

# *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development

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*Pseudomonas syringae* relies on type III secretion system to deliver effector proteins into the host cell for parasitism. Type III genes are induced *in planta*, but host factors affecting the induction are poorly understood. Here we report on the identification of an *Arabidopsis* mutant, *att1* (for aberrant induction of type three genes), that greatly enhances the expression of bacterial type III genes *avrPto* and *hrpL*. *att1* plants display enhanced disease severity to a virulent strain of *P. syringae*, suggesting a role of *ATT1* in disease resistance. *ATT1* encodes CYP86A2, a cytochrome P450 monooxygenase catalyzing fatty acid oxidation. The cutin content is reduced to 30% in *att1*, indicating that CYP86A2 plays a major role in the biosynthesis of extracellular lipids. *att1* has a loose cuticle membrane ultrastructure and shows increased permeability to water vapor, demonstrating the importance of the cuticle membrane in controlling water loss. The enhanced *avrPto-luc* expression is specific to *att1*, but not another cuticle mutant, *wax2*. The results suggest that certain cutin-related fatty acids synthesized by CYP86A2 may repress bacterial type III gene expression in the intercellular spaces.

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## Introduction

The ability of plant-associated microbes to sense the host environment is crucial for their successful establishment in plants. Plant-sensing triggers virulence/pathogenicity gene expression, enabling the microbes to suppress, tolerate, or evade host defenses and exploit nutrients for their multiplication (Lee and Camilli, 2000). Conversely, plant extra-

cellular factors affecting bacterial gene expression, either positively or negatively, may play important roles in disease susceptibility or resistance.

Many Gram-negative plant bacterial pathogens, including *Pseudomonas syringae*, *Xanthomonas*, *Erwinia*, and *Ralstonia solanacearum*, use the type III secretion system (TTSS) to deliver a repertoire of effector proteins into their host cells for parasitism (Galan and Collmer, 1999; He and Jin, 2003). These bacteria sense host-derived signals to activate coordinately genes encoding TTSS and effectors. However, host signals and bacterial mechanisms that regulate these genes are poorly understood.

Certain carbon sources and amino acids are known to affect type III gene expression in culture media (Huynh *et al*, 1989; Arlat *et al*, 1992; Rahme *et al*, 1992; Wei *et al*, 1992). However, it is unclear if these truly mimic the apoplast environment of plants, and little is known about biochemical and physical features of the apoplast involved in type III gene regulation. It is therefore imperative to use living plants for host signal studies.

Animal bacterial type III genes are activated upon contact with host cells (Francis *et al*, 2002), first described in *Yersinia pseudotuberculosis* (Pettersson *et al*, 1996) and later in *Pseudomonas aeruginosa* (Vallis *et al*, 1999), *Yersinia enterocolitica* (Stainier *et al*, 1997), and *Shigella flexneri* (Demers *et al*, 1998). In *Y. pseudotuberculosis*, the contact with HeLa cells is believed to trigger the release of LcrQ, a TTSS secreted protein that negatively regulates other type III gene expression (Pettersson *et al*, 1996). Plant bacterial type III genes are also induced by the host. However, the molecular basis of the contact-dependent gene induction remains largely unknown.

A series of recent studies elegantly demonstrated that *R. solanacearum* type III genes are induced when the bacterium is in direct contact with the plant cell (Aldon *et al*, 2000). The induction is mediated by PrhA, a membrane protein with significant similarities with siderophore receptors (Marenda *et al*, 1998). The *prhA* mutation does not affect the induction by an inducing medium, suggesting distinct signaling mechanisms in the perception of signals derived from host cell and culture medium. Additional components acting downstream of PrhA are PrhR, PrhI, PrhJ, HrpG, and HrpB, which function in a sequential order (Brito *et al*, 1999, 2002). The plant signal perceived by PrhA is likely a nondiffusible wall component that is resistant to protease and heat (Aldon *et al*, 2000), but the chemical nature of the signal remains unknown.

