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Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in Arabidopsis

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SUMMARY

The deposition of lignin during plant–pathogen interactions is thought to play a role in plant defence. However, the function of lignification genes in plant disease resistance is poorly understood. In this article, we provide genetic evidence that the primary genes involved in lignin biosynthesis in Arabidopsis, *CAD-C* and *CAD-D*, act as essential components of defence to virulent and avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, possibly through the salicylic acid defence pathway. Thus, in contrast with cellulose synthesis, whose alteration leads to an increase in disease resistance, alteration of the cell wall lignin content leads directly or indirectly to defects in some defence components.

INTRODUCTION

Plants develop immune responses to react to microbial attack. These responses are initiated by recognition events which result in the activation of an array of defence mechanisms, integrated by a complex signalling system. They include the generation of signals such as reactive oxygen intermediates, synthesis of antimicrobial compounds, lignification of cell walls, and expression of pathogenesis-related (PR) proteins (Glazebrook, 2005). In addition to these general defence mechanisms, one of the most spectacular manifestations of plant resistance is the hypersensitive response (HR), a form of programmed cell death occurring in a limited area at the site of infection (Heath, 2000).

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Lignins, which are complex aromatic polymers resulting from the oxidative polymerization of hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl alcohols), are not only associated with plant growth and development, but also with defence responses to environmental stresses (Lange *et al*., 1995; Nicholson and Hammerschmidt, 1992; Vance *et al*., 1980). In many cases of plant–pathogen interactions, transcriptional profiling studies have revealed the activation of genes whose products are involved in the biosynthesis and modification of cell wall components (Cheong *et al*., 2002; Rinaldi *et al*., 2007; Schenk *et al*., 2000). The deposition of lignin during plant–pathogen interactions is thought to play a role as a physical barrier against infection. Unpolymerized monolignols may also have antimicrobial activity (Keen and Littlefield, 1979). However, although a number of reports have genetically defined the role of individual lignification genes during plant development, their function in plant disease resistance is poorly understood (Boerjan *et al*., 2003). Recently, Kawasaki *et al*. (2006) demonstrated that cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defence signalling in rice. This interesting finding suggests a more sophisticated regulation of lignin biosynthesis than expected during defence responses.

Cinnamyl alcohol dehydrogenase (CAD) is a specialized enzyme involved in the reduction of cinnamaldehydes into cinnamyl alcohols, the last step of monolignol biosynthesis before oxidative polymerization in the cell wall. These enzymes are encoded by complex gene families in plants. The complete *CAD* gene family has been surveyed in Arabidopsis (Kim *et al*., 2004; Sibout *et al*., 2003), demonstrating the existence of nine CAD-like proteins distributed into four different classes based on their amino acid similarity. Of the nine putative CADs, two exhibit the highest activity and homology to *bona fide* CADs from other species: CAD-C and CAD-D. A second class contains CAD-A, CAD-B1 and CAD-B2, which are most closely related to the poplar sinapyl alcohol dehydrogenase (Li *et al*., 2001), and which

are catalytically less active, at least by an order of magnitude, compared with CAD-C and CAD-D. CAD-1, CAD-E and CAD-F are closely related to the alfalfa CAD2 (Brill *et al*., 1999), and display extremely low activity on cinnamyl aldehydes. Finally, the fourth class contains CAD-G, which does not have well-identified homologues. Interestingly, although the characterization of *cad-C* and *cad-D* single mutants in Arabidopsis has revealed only a modest reduction in lignin content and a weak alteration in lignin quality, *cad-C/cad-D* double mutants have an extremely strong phenotype (Sibout *et al*., 2003, 2005). Lignin content in the stem is reduced to 40% and coniferyl and sinapyl alcohol incorporation is reduced by 94%, indicating that *CAD-C* and *CAD-D* act as the primary genes in lignin biosynthesis in *Arabidopsis thaliana*. Thus, these genes constitute an interesting target to address the question of the role of lignin biosynthesis in plant–pathogen interactions. In this study, we used a genetic approach to determine whether the Arabidopsis *CAD-C* and *CAD-D* genes act as essential components of defence to virulent and avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. As the two genes may be redundant, we also used the mutant which is knock out for both genes to assess the resistance to *P. syringae* pv. *tomato*. In addition, we studied the expression pattern of the different members of the *CAD* gene family during the interaction, when compared with the other lignin biosynthesis genes.

