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ORIGINAL ARTICLE

Evidence for a role of *AtCAD* 1 in lignification of elongating stems of *Arabidopsis thaliana*

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Abstract The cinnamyl alcohol dehydrogenase (*AtCAD*) multigene family in *Arabidopsis* is composed of nine genes. Our previous studies focused on the two isoforms AtCAD C and AtCAD D which show a high homology to those related to lignification in other plants. This study focuses on the seven other *Arabidopsis* CAD for which functions are not yet elucidated. Their expression patterns were determined in different parts of *Arabidopsis*. Only CAD 1 protein can be detected in elongating stems, flowers, and siliques using Western-blot analysis. Tissue specific expression of *CAD* 1, B1, and G genes was determined using their promoters fused to the *GUS* reporter gene. *CAD* 1 expression was observed in primary xylem in accordance with a potential role in lignification. *Arabidopsis* T-DNA mutants knockout for the different genes *CAD* genes were characterized. Their stems displayed no substantial reduction of CAD activities for coniferyl and sinapyl alcohols as well as no modifications of lignin quantity and structure in mature inflorescence stems. Only a small reduction of lignin content could be observed in elongating stems of *Atcad* 1 mutant. These *CAD* genes in combination with the *CAD D* promoter were used to complement a *CAD* double mutant severely altered in lignification (*cad c cad d*). The expression of *AtCAD* A, B1, B2, F, and G had no effect on restoring a normal lignin profile of this mutant. In contrast, *CAD* 1 complemented partly this mutant as revealed by the partial restoration of conventional lignin units and by the decrease in the frequency of β-O-4 linked *p*-OH cinnamaldehydes.

Keywords *Arabidopsis* · Cinnamyl alcohol dehydrogenase · Gene family · Lignin · Stem

Abbreviations
CAD Cinnamyl alcohol dehydrogenase
GUS Beta-glucuronidase
WT Wild-type

Introduction

The availability of the complete sequence of the model plant, *Arabidopsis thaliana* (*Arabidopsis* Genome Initiative 2000) allows the identification of all the related genes for a determined function. The genome of *Arabidopsis* is partly duplicated and various studies have shown that most genes belong to multigene families. This is the case for different genes encoding enzymes of the monolignol biosynthetic

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pathway (Costa et al. 2003; Goujon et al. 2003a; Raes et al. 2003) including the last step catalyzed by cinnamyl alcohol dehydrogenase (CAD). Nine putative *CAD* genes were identified in previous studies (Costa et al. 2003; Goujon et al. 2003a; Raes et al. 2003; Sibout et al. 2003; Kim et al. 2004). They were named *AtCAD* 1 (At4g39330; Somers et al. 1995), A (At4g37970), B1 (At4g37980), B2 (At4g37990), C (At3g19450), D (At4g34230), E (At2g21730), F (At2g21890), and G (At1g72680) according to the classification of Tavares et al. (2000) that was subsequently completed by Sibout et al. (2003). *Eli-3-2* (Somssich et al. 1996) was renamed *AtCAD* B2 because of its high identity with *AtCAD* B1. Two other classifications were proposed by Costa et al. (2003) and Raes et al. (2003); however, due to previous nominations of some *CAD* genes, the classification of Tavares et al. (2000) was conserved (Table 1). These genes are localized on four different chromosomes and some of these genes are grouped in clusters (*AtCAD* E and F on chromosome 2; *AtCAD* A, B1, and B2 on chromosome 4). Analysis of the nine *Arabidopsis* CAD at the amino acid level revealed a diversified small family with highly conserved clusters. Some AtCAD are rather closely related such as AtCAD B1/AtCAD B2 (85% identity), AtCAD C/AtCAD D (75% identity), and AtCAD E/AtCAD F (98% identity). In this family, AtCAD G is the most distant protein when compared to the others and shares less than 50% identity with the closest groups. When CADs previously identified and studied in other plant species were taken into consideration, phylogenetic analysis based on amino acid sequence comparison (Fig. 1) showed that *Arabidopsis* CAD are organized in four classes. Interestingly, at least one CAD previously identified in other plants is present in each of the *Arabidopsis* classes. AtCAD C and AtCAD D (class I) belong to the well-characterized CAD group (Sibout et al. 2003). AtCAD B1, AtCAD B2, and AtCAD A (class II) belong to the same subfamily as poplar PtSAD (Li et al. 2001). AtCAD 1, E, and F (class III) make up a subfamily with alfalfa MsaCAD1 (Brill et al. 1999). AtCAD G constitutes another subfamily (class IV) with a *Medicago truncatula* putative 2-hydroxyacid dehydrogenase and two rice OsCAD (data not shown).

Our previous studies on *Arabidopsis CAD* (Sibout et al. 2003, 2005) focused on two members (*AtCAD* C and D) of the class I showing high similarity to tobacco, poplar, and alfalfa CADs, with dominant roles in developmental lignification (Halpin et al. 1994; Baucher et al. 1996, 1999). All of these orthologs present similar patterns of expression with *AtCAD* D which is mainly expressed in *Arabidopsis* inflorescence stems (Fig. 2; supplementary data 1). The expression of *CAD* C and *CAD* D in *Escherichia coli* and the kinetic characterization of these recombinant proteins suggested that these enzymes could be major determinants for *p*-OH cinnamylaldehyde reduction (Kim et al. 2004). This is in concordance with the high expression level of these genes in lignified parts of stems (Sibout et al. 2003). More recent work has demonstrated the redundancy of expression of these two genes since CAD activity and lignin content were only highly reduced in stem (about 90 and 30%, respectively) in a double *cad c cad d* null mutant (Sibout et al. 2005). In this double mutant, lignin structure was severely modified by the incorporation of unusually high amounts of coniferaldehyde and sinapaldehyde. However, in spite of the complete knockout of *AtCAD* D and C genes, lignin analysis revealed that this mutant still contained weak but noticeable amounts of conventional G and S lignin units that originate from coniferyl and sinapyl alcohols, respectively. This result suggests that other CADs may be involved in stem lignification and prompted us to identify which of the other *CAD* genes has also a role in constitutive lignification. With this objective, we performed a detailed analysis of the expression profile of the *AtCAD* 1, A, B1, B2, E, F, and G genes and we comprehensively characterized the corresponding knockout mutants for all these genes except *AtCAD* F. The ability of these enzymes to restore CAD activity in the *cad c cad d* mutant was also considered.

Materials and methods

Plant materials and growth conditions

The ecotypes Wassilewskija (WS) and Columbia 0 (Col0) were used in this work. Mutants were identified in the *A. thaliana* T-DNA insertion collection of Versailles (Bouché and Bouchez 2001) and in the Salk Institute Genomic Analysis Laboratory (Alonso et al. 2003). The *Atcad* B2, E, and G mutants were selected using the systematic border sequencing program (http://www.flagdb-genoplante-info.infobiogen.fr) of the Versailles collection of mutants (Samson et al. 2004). The *Atcad* 1, A, and B1 mutants were selected using the systematic border sequencing program of the Salk Institute (San Diego, USA) on the TAIR site (http://www.*Arabidopsis*.org). The *shp1 shp2* line expressing the *pCAD 1::GUS* fusion was obtained by crossing a selected GUS line with this double mutant (Liljegren et al. 2000).