The cuticle envelops the aerial parts of the plant. It serves as a protective barrier that prevents water and solute losses (Jenks, 2002) and pathogen penetration (Jenks *et al*, 1994). The cuticle is composed of two basic layers, an outermost epicuticular wax layer and an underlying cuticle membrane layer (Jeffree, 1996). In addition to the external epidermal surface, the cuticle membrane extends into the substomatal chamber (Esau, 1977). This notion is potentially interesting,

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because the substomatal chamber is where bacteria reside immediately after entering the stomatal opening. The cuticle membrane is primarily an insoluble polyester of hydroxyl and epoxy hydroxyl fatty acids (Kolattukudy, 1981), whereas the epicuticular waxes and intracuticular waxes (embedded in the cutin matrix) are primarily soluble long-chain aliphatic molecules. Cutin content has been analyzed in a number of plant species (Kolattukudy, 2001), but not *Arabidopsis*. Typical cutin monomers reported in the literature are 10,16-dihydroxy hexadecanoic acid, 18-hydroxy-9,10 epoxy octadecanoic acid, and 9,10,18-trihydroxy octadecanoic acid. Diacids, monohydroxy diacids, and unsaturated acids have also been reported (Holloway, 1982). While biochemical studies have established the cutin biosynthetic pathway, genes encoding the key enzymes have not been unequivocally identified (Kolattukudy, 2001; Nawrath, 2002).

Here we describe the use of a genetic approach to investigate host signals involved in the regulation of *Pseudomonas* type III gene expression. We developed a highly sensitive reporter assay for *P. syringae* type III gene expression in plants. As a first step to understand host factors affecting bacterial type III gene expression, we identified a genetic locus in *Arabidopsis*, named *ATT1* (for *Aberrant* induction of *Type Three* genes), that negatively regulates the expression of type III effector gene *avrPto* and regulatory gene *hrpL*. The latter encodes an ECF family alternate  $\sigma$  factor that recognizes the 'hrp box' motif conserved in the promoters of type III genes including *avrPto* (Salmeron and Staskawicz, 1993; Xiao *et al*, 1994). *ATT1* encodes CYP86A2, a cytochrome P450 monooxygenase catalyzing fatty acid oxidation. *att1* contains only 30% of the cutin in the wild-type plants and has a loosely structured cuticle membrane. The results led us to suggest that CYP86A2 is involved in the biosynthesis of hydroxylated fatty acids that function in both cuticle development and repression of bacterial type III gene expression.

## Results

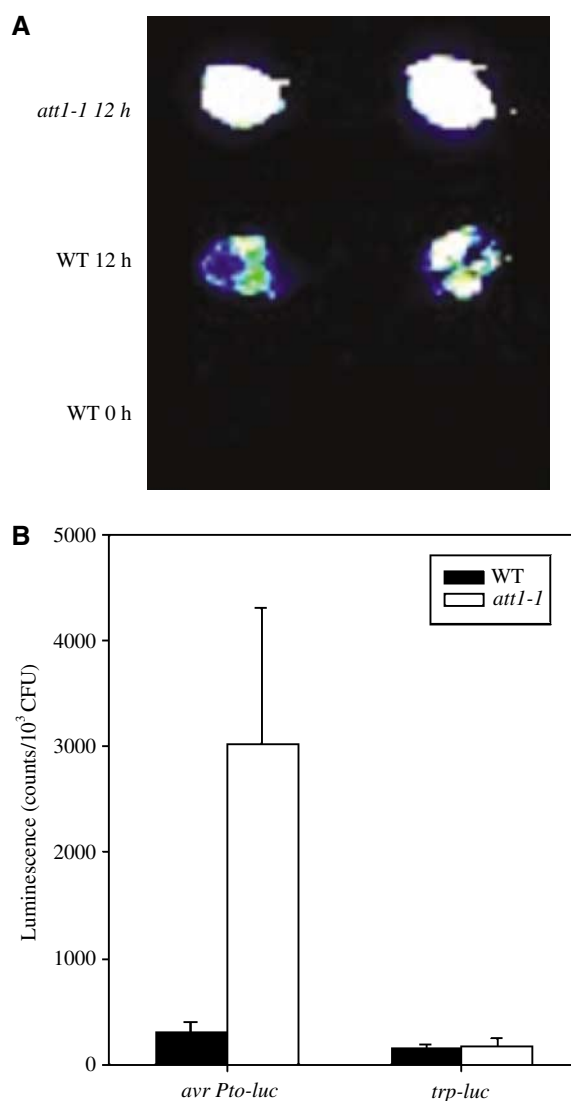
### Isolation of *att1* mutant by using *avrPto-luc* reporter gene

