemal et al. 1998). An antisense strategy aimed at CCR has only been used in tobacco. CCR-down-regulation in tobacco drastically affects the plant phenotype (Piquemal et al. 1998; Chabannes et al. 2001a, 2001b; Pinçon et al. 2001).

Up to now the model plants used to study lignin chemistry and biosynthesis have been woody species (poplar, sweetgum) and some annual plants (tobacco, maize, alfalfa, festuca). However, recently, *Arabidopsis thaliana* has been proposed as a good model system for lignification studies (Meyer et al. 1998; Boudet 2000). Even though the vegetative apparatus of *A. thaliana* is a ground rosette, it develops a lignified flowering stem (Dharmawardhana et al. 1992). Besides the xylem vessels, which are lignified, the interfascicular parenchyma of the flowering stem differentiates into highly lignified fibers with growth. Under certain growth conditions, lignins in *Arabidopsis* can represent up to 18% of the dry weight of the extractive-free mature stem and are typical of angiosperms since they contain both S and G units (Dharmawardhana et al. 1992).

In this work *A. thaliana* was used in an antisense strategy aimed at CCR to determine the consequences of down-regulation of this enzyme. The first part deals with the generation and characterization of antisense plants. The second describes the effects of down-regulation on the phenotype, lignin content and structure, and cell wall ultrastructure. In vitro cell wall degradability was investigated to assess the potential use of CCR down-regulation in other species to improve digestibility of forage crops.

Materials and methods

Plant material

Wild-type and transformant *Arabidopsis thaliana* (L.) Heynh. plants, ecotype Wassilevskija (WS), were generated in Versailles and grown in the greenhouse under four culture conditions (CC1, CC2, CC3 and CC4):

1. CC1, light 16 h, relative humidity (RH) 40%, temperature 30 °C; dark 8 h, RH 80%, temperature 15–20 °C;
2. CC2, light 16 h, RH 60%, temperature 25 °C; dark: 8 h, RH 80%, temperature 20 °C;
3. CC3, light 8 h, RH 70%, temperature 24 °C; dark 16 h, RH 70%, temperature 16 °C;
4. CC4, light 8 h, RH 85%, temperature 20 °C; dark; 16 h, RH 85%, temperature 16 °C.

Lignin analyses were performed on the wild type (WS) and transformant plants grown under these four culture conditions. RNA analyses, and biochemical and histochemical analyses were performed under the first culture conditions (CC1). For root analysis, plants were grown in aeroponic devices.

DNA construct

Plasmid pSPORT1 (Life technologies), containing the expressed sequence tag (EST) 67E6T7, was digested with *Bam*H I and *Sma* I. The 1.3-kb fragment was subsequently cloned into linearized pLBR19 digested with the same restriction endonucleases. The P70::antisense *AtCCR1* cDNA cassette was excised by a double *Sph* I and *Sma* I digestion. The cassette was cloned into pBIBKAN (Becker 1990) in the *Sst* I and *Sma* I sites. The latter construct was finally transferred to *Agrobacterium tumefaciens* strain C58pMP90 for subsequent plant transformation experiments (Koncz and Schell 1986).

Plant transformation

Arabidopsis thaliana plants (WS ecotype) were transformed by vacuum-infiltration of whole plants in an *Agrobacterium* solution, according to Bechtold et al. (1993). Plants were left to self-pollinate and seeds were harvested after complete drying of the stems. Primary transformants (T1) were selected on *Arabidopsis* medium (Estelle and Somerville 1987) containing 50 mg l^{-1} kanamycin. Primary transformants were self-pollinated to obtain homozygous plants for the insertion. Only lines segregating 3:1 for the resistance to kanamycin were selected for further analyses. Analyses were performed on monoinsertional homozygous lines, verified by Southern blot experiments (data not shown).

RNA analysis

Total RNA was isolated from 1 g of frozen tissue (1-week-old seedlings, young leaf blades, apical and basal parts of the stem harvested from 4-week-old plants) according to Verwoerd et al. (1989). RNA samples (20 µg) were separated on formaldehyde/agarose gels, transferred onto nylon filters (NEN, Boston, Mass., USA) and hybridized with randomly primed double-stranded DNA probes. The *At*CCR1 probe was obtained by digestion of plasmid containing the EST 67E6T7 by *Bam*HI and *Sma*I. A cDNA corresponding to the 18S rRNA from *Raphanus sativus* was used to quantify RNA deposition on the blots.

CCR activity assays

CCR activity was assayed as described in Chabannes et al. (2001b).

Histochemical staining and immunocytolabelling

Wiesner and Mäule reactions were performed on hand-cut cross-sections of mature stems of *A. thaliana* from wild-type and down-regulated lines according to standard protocols.

For the safranin–astra blue staining, stems from wild-type, ASCCR2 and ASCCR7 lines were fixed in 1.25% glutaraldehyde plus 2% paraformaldehyde and embedded in LR White resin as previously described (Burlat et al. 2001). Semi-thin cross-sections of stems (1 µm) were stained with safranin–astra blue according to the protocol of Srebotnik and Messner (1994) with slight modifications. Sections were immobilized on a single microscopy slide, and sequentially incubated for 30 min each in 1% aqueous safranin solution and 1% aqueous astra blue solution, respectively, with thorough rinsing in distilled water after each incubation step.

For transmission electron microscopy (TEM), the polysaccharide moiety of the walls was contrasted on ultra-thin sections by the periodic acid–thiocarbohydrazide–silver proteinate (PATAg) method (Thiery 1967) modified for secondary walls by Ruel et al. (1981). Briefly, the periodate oxidation was carried out with a 5% solution of periodic acid in water for 90 min (instead of with a 1% solution for 30 min), thiocarbohydrazide was applied for 24 h and silver proteinate for 30 min, in the dark.

For light-microscopic immunolabelling, serial semi-thin cross-sections from the series used for safranin–astra blue staining were also utilized for immunolabelling with anti-GSzt lignin antibody, specific for a mixed guaiacyl/syringyl (GS) lignin polymer enriched in non-condensed inter-unit linkages (ß-O-4; Joseleau and Ruel 1997). The sections were supported on plastic rings using the same protocol as for TEM immunolabelling (Joseleau and Ruel, 1997)

with a few modifications. The antiserum was diluted to 1/200. All washing steps were done on 100-μl drops, and the silver-enhancement step was performed for 15 min at room temperature using the IntenSE kit (Amersham). As a negative control, the corresponding pre-immune serum was used in the same conditions. Finally, sections were transferred to microscopy slides, allowed to dry and counter-stained with safranin.