RESULTS

Three members of the *CAD* gene family are induced in response to *Pseudomonas*

Arabidopsis *CAD* gene expression was studied during plant development (Sibout *et al*., 2003). To evaluate the possible role of lignin biosynthesis in plant defence, the expression of all nine members of the Arabidopsis *CAD* gene family was analysed after challenge with virulent or avirulent strains of *P. syringae* pv. *tomato* DC3000 by quantitative real-time polymerase chain reaction (Q-RT-PCR) (Lorrain *et al*., 2004). Specific primers were designed for each member of the family, with the exception of the *CAD-E* and *CAD-F* genes, which are 98% identical at the nucleotide level. In this specific case, common primers were designed to amplify both genes (Table S1, see Supporting Information). After inoculation with virulent or avirulent *P. syringae* pv. *tomato* strains expressing the avirulence gene *avrRpt2*, the expression of *CAD-D*, *CAD-B2* and *CAD-G* was strongly induced when compared with the control treated with water, whereas the expression of *CADC*, *CADA*, *CADB1* and *CADE-F* was not significantly changed, at least at the early time points (Fig. 1). Two genes (*CAD-C* and *CAD-EF*) were expressed at very low levels. Interestingly, in the case of *CAD-A*, *CAD-1* and *CAD-B1*, gene expression was clearly induced by the inoculation procedure and appeared repressed 24 h after inoculation when compared with the water control. *CAD-D*, *CAD-B2* and *CAD-G* showed similar expression profiles, with a strong increase between 9 and 24 or 48 h, which was more rapid in response to the avirulent strain, especially for *CAD-B2* and *CAD-G*. In leaves treated with water, these three genes were weakly expressed, whereas the other *CAD* genes were induced. These results indicate that three members of the *CAD* gene family are strongly induced on inoculation with a bacterial pathogen.

CAD-C and CAD-D act redundantly as components of basal resistance

To further elucidate the role of lignin biosynthesis in plant resistance, we used previously characterized mutant lines affected in the different *CAD* genes (Eudes *et al*., 2006; Sibout *et al*., 2003, 2005). The other Arabidopsis mutant lines (used as controls) were obtained from the following sources: the mutant *eds1* from Jane Parker (Parker *et al*., 1996) and the mutant *pad-4* from Jane Glazebrook (Glazebrook *et al*., 1997). We focused our analysis on mutants in the *CAD-D*, *CAD-B2* and *CAD-G* genes because of the activation of these genes in response to pathogen attack, and on the *cadC* and *cadD* single and *cad-C/cad-D* double mutants, as *CAD-C* and *CAD-D* are the primary genes for lignin biosynthesis (Sibout *et al*., 2005). *cadG*, *cadB1* and *cad1* plants behaved like wild-type plants in both incompatible and compatible interactions with *P. syringae* pv. *tomato*, as shown by the evaluation of bacterial colonization and the observation of disease symptoms in these mutants (Fig. 2). In contrast, *cad-D*, *cad-B2*, the double mutant *cad-C/cad-D* and, in certain cases, *cad-C* exhibited decreased resistance to both virulent and avirulent *P. syringae* pv. *tomato*. Interestingly, the double mutant *cad-C/cad-D* displayed even more decreased resistance than *cad-C* or *cad-D* single mutants in compatible and incompatible interactions (Figs 2 and S1, see Supporting Information). Bacterial colonization of *cad-C* and *cad-D* single mutants was 2–10-fold increased, whereas colonization of *cad-C/cad-D* was 5–60-fold increased when compared with the wild-type (Fig. 2A,B). The susceptibility of the double mutant is similar to the enhanced disease phenotype of the *eds1* mutant (Parker *et al*., 1996) in response to DC3000, and to the susceptible phenotype of the *La-er* ecotype in response to the strain containing the *avrPphB* avirulence gene. The *cadB2* mutant also displayed a clear phenotype, close to the phenotype of the double mutant *cad-C/cad-D*. Consistent with these data, *cad-C/cad-D* plants and, to a lesser extent, *cad-B2* plants, displayed markedly increased disease symptoms compared with the wild-type in the interaction with the two strains of *P. syringae* pv. *tomato* (Fig. 2C). Taken together, these results suggest that *CAD-C*, *CAD-D* and *CAD-B2* act as positive components of basal resistance and effector-triggered resistance to *P. syringae* pv. *tomato*.