The *avrPto* gene of *P. syringae* pv. *tomato* contains a typical *hrp* box in the promoter and is induced strongly *in planta* (Salmeron and Staskawicz, 1993). *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato* DC3000 strains carrying *avrPto-luc* were constructed to monitor noninvasively the activation of a type III gene promoter *in planta*. The reporter strains displayed strong luciferase activity when infiltrated into *Arabidopsis* plants, which can be detected as luminescence by a cooled charge-coupled device (CCD). *P. syringae* pv. *phaseolicola* is a nonhost strain, whereas DC3000 is a virulent strain on *Arabidopsis*. Both strains displayed similar induction of *avrPto-luc*, consistent with the finding that the host signal(s) for type III genes are not determinants of host specificity (Rahme *et al*, 1992).

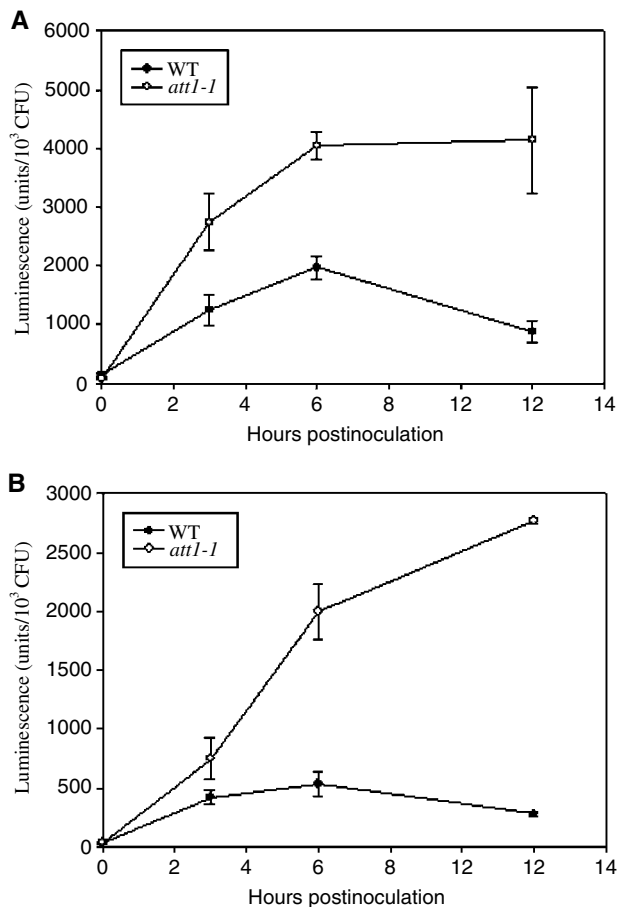
In an attempt to identify host factors involved in type III gene induction, we screened for *Arabidopsis* mutants displaying altered induction of *luc*. The use of *P. syringae* pv. *phaseolicola* (*avrPto-luc*), which is unable to multiply in *Arabidopsis*, avoids potential confusion between bacterial growth with *avrPto-luc* transcription. From approximately 4000 ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis* Col-*gl* M<sub>2</sub> plants, a single mutant that hyperin-

duced the *avrPto-luc* reporter gene was isolated (Figure 1A). This mutant is named *att1-1* (for aberrant induction of a type three gene). *att1-1* plants are morphologically identical to wild-type plants when grown in the growth chamber. At 12 h after infiltration, *avrPto-luc* was 10 times more active in *att1-1* than in wild-type plants. In contrast, the constitutive reporter gene *trp-luc* was not affected in this mutant (Figure 1B), suggesting that the effect of *att1-1* was specific to the type III gene.

We next examined the kinetics of *avrPto-luc* induction in wild-type and *att1-1* plants. Figure 2A shows that while the wild-type plants transiently activated *avrPto-luc* in the bacterium, the *att1-1* mutant enabled a much greater and prolonged induction of *avrPto-luc*. The observed values (normalized to leaf bacterial numbers) reflect the reporter gene expression,



**Figure 1** *att1-1* specifically enhances type III gene expression. (A) CCD image of *avrPto-luc* expression in wild-type Col-*gl* (WT) and *att1-1* leaves. (B) Activity of *avrPto-luc* and *trp-luc* in wild-type Col-*gl* (WT) and *att1-1* leaves. Plants were inoculated with *P. syringae* pv. *phaseolicola* carrying *avrPto-luc* or *trp-luc*, and leaves were detached at 0 or 12 h for luciferase assay. Error bars indicate standard errors. The experiments were repeated three times with similar results.