All sections, either with safranin–astra blue staining or for immunolabelling, were mounted under a coverslip in immersion oil and observed with a Zeiss Axioplan 2 photomicroscope using Kodak Gold 100 film.

Transmission electron microscopy and immuno-topochemical analysis

Small slices (1 mm in thickness) taken from the base of the stem by freehand sectioning with a razor blade were fixed in a freshly prepared mixture of 0.1% glutaraldehyde (v/v) and 2% para-formaldehyde (w/v) in 0.01 M phosphate buffer (pH 7–7.2). After rinsing in phosphate buffer, they were dehydrated through a graded ethanol series up to 80% (v/v), then infiltrated and embedded in LR White resin (hard mixture; TAAB) and polymerized for 24 h at 50 °C, as described earlier by Joseleau and Ruel (1997). For TEM, sections were immunolabelled as described by Ruel et al. (2002).

Lignin characterization

The lignin content of ground extractive-free mature dry stems was estimated by the standard Klason procedure (Dence 1992). Lignin structure was investigated using thioacidolysis and its most recent developments (Lapierre et al. 1995). The lignin-derived monomers were identified by GC–MS of their trimethylsilyl (TMS) derivatives.

Digestibility experiments

Stems of 25–30 plants were harvested after 3.5 months of growth under CC4 conditions. Two independent determinations of the digestibility parameters were obtained. Stems were manually shaken in order to eliminate parts of leaves and siliques. Stems were then oven-dried at 65 °C, and ground with a hammer mill to pass through a 1-mm screen for subsequent analyses. Neutral detergent fiber (NDF) was estimated according to Goering and van Soest (1970). The in vitro dry matter digestibility (IVDMD) was estimated according to enzymatic solubility (Aufrère and Michalet-Doreau 1983) and the in vitro NDF digestibility (IVNDFD) was computed assuming that the non-NDF part of the plant material was completely digestible, according to Struik (1983) and Dolstra and Medema (1990).

Results

Expression of the *AtCCR1* gene in *Arabidopsis thaliana*

AtCCR1 expression was monitored in different parts of wild-type *A. thaliana* by Northern blot analyses, using the *AtCCR1* cDNA as a probe. Three different parts of the plants were tested according to their lignification pattern: the leaf sheaths (non-lignified or poorly lignified tissues), the basal part of the stem (highly lignified tissue with totally differentiated interfascicular fibers) and the apical part of the stem (tissue undergoing lignification with differentiating interfascicular fibers; Fig. 1). The
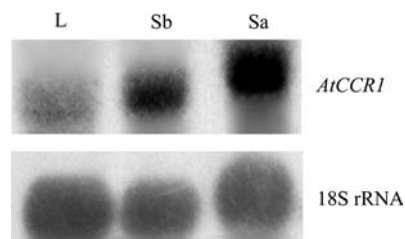

Fig. 1 Northern analysis performed on wild-type *Arabidopsis thaliana* (total RNA from: *L* leaf blade, *Sb* basal part of the stem, *Sa* apical part of the stem). Probes correspond to the cDNA of the18S rRNA from *Raphanus sativus* (18S) and to the full-length *AtCCR1* cDNA from *A. thaliana*

Northern experiment showed differential expression of this gene in the three tested tissues. The gene was weakly expressed in the leaf blade, moderately expressed in the basal part of the stem and highly expressed in the apical part.

DNA construct, plant transformation and selection of lines of interest

The *AtCCR1* cDNA, under the control of the CaMV 35S promoter with a double enhancer sequence was cloned into the pBIBKAN binary vector (Becker 1990). This vector contains a kanamycin-resistance gene to select transformed cells. *A. thaliana* (ecotype WS) was transformed and 42 independent transformed lines were obtained. Ten of them, segregating 3:1 for resistance to kanamycin, were selected. Homozygous lines used for further analyses were selected in the self-progenies of the primary transformed plants. CCR activity was measured on the total mature stems of these ten ASCCR lines and compared to controls, as described by Chabannes et al. (2001b). ASCCR2 and ASCCR7 lines displayed 19.2% and 24.4% residual activity of the wild type, respectively (Table 1). The floral stems of these lines were shorter than those of wild-type plants when grown in greenhouse conditions. Preliminary analyses demonstrated a reduction in lignin content in stems of these lines (Table 1). Lines with higher residual CCR activity (ASCCR1 and ASCCR8) also had a lower lignin content than the control but no difference in the height of stems when grown in greenhouse conditions.

Anatomy and histochemistry of the ASCCR lines in standard growth conditions

The 10 homozygous lines were grown in the greenhouse. Interestingly, lignin-specific staining of hand-cut stem sections revealed that some lines, which at the macroscopic level presented normal phenotypes, exhibited on microscopic examination particular lignification patterns. For instance, the ASCCR3 line showed differential Mäule staining within a single

Table 1 CCR activity and lignin content of wild-type (*WS*) and ASCCR lines (*AS*) of *Arabidopsis thaliana*. CCR activity (percentage of the wild type) was determined on crude extracts of stems. The experiments were performed three times on plants grown in CC2 (SE does not exceed 5%). Lignin content (% *KL* Klason lignin as a percentage of extract-free dry weight) in WS and AS stems was determined in two successive cultures in the greenhouse (SEs between duplicate measurements are indicated in parentheses). *ND* Not determined

Line	CCR activity	%KL	
		(12/1998)	(06/2000)
WS	100	17.5 (0.2)	13.5 (0.2)
AS1	35.8	ND	11.5 (0.1)
AS2	19.2	ND	8.6
AS3	90.9	16.15 (0.1)	ND
AS4	109.2	16.5 (0.1)	ND
AS5	107.4	ND	ND
AS7	24.4	11.2 (0.2)	9.4 (0.2)
AS8	49.7	12.3 (0.2)	8.35 (0.1)
AS9	84.7	16.9 (0.1)	ND
AS10	143.7	17.2 (0.1)	ND
AS11	106.2	16.1 (0.2)	ND