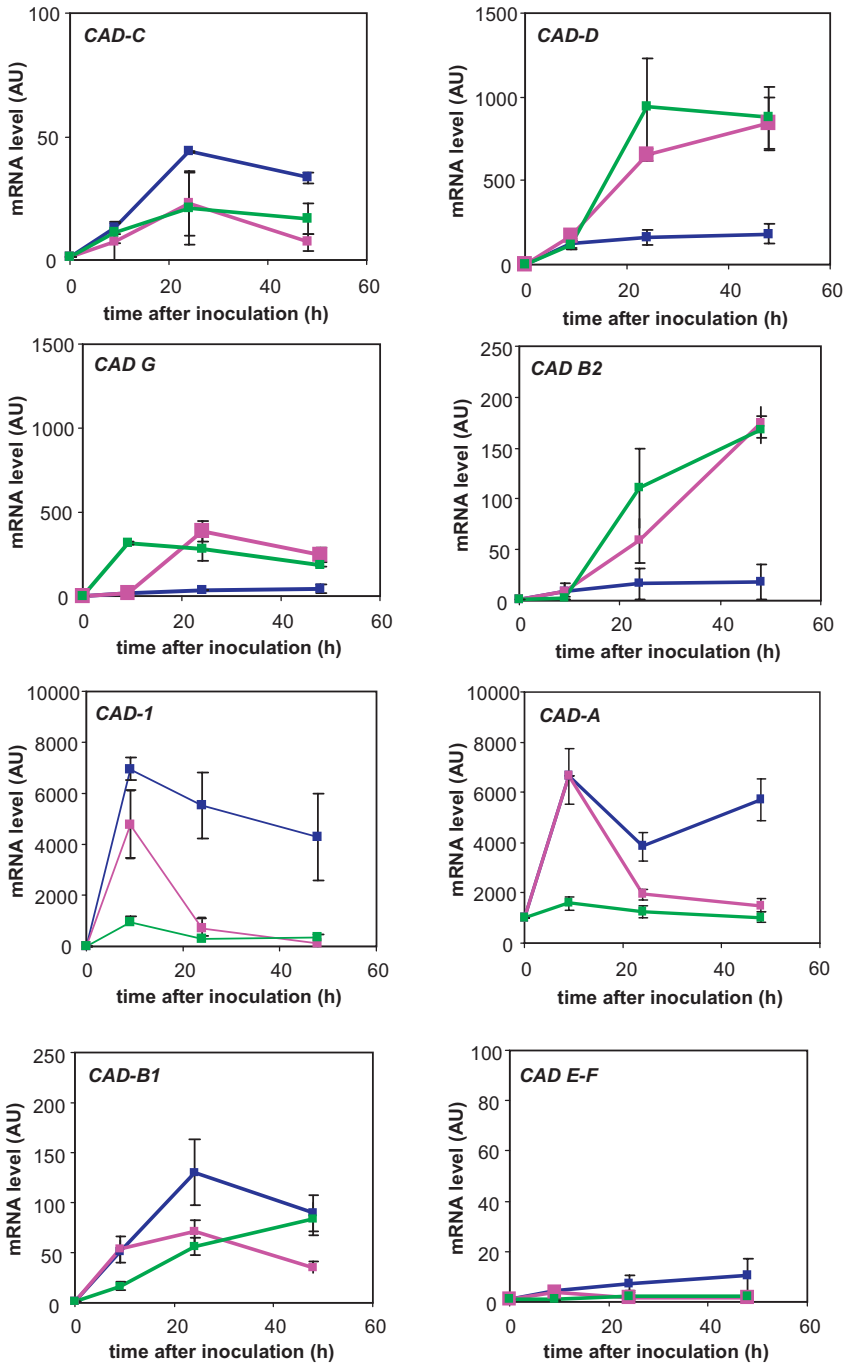

Fig. 1 Expression pattern of cinnamyl alcohol dehydrogenase (*CAD*) family members in response to pathogen inoculation. Gene expression level [determined by quantitative real-time polymerase chain reaction (Q-RT-PCR) analysis] in leaves of 4-week-old Arabidopsis plants inoculated with water (blue), *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (pink) and *Pst* DC3000/*avrRpt2* (green). The expression values of each gene were normalized using the expression level of *β-tubulin4* as an internal standard. The mean values and standard errors were calculated from two independent experiments. AU, arbitrary units.

Defence gene regulation is altered in the double mutant *cad-C/cad-D*

The balance between signalling components, such as ethylene (ET), jasmonates (JA) and salicylic acid (SA), is crucial for the modulation and adaptation of the various defence mechanisms to pathogens. For this reason, we investigated whether the decreased resistance phenotype of *cad-C/cad-D* plants is correlated with an alteration in these signalling pathways. For this purpose, we analysed the expression of defence marker genes in