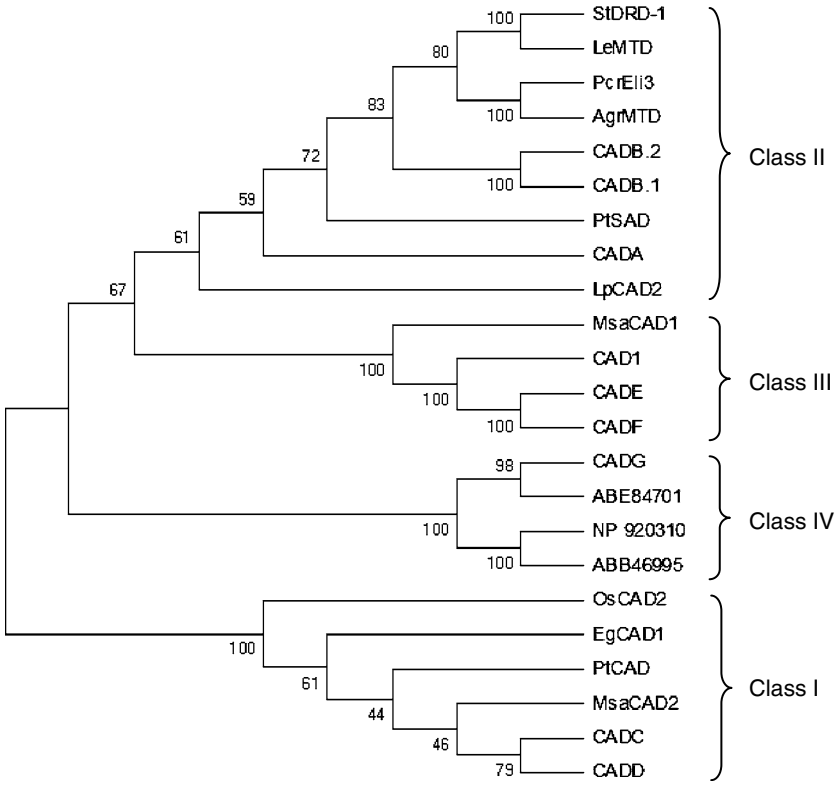

Fig. 1 Phylogenetic analysis of the CAD protein family. Accession number of proteins used to build the tree are: *Arabidopsis thaliana* AtCAD1 (NP_195643); AtCADA (NP_195510); AtCADB1 (CAA48027); AtCADB2 (NP_195512); AtCADC (NP_188576); AtCADD (NP_195149); AtCADE (NP_179765); AtCADF (NP_179780); AtCADG (NP_177412); *Medicago sativa* MsaCAD1 (AAC35846); MsaCAD2 (AAC35845); *Populus tremuloides* PtCAD (AAF43140); *Populus tremuloides* PtSAD (AAK58693); *Petroselinum crispum* PcrELI3 (X67817); *Apium graveolens* AgrMTD (mannitol deshydrogenase, AAC15467); *Lycopersicum esculentum* LeMTD (mannitol dehydrogenase, S72477); *Solanum tuberosum* StDRD-1 (CAD29291); *Lolium perenne* LpCAD2 (AAL99536). *Oryza sativa* OsCAD2 (DAA02237). Accession numbers ABB46995 and NP_920310 stand for two protein sequences from *Oryza sativa*, and accession number ABE84720 stands for a protein sequence from *Medicago truncatula*

Table 1 *CAD* gene names according to different classifications, origin and T-DNA insertion of *Atcad* mutant lines analyzed in this work

Gene name	Costa et al. classification	Raes et al. classification	Mutant name	Ecotype	N° AGI	FST number
CAD 1	*CAD9*	*CAD1*	*Atcad* 1	Col 0	At4g39330	S_037853
CADA	*CAD6*	*CAD3*	*Atcad* A	Col 0	At4g37970	S_030496
CADB1	*CAD7*	*CAD4*	*Atcad* B1	Col 0	At4g37980	S_083037
CADB2	*CAD8*	*CAD5*	*Atcad* B2	WS	At4g37990	V_181G09
CADE	*CAD3*	*CAD8*	*Atcad* E	WS	At2g21730	V_124H09
CADG	*CAD1*	*CAD9*	*Atcad* G	WS	At1g72680	V_194E03

V Versailles collection; *S* Salk Institute collection

The transformed and wild-type *Arabidopsis* plants were grown together in the same greenhouse to ensure uniform environmental conditions. For stem sections and GUS assays, plants were grown in growth chambers at 23°C with 12 h light for 5 weeks. Young stems (4–5 cm in length) of WT and *Atcad* 1 were harvested and used for CAD activity and RNA expression after freezing or dried in an oven at 50°C for determination of lignin parameters.

RNA analyses

RT-PCR and RFLP-RT-PCR

Total RNA was extracted from several *Arabidopsis* tissues as described by Verwoerd et al. (1989). RNA was then redissolved in DEPC-treated water and RNA concentrations determined by absorbance at 260 nm. Reverse transcriptase reaction was carried out on 10 µg

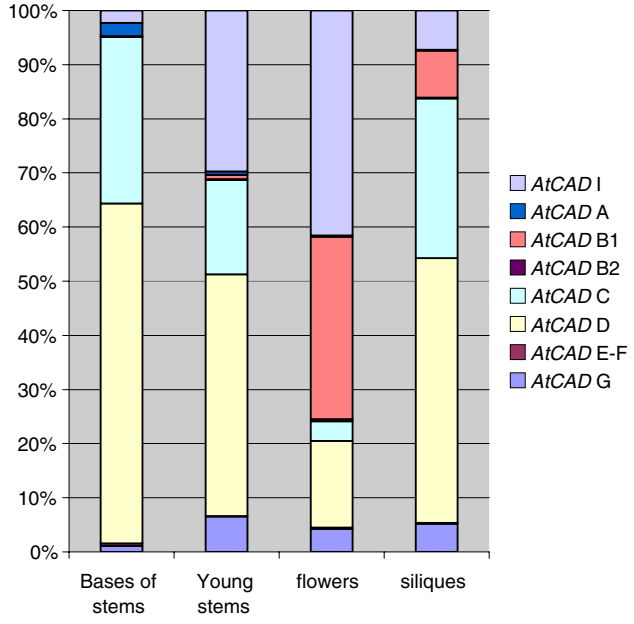

Fig. 2 Percentage of *AtCAD* cDNA detected in siliques, flowers apex, bases of stem, and elongating stems analyzed by real-time PCR (see Sect. "Materials and methods" for details on the technique)

DNAse treated (Promega, Madison, WI, USA) total RNA in 50 µl volume. Five microliters of the reverse transcription reactions were used as template for a PCR by using primers specific for each gene (supplementary data 2). Due to the large homology between *AtCAD* E and F, RFLP-RT-PCR (Lurin and Jouanin 1995) was used to differentiate the potential transcript of these genes. The PCR product was digested by the restriction enzymes *Mly* I and *Bsa* WI (which cut *AtCAD* E and F, respectively) then run on an agarose gel.

Quantitative RT-PCR

RNA extraction and real-time retro-transcriptase-polymerase chain reaction

Total RNA was isolated from stem and flowers tissues using the RNAeasy plant kit (Qiagen, Valencia, CA, USA) with a slightly modified protocol, particularly, with a DNAse (Qiagen, Valencia, CA, USA) step repeated twice to eliminate any genomic DNA. For siliques, total RNA was extracted using the method described in Chang et al. (1993) and purified according to the Rneasy Mini Protocol for RNA cleanup (Qiagen, Valencia, CA, USA) with a DNAse step repeated twice as well. Reverse transcriptase (superscript II; Invitrogen, Carlsbad, CA, USA) reaction was carried out using 1 µg of total RNA in 20 µl volume with poly-dT oligonucleotide primers according to manufacturer's instructions. All samples were treated simultaneously. The reverse transcript cDNA were treated with RNAse H and diluted fivefold prior to PCR analysis.

Real-time PCR analyses were carried out using the QuantiTectTM Syber® Green PCR Kit (Qiagen Inc.) and the Opticon PCR apparatus (MJ Research, Waltham, MA, USA). Data were treated using the Opticon 2 software provided by MJ research. Most of primers sequences for real-time RT-PCR are available in supplemental data of Sibout et al. (2005). Primers used for *CAD* D and *CAD* C amplification are indicated in supplementary data 2. The PCR amplification reaction is a two-step reaction with a step of denaturation of DNA at 94°C for 10 s and an elongation step at 62°C for 2 min. The fluorescence was evaluated at the end of the 2 min elongation. The PCR reaction was maintained for 45 cycles. Analysis of dissociation curves (melting curves) in addition to gel electrophoresis were systemically done to verify nature of amplicons. Finally, to verify absence of genomic DNA, total RNA of the *Atcad c* and *Atcad d* mutants which show null expression for the corresponding genes (Sibout et al. 2003) were subjected to the same approach using specific *AtCAD* D and *AtCAD* C primers (data not shown).

Absolute quantity of transcripts was calculated using DNA standard curves a described in Rutledge and Côté (2003). The results are presented in percentage of total amount of *CAD* cDNA detected (Fig. 2) or in absolute values standardized using *Ef1* gene as a housekeeping gene (supplementary data 1). Therefore, results are expressed in number of molecules of the *AtCAD* gene for 100,000 molecules of Ef1.

Western-blot analysis and enzyme activities

Total protein extracts were obtained by homogenization of fresh tissues in 100 mM Tris–HCl (pH 7.5) containing 0.4% PVPP, 0.5% PEG, 15 µM β-mercaptoethanol and quantified according to Bradford (1976).

Western-blot analysis

Protein samples (15 µg) were heated at 95°C for 5 min in Laemli buffer, cooled, and centrifuged briefly prior to loading on a 12% acrylamide SDS-PAGE with a 10% resolving gel using a Bio-Rad Protean II apparatus and run at 50 V for 2 or 4.5 h. Proteins were transferred onto 0.45 µm nitrocellulose membrane (Amersham, Piscataway, NJ, USA) using an electroblotting apparatus (Bio-Rad, Hercules, CA, USA). The polyclonal antibodies raised against tobacco xylem CAD 2 (Halpin et al. 1994) and the poplar SAD

(Li et al. 2001) were used at a 1:2,000 and 1:1,000 dilution, respectively. Blots were developed using the ECL™ Western-blotting analysis system (Amersham, Piscataway, NJ, USA).