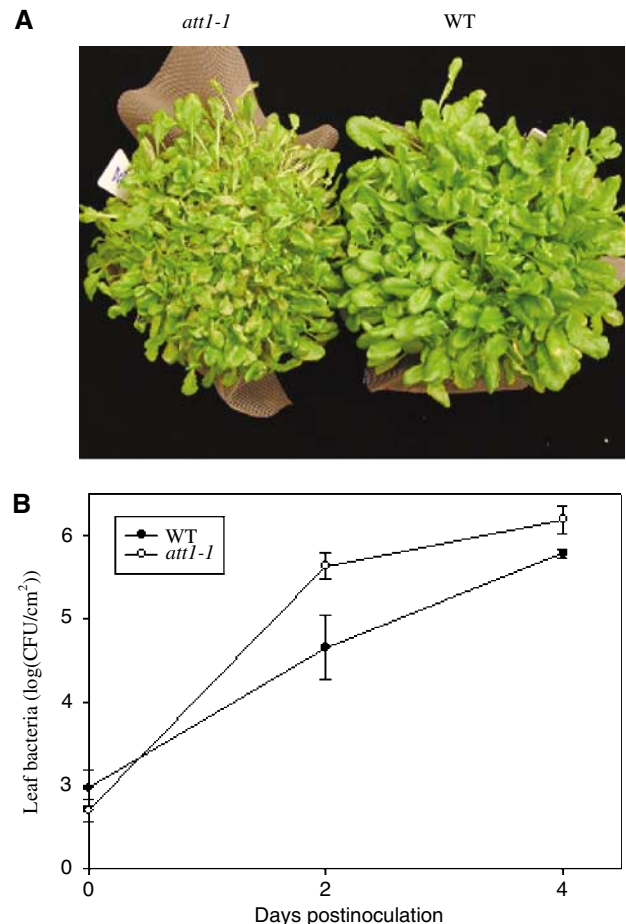


**Figure 2** Kinetics of type III gene expression in wild-type *Col-gl* (WT) and *att1-1* leaves. Plants were inoculated with *P. syringae* pv. *phaseolicola* carrying *avrPto-luc* (A) or *hrpL-luc* (B), and leaves were detached at the indicated times for luciferase assay. Error bars indicate standard errors. The experiments were repeated three times with similar results.

because bacteria did not grow in either wild-type or *att1-1* plants (Supplementary Figure S1; data not shown). We also determined if *att1-1* affected the expression of *hrpL-luc* in the plant. Similar to *avrPto-luc*, *hrpL-luc* was strongly induced in wild-type plants, and the induction was dramatically enhanced and long lasting in *att1-1* (Figure 2B). The differential induction in *att1-1* and wild-type plants was also observed in DC3000 (data not shown).

#### ***att1* enhances disease severity in plants**

To determine if the increased type III gene induction altered disease susceptibility, we dip-inoculated plants with DC3000 (Figure 3A). Typical disease symptoms were observed in wild-type plants, but they were much more severe in *att1-1* plants. A small but reproducible increase of bacterial growth (~5-fold) was observed in *att1-1* plants 2–4 days after dipping inoculation (Figure 3B). However, the increased bacterial growth was not observed when plants were inoculated by syringe infiltration (Supplementary Figure S1). The cause of different bacterial growth results between dipping and infiltration inoculations is not understood, but similar results have also been reported for strains of DC3000 lacking the phytotoxin coronatine (Mittal and Davis, 1995).