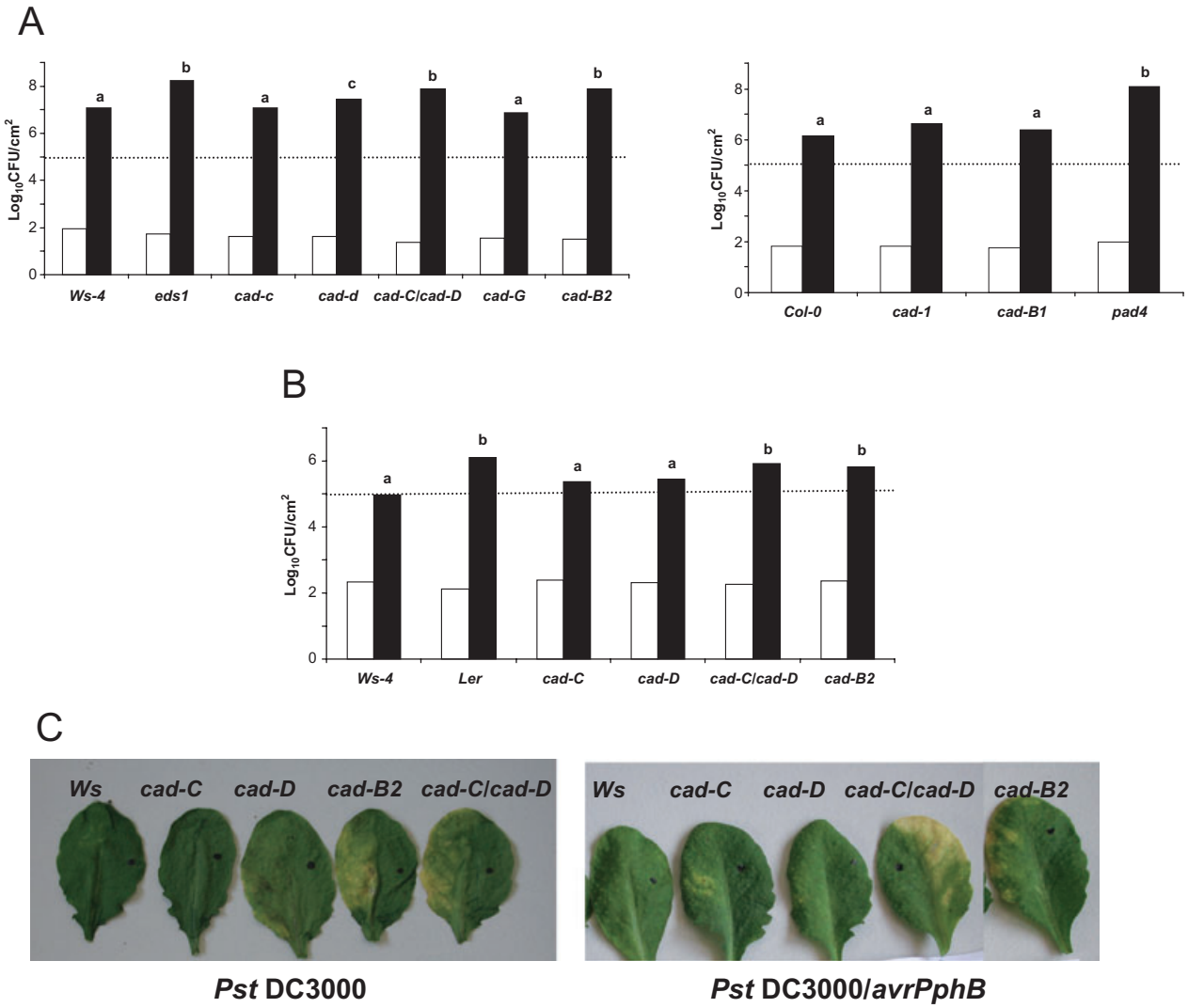

Fig. 2 Phenotypes of *cad-C*, *cad-D*, *cad-G*, *cad-B2*, *cad-1*, *cad-B1*, *eds-1*, *pad-4* single mutants, *cad-C/cad-D* double mutant and the wild-type ecotypes Ws and La-er, after inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*). Growth of *Pst* DC3000 (A) and *Pst* DC3000/*avrPphB* (B) in wild-type and mutant plants, determined at 0 days (white bars) and 3 days (black bars) post-inoculation with a bacterial suspension of 10^5 cfu/mL. The mean bacterial densities were calculated from 9–12 replicates and are representative of two independent experiments. According to analysis of variance (ANOVA) test, means of colony-forming units (cfu) do not differ significantly if they are indicated by the same lowercase letter. (C) Representative mutant and wild-type leaves 3 days after inoculation with a suspension of the bacterial strain indicated at a density of 10^7 cfu/mL.

the double mutant. Marker genes of the SA pathway, *ICS* (isochorismate synthase, involved in the biosynthesis of SA), *PR1* and *PR5* (pathogenesis-related), were tested. The expression level of *ICS* was induced on inoculation with virulent and avirulent *P. syringae* pv. *tomato* strains, and was significantly higher in the double mutant when compared with the wild-type (Fig. 3A). Surprisingly, the expression of *PR1* and, to a lesser extent, *PR5* was reduced in *cad-C/cad-D* in response to the virulent strain, suggesting a negative effect of the mutations on (or downstream of) SA production. No significant effect could be detected in response to the avirulent strain of *P. syringae* pv. *tomato*. The measurement of SA levels showed that SA production was also reduced in the double mutant when compared with the wild-type during the compatible interaction (not in response to the avirulent strain) (Fig. 3B). The expression of the marker gene for JA- and ET-dependent signalling pathways, *PR3* (pathogenesis-related), was also affected in the double mutant, but its expression was, as *ICS*, higher in the double mutant when compared with the wild-type. Hence, *CAD-C* and *CAD-D* depletion may be critical for the regulation of the defence signalling pathways, affecting negatively the SA pathway, at least in the context of the compatible interaction. As this pathway plays an