CAD activities

Crude extracts were assayed spectrophotometrically (Ultra microplate BioTek Instruments, Winooski, VT, USA) for aromatic alcohol dehydrogenase activity by oxidation of coniferyl alcohol dehydrogenase (conAD) activity or sinapyl alcohol dehydrogenase (sinAD) activity. Assays were carried out at 25°C for 30 min in 250 µl of 100 mM Tris–HCl (pH 8.8), 20 mM NADP, and 50 mM of coniferyl or sinapyl alcohols using micro-ELISA plate. Twenty micrograms of total protein for stem extracts, 40 µg for siliques, and 80 µg for flowers were used for these reactions. Formation of *p*-OH cinnamaldehydes was monitored at 400 nm using the following molar extinction coefficient: coniferaldehyde $2.10\times10^4\,M^{-1}\,cm^{-1}$, sinapaldehyde $1.68\times10^4\,M^{-1}\,cm^{-1}$ as described in Mitchell et al. (1994). An assay without NADP was used as a control. Resulting units are defined as the amount of activity which converts 1 nM of hydroxy-cinnamyl alcohol into the corresponding aldehyde per second (1 nkatal), per milligram of crude protein extract. Pools of plant organs from each line were used for the measurements. Values obtained from three biological replicates corresponding to three independent cultures were used to calculate SEMs. For all organs, values obtained from wild-type ecotype Col0 did not differ significantly from values obtained from wild-type ecotype WS.

Promoter cloning and *uidA* fusion

pCAD 1::GUS and pCAD G::GUS constructs

Gene fusion products with the gene coding for beta-glucuronidase gene (*uidA* or *GUS*), under the control of *AtCAD* 1 and *AtCAD* G promoters were constructed for monitoring expression of these genes in different organs and tissues. PCR products corresponding to 2 kb of the promoter regions were cloned using the GATEWAY™ cloning technology according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). This approach was used to transfer the promoters of *AtCAD* G and *AtCAD* 1 from an entry clone in a destination vector for GUS expression. The two promoters were amplified by PCR using the primers indicated in supplementary data 2, and the two PCR products were re-amplified with the same promoter specific primers with addition of the *att*B1 and *att*B2 recombination sites. The *att*B-PCR products were inserted into the vector pDONR™ 207 (Invitrogen, Carlsbad, CA, USA) by an *att*B ×*att*P (BP) reaction. In this reaction, the *att*B-PCR product recombined with the vector pDONR 207 containing *att*P sites and a *ccd*E gene lethal for *E. coli*. After transformation of DH10B competent cells, only recombined plasmid, in which the *ccd*B gene was replaced by the *att*B-PCR product, yielded colonies. For each construct, this resulted in an entry clone containing the promoter flanked by *att*L recombination sites. As described previously, the two promoters were then recombined into the GATEWAY-modified binary vector pGWB3 (no promoter, C-GUS) derived from the vector pABH-Hm1 by an *att*L × *att*R (LR) reaction. The vector pGWB3 was a gift from Tsuyoshi Nakagawa (Research Institute of Molecular Genetics, Shimane University, Matsue 690, Japan). This resulted in the two following constructs, *AtCAD* G::*GUS* and *AtCAD* 1::*GUS*. All the GATEWAY-modified vectors were propagated using DB3.1™ competent cells that were suitable for propagation of the control of cell death (*ccd*B) gene-containing plasmids (Invitrogen, Carlsbad, CA, USA).

pCAD B1::GUS construct

Genomic DNA from *A. thaliana*, ecotype WS, was used to amplify the PCR fragment including the promoter region of *CAD B1* using primers listed in supplementary data including *Xba* I and *Bam* H1 sites, respectively (in bold). This *CAD B1* fragment was subcloned upstream of the *GUS* coding region in pBI101.2 (Clontech, Palo Alto, CA) after *Xba* I/*Bam* HI digestion. The resulting construct contained a 0.5 kb region upstream of the *CAD* B1 translational start site, and a 20 bp of the coding sequence fused in frame with the *uid*A gene.

Expression of *AtCAD* genes in the *cad c cad d* mutant

The binary plasmid CHIMpCAMBIA-1390 (Hyg®) already used in a previous study (Sibout et al. 2005) was used. This chimeric expressing system was made to express specifically different *AtCAD* genes under the control of the *AtCAD D* promoter and the terminator of the nopaline synthase gene. cDNA corresponding to *AtCAD* A was cloned using RT-PCR on *Arabidopsis* total mRNA (ecotype WS). Genomic DNA sequences corresponding to *AtCAD* 1 and *AtCAD* G were cloned using PCR on *Arabidopsis* genomic DNA (ecotype WS). Genomic DNA sequences corresponding to *AtCAD* B1 and *AtCAD* B2 were cloned using PCR on *Arabidopsis* BAC F20D10. *Arabidopsis* BAC F7D8

was used to clone *AtCAD* F (ecotype Col0). Besides a *Spe* I site inserted in both primers used to amplify each cDNA, a KOZAK ATG START was made by adding five adenosines between the *Spe* I site and the ATG start of each gene (only the translated region and not the 5′ and 3′ untranslated region was amplified). The primers used are indicated in supplementary data 2. Resulting amplicons were cloned into the pGEM-T easy vector, then sequenced, digested by *Spe* I, and inserted into the binary vector CHIMpCAMBIA 1390. Clones with the appropriate sense of open reading frame (ORF) were selected after sequencing.

For example, the resulting construction was named *ChimAtCAD* 1 when a fragment corresponding to the ORF of the *Arabidopsis CAD* 1 gene was inserted in the CHIMpCAMBIA 1390. Same designation was done for *ChimAtCAD* A, *ChimAtCAD* B1, *ChimAtCAD* B2, *ChimAtCAD* F, and *ChimAtCAD* G. Sixteen lines were regenerated per construct. RT-PCR was performed on stem extracts to identify lines expressing the transgenes. Whole dry stem extracts of several primary transgenic lines expressing the different *CAD* genes at the highest level were analyzed by gas chromatography–mass spectrometry (GC–MS) after thioacidolysis.

Arabidopsis transformation

The binary vectors were introduced in the *Agrobacterium* strain C58pMP90 (Koncz and Schell 1986) by electroporation. Plants were transformed by the flower infiltration protocol (Bechtold and Pelletier 1998). T1 transgenic plants were selected on Estelle and Somerville (1987) medium containing kanamycin (100 mg l^{-1}) or hygromycin (50 mg l^{-1}). T1 or T2 plants were used for the GUS bioassays and for *cad c cad d* potential complementation.

GUS staining

Entire leaves, flowers, and seedlings were harvested and immediately incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid reaction medium as described in Jefferson et al. (1987) for 2 h or overnight depending on the rate of staining and were then dehydrated in 95% ethanol. Stem samples (about 1 cm long) were stained as described for entire organs then cut with a vibratome before observation.

Lignin analysis

Dried mature stems or siliques were collected after removal of leaves. Extract-free samples were prepared using a Soxhlet apparatus by sequentially extracting the ground material with toluene–ethanol (2:1, v/v), ethanol, and water. The determination of lignin content was carried out on the extract-free samples using the standard Klason procedure (Dence 1992). The evaluation of acido-soluble lignin was performed by measuring the absorbance at 205 nm of the supernatant recovered from the Klason lignin procedure and according to Dence (1992). The evaluation of lignin structure was carried out on whole plant material or on extract-free material, using the thioacidolysis procedure (Lapierre et al. 1995, 1999). The lignin-derived monomers were identified by GC–MS as their trimethyl-silylated derivatives. For the thioacidolysis of lines expressing *AtCAD* 1, A, B1, B2, F, or G under the control of the *AtCAD* D promoter in the *cad c cad d* background, two or four primary transformants (kanamycin resistant) were selected on the basis of a high expression of the transgene were selected. For each construct, the data represent the mean value and standard error calculated for these primary transformants.

Screening databases, DNA sequence analysis, protein alignments, and statistics

Databases were screened with BLAST algorithms (Altschul 1990). DNA and protein alignments were carried out with GCG, BioEDIT, and CLUSTALW. For phylogenetic analysis, an alignment of protein sequences was made with CLUSTAL W version 1.83 (Thompson et al. 1994). An unrooted dendrogram was then generated using MEGA version 3.1 (Kumar et al. 2004) with the neighbor-joining method. The numbers at the branching points (see Fig. 1) indicate the percentage of times that each branch topology was found during bootstrap analysis (n=1,000).

Results

Transcription pattern of *AtCAD* 1, A, B1, B2, E, F, and G genes

Northern-blot analyses performed on different tissues were successful only for *AtCAD* 1 (data not shown), in addition to *AtCAD* C and D already published (Sibout et al. 2003). *CAD* 1 was expressed mainly in elongating stems (2–4-week-old plants), green siliques, and flowers. Other transcripts of the *CAD* gene family could not be detected with this method. In order to accurately determine the relative expression level of the various *CAD* genes, quantitative RT-PCR was performed on total RNAs extracted from different organs

with various levels of lignification: young stems, base of mature stems, floral apex, and siliques (Fig. 2 and supplementary data 1). In agreement with previous data, this experiment confirmed the high expression level of *AtCAD* D in lignified organs. *AtCAD* 1 was also highly expressed in young stems, flowers, and siliques but lower levels were observed in the base of stem which is in agreement with northern experiments. *AtCAD* 1 transcript level was found to be the highest in young stems and flower apex. *AtCAD* B1 was found weakly expressed in stems, but strongly present in the floral apex. *AtCAD* B2 displayed the same pattern as *AtCAD* B1, but with an overall lower expression level. Transcript level of *AtCAD* G was found low in all tissues. *AtCAD* A and/or *AtCAD* E/F mRNAs were mainly expressed at the base of the stem whereas they were poorly expressed in other tissues. These results are in good accordance with the number of EST identified in different cDNA libraries as reported in the Flagdb database (http://www.flagdb-gpi.infobiogen.fr). RFLP-RT-PCR was compulsory to distinguish *AtCAD* E from *AtCAD* F transcripts. *AtCAD* F could not be detected in the tested organs (roots, stems, leaves, flowers, siliques), which suggests that the transcripts revealed by quantitative RT-PCR correspond to *AtCAD* E. In addition, *CAD* transcripts corresponding to *AtCAD* 1, B1, and B2 were detected by RT-PCR in seeds 6 days after pollinization (dap) while those of *AtCAD* G were evidenced at 15 dap (data not shown).