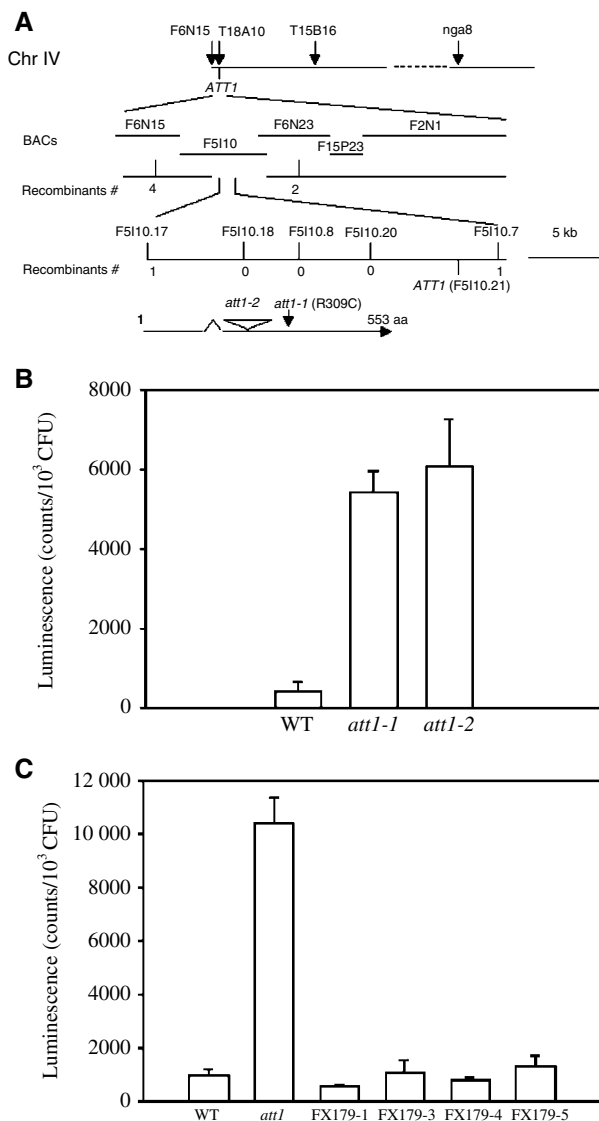


**Figure 3** *att1* displays enhanced disease severity. (A) Disease symptoms. (B) Bacterial growth assay. Wild-type *Col-gl* (WT) and *att1-1* plants were dip-inoculated with DC3000 at  $1 \times 10^8$  CFU/ml. Disease symptoms were photographed 7 days after inoculation. Leaf bacterial number was determined at 0 and 4 days after inoculation. The experiment was repeated twice with similar results.

We also inoculated *att1-1* plants with the nonhost strain *P. syringae* pv. *phaseolicola* and the incompatible strain DC3000 (*avrB*) by infiltration. No difference in bacterial growth (Supplementary Figure S1) or hypersensitive response was observed between *att1-1* and wild-type plants, indicating that the enhanced type III gene induction by itself does not convert the plant from resistant to susceptible. This is consistent with a previous report that the expression level of type III gene does not determine host range (Rahme *et al*, 1992).

#### **Isolation of *ATT1***

To determine the nature of mutation, the *att1-1* mutant was back-crossed with wild-type *Col-0* plants. All five *F*<sub>1</sub> plants tested supported normal *avrPto-luc* induction. *F*<sub>2</sub> plants segregated in a 1:3 ratio (17 mutant to 55 wild-type,  $\chi^2 = 0.07$ ), indicative of a single recessive mutation. The *att1-1* mutant was crossed with ecotype Nossen to generate an *F*<sub>2</sub> mapping population. Simple sequence-length polymorphism (SSLP) markers located *ATT1* to the top of chromosome IV. Further mapping using approximately 1000 *F*<sub>2</sub> plants placed *ATT1* on the BAC clone F5I10 in a 50 kb interval containing six genes (Figure 4A). Sequence analysis of the six genes in the *att1-1* mutant revealed a single nucleotide substitution (C to T at



**Figure 4** Map-based cloning of *ATT1*. (A) Physical mapping of *ATT1*. *ATT1* was mapped to the upper arm of chromosome IV and positioned on the BAC clone F5110. (B) T-DNA insertion in *att1* phenotype. Col-*gl* (WT), *att1-1*, and the T-DNA line (Salk-005826; *att1-2*) were infiltrated with *P. syringae* pv. *phaseolicola* carrying the *avrPto-luc* reporter. Relative LUC activity was measured 12 h after inoculation. Error bars represent standard error. (C) At4g00360 complements the *att1-1* mutation. An 8 kb *Pvu*II fragment containing At4g00360 was mobilized from the BAC clone F5110 to pBI121 (by replacing the 35S-*GUS* fragment) and transformed into *att1-1* mutant plants. Four of the five putative primary transgenic plants tested contained the transgene. These plants (FX179-1, -3, -4, -5) were tested for *avrPto-luc* activation and compared with wild-type and untransformed *att1-1* plants. Error bars indicate standard error.