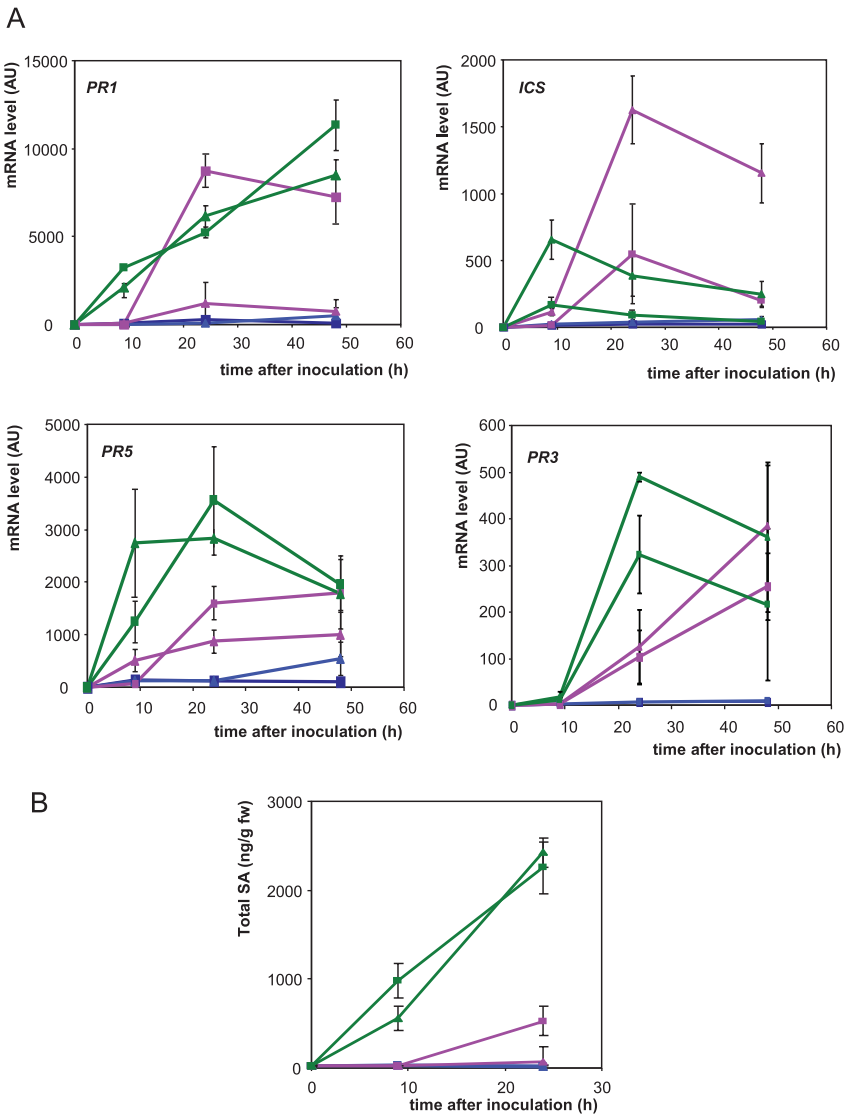

Fig. 3 Defence gene expression (A) and salicylic acid (SA) production (B) in the double mutant *cad-C/cad-D* (triangles) and wild-type (squares) after inoculation with water (blue), *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (pink) and *Pst* DC3000/avrRpt2 (green). (A) The expression values of each gene were normalized using the expression level of *β-tubulin4* as an internal standard. The mean values and standard errors were calculated from two independent experiments. AU, arbitrary units. (B) For SA measurement, the mean values and standard errors were calculated from two independent experiments.

essential role in resistance to *P. syringae* pv. *tomato*, this defect could explain, at least in part, the alteration in basal resistance observed previously (Fig. 2).

Effect of CAD-C and CAD-D depletion on the expression of the other members of the *CAD* gene family and of other lignin biosynthesis-related genes in response to *Pseudomonas*

To assess the specificity of the effect of the absence of *CAD-C* and *CAD-D* on lignin biosynthesis, we tested whether any alteration of the expression of the genes involved in lignin biosynthesis could occur in the double mutant in the context of the interaction with *P. syringae* pv. *tomato*. *CAD-B2* and *CAD-G*, previously demonstrated to be induced in response to *P. syringae* pv. *tomato*, were studied first (Fig. 4). In response to *P. syringae* pv. *tomato*, the expression of these two genes was differentially affected in the double mutant *cad-C/cad-D* when compared with the wild-type: *CAD-G* was over-expressed (about twofold increased) and *CAD-B2* was down-regulated (two- to three-fold decreased). For comparison, the expression profile of *CAD-B1* was similar in the double mutant and the wild-type. Finally, the analysis of other genes encoding proteins involved in lignin biosynthesis did not reveal substantial alterations (Fig. S2, see Supporting Information). Only *CCR-2*, which has been shown to be induced by pathogen infection (Lauvergeat *et al*., 2001), was slightly affected in the double mutant (Fig. 4).

Based on these findings, we can assume that the resistance and defence phenotypes of the double mutant *cad-C/cad-D* result from the primary effect of the absence of the two proteins CAD-C and CAD-D.