stem, at different levels and on independent individuals of the same line, suggesting that the efficiency with which the antisense construct affected lignin quality was heterogeneous (Fig. 2a, b). Coloration with Wiesner reagent did not reveal any difference between the wild type and these ASCCR lines. ASCCR4 showed ectopic lignification of the pith in some parts of the stem (revealed by both Wiesner and Mäule stainings). The lignins of the lignified pith cells (medullar parenchyma) are rich in S units (revealed by Mäule staining), as already described for ectopic lignification in the pith of the stem (Zhong et al. 2000; Fig. 2c). However, only ASCCR2 and ASCCR7 lines presented a stable altered phenotype throughout development and were selected for further analyses. The most striking anatomical modifications displayed by these two lines were collapsed xylem vessels in the stems together with a
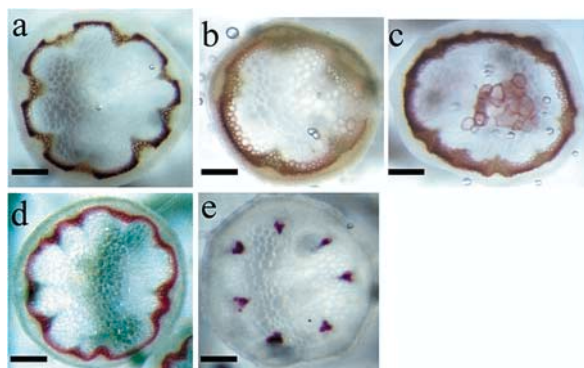

Fig. 2a–e Screening of *A. thaliana* CCR antisense (ASCCR) lines using lignin histochemical techniques. Hand-cut stem cross-sections of wild-type (*WS*) and different ASCCR lines were stained either with Mäule reagent (**a–c**) or with Wiesner reagent (**d, e**). **a** WS; **b** ASCCR3; **c** ASCCR4; **d** WS; **e** ASCCR2. Bars = 0.5 mm

very weak Wiesner coloration of the vessels and no, or faint, coloration of the interfascicular fibers (Fig. 2d, e, for wild-type and ASCCR2 lines). A more accurate comparison of ASCCR2 and ASCCR7 lines with the wild type was then performed on semi-thin cross-sections of resin-embedded stems using the safranin–astra blue co-staining technique for polysaccharides and lignins (Fig. 3a–c, j–l). The safranin–astra blue technique stains polysaccharides in blue only in the absence of lignins, and the lignins in pink-red both in the presence and absence of polysaccharides (Srebotnik and Messner 1994). In the wild-type stem, the pinkish lignin-specific coloration was present in the interfascicular fibers (Fig. 3a) and xylem vessel elements (Fig. 3j). In ASCCR2 and ASCCR7, besides the apparent thinning of fiber cell walls in the interfascicular area (Fig. 3b, c) and the collapsing of vessels (Fig. 3k, l), the lignin-specific pink coloration was less intense whereas the polysaccharide-specific blue coloration was highly visible in tissues (xylem and interfascicular fibers) that are normally lignified in the wild type. This showed that the polysaccharides were the predominant components of xylem and fiber walls in these lines (Fig. 3b, c, k, l).

Lignin immunolocalization

The non-condensed G–S sub-units which are the most frequent structures in dicots and hardwoods (Terashima et al. 1993) were identified with the specific anti-GSzt antibody previously described (Ruel et al. 1994, 1999). Immuno-gold labelling was performed on serial sections from the series used for the safranin–astra blue staining. This specific labelling showed the presence in high proportions of this type of lignin epitope in the walls of inter-fascicular and xylem fibers, and vessels of the wild type (Fig. 3d, m). This result demonstrates the presence of non-condensed GS epitopes in secondary walls, as already shown by TEM (Joseleau and Ruel 1997; Ruel et al. 1999). The same antiserum used either on ASCCR2 (Fig. 3e, n) or ASCCR7 (Fig. 3f, o) indicated a reduction in non-condensed lignin structures in interfascicular fibers and xylem. No labelling was observed in the negative controls when using the pre-immune serum (Fig. 3g–i). Regarding vessel collapsing and lignin-modification patterns, ASCCR2 displayed slightly more drastic alterations than ASCCR7. These microscopic observations are in agreement with the lower residual CCR activity in ASCCR2 than in ASCCR7, indicating that the lower the residual CCR activity, the stronger the micro-morphological effects.

Micromorphology and lignin immunolabelling in TEM

Transmission electron micrographs revealed differences between wild-type plants and ASCCR2 lines. Walls from the xylem and interfascicular regions were

