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

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

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

INTRODUCTION

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

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

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

RESULTS

Identification and Phenotyping of the *ccc* Triple Mutant

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

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

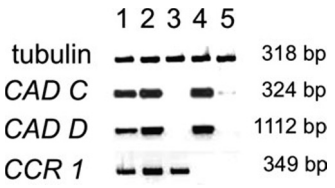

Figure 1. Expression of the Target Genes in the *ccc* Triple Mutant.
RT–PCR amplifications were performed for different genes (tubulin, *CAD C*, *CAD D*, and *CCR1*) on total RNA extracted for stems of WS, Col 0, *cad c cad d*, *ccr1g*, and *ccc* plants.
Lane 1, WS; 2, Col 0; 3, *cad c cad d*; 4, *ccr1g*;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

The *ccc* Triple Mutant Possesses Non-Dehiscent Anthers

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