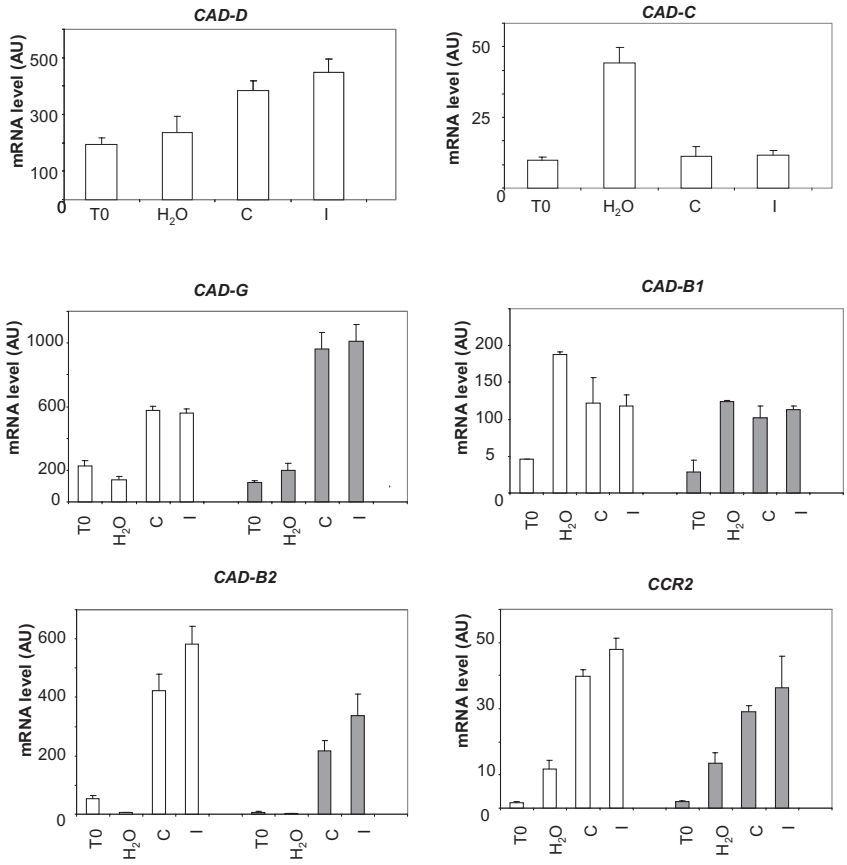

Fig. 4 Effects of *CAD-C* and *CAD-D* depletion on the expression of the other members of the cinnamyl alcohol dehydrogenase (*CAD*) gene family and of other lignin biosynthesis-related genes in response to *Pseudomonas syringae* pv. *tomato (Pst)*. Gene expression in the wild-type (white bars) and double mutant (gray bars), 0 h (T0) and 24 h after inoculation with water, *Pst* DC3000 (C) and *Pst* DC3000/*avrRpt2* (I). The expression values of each gene were normalized using the expression level of *β-tubulin4* as an internal standard. Mean expression values and standard errors were calculated from three replicates of a representative experiment.

DISCUSSION

Lignins and lignin-related compounds are known to be induced at sites of pathogen infection (Reimers and Leach, 1991). The biological significance of this lignin deposition is thought to be reinforcement of the cell wall, lignins being extremely resistant to microbial degradation. However, no genetic evidence has been clearly produced in favour of this hypothesis. In this article, we provide genetic and molecular evidence that: (i) some members of the *CAD* gene family are induced on pathogen attack; (ii) *CAD-C* and *CAD-D* genes, which have been demonstrated to be the primary genes involved in lignin biosynthesis, act as essential components of defence to virulent and avirulent strains of the bacterial pathogen *P. syringae* pv. *tomato*; and (iii) SA-dependent defence gene regulation, possibly as a consequence of the alteration of SA biosynthesis during a compatible interaction, is affected in the double mutant *cad-C/cad-D*.

Different studies have highlighted that CAD proteins are encoded by a multigene family comprising nine members in Arabidopsis (Sibout *et al*., 2003), corresponding to six *bona fide* catalytically active CADs and three displaying very low enzymatic activity (Kim *et al*., 2004). This suggests that some CADs may display alternative metabolic roles, for example in plant defence. This hypothesis is reinforced by the observation of the induced expression of such CADs on pathogen attack. Among the less active CADs (CAD-E, CAD-F, CAD-B1 and CAD-B2), CAD-B1 has been shown to be induced in response to *P. syringae* pv. *tomato* inoculation (Kiedrowski *et al*., 1992; Somssich *et al*., 1996). We confirmed this observation here and showed that the other less active CADs are not induced on *P. syringae* pv. *tomato* inoculation, suggesting other metabolic roles, probably in response to mechanical wounding or abiotic stresses, as suggested by their clear induction by the inoculation procedure. In the case of CAD-B2, the corresponding mutant exhibits decreased resistance to *P. syringae* pv. *tomato*, despite the absence of visible impact of this mutation on lignin biosynthesis (Eudes *et al*., 2006). As CADB2 acts as a benzyl alcohol dehydrogenase accepting various benzaldehyde substrates, the mutant phenotype can be interpreted as an absence of accumulation of compounds involved in defence.