GUS analysis of *pAtCAD 1::GUS*, *pAtCAD::GUS*, and *pAtCAD G::GUS* lines

In a previous study (Sibout et al. 2003), the expression profile of both members of the first class (*CAD* C and *CAD* D) was investigated. However, no data were available concerning the tissue specificity of the other classes of *CAD* genes. In order to localize the expression of *CAD* 1 (class III), B1 (class II), and G (class IV) genes, promoter functional analysis of the corresponding genes was performed using the GUS reporter system. The analysis of the GUS patterns in *Arabidopsis* plants showed that *AtCAD* 1 (*pAtCAD 1::GUS* lines), *AtCAD* B1 (*pAtCAD B1::GUS* lines), or *AtCAD* G (*pAtCAD G::GUS* lines) promoter analyses corroborated results obtained by mRNA investigations (Fig. 3). GUS staining in *pAtCAD* 1::*GUS* lines (Fig. 3a–j′) was observed in plantlets (crown, root tips, emergence zone of lateral roots, and foliar veins; Fig. 3a, b″), leaves (veins; Fig. 3c, d), flowers (stigma; Fig. 3e), siliques (replum and internode; Fig. 3f, g), and seed funicule (Fig. 3h). On stem transverse sections, GUS staining could be observed in the xylem, the phloem, and the epidermal layer of young stem (Fig. 3i, i′) whereas no staining was observed in the xylem of mature stem and in interfascicular fibers (Fig. 3j, j′).

Tissues from *pAtCAD* G::*GUS* lines stained slower than tissues of *pAtCAD* 1::*GUS* lines. GUS expression was observed in the roots of plantlets (Fig. 3k, l), in the vascular tissues of leaves (Fig. 3 m), in the replum and internode of siliques (Fig. 3o–p), in flowers (stigma and pollen, Fig. 3o), and in the seed funicule and testa (Fig. 3q). On stem transverse sections, a weak GUS staining was detected in phloem and fiber tissues (Fig. 3n, n′).

GUS staining was observed in *pAtCAD* B1::*GUS* plantlets (Fig. 3r, s), in flowers (petal, stigma, pollen; Fig. 3t), in the internode zone of siliques (Fig. 3u), in the senescing zones of leaves (Fig. 3v), and in the seed testa (Fig. 3x). No GUS staining was observed in veins of leaves nor in xylem and fibers of inflorescence stems (Fig. 3v, w, respectively) suggesting no relation of the expression of this gene with developmental lignification.

Most of the *CAD* genes were expressed in the replum and internode zones of siliques. However, a more detailed study revealed that only *pCAD 1::GUS* lines displayed a GUS staining in valves (Fig. 4b, c), which suggests that *AtCAD* 1 could be involved in fruit lignification. In parallel, lignification in these tissues was revealed using phloroglucinol staining (Fig. 4a). The replum is a lignified and rigid structure that separates the valves, and the two valves themselves are lignified at the level of the valve margin cells and in an internal valve cell layer. The *pAtCAD 1::GUS* construct was introduced in the *shatterproof1*/*shatterproof2* (*shp1*/*shp2*) double mutant (Liljegren et al. 2000) in order to determine the potential role of *CAD* 1 in the lignification of valves of *Arabidopsis* siliques. This *shp1*/*shp2* double mutant lacks the wild-type MADs-box transcriptional regulator alleles necessary for the control of lignification in the dehiscence zone. Such a mutation leads to siliques that are unable to shatter at maturity. *pAtCAD 1::*GUS expression was absent in a *shp1*/*shp2* double mutant background (Fig. 4d), suggesting a regulation of *AtCAD* 1 transcript by *SHP1*/*SHP2*.

Isolation of *Atcad* mutants

With the goal to define the role of the *CAD* 1, A, B1, B2, E, and G genes in *Arabidopsis* lignification, we looked for corresponding knockout mutant lines in the Versailles and the SALK Institute T-DNA insertion collections (Table 1). The localization of the T-DNA insertion was variable according to the lines

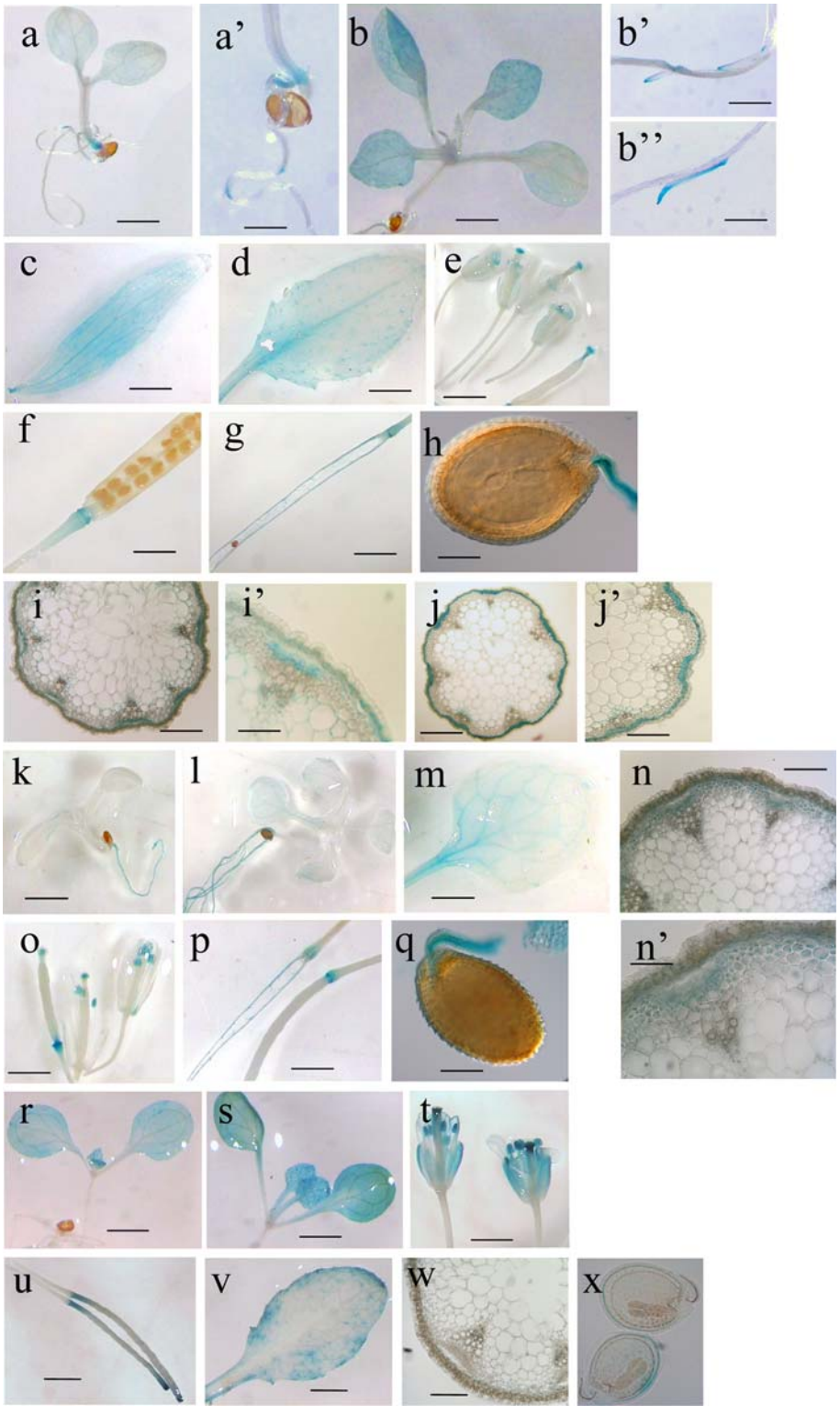

Fig. 3 GUS expression profiles of *AtCAD* 1, *AtCAD* G, and *AtCAD* B1 promoters fused to the *uid*A reporter gene. GUS assays were performed on lines harboring the *pAtCAD 1::GUS* (**a–j′**), *pAtCAD G::GUS* (**k–q**) and *pAtCAD B1::GUS* (**r–x**) constructs. GUS staining in *pAtCAD G::GUS* lines was lower than in *pAtCAD 1::*GUS and *pAtCAD* B1::*GUS* lines (an overnight incubation compared to a 2-h incubation was necessary to observe staining). **a**, **a′**, **k**, **r** Seedlings, 2 cotyledons stage. **b**, **b′**, **b″**, **l**, **s** Seedlings, stage of 2 first leaves. **c**, **d**, **m**, **v** Leaves (cauline and rosette). **e**, **o**, **t** Flowers. **f**, **g**, **p**, **u** Siliques. **h**, **q**, **x** Seeds 15 days after fertilization. **i**, **i′**, **j**, **j′**, **n**, **n′**, **w** Transverse sections of elongating (**i**, **i′**) and mature (**j**, **j′**, **n**, **n′**, **w**) stems. Scale bars=5 mm (**a**, **b**, **e**, **g**, **k**, **l**, **o**, **p**, **r–u**), 2.5 mm (**a′**, **b′**, **b″**, **c**, **d**, **f**, **m**, **p**, **v**), 500 μm (**i**, **j**), and 200 μm (**h**, **i′**, **j′**, **n**, **n′**, **q**, **w**, **x**)

(Fig. 5). Each line was shown to contain one T-DNA insertion. Flanking regions of each T-DNA border were sequenced. No important deletions in the vicinity of the insertions were observed except for the *Atcad* E mutant where a deletion of 1,500 bp upstream the T-DNA was detected (data not shown). However, this deletion had no impact on the neighboring genes. Therefore, only the *CAD* genes were targeted in the *Atcad* mutant lines selected in this work.