nucleotide 157 143 of chromosome IV) in the At4g00360 gene, causing a single amino-acid substitution (R309C). No mutation was found in the remaining five genes. At4g00360 contains a single intron and encodes a protein of 553 amino acids. To further confirm if this mutation is responsible for the *att1-1* phenotype, we identified a T-DNA insertion line, Salk\_005826, from the Salk T-DNA database. This line contained a T-DNA insertion disrupting the second exon of At4g00360 and supported identical *avrPto-luc* induction compared to *att1-1* (Figure 4B), providing further evidence that

At4g00360 was the *ATT1* gene. The allele in the T-DNA line is thus named *att1-2*. To assign unequivocally At4g00360 as *ATT1*, we introduced an 8 kb *Pvu*II fragment containing At4g00360 into *att1-1* by *Agrobacterium*-mediated transformation. Four transgenic plants were tested for the *avrPto-luc* induction *in planta*, and all displayed normal expression of *avrPto-luc*, demonstrating that At4g00360 is *ATT1* (Figure 4C).

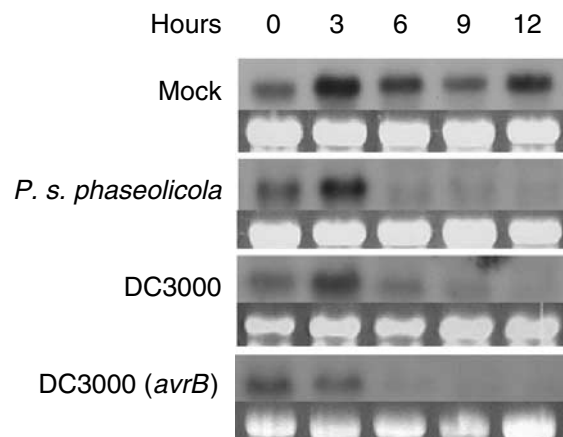
### *ATT1* mRNA is repressed by bacterial infection

We next determined if the *ATT1* gene expression is affected by bacterial infection. Northern analyses indicated that the *ATT1* transcripts were nonspecifically induced at 3 h by buffer infiltration (Figure 5). However, inoculation with *P. syringae*, regardless of whether compatible, incompatible, or nonhost strains were used, repressed the gene expression at later time points.

### *ATT1* encodes a putative fatty acid hydroxylase

At4g00360 encodes the cytochrome P450 monooxygenase CYP86A2. For consistency, the *ATT1* gene is referred to as CYP86A2 in the rest of the paper. *Arabidopsis* contains 246 cytochrome P450 genes, which are divided into different families based on sequence and function (Werck-Reichhart *et al*, 2002). The *Arabidopsis* CYP86 family is highly homologous to the animal and fungal P450 families CYP52 and CYP4, which catalyze the metabolism of fatty acids and alkanes through  $\omega$ -hydroxylation (Werck-Reichhart *et al*, 2002; Figure 6). CYP86A2 shows 87% amino-acid sequence identity with CYP86A8 and 73% with CYP86A1. Both CYP86A1 and CYP86A8 catalyze the  $\omega$ -hydroxylation of C<sub>12</sub> to C<sub>18</sub> saturated and unsaturated fatty acids *in vitro* (Benevise *et al*, 1998; Wellesen *et al*, 2001). Thus these results suggest that CYP86A2 is a fatty acid  $\omega$ -hydroxylase.

The *cyp86a2-1* (R309C) is phenotypically indistinguishable from *cyp86a2-2*, a likely null mutant because of an early truncation caused by the T-DNA insertion. R309 is a variant residue in the oxygen binding/activation motif of P450 proteins (AGRDTs; Werck-Reichhart *et al*, 2002), but is conserved among members of the CYP86A subfamily. This



**Figure 5** Bacteria repress *ATT1* expression. Wild-type plants were infiltrated with water (mock) or the indicated bacterial strains at a concentration of  $2 \times 10^6$  CFU/ml, and tissues were harvested at the indicated times for RNA isolation. RNA blots were hybridized with a radiolabeled *ATT1* probe. The rRNA gel pictures indicate equal loading of RNA in lanes. The Northern analysis was repeated three times with similar results.