Analysis of the expression pattern of the primary and most active CADs, *CAD-C* and *CAD-D*, showed that *CAD-C* is not induced by pathogen attack, whereas *CAD-D* is strongly induced 24 h post-inoculation. These results confirm and extend the data available in public databases from microarray analyses (Arabidopsis eFP Browser at bar.utoronto.ca) in response to diverse

pathogens, such as different strains of *Pseudomonas* or *Phytophthora infestans*. Interestingly, *CAD-D* is also induced by pathogen-associated molecular pattern (PAMP) treatment (flagellin flg22; Gomez-Gomez and Boller, 2002) and in response to an hrp$^-$ bacterial mutant (hrcC$^-$; Yuan and He, 1996) (NASCarrays, experiment reference number: -NASCarrays 120), suggesting that CAD-D induction is part of PAMP-triggered immunity, the plant's first active response to microbial perception, initiated on recognition of conserved microbial features by plant cell surface receptors (Chisholm *et al*., 2006). Moreover, the differential regulation of *CAD-C* and *CAD-D* genes in response to pathogen attack is reminiscent of the previous observations that *AtCCR1* and *AtCCR2* are differentially regulated in response to *Xanthomonas* inoculation (Lauvergeat *et al*., 2001), suggesting that, beyond their functional redundancy demonstrated previously (Sibout *et al*., 2005), and their similar pattern of expression during development (Kim *et al*., 2007), each *CAD* gene might have a specific role in development vs. pathogen responsiveness. However, the loss of either CAD-C or CAD-D function results in a partial (or no) loss of resistance to virulent and avirulent strains of *P. syringae* pv. *tomato*, and the depletion of both genes confers a clear susceptible phenotype. Similar observations were made in response to different avirulent strains of *P. syringae* pv. *tomato* (Fig. S1), suggesting a general and non-race-specific effect of the mutations. As is the case during development, these data confirm the functional redundancy of these genes in the context of plant–pathogen interactions, and demonstrate their essential role in plant defence. However, different possibilities, which are not exclusive, can be proposed to explain this role: (i) a direct role of lignin accumulation during plant defence, acting as a barrier to infection; (ii) a function in plant resistance for the accumulation of soluble aldehydes, and/or the absence of lignin-related compounds, observed in the double mutant (Rutten and Gocke, 1988; Sibout *et al*., 2005; Utama *et al*., 2002); or (iii) an indirect role of CAD-C and CAD-D as possible regulators of the SA defence pathways. Concerning the first possibility, histological and biochemical analyses were performed as described previously by Sibout *et al*. (2005) on inoculated leaves of the wild-type and the double mutant *cad-C/cad-D* (data not shown). Unfortunately, these analyses did not enable this point to be clarified, probably because of the very small amounts of lignins accumulated at the inoculation sites, and the limited number of cells influenced by this process. However, we observed that both SA production and the SA defence pathway are altered in the *cad-C/cad-D* mutant, at least in the case of the compatible interaction, which is in good agreement with the role of SA as a central regulator of defence to *P. syringae* pv. *tomato* in Arabidopsis. In the case of the incompatible interaction, another hypothesis, including a possible delay in SA accumulation in the mutant when compared with the wild-type, can be envisaged. Surprisingly, for the compatible interaction, although genetic and biochemical evidence demonstrated that, in *A. thaliana*, the biosynthetic pathway for SA is dependent on ICS (Nawrath and Metraux, 1999; Strawn *et al*., 2007; Wildermuth *et al*., 2001), we observed here that *ICS* expression was induced at high levels in the double mutant, but did not lead to SA biosynthesis. Different hypotheses can be proposed to explain this result: (i) some metabolites produced in the double mutant could interfere with SA biosynthesis downstream of ICS expression (inhibition of ICS activity or alteration of downstream enzymes); or (ii) SA could be synthesized *via* a derived route, possibly through AtCM1 (chorismate mutase 1) and phenylalanine, which could be altered in the double mutant. Previous studies in different plant species have indicated that SA can be synthesized from phenylalanine. However, although *AtCM1* is induced by bacterial pathogens (Eberhard *et al*., 1996; Mobley *et al*., 1999) and has a high reported K_m for chorismate (Mobley *et al*., 1999), the dependence of the SA biosynthetic pathway on ICS is well established in *A. thaliana* (Wildermuth *et al*., 2001) and, more recently, in tobacco (Catinot *et al*., 2008). In addition, phenylalanine ammonia lyase (PAL) expression has been shown to be unaffected in the double mutant when compared with the wild-type (data not shown). Thus, we propose that CAD-C and CAD-D act as components of defence mechanisms, primarily as key effectors of lignin biosynthesis, and, secondarily, as possible regulators of the SA defence pathways.

At this point, it should be noted that, in mutants impaired in cellulose synthesis and displaying similar phenotypic characteristics (Cano-Delgado *et al*., 2003; Hernandez-Blanco *et al*., 2007; Turner and Somerville, 1997), disease resistance and JA levels are enhanced. This phenotype is interpreted as an alteration of cell wall integrity, resulting in the activation of novel defence pathways. These defence pathways probably include lignin biosynthesis. Thus, the alteration of lignin biosynthesis has an opposite effect on defence when compared with the reduction in cellulose synthesis.