Homozygous mutant lines for each insertion were obtained and the impact of the T-DNA insertion on

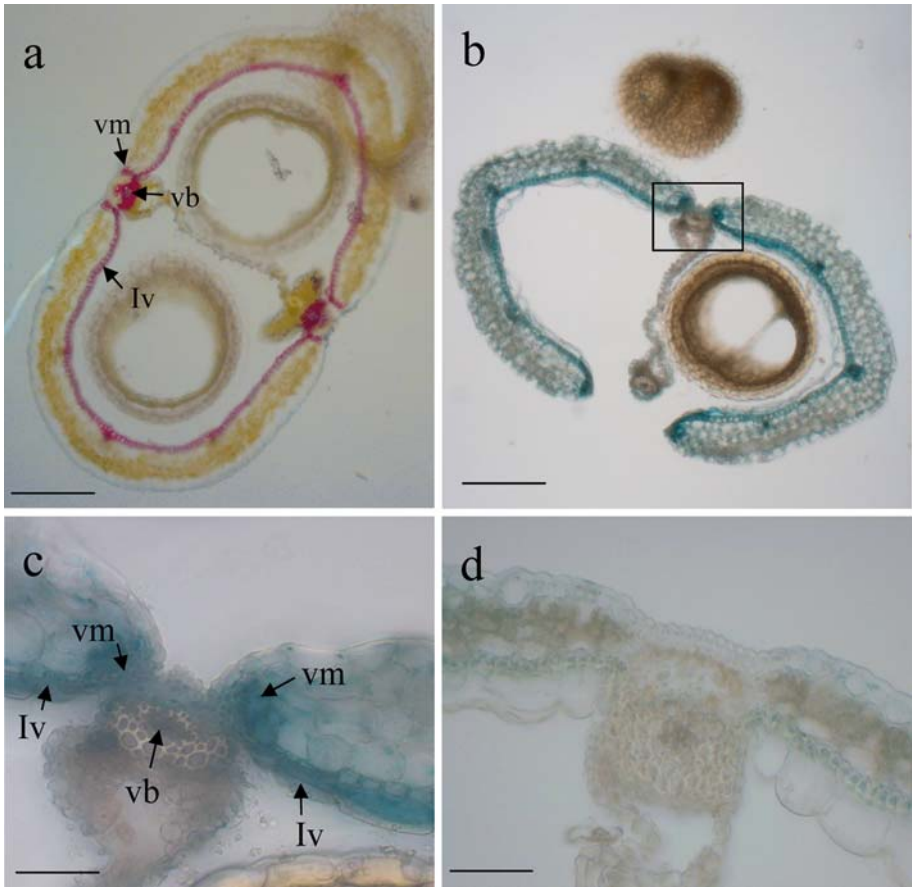

Fig. 4 Expression of *pCAD* 1::GUS in silique. **a** Transverse sections of wildtype fruit (stage 17) stained with phloroglucinol. The internal valve cell layer (Iv), the valve margin (vm), and the vascular bundle (vb) are lignified. **b** Expression of *AtCAD* 1 in a transverse section of a wildtype fruit. **c** Close up of the valve margin region of the transverse section boxed in (**b**). GUS activity is retrieved in the internal valve cell layer (Iv) and in the valve margin (vm) cells immediately adjacent to the dehiscence zones. The vascular bundle does not show any GUS activity. **d** Expression of *AtCAD* 1 in a transverse section of a *shp1*/*shp2* mutant fruit. No GUS activity can be detected in the valves of the silique. Scale bars=200 μm (**a**, **b**), 50 μm (**c**, **d**)

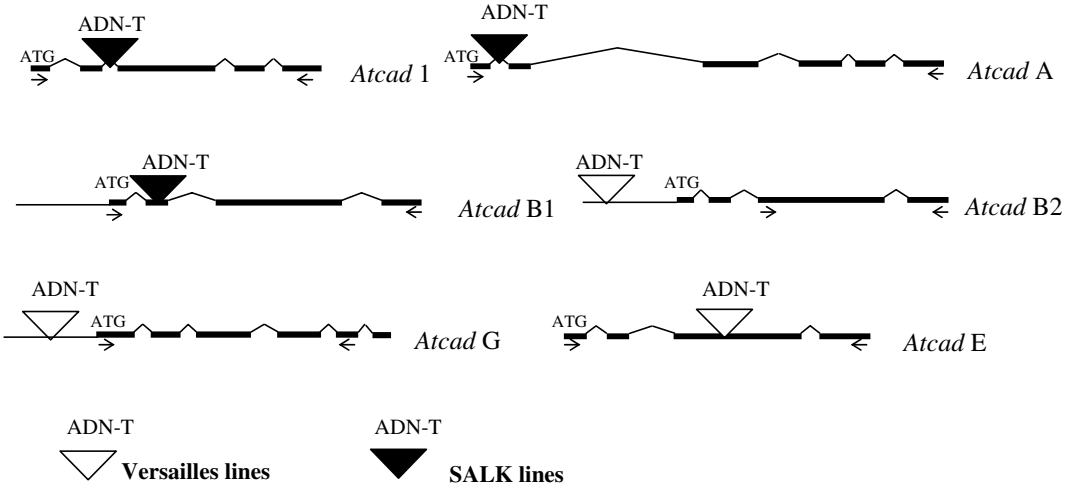

Fig. 5 Diagrammatic representation of *AtCAD* genes and localization of the T-DNA insertions (*triangle*) for each mutant line. The figure indicates the position of the primers (*arrows*) used to verify the absence of mRNA expression of *AtCAD* genes

mRNA detection was determined by RT-PCR experiments on total RNA of each mutant using specific primers. The absence or presence of a truncated mRNA signal for the target *CAD* genes was confirmed for each mutant (data not shown). No visual phenotypes were observed when these mutant lines were grown in greenhouse conditions. However, the seeds of *Atcad* B1 and *Atcad* B2 mutants were very sensitive to the hypochlorite sterilization which impeded their germination to a large extent whereas no difference was observed on germination ability when the seeds were sown without sterilization.

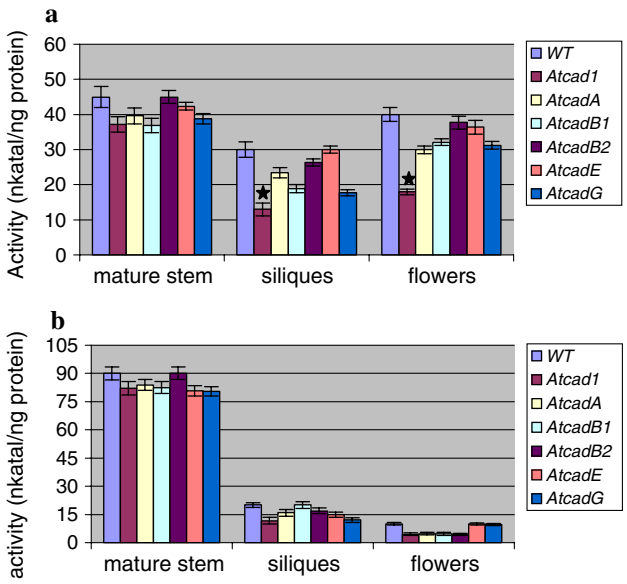

Fig. 6 Coniferyl alcohol dehydrogenase (**a**) and sinapyl alcohol dehydrogenase (**b**) activities in mature inflorescence stems, flowers, and siliques from wild-type and *CAD* mutant plants. *Bars* indicate SE of the mean of three assays (see Sect. "Materials and methods"). *Stars* indicate values statistically different from WT (P=0.05)

CAD activities and changes in CAD protein quantity in *Atcad* mutants

The impact of mutations on CAD proteins was assessed first by enzymatic assays. conAD or sinAD activities were quantified in protein extracts from stems, flowers, and siliques of the mutant lines and compared to those of the corresponding wild-type ecotypes (WS or Col0).