Fig. 3 Histochemical and immunocytochemical analysis of wild-type (**a, d, g, j, m**), ASCCR2 (**b, e, h, k, n**) and ASCCR7 (**c, f, i, l, o**) lines. Serial semi-thin cross-sections of resin-embedded stems from the three *A. thaliana* lines were stained with safranin–astra blue (**a–c, j–l**) or immuno-stained with anti-GSzt lignin-specific antiserum (**d–f, m–o**) and the corresponding pre-immune serum (**g–i**). *Co* Cortex, *En* endodermis, *Ep* epidermis, *IF* interfascicular fibers, *Ph* phloem, *Pi* pith, *X* xylem vessels. Bar = 5 μm
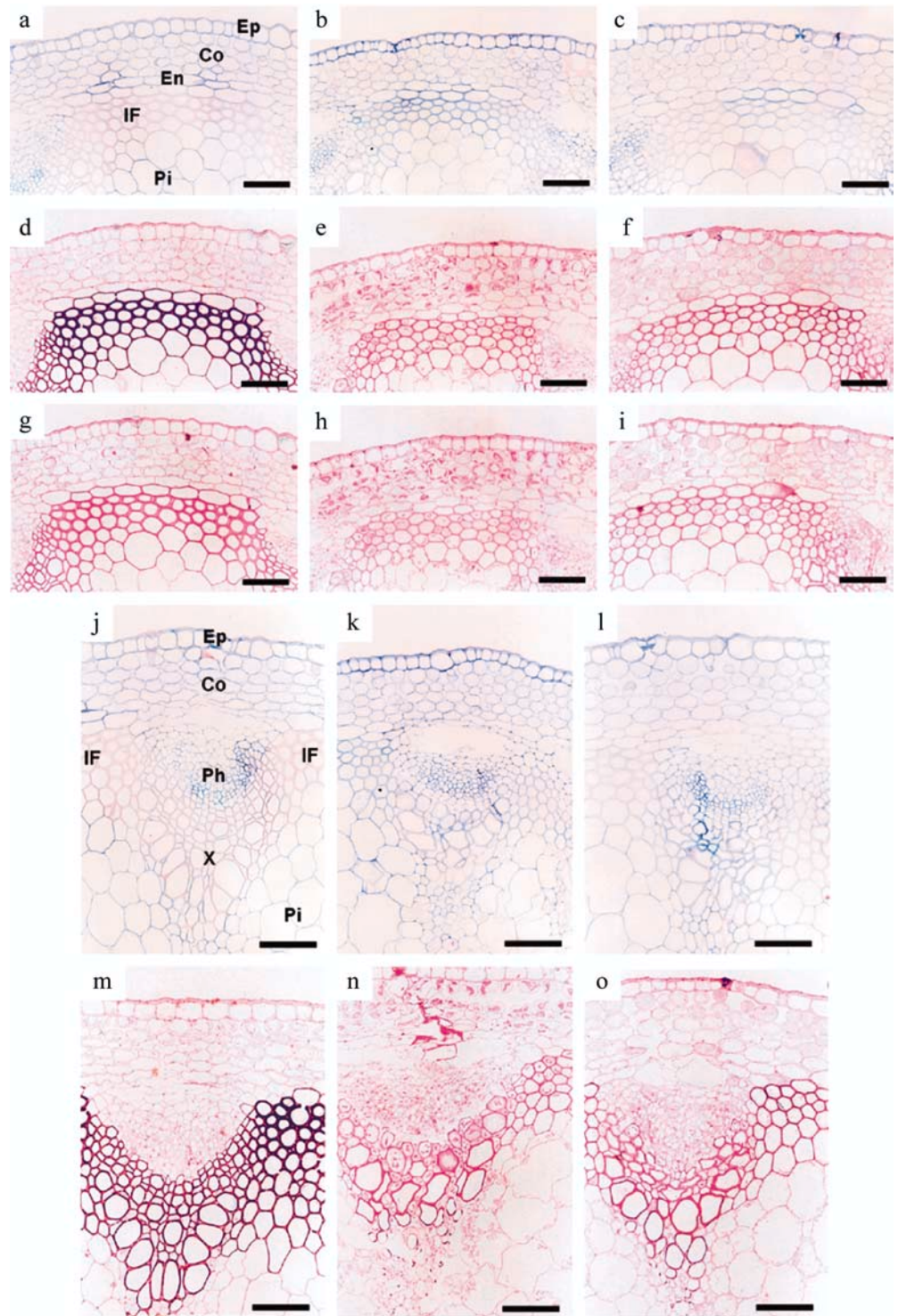
examined after PATAg staining, a general stain for polysaccharides which gives images of the macromolecular arrangement of the cell walls. In down-regulated ASCCR2, the collapsed xylem cells exhibited ultrastructural modifications consisting of an unmasking of the cellulose framework and a loosening of the cellulose microfibrils. This was particularly evident in the S2 layer of vessels (Fig. 4a, b). In walls of adjacent fibers, concentric lamellae of microfibrillar material were detached. As for cell corners, they differed from the normal plant in being filled with a very loose and non-organized polysaccharide material. Fibers forming the interfascicular areas were modified in the inner part of S2, whereas S1 and outer-S2 layers kept their normal micromorphological aspect. S2 displayed a gradient in its disorganization, which was

Fig. 4a–j Electron micrographs of cross-sections through *A. thaliana* stems of wild-type and ASCCR2 lines. **a–d** Periodic acid–thiocarbohydrazide–silver proteinate (PATAg) staining. **a, b** Part of a vessel and adjacent fiber walls of wild-type (**a**) and ASCCR2 (**b**) lines. **c, d** Fibers in the inter-fascicular zone of wild-type (**c**) and ASCCR2 (**d**) lines. **e, f** Distribution of non-condensed GS lignin sub-units in the inter-fascicular zone of wild-type (**e**) and ASCCR2 (**f**) lines. *CC* Cell corner; *F* fiber; *ML* middle lamella; S_1, S_2, secondary wall layers; oS_2, iS_2, outer- and inner-S_2 sub-layers, respectively; *V* vessel. Bar = 0.5 µm
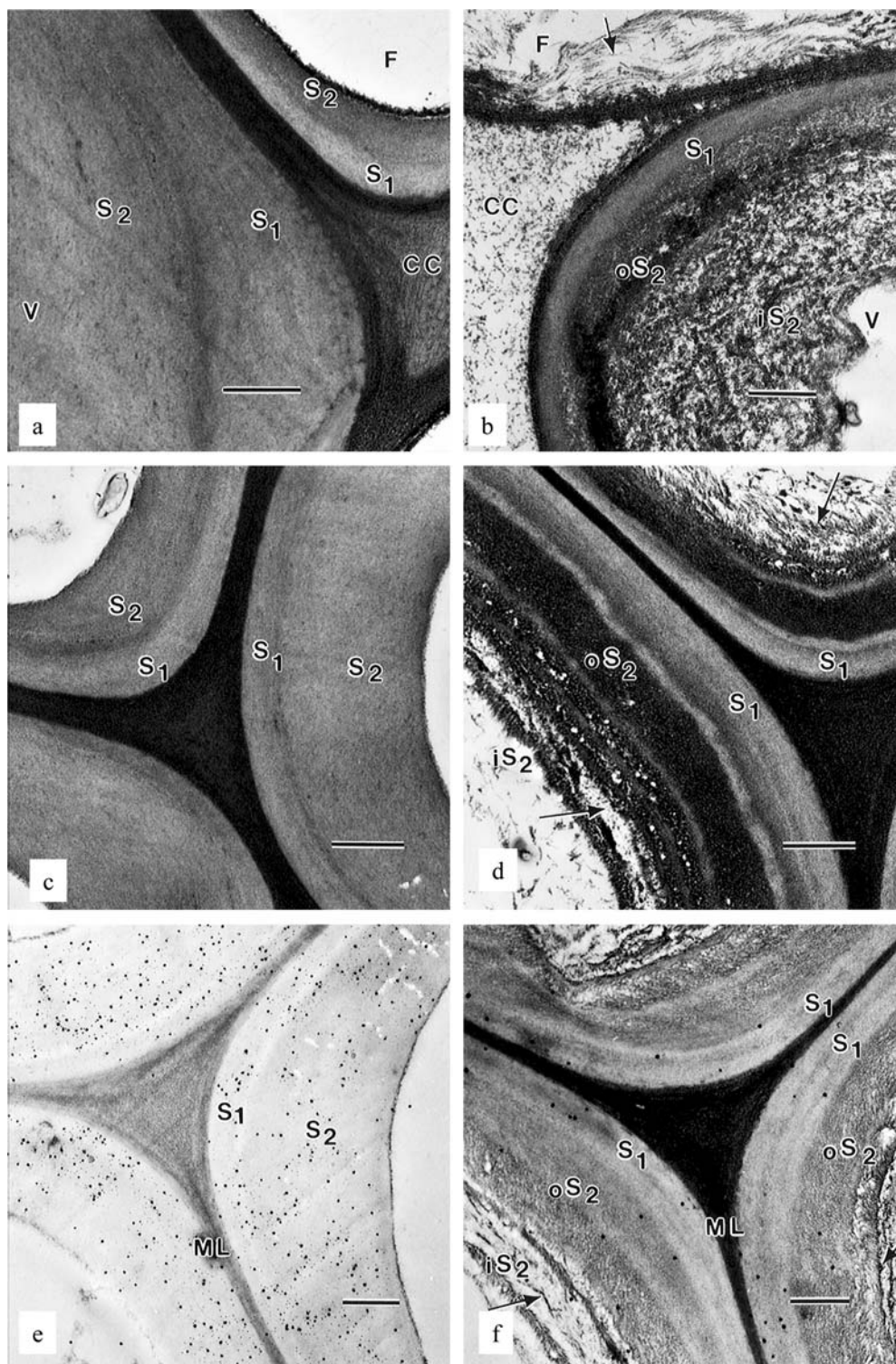
more important in the newly formed inner sub-layers (Fig. 4c, d). Another characteristic of ASCCR2 is its particular reactivity to the PATAg staining which underscored a succession of concentric sub-layers of different intensities in S2. This is different from the normal plant in which the PATAg reagent shows a homogeneous pattern of staining throughout S1 and S2 (Fig. 4c, d).