EXPERIMENTAL PROCEDURES

Plant material and pathogen infection

Arabidopsis plants were grown under controlled conditions (Lacomme and Roby, 1996). Depending on the experiment, we used the wild-type ecotypes Col-0, Ws-4 and La-er, and the mutants *cad-C* (*Ws-4*), *cad-D* (*Ws-4*) (Sibout *et al*., 2003), *cad-C/cad-D* (*Ws-4*) (Sibout *et al*., 2005), *cad-B1*(*Col-0*), *cad-B2* (*Ws-4*), *cad-1*(*Col-0*) and *cad-G* (*Ws-4*) (Eudes *et al*., 2006), *eds1* (*Ws-0*) (Parker *et al*., 1996) and *pad-4* (*Col-0*) (Glazebrook *et al*., 1997).

Plant inoculations, using *P. syringae* pv. *tomato* strain DC3000 carrying the *avrPphB*, *avrRpt2* or *avrRpm1*gene, and *in planta* bacterial growth analysis were performed as described pre-

viously (Lorrain *et al*., 2004). Briefly, the ecotypes Ws-4 and Col-0 behave similarly to these strains (susceptible to DC3000 and resistant to DC3000/*avrPphB*), whereas the ecotype La-er is susceptible to the strain containing *avrPphB*. The virulent and avirulent *P. syringae* pv. *tomato* strains were grown at 28 °C on King B medium supplemented with the appropriate antibiotics. Four- or five-week-old plants were used for bacterial inoculation. For this objective, they were kept at high humidity 12 h before the experiments and then infiltrated with a bacterial suspension of 10^5 colony-forming units (cfu)/mL for the determination of *in planta* bacterial growth, and 10^7 cfu/mL for gene expression analysis.

Gene expression analysis

Total RNA extraction and Q-RT-PCR were performed as described by Bouchez *et al*. (2007). Total RNA extraction was performed from leaves with a Nucleospin RNA kit following the manufacturer's instructions (Macherey-Nagel, Hoerdt, France). Total RNA (1.5 μg) was subjected to cDNA synthesis (Superscript II reverse transcriptase, Invitrogen, Cergy Pontoise, France), which was used as template in the Q-PCR analysis. Q-PCR was performed using gene-specific primers (Table S1), LightCycler reagents and apparatus (Roche Diagnostics, Meylan, France). Q-PCRs were performed with two independent biological assays. *β-tubulin4* and a gene (At2g28390) whose expression has been shown to be extremely stable under different physiological conditions (Czechowski *et al*., 2005) were used as biological controls. The expression values of individual genes were normalized using the expression level of *β-tubulin4* as a control, and the data were expressed for each point as the fold induction compared with the wild-type Ws-4 (arbitrary units). The mean expression values were calculated from the results of two independent experiments. The same results were obtained after normalization with At2g28390 (data not shown).

SA extraction and analysis

Three hundred milligrams of tissue were ground in liquid nitrogen, and 50 ng of an internal standard (*o*-anisic acid, oANI) were added before the extraction. Total SA (free SA plus SA conjugates) was extracted and analysed according to Mercier *et al*. (2001) with some modifications. After extraction, analysis was performed with a high-performance liquid chromatography (HPLC) apparatus (Ultimate 3000, DIONEX SA, Voisins-le-Bretonneux, France). Total SA and oANI (internal standard) were quantified with a spectrofluorimeter (Jasco FP-920 Bouguenais, France), with excitation and emission wavelengths of 305 and 365 nm, respectively. The data were analysed using Chromeleon 6.8 chromatography software (DIONEX SA). Experiments were repeated twice and the data represent the mean values ± standard error.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phenotypes of *cad-C* and *cad-D* single mutants, *cad-C/cad-D* double mutant and the wild-type ecotype Ws after inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*). Growth of *Pst* DC3000/*avrRpt2* (A) and *Pst* DC3000/*avrRpm1* (B) in wild-type and mutant plants, determined 0 days (white bars) and 3 days (black bars) post-inoculation with a bacterial suspension of 10^5 cfu/mL. The mean bacterial densities were calculated from 9–12 replicates and are representative of two independent experiments. (C) Representative mutant and wild-type leaves 3 days after inoculation with a suspension of DC3000/*avrRpt2* at a density of 10^7 cfu/mL.

Fig. S2 Quantitative real-time polymerase chain reaction (Q-RT-PCR) analysis of lignin biosynthesis-related gene expression in Arabidopsis wild-type plants (squares) and in the double mutant *cad-C/cad-D* (triangles) after inoculation with water (blue), *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (pink) and *Pst* DC3000/*avrRpt2* (green). The expression values of each gene were normalized using the expression level of *β-tubulin4* as an internal standard. These results are representative of two independent experiments.

Table S1 Primers used for real-time polymerase chain reaction experiments.

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