Cinnamyl alcohol dehydrogenase activities were detected in all tested organs using both coniferyl and sinapyl alcohols. However, sinAD activity was found very low and poorly repeatable in siliques and flowers. Mature stems showed the highest conAD and sinAD activities, but no substantial decrease of CAD activities were found in the mutants studied herein (Fig. 6). The strong and redundant expression of CAD C and D in mature stems may explain these results (Sibout et al. 2003). conAD activity was approximately 50% reduced in flowers and siliques of *Atcad* 1. conAD activity was also slightly reduced in siliques of *Atcad* B1 and G mutants. In conclusion of these CAD activity measurements, the mutant presenting a significant reduction of CAD activity in several organs was *Atcad* 1.

Considering the high amino acid homology between poplar SAD and *AtCAD* A, 1, B1, B2, and E proteins, we carried out western-blot analyses using an antiserum containing antibodies directed against

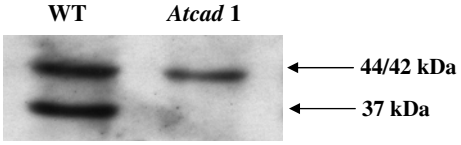

Fig. 7 Western-blot analysis of crude extracts from flowers of wild-type plant (WT) and *Atcad* 1 plants. Crude extracts were assayed by immuno-blotting using anti-poplar SAD antibody (adapted from Li et al. 2001). The apparent molecular masses of AtCAD D, AtCAD C (44/42 kDa) and AtCAD 1 (37 kDa) are indicated

the poplar SAD (Li et al. 2001). In addition, western-blot analyses were performed with an antiserum containing antibodies directed against tobacco CAD (Halpin et al. 1994). Long migrations on acrylamide gel allowed us to identify two proteins with an apparent molecular weight of 44 and 42 kDa. These proteins were recognized by both antisera and identified as AtCAD C and D (data not shown). In addition to this 44/42 kDa doublet, a band at 37 kDa was detected in young stem, flower, and silique protein extracts. Both antisera recognized this additional band but the intensity was higher with the antiSAD one (results not shown). This band was absent when western-blot experiments were performed with flower extracts from *Atcad* 1 (Fig. 7). This result establishes that the 37 kDa protein corresponds to AtCAD 1. No difference on western-blot analyses could be detected for *Atcad* B1, B2, E, and G mutants, as compared to the control. This result suggests that these CAD proteins are not expressed at sufficient levels to allow their detection in the control with the two antisera used herein (data not shown).

Lignin modification in mutants

The histochemical analyses of lignified stems using the Wiesner (phloroglucinol–HCl) or the Mäule reagents did not reveal any perturbation of lignification between the control and the *Atcad* mutants (data not shown). The acido-insoluble lignin content of the extract-free floral mature stems was determined by the Klason standard method (Dence 1992) which systematically includes the removal of extractives before analysis and the correction for ash content, if any. The wild-type and mutant lines displayed similar amount of extractives and negligible ash levels in the Klason lignin fraction. As already observed for other *Arabidopsis* lines (Goujon et al. 2003b), Klason lignin amount in extractive-free mature stems were found to display substantial variations according to growth conditions. Nevertheless, different replications did not reveal any important changes in the Klason lignin

amount of all the mutants studied herein (Table 2 for one representative sampling of each line). Acido-soluble lignin amount was found to be in the 2–3% range whatever the sample of extract-free floral stem (WT and *Atcad* lines; data not shown). However, the Klason lignin content of elongating stems (10–15 cm) of the *Atcad* 1 mutant was slightly reduced (10–20%; Table 2) suggesting a role for AtCAD 1 in the first steps of xylem lignification. This reduction in xylem lignin level was confirmed by microscopy FT-IR performed in cross sections of elongating stems (data not shown).

The *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin-derived monomers released by thioacidolysis of wild-type and homozygous mutant lines were analyzed by GC–MS. The data reported in Table 2 show that the thioacidolysis yield, when expressed on the basis of the Klason lignin content of the extract-free stems, did not clearly discriminate the control and the mutant lines. The S/G ratio of the β-O-4 lignin fraction did not vary in stem extracts from different cultures of these mutants (Table 2) even in young stems of *Atcad* 1.

Table 2 Comparison of lignin characteristics in stems of *Atcad* and WT lines

Line	Lignin Klason[a]	Monomers[b]	S/G[b]
WT			
Elongating stem	11.1[c]	224 ± 9	0.11
Mature stem	15.5 ± 0.2	1030 ± 82	0.40
Atcad 1			
Elongating stem	8.6[c]	291 ± 4	0.11
Mature stem	15.65 ± 0.1	1031 ± 7	0.40
WT	15.4 ± 0.1	1113 ± 36	0.37
Atcad A	16.6 ± 0.1	905 ± 105	0.38
WT	18.4 ± 0.1	1243 ± 130	0.42
Atcad B1	17.3 ± 0.1	1251 ± 6	0.41
WT	20.0 ± 0.2	1574 ± 131	0.42
Atcad B2	20.3 ± 0.1	1373 ± 39	0.38
WT	18.6 ± 0.1	1349 ± 15	0.38
Atcad E	18.2 ± 0.25	1438 ± 118	0.38
WT	17.5 ± 0.2	1167 ± 76	0.375
Atcad G	17.15 ± 0.5	1119 ± 15	0.38

The lignin parameter determination was performed at least twice for each mutant and WT lines grown in the same conditions. Only the results of one representative sampling are shown

[a] Klason lignin content was calculated as weight percentage of extractive free stem extract

[b] Main lignin-derived conventional S and G monomers obtained by thioacidolysis, expressed in μmol (G+S) /Klason lignin (μmol g^{-1}) and S/G ratio

[c] No repetition of the measurement was performed for elongating stems due to the small amount of material but two different samplings were analyzed and FT-IR analysis of primary xylem confirmed the lower lignin level of the *Atcad* 1 sample

G and S indene marker compounds, diagnostic for coniferaldehyde or sinapaldehyde units linked at Cβ in CAD deficient plants (Kim et al. 2002) and in the *cad c cad d Arabidopsis* mutant (Sibout et al. 2005), were detected as trace components in the lignins of these different mutants. Their levels did not exceed the control levels even in the *Atcad* 1 mutant (data not shown).

Siliques are lignified in the valve and the replum zones (Ferrandiz et al. 1999) and this lignification is involved in the dehiscence process (Liljegren et al. 2000). As compared to mature stems lignins, compositional analyses revealed that siliques contain less lignins and that these lignins are enriched in G units and in resistant inter unit bonds (as revealed by a lower recovery of thioacidolysis monomers when expressed on the basis of the lignin content). No reduction of Klason lignin was observed in the siliques of the mutants (data not shown) including the *Atcad* 1 mutant.

Complementation of the *cad c cad d* mutant

The double *cad c cad d* mutant analyzed in a previous study (Sibout et al. 2005) was used to test the potential CAD activity of the six *CAD* genes in vivo. The corresponding coding sequences were fused to the promoter region of the *CAD-D* gene. This tissue-specific promoter is strongly expressed in xylem and in interfascicular fibers (Sibout et al. 2003). The binary plasmid CHIMpCAMBIA-1390 (Hyg) already used in a previous study (Sibout et al. 2005) was used to introduce the cDNA or genomic sequences corresponding to *AtCAD* 1, A, B1, B2, F, G in the *cad c cad d* mutant. The obtained lines were named *ChimAtCAD* 1, *ChimAtCAD* A, *ChimAtCAD* B1, *ChimAtCAD* B2, *ChimAtCAD* F, and *ChimAtCAD* G. The phenotype of the regenerated lines (16 primary transformants per construct) was observed in order to determine the potential disappearance of the typical reddish color of the double mutant stem as a first sign of efficient complementation. However, no reversion to the WT phenotype was observed for all the regenerated lines which, similar to the double mutant, did not react to the Mäule staining. Therefore, RT-PCR experiments were performed to identify lines expressing the introduced *CAD* genes at the highest level (data not shown). The lignins of inflorescence stems of the selected primary regenerants (four or three lines per gene) were analyzed by GC–MS after thioacidolysis (Table 3). These sensitive analyses applied to the low sample amounts which were available revealed that the dramatically altered lignification of the *cad c cad d*

line was significantly modified by the *ChimAtCAD* 1 construct. Whereas the total yields in thioacidolysis monomers released from the mature floral stems of *cad c cad d*, *ChimAtCAD* A, *ChimAtCAD* B1, *ChimAtCAD* B2, *ChimAtCAD* F, and *ChimAtCAD* G lines were found similarly weak, this yield was significantly increased by the *ChimAtCAD* 1 construct, even though it was not entirely restored to the wild-type level (Table 3). This result means that the *ChimAtCAD* 1 construct significantly increased the level of β-O-4 linked H, G, or S units in mature floral stems and relative to the other mutant lines. The relative frequencies of the G and S monomers did not discriminate the various complemented lines from the double mutant, with G and S monomers representing 91–93% and 5–6% of the total, respectively. Surprisingly enough, the frequency of the H minor thioacidolysis monomers was another effect of the *ChimAtCAD* 1 construct: whereas this frequency was in the 1–2% range in the hypolignified stems of the *cad c cad d*, *ChimAtCAD* A, *ChimAtCAD* B1, *ChimAtCAD* B2, *ChimAtCAD* F, and *ChimAtCAD* G lines, this frequency was significantly lower in the *ChimAtCAD* 1 and wild-type lines (0.60 and 0.65%). The most specific signature of CAD deficiency in the lignin of the double mutant certainly was the high molar proportion of indenes relative to the conventional thioacidolysis monomers (Table 3). While this signature was maintained in the 35–55% range for the *cad c cad d*, *ChimAtCAD* A, *ChimAtCAD* B1, *ChimAtCAD* B2, *ChimAtCAD* F, and *ChimAtCAD* G lines, it was significantly reduced in the case of *ChimAtCAD* 1 line (10% of the conventional monomers level; Table 3). Taken together, these results demonstrate that CAD A, B1, B2, F, and G are not able to efficiently reduce coniferaldehyde or sinapaldehyde.