The immunolocalization in TEM showed that non-condensed epitopes, which are homogeneously distributed in the fiber walls of the wild-type plant (Fig. 4e), appeared to be reduced in the transformant, with their

highest concentration in S1 and a very low density in S2 (Fig. 4f). In vessels, the abundant labelling observed in S2 of the control plant was absent in ASCCR2 (data not shown).

Influence of culture conditions on growth

The consequences of *AtCCR1* down-regulation were examined under different culture conditions (CC1 to CC4), to assess the impact of environmental conditions on these transformed plants. CC1 consisted of a long photoperiod (16 h, 30 °C/8 h, 15–20 °C) and relatively dry and warm conditions. CC2 consisted of a long photoperiod (16 h, 25 °C/8 h, 20 °C) and a higher degree of humidity (automatic water spraying). CC3 consisted of a short photoperiod (8 h, 24 °C/16 h, 16 °C) and high degree of humidity. CC4 consisted of a short photoperiod (8 h, 20 °C/16 h, 16 °C), high degree of humidity and relatively cold temperatures. The maximum height reached by each line was determined in CC1, CC2 and CC3. The height of mature stems of the wild type varied from 33 cm in CC2 to 45 cm in CC3 (38% variation). The maximum height of the ASCCR2 line ranged from 17 to 25 cm (32% variation). Regardless of the culture conditions, the mature stem height of the ASCCR2 and ASCCR7 lines was always lower than that of the wild type. ASCCR2 and ASCCR7 lines were unable to keep an upright growing position and their flowering stems crept along the ground. The most important difference was observed in CC3, where the height difference between WS and ASCCR2 and ASCCR7 reached 44% (Fig. 5). Seed production was not affected to a great extent, but viability of seeds was impaired in the ASCCR2 and ASCCR7 lines with 63% and 75% germination, respectively, compared to 82% for the wild type (measured in CC4).
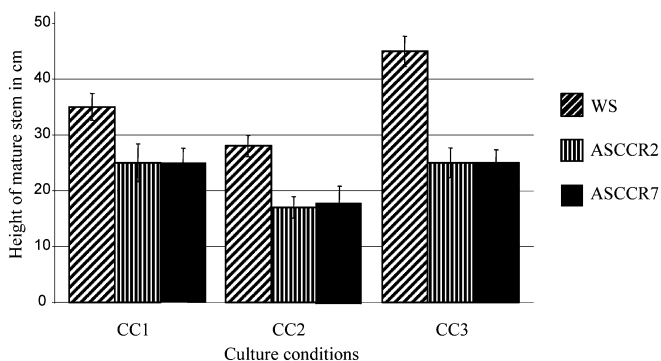

Fig. 5 Comparison of maximum heights reached by wild-type (*WS*), ASCCR2 and ASCCR7 lines of *A. thaliana* in three different culture conditions (CC1, CC2, CC3). Data are means ± SD (*n* = 10 plants)

Table 2 Lignin contents of stems of wild-type (*WS*), ASCCR2 and ASCCR7 lines of *A. thaliana* cultivated in four different cultureconditions. *%KL* Klason lignin as a percentage of extract-freedry weight (SEs between duplicate measurements are indicated in parentheses)

Conditions	%KL		
	WS	ASCCR2	ASCCR7
CC1	15.1 (0.1)	8.2 (0.2)	9.5 (0.1)
CC2	13.0 (0.1)	8.1 (0.1)	8.5 (0.2)
CC3	13.5 (0.1)	8.6 (0.1)	9.4 (0.2)
CC4	15.0 (0.2)	9.35 (0.1)	10.8 (0.1)

Lignin content and structure in wild-type, ASCCR2 and ASCCR7 lines in different culture conditions

The lignin content of the extractive-free dry stems was determined using the Klason method for each line grown in the same culture conditions. The lignin contents of both ASCCR2 and ASCCR7 lines were dramatically reduced relative to the wild type whatever the culture conditions (Table 2). The highest reduction in lignin content relative to the control line was observed in CC1 (46% reduction).

Lignin-derived monomers and dimers released by thioacidolysis of the extractive-free cell wall fraction of the stems were analyzed by GC–MS of their trimethylsilyl (TMS) derivatives (Lapierre et al. 1995). Thioacidolysis is an analytical method that proceeds by cleavage of labile β-O-4 bonds (i.e. non-condensed linkages). G and S lignin-derived monomers are generated from G and S units involved in β-O-4 bonds, without any interference from non-lignin phenolics. Components such as ferulic acid incorporated in the cell wall through thioacidolysis-cleavable bonds are also detected with this method. The thioacidolysis yields from ASCCR2 and ASCCR7 lines revealed that their lignins were enriched in carbon–carbon resistant bonds (i.e. condensed linkages) in all culture conditions, indicated by a decrease in total yields (Table 3). The proportion of β-O-4 linked G units, giving rise to the G thioacidolysis monomers, was reduced in CC1 and CC2, but not in CC3. The yield difference between the wild type and ASCCR2 lines reached 44% (1,284 vs. 721) in CC1 and 46% (1,101 vs. 591) in CC2, but soared to 66% (1,507 vs. 511) in CC3. The S-to-G ratio was increased in the ASCCR lines in CC1, whereas it was not clearly changed in CC2. This ratio was decreased in CC3.

Abnormal components, such as ferulic acid also accumulate in the cell walls of the ASCCR2 and ASCCR7 lines (Table 3). There is substantially more ferulic acid incorporated in the cell walls of the two ASCCR lines than in the control whatever the culture conditions.

Albeit H units are systematically recovered in low amounts, down-regulation of CCR induced a significant decrease in H monomers in the stems. Thioacidolysis analyses were performed in the roots of wild

Table 3 Yields of lignin-derived-thioacidolysis main monomers and of thioacidolysis-released ferulic acid from wild-type (*WS*) *A. thaliana* and ASCCR2 and ASCCR7 lines grown indifferent culture conditions (CC1, CC2 and CC3). The SEs for total yields and between duplicate experiments are indicated in parentheses

	Total yield H + G + S [μmol (g KL)$^{-1}$]	Molar percentage H:G:S	Ferulic acid released by thioacidolysis [μmol (g KL)$^{-1}$]
CC1			
WS	1,284 (16)	0.7:71.6:28.2	1.7
ASCCR2	721 (12)	0.4:65.5:34.1	5.0
ASCCR7	865 (20)	0.3:63.4:36.3	6.1
CC2			
WS	1,101 (1)	0.9:73.1:26.0	2.1
ASCCR2	591 (11)	0.6:70.2:29.2	5.1
ASCCR7	697 (6)	0.5:69.5:30.0	8.5
CC3			
WS	1,507	0.5:67.0:32.3	1.4
ASCCR2	511	0.3:72.9: 26.5	3.6
ASCCR7	855	0.4:70.4:29.0	5.4

type plants and ASCCR7 grown in aeroponic conditions. The results showed that there was also a strong decrease in H monomers in the roots (data not shown). These results mean that H units are less abundant in the lignins and/or that H units are more involved in the carbon–carbon inter-unit-bonds in the ASCCR lines.

Digestibility assays

Digestibility assays were performed with plants grown under culture condition CC4. Stem dry matter yield per plant was unchanged between the wild type and ASCCR (Table 4). The reduction in height seemed to be compensated by a higher degree of branching in the ASCCR2 and ASCCR7 lines, and the total biomass was therefore similar in the wild type and the ASCCR lines. The NDF (neutral detergent fiber which mainly comprises cell wall components) content and in vitro dry matter digestibility (IVDMD, enzymatic solubility) were estimated on ground dry mature stems. In vitro NDF digestibility (IVNDFD) was computed assuming that the entire non-NDF fraction was completely digestible. ASCCR2 and ASCCR7 lines had a lower NDF content in dry matter, possibly due to a higher soluble carbohydrate content and/or different resistance of cell wall to neutral detergent attack, and a lower lignin content in the cell wall (estimated as NDF) than the wild type. ASCCR2 and ASCCR7 lines had a higher IVDMD and a 2-fold

Table 4 Dry matter yield of *A. thaliana* stems, cell wall content and digestibility of wild-type (*WS*), ASCCR2 and ASCCR7 lines grown in CC4 conditions. *NDF* Neutral detergent fiber, *IVDMD* in vitro dry matter digestibility, *IVNDFD* in vitro NDF digestibility

	WS	ASCCR2	ASCCR7
Stem yield (g/plant)	0.3	0.4	0.4
NDF (%)	60.8	47.6	52.0
IVDMD (%)	55.8	79.0	74.9
IVNDFD (%)	27.4	55.9	51.7

higher IVNDFD than the wild type. The ASCCR2 line, which had a lower residual CCR activity and a lower lignin content than ASCCR7, also had the highest cell wall digestibility.

Discussion

AtCCR1 expression in the wild type is correlated with lignification

The expression pattern of *AtCCR1* (At1g15950) can be compared to the expression pattern observed for the *CCR1* gene of *Eucalyptus gunnii* (Lacombe et al. 1997). *AtCCR1* was highly expressed in the apical part of the stem, a tissue undergoing active lignification, therefore indicating a strong correlation between lignification and *AtCCR1* gene transcription. Our study completes the work initiated by Lauvergeat et al. 2001, which attributes to *AtCCR1* a role in constitutive lignification. This gene was also weakly expressed in the leaf blade. One part of this expression should be correlated with the formation of vascular tissue in secondary veins. However, it cannot be excluded that *At*CCR1 also participates in the formation of lignans and/or neo-lignans involved in plant defence (Lewis and Davin 1994).

*At*CCR1 down-regulation has a strong impact on the phenotype

CCR-down-regulated plants were obtained using the antisense strategy. The entire *AtCCR1* cDNA in an antisense orientation was expressed under the control of the 35S CaMV promoter, previously used in other antisense strategies aimed at reducing monolignol biosynthetic gene expression (Piquemal et al. 1998; Zhong et al. 1998; Chabannes et al. 2001b; Pinçon et al. 2001). Observation of the phenotype of homozygous antisense transgenic plants demonstrated that *At*CCR1 plays a major role in normal development of *Arabidopsis*. Whatever the culture conditions, the floral stems of the

ASCCR lines were shorter than those of wild-type plants. The height variations observed for the wild type in different culture conditions were not followed by the ASCCR lines. This suggests that the transformed plants have a limitation in their growth. Recently, the identification of an ethyl methane sulfonate (EMS) *AtCCR1* null mutant (*irx4*) has been reported (Jones et al. 2001). This mutant has an altered phenotype, similar to the ASCCR lines, with collapsed xylem vessels and displays 50% residual lignin content (Jones et al. 2001). Phenotype comparison between *irx4* and these CCR down-regulated lines shows that in our conditions the *AtCCR1* antisense strategy is fully efficient. However, the 50% reduction in lignin content is not in itself sufficient to explain the dwarf phenotype associated with the low CCR activity. Zhong et al. (1998) have shown that antisense tobacco plants down-regulated for both cinnamoyl-coenzyme A *O*-methyltransferase (CCoAOMT) and caffeic acid *O*-methyltransferase (COMT), despite a 50% lignin reduction, grow normally in greenhouse conditions. The growth defects could be explained by the possible accumulation of free phenolics in the antisense plants (Piquemal et al. 1998; Chabannes et al. 2001b), having a toxic effect on the general metabolism and interfering with other pathways. Such accumulation of toxic free phenolics could account for the lower seed viability observed in *Arabidopsis*, as already reported for tobacco by Piquemal et al. (1998). The residual CCR activity in ASCCR lines (about 20%) could be explained by the remaining *AtCCR1* expression or by other CCR-like enzymes (Jones et al. 2001) that could metabolize the *At*CCR1 substrate used in the in vitro assay.