Discussion

AtCADs belong to a small multigene family in *Arabidopsis*

Different studies have highlighted that *CAD* genes, which have been relatively well studied, could occur in more than one copy in various plant species. Moreover, several *CAD* genes have been identified in different plants (alfalfa: Van Doorsselaere et al. 1995; Brill et al. 1999; ryegrass: Lynch et al. 2002; strawberry: Blanco-Portales et al. 2002; poplar: Van Doorsselaere et al. 1995; Li et al. 2001; rice: Tobias and Chow 2005) and therefore *CAD* genes are usually part of small multigene family. Recently, three distinct publications unraveled the *CAD* genes in *Arabidopsis* (Costa et al. 2003; Goujon et al. 2003a; Raes et al. 2003). Some of our previous studies on *Arabidopsis CAD* (Sibout et al. 2003, 2005) were focused on *CAD*s of class I which is composed of two members (*AtCAD* C and D). Lignin content of the corresponding double mutant is severely decreased. Nevertheless, the low residual G conventional units derived from coniferyl alcohol could be still detected in weak but noticeable amount. In addition, the flower and the silique extracts of the *cad c cad d* mutant were still able to reduce either coniferaldehyde or sinapaldehyde, which demonstrates the remaining occurrence of CAD activities in these organs (our unpublished results). It is thus obvious that some of other members of the CAD family play a significant role in developmental lignification.

In a recent paper, Kim et al. (2004) used heterologous expression in *E. coli* to determine substrate specificity of all *Arabidopsis CAD* genes. These authors deduce from this study that CAD D and CAD C showed high substrate affinities for cinnamyl alcohols.

Table 3 Analysis of lignin-derived monomers by thioacidolysis

Line (n = number of plants)	Total yield (H+G+S) in μmol/g DM	%H	%G	%S	Relative importance of indenes
WT ($n = 4$)	181 ± 15^{a}	0.65 ± 0.09^{a}	70.85 ± 1.19^{a}	28.53 ± 1.10^{a}	0.051 ± 0.028^{a}
cad c cad d ($n = 4$)	6.55 ± 1.9^{b}	1.90 ± 0.34^{b}	92.48 ± 1.01^{b}	5.63 ± 0.79^{b}	54.43 ± 8.49^{b}
ChimAtCAD1 ($n = 4$)	37.53 ± 9.62^{c}	0.60 ± 0.08^{a}	93.73 ± 1.50^{b}	5.65 ± 1.42^{b}	10.41 ± 3.85^{c}
ChimAtCADA ($n = 3$)	5.80 ± 1.99^{b}	2.80 ± 0.44^{b}	91.03 ± 0.87^{b}	6.17 ± 1.20^{b}	39.97 ± 14.14^{b}
ChimAtCADB1 ($n = 3$)	10.66 ± 1.85^{b}	1.87 ± 0.40^{b}	92.27 ± 0.25^{b}	5.83 ± 0.15^{b}	37.20 ± 0.90^{b}
ChimAtCADB2 ($n = 3$)	8.93 ± 3.23^{b}	1.40 ± 0.35^{b}	92.03 ± 1.63^{b}	6.57 ± 1.50^{b}	38.89 ± 1.47^{b}
ChimAtCADG ($n = 4$)	6.30 ± 0.83^{b}	2.27 ± 0.15^{b}	91.77 ± 0.74^{b}	5.93 ± 0.71^{b}	51.31 ± 10.19^{b}
ChimAtCADF ($n = 2$)	7.70 ± 0.28	1.35 ± 0.64	92.55 ± 0.92	6.10 ± 0.28	34.98 ± 4.23

Thioacidolysis was performed on mature inflorescence stems of wild type, *cad c cad d* double mutant and *cad c cad d* lines complemented with *AtCAD* cDNAs. The total yield in main lignin-derived conventional monomers (H+G+S) is expressed in μmoles per gram of dry mater. The relative importance of the indene monomers released from *p*-OH cinnamaldehydes linked at Cβ by β-O-4 bonds is expressed as molar percentage of the conventional (H+G+S) main monomers. The data are means ± SE between assays on different primary transformants ($n = 4$, 3 or 2) for the complemented lines and on different plants ($n = 4$) for the WS and *cad c cad d* lines. Values in the same column with different superscript letters are significantly different ($P < 0.01$, Student's test applied when $n = 3$ or 4)

Since the publication of Sibout et al. (2005), we hypothesized that other *CAD* genes may contribute to lignin biosynthesis in *Arabidopsis*. By combining different approaches, the present study investigates each class of *Arabidopsis CAD* genes with the goal to figure out candidate genes that may assume this role *in planta*.

AtCAD 1, E, and F (class III)

Phylogenetic classification of the *CAD* gene family shows that they belong to four subfamilies (Raes et al. 2003; Sibout et al. 2003). One subfamily contains *AtCAD* 1, E, and F which are close to MsaCAD1, a *Medicago sativa* CAD (Brill et al. 1999). MsaCAD1 was shown to have affinity not only for sinapaldehyde and coniferaldehyde, but also for benzaldehyde (Brill et al. 1999). *AtCAD* 1 is expressed to a relatively high level since it can be detected in northern analysis experiments and is well represented in EST databases. This gene codes for a 37 kDa protein detectable by Western experiments in young stems, flowers, and siliques. Promoter *AtCAD* 1::GUS fusion demonstrated the *CAD* 1 expression in young xylem, the first lignified tissues of stem (Fig. 3i), but not in interfascicular fibers, which are the late sites of stem lignification. Nevertheless, other sites of expression (epiderm, phloem, roots...) of the At*CAD* 1 do not coincide with lignification. CAD activities in the *Atcad* 1 insertional mutant were reduced in flowers and siliques (about 50% residual activity for coniferyl alcohol). This CAD activity reduction is detected despite the low affinity for *p*-OH cinnamaldehydes observed in vitro by Kim et al. (2004). The absence of CAD 1 has a small but detectable impact on lignin content in elongating floral stem whereas no difference was detected when the Klason analysis was performed on dried mature stems. This lignin reduction was confirmed by FT-IR analysis performed on stem sections. It was already observed by Kim et al. (2004) on the same mutant during stem development. A partial complementation of the *cad c cad d* mutant at the G unit level was observed when this CAD was expressed under the control of the *CAD* D promoter, therefore present in tissues containing high coniferaldehyde and/or sinapaldehyde content. This involvement of *AtCAD* 1 in the biosynthesis of coniferyl alcohol in young developing stem could explain the presence of residual conventional G units in the lignins of the double mutant (Sibout et al. 2005). However, CAD-like genes such as At5g19440, the ortholog of the tobacco *CAD* 1, could be also partly responsible for biosynthesis of lignin precursors (Damiani et al. 2005). An increase in transcript level of *AtCAD* 1 was detected in stem of *cad c cad d* mutant (Sibout et al. 2005). This could be related to a compensatory mechanism for the biosynthesis of coniferyl alcohol. *AtCAD* 1 expression is also specifically related to lignification in silique, in particular in the dehiscence zone. The dehiscence zone is represented by a thin band of cells that develops between the replum and the valves, and lignification of the valves has been proposed to mechanically contribute to fruit opening (Ferrandiz et al. 1999). Lignification of valve margin cells is strongly affected in the *shatterproof1*/*shatterproof2* (*shp1*/*shp2*) double mutant whereas the dehiscence zone fails to develop, leading to siliques that are unable to shatter at maturity (Liljegren et al. 2000). Our data suggest that *CAD* 1 could be transcriptionally regulated by either one or both MADs-box proteins encoded by *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*). It must be noticed that *Atcad* 1 siliques are dehiscent which suggests that the lost of function of *AtCAD* 1 does not change the silique mechanical properties enough to prevent the internal tension causing the shattering. Several CADs are expressed in siliques at the replum level (such as AtCAD D) and therefore no impact on lignin content was detected since the valves constitute a very small proportion of the lignified cells of the silique. Conversely, the high transcript level of *AtCAD* 1 in flowers is probably not related to lignification since it is mainly expressed at the stigma extremity, a non-lignified flower part. *MsaCAD*1 was also highly expressed in flowers (Brill et al. 1999) and a related EST was identified as epiderm specific in maize (Nakazono et al. 2003). The expression in epiderm could suggest a role of this CAD in the biosynthesis of soluble phenolics since these compounds accumulate in this tissue; however, no modification of the soluble phenolic pool could be observed in plantlets of *Atcad* 1 (data not shown). Griffiths et al. (1999) identified cinnamyl alcohol in the epicuticular waxes of faba bean flowers and this alcohol could be present in *Arabidopsis*.