*At*CCR1 down-regulation has a strong impact on lignin topochemistry

Several ASCCR lines showed phenotypic alterations consisting of limited height and tissue disorganization in the stems. These traits were not systematically observed for each line over the course of stem development. Such heterogeneity may be ascribed to an incomplete efficiency of the antisense construct in each tissue and at each stage of development and is correlated with a relatively high residual CCR activity. Only two lines, ASCCR2 and ASCCR7, presenting a low residual CCR activity (about 20%), showed a constant phenotype throughout their development. The collapsing of the xylem vessels in these two lines must be correlated with the general decrease in lignin content, as this was also observed in the *CCR* antisense tobacco plants (Piquemal et al. 1998) and the *irx4* mutant (Jones et al. 2001). This collapsing reveals a mechanical weakness of the xylem cell walls relative to the forces of pressure linked to the transport of water through the plant. It is important to note that the *irx4* mutant was identified due to its irregular xylem (Turner and Sommerville 1997).

Lignin-specific staining evidenced a reduction in the lignin content in xylem vessels and interfascicular fibers.

Immunolocalization of non-condensed GS lignin epitopes, observed in light microscopy, showed that the normally lignified tissues (xylem and fibers) of the wild type were not labelled in the ASCCR2 and ASCCR7 lines. This was an indication that not only was the lignin content in the secondary walls of vessels and fibers abnormally low but also that the lignin that had been synthesized was almost devoid of non-condensed sub-units. This observation is substantiated by the results of thioacidolysis showing the low level of non-condensed units in the ASCCR lines. With the resolution of TEM at the ultrastructural level it was demonstrated that the highly unorganized inner S2 layer in these lines is almost negative to the labelling for non-condensed sub-unit epitopes. Conversely, the S1 layer and the primary wall retained an almost normal labelling, showing that the occurrence of non-condensed substructures had not been appreciably affected in these areas of the wall of the transformants. Thus, lignification in the layers and sub-layers deposited before internal-S2 is less affected by the CCR down-regulation.

The low lignin content, together with the profound secondary wall loosening and the concomitant absence of non-condensed forms of lignins have already been described as characteristic of the CCR down-regulated transformation in tobacco (Chabannes et al. 2001a; Ruel et al. 2001). The results obtained with the two ASCCR *Arabidopsis* lines constitute additional evidence that in the absence of non-condensed lignin, the inner S2 layer of the secondary wall is not able to assemble in a proper way. The involvement of non-condensed forms of lignin during secondary wall assembly was further supported by a topochemical analysis of lignin in developing *A. thaliana* plants (Ruel et al. 2002). These results suggest that *At*CCR1 is important for lignification and assembly of the inner secondary cell wall. The mechanisms that control the supramolecular arrangements between lignin and cellulose are not known, but it appears that mutants with altered spatial patterns of wall macromolecule distribution (Turner and Somerville 1997) are useful tools for the study of cell wall formation and assembly.

Lignin content and structure are altered in the CCR antisense lines and vary according to culture conditions

A. thaliana stems possess G lignins in xylem and GS lignins in the interfascicular fibers (Chapple et al. 1992). Therefore, the S-to-G ratio could be in part representative of the fiber-to-xylem ratio, which seems to vary according to growth conditions. Dry culture conditions (CC1) induce a high accumulation of lignins in the stem of wild-type plants. In these conditions wild-type plants may compensate for a loss of turgor by synthesizing more lignins in fibers (the proportion of β-O-4 linked S units is higher in CC1 than in CC2) to keep the stems growing upright. The wild type was less lignified when grown in CC2 conditions, which are more favorable to

limit water loss. In CC3, wild type plants developed a stronger stem and more fibers differentiated in these conditions than in the two others (S:G = 0.46 in CC3 compared to S:G = 0.37 in CC2). These results demonstrate that lignin content and structure are correlated with growth and development in normal plants.

Total lignin content was strongly decreased in the ASCCR2 and ASCCR7 lines in all culture conditions. These data are in accordance with what was observed in the *irx4* mutant (Jones et al. 2001). The decrease in lignin content could be directly correlated with the down-regulation of CCR activity in the stems. This result demonstrates that CCR is a limiting enzyme that regulates lignin content in *A. thaliana*. This is in agreement with the results obtained with the CCR antisense tobacco (Piquemal et al. 1998). The highest difference in lignin content between the wild type and the ASCCR lines was observed in CC1, whereas in CC2 and in CC3 the differences between the wild type and the ASCCR lines were lower. It thus appears that phenotypic differences between the wild type and CCR-down-regulated lines could be accentuated under extreme culture conditions (CC1), and especially temperature and hygrometric variations.

Lignin structure was significantly altered in the ASCCR2 and ASCCR7 lines whatever the culture conditions. Lignins were enriched in condensed inter-unit bonds, more specifically established at the level of G and H units. In CC3, the S-to-G ratio in the ASCCR lines is lower than in the wild type (higher in CC1 and CC2). This shows that the deposition of lignins, and especially of S units, is also impaired in the CCR down-regulated lines in CC3. This suggests a substantial influence of culture conditions on the rate of incorporation of S and G units both in the wild type and CCR down-regulated plants. The alteration of lignification was observed not only in the stems, but also in roots. In addition, it was found that ferulic acid was incorporated, through thioacidolysis-labile bonds, into the walls of the ASCCR transformed lines. It is very likely that ferulic acid, which builds up as a consequence of *At*CCR1 down-regulation, participates in lignin cross-linking together with G and H units. Incorporation of ferulic acid was also observed in CCR antisense tobacco plants (Piquemal et al. 1998) and therefore could be considered as a marker of CCR down-regulation.

AtCCR1 down-regulation has a positive impact on digestibility

To our knowledge, this experiment demonstrated, for the first time, the improvement of cell wall digestibility of plants down-regulated on a *CCR* gene. Due to the important reduction in lignin content, an improvement in digestibility could be anticipated. The increase in IVNDFD was of the same order of magnitude as that previously observed in mutants such as *bm-3* maize whose caffeic acid *O*-methyltransferase (COMT) activity was reduced to almost zero and had a similar decrease in lignin content (Méchin et al. 2000).

The consequences of *At*CCR1 down-regulation on lignification in *Arabidopsis thaliana* are similar to those shown in tobacco, and in the recently reported *irx4 A. thaliana* mutant. Using the model plant *A. thaliana* will enable us to perform a more exhaustive study of the consequences of deregulation of the lignin pathway genes.

References

Aufrère J, Michalet-Doreau B (1983) In vivo digestibility and prediction of digestibility of some by-products. European Economic Community seminar, 26–29 September 1983. Mlle Gontrode, Belgium

Baucher M, Monties B, Van Montagu M, Boerjan W (1998) Biosynthesis and genetic engineering of lignin. Crit Rev Plant Sci 17:125–197

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. CR Acad Sci Paris, Sci Vie 316:1194–1199

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res 18:203

Boudet AM (2000) Lignins and lignification: selected issues. Plant Physiol Biochem 38:81–96

Burlat V, Kwon M, Davin LB, Lewis NG (2001) Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry 57:883–897

Chabannes M, Ruel K, Yoshinaga A, Chabbert B, Jauneau A, Joseleau JP, Boudet AM (2001a) In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. Plant J 28:271–282

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001b) 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

Chapple CCS, Vogt T, Ellis BE, Somerville CR (1992) An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. Plant Cell 4:1413–1424

Dence C (1992) Lignin determination. In: Dence C, Lin S (eds) Methods in lignin chemistry. Springer, Berlin Heidelberg New York, pp 33–61

Dharmawardhana DP, Ellis BE, Carlson JE (1992) Characterization of vascular lignification in *Arabidopsis thaliana*. Can J Bot 70:2238–2244

Dolstra O, Medema JH (1990) An effective screening method for genetic improvement of cell-wall digestibility in forage maize. In: Proceedings of the 15th congress maize and sorghum section of Eucarpia, Baden, Austria, June 4–8, pp 258–270

Estelle M, Somerville CR (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet 206:200–206

Goering HK, Van Soest PJ (1970) Forage fiber analysis (apparatus, reagents, procedures, and some applications). USDA ARS

Agricultural handbook 379. US government Printing Office, Washington, DC

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Curr Opin Plant Biol 5:224–229

Humphreys JM, Hemm MR, Chapple C (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc Natl Acad Sci USA 96:10045–10050

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

Joseleau J-P, Ruel K (1997) Study of lignification by noninvasive techniques in growing maize internodes—an investigation by Fourier transform infrared, cross-polarisation-magic angle spinning ^{13}C-nuclear magnetic resonance spectroscopy and immunocytochemical transmission electron microscopy. Plant Physiol 114:1123–1133

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204:383–396

Lacombe E, Hawkins S, Van Doorselaere J, Piquemal J, Goffner D, Poeydomenge O, Boudet AM, Grima-Pettenati J (1997) Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. Plant J 11:429–441

Lapierre C, Pollet B, 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, 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

Lewis NG, Davin LB (1994) Evolution of lignan and neolignan biochemical pathways. In: Nes WD (ed) Isopentonoids and other natural products: evolution and function. ACS Symposium Series, No 562, Washington DC, pp 202–246

Li L, Popko JL, Umezawa T, Chiang VL (2000) 5-hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. J Biol Chem 275:6537–6545

Li L, Cheng XF, Leshkevich J, Umezawa T, Harding SA, Chiang VL (2001) The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13:1567–1585

Méchin V, Argillier O, Barrière Y, Mila I, Polet B, Lapierre C (2000). Relationships of cell-wall composition to in vitro cell-wall digestibility of maize inbred line stems. J Sci Food Agric 80:574–580

Meyer K, Shirley AM, Cusumano JC, Bell-Lelong DA, Chapple CCS (1998) Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. Proc Natl Acad Sci USA 95:6619–6623

Osakabe K, Tsao CC, Li L, Popko JL, Umezawa T, Carraway DT, Smeltzer RH, Joshi CP, Chiang VL (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc Natl Acad Sci USA 96:8955–8960

Pinçon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-*O*-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiol 126:145–155

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

Ruel K, Barnoud F, Eriksson KE (1981) Micromorphological and ultrastructural aspects of spruce wood degradation by wild type *Sporotrichum pulverulentum* and its cellulase-less mutant Cel 44. Holzforschung 35:157–171

Ruel K, Faix O, Joseleau JP (1994) New immunogold probes for studying the distribution of the different lignin types during plant cell wall biogenesis. J Trace Microprobe Tech 12:247–265

Ruel K, Burlat V, Joseleau JP (1999) Relationship between ultrastructural topochemistry of lignin and wood properties. Int Assoc Wood Anat J 20:203–211

Ruel K, Chabannes M, Boudet A-M, Legrand M, Joseleau J-P (2001) Reassessment of qualitative changes in lignification of transgenic tobacco plants and their impact on cell wall assembly. Phytochemistry 57:875–882

Ruel K, Montiel MD, Goujon T, Jouanin L, Burlat V, Joseleau JP (2002) Inter-relation between lignin deposition and polysaccharide matrices during the assembly of the plant cell walls. Plant Biol 3:1–7

Sarkanen KV, Hergert HL (1971) Classification and distribution. In: Sarkanen KV, Ludwig CH (eds) Lignins: occurrence, formation, structure and reactions. Wiley-Interscience, New York, pp 43–94

Srebotnik E, Messner K (1994) A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. Appl Environ Microbiol 60:1383–1386

Struik PC (1983) Physiology of forage maize (*Zea mays* L.) in relation to its productivity. PhD thesis, Wageningen, The Netherlands

Terashima N, Fukushima K, Takabe K (1993) Comprehensive model of the lignified plant cell wall. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) Forage cell wall structure and digestibility. ASA Madison, Wis, pp 247–270

Thiery JP (1967) Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J Microsc 6:987–1017

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

Verwoerd TC, Dekker BMM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res 17:2362

Zhong R, Morrison WH, Negrel J, Ye ZH (1998) Dual methylation pathways in lignin biosynthesis. Plant Cell 10:2033–2045

Zhong R, Ripperger. A, Ye ZH (2000). Ectopic deposition of lignin in the pith of stems of two *Arabidopsis* mutants. Plant Physiol 123:59–70