AtCAD E and F belong to the same subfamily and their sequences are very similar (98% identity). RFLP-RT-PCR was necessary to differentiate these two genes. Using this technique, no expression of *AtCAD* F was detected in the parts of *Arabidopsis* plants used for RT-PCR and *AtCAD* E was expressed at a very low level in stems, flowers, and siliques. In addition, no mRNA corresponding to *AtCAD* F was detected in the *Atcad* E null mutant. CAD activity was not significantly reduced in the *Atcad* E mutant. No modification of lignin content/structure in stems of the *Atcad* E mutant was detected. In addition, no complementation of the *cad c cad d* mutant following expression of

AtCAD F was observed. *AtCAD* F expression was detected in a transcriptional study on secondary growth in hypocotyls of *A. thaliana* (Oh et al. 2003). According to the very low expression level of *AtCAD* E and F in lignified tissues and to the lack of affinity of the corresponding enzymes for monolignol specific substrates (Kim et al. 2004; this study) these genes do not participate significantly to developmental lignification in *Arabidopsis*.

AtCAD A, B1, and B2 (class II)

In the phylogenetic analysis, AtCAD A, B1, B2 fall within the same cluster as PtSAD which has been identified and characterized in poplar (Li et al. 2001) and *CAD*-related and pathogen-induced genes identified in parsley (Somssich et al. 1989), celery (Williamson et al. 1995), and potato (Montesano et al. 2003). PtSAD was shown to be highly specific for sinapaldehyde and was proposed to be responsible for S unit deposition in lignins of poplar fibers (Li et al. 2001). The active site topology revealed by the crystal structure substantiates kinetic results and indicates the high specificity for sinapaldehyde (Bomati and Noel 2005). This PtSAD was included in the plant alcohol dehydrogenase family that includes cinnamaldehyde and benzaldehyde dehydrogenases. Substrate affinity was correlated with amino acid substitution in the active site and little changes in the active sites were related through substrate affinity modification (Bomati and Noel 2005). The celery CAD was classified as a mannitol dehydrogenase (Williamson et al. 1995) and the potato CAD as a benzyl alcohol dehydrogenase (Montesano et al. 2003) so was the *Arabidopsis* CAD B2 (Somssich et al. 1996).

In *Arabidopsis*, *AtCAD* A was expressed at a very low level in mature stems. CAD activity in *Atcad* A mutant was not reduced in inflorescence and lignin characteristics were not modified. The absence of complementation of the *cad c cad d* mutant by the *AtCAD* A gene under the control of the *CAD* D promoter definitively excluded a role of this gene in lignification. The transcript level of *AtCAD* A was increased by two-fold in the *cad c cad d* mutant but the origin of this compensatory mechanism is not known (Sibout et al. 2005).

AtCAD B1 and B2 were previously identified for their induction after pathogen infestation (Kiedrowski et al. 1992) and called *ELI3* 1 and 2. Expression of these genes was quite low in stem and CAD B1 was mainly observed in flowers. They were also detected at the early stage of seed development. *AtCAD* B1 was expressed at a higher level than *AtCAD* B2 in quantitative RT-PCR and its main sites of expression, determined by promoter–GUS fusion, are plantlets and flowers. AtCAD B1 could have affinity for aldehydes present in flowers. *AtCAD* B1 and *AtCAD* B2 were originally identified as part of the defense response in parsley and in *Arabidopsis* (Somssich et al. 1989; Kiedrowski et al. 1992). The identification of transcripts with significant homology to *AtCAD* B2 in celery, parsley, and potato in response to pathogen infections suggested that expression of *AtCAD* B1 and B2 genes could be increased by pathogens as already shown by Kiedrowski et al. (1992). In a recent study (Marathe et al. 2004) *AtCAD* B2 expression was shown to increase in response to cucumber mosaic virus infection. In addition, these genes could also be induced by abiotic stresses since the expression of a transcript homolog to *AtCAD* B was induced in root upon exposure of the shoot to light in tomato (Lauter 1996). Specific transcript accumulation for *AtCAD* B2 was shown during leaf senescence (Quirino et al. 1999) and after wounding (Cheong et al. 2002). Therefore, *AtCAD* B1 and B2 seem to be related to response to stresses but not to the neosynthesis of lignin in stress conditions since they were not able to complement the *cad c cad d* mutant. Conditions of induction of *AtCAD* B1 and B2 by biotic and abiotic stresses will be extensively studied in the future using *Arabidopsis* lines expressing promoter–GUS fusions. The expression of *AtCAD* 1 in seed testa and the low resistance to sterilization of seeds of *Atcad* B1 and B2 mutants suggests a role of *AtCAD* B1 and B2 in the synthesis of a protective compound in seeds. Presence of *CAD* B1 and B2 orthologs in seeds of *Sesamun indicum* suggested their possible involvement in lignan biosynthesis (Suh et al. 2003). Lignans are probably present in *Arabidopsis* seeds and *AtCAD* B1 and B2 could be involved in this phenylpropanoid-related pathway.

AtCAD G (class IV)

AtCAD G is the only member of the class IV *CAD* subfamily. Two rice and a *M. truncatula* proteins belong to the same class (Fig. 1). Another gene with good level of sequence similarity was also identified in celery and is specifically accumulated in phloem (Vilaine et al. 2003). Presence in low amount of the *AtCAD* G transcript in all plant parts tested in this study, together with data from EST databases pushed us for a deeper analysis. We then performed promoter–GUS fusion analysis in transgenic *Arabidopsis*. *AtCAD* G expression profile does not coincide exclusively with lignification since GUS expression was detected in interfascicular fibers of stems as well as in replum and

internodes of siliques (Fig. 3) and some lignified parts of the plant but also in phloem. The affinity of AtCAD G for cinnamaldehydes was very low according to Kim et al. (2004). In accordance with the lack of impact on CAD activity, lignin content, and composition were not modified in inflorescence stems of *Atcad* G. The lack of complementation of the *cad c cad d* mutant clearly excluded a role in lignification for this gene. *AtCAD* G expression was detected in developing seeds (16 dap) and transcript accumulation of orthologous genes were detected in seeds of *S. indicum* (Suh et al. 2003) suggesting as for AtCAD G a role in lignan biosynthesis. Baerson et al. (2005) demonstrated recently induction of *AtCAD* G in *Arabidopsis* seedlings exposed to the allelochemical benzoxazolin-2 (3*H*)-one. A high increase (fivefold) of transcript accumulation of AtCAD G was observed in the double *cad c cad d* mutant (Sibout et al. 2005). The introduction of the *pCAD* G::GUS construct in this double mutant revealed the increased GUS expression in vascular tissues with, in addition, its expression in interfascicular cambium (our unpublished results). The induction of *AtCAD* G could suggest a role in mechanical stress response or in detoxification processes resulting from compromised stem rigidity or accumulation of aldehydes in the *cad c cad d* mutant.

Conclusion

Nine *CAD* genes are present in the *Arabidopsis* genome according to bioinformatic studies and many orthologs to *AtCAD* genes have been identified in different plant species demonstrating the presence of a CAD multigene family in plant genomes. CADs are generally considered to be involved in the lignin biosynthetic pathway, however in most cases, a role in this pathway could not be clearly demonstrated. Antisense strategies have demonstrated the role of *CAD* genes of the class I in lignin biosynthesis (Halpin et al. 1994; Baucher et al. 1996, 1999). In *Arabidopsis*, previous studies have proposed a major role of two genes (*AtCAD* C and D) in lignin biosynthesis in accordance with their profile of expression (Sibout et al. 2003) and their substrate specificity (Kim et al. 2004). A double *cad c cad d* mutant synthesizes lignins unusually enriched in coniferaldehyde and in sinapaldehyde units, both aldehydes being CAD substrates (Sibout et al. 2005), but still containing substantial amounts of G lignin units derived from coniferyl alcohol. The present work proposes that CAD 1 is involved in constitutive lignification by reducting *p*-OH cinnamaldehydes in the primary xylem of elongating stems. Moreover, an additional role of *CAD* 1 in the biosynthesis of monolignols in siliques cannot be excluded. In contrast, the other *CAD* A, B1, B2, E, F, and G genes may not be related to lignification since the corresponding enzymes do not seem to have any significant affinity for coniferaldehyde and sinapaldehyde in vivo. Obtaining a triple *cad c cad d cad* 1 mutant is underway in order to determine if other *CAD*-like genes such as the ortholog of the tobacco *CAD* 1 (Damiani et al. 2005) contribute to the biosynthesis of lignin precursors in *Arabidopsis*.

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