Figure 6 Sequence alignment of *ATT1* with other CYP86A subfamily members. Sequences underlined with a solid line, a dashed line, and a double line indicate oxygen binding and activation motif, ERR triad, and heme binding motif, respectively.

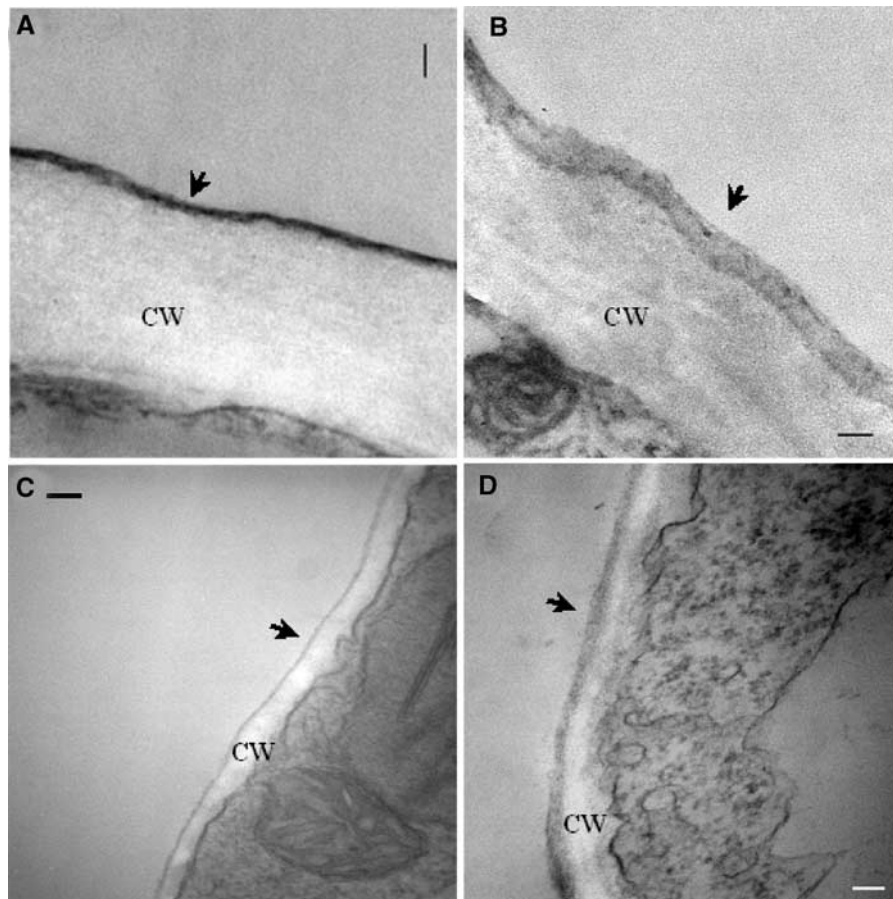
suggests that R309 plays a critical role specific to the CYP86A subfamily.

**CYP86A2 is required for the biosynthesis of cutin and cuticle development**

ω-Hydroxy fatty acids are exported to outside of plant cells through an unknown mechanism. The most abundant form of plant extracellular lipids in leaves is cutin, which is formed upon interesterification of ω-hydroxy fatty acids (Kolattukudy, 2001). We therefore tested if CYP86A2 is involved in the synthesis of hydroxy fatty acids *in vivo* by examining leaf cuticle membrane structure of *cyp86a2-1* using transmission electron microscopy. Figure 7A and B shows that the *cyp86a2-1* epidermis had a cuticle membrane that was less osmiophilic, as indicated by the reduced electron density, but more than twice as thick compared with that of wild type. Osmium tetroxide stains lipid components. The lower electron density in *cyp86a2-1* suggests a reduction of fatty acids in the cuticle membrane. Interestingly, we found that the *Arabidopsis* cuticle membrane covered cells surrounding the entire substomatal chamber including mesophyll cells, although it was much thinner than that on the epidermal surface. This mesophyll cuticle membrane of

*cyp86a2* was more than twice as thick as that in the wild-type plants (Figure 7C and D), indicating that the defects are not limited to epidermal cells. Similar observations were made in *cyp86a2-2* (data not shown).

We next determined cutin composition in the wild-type and *cyp86a2-1* plants. The cuticle membrane consists of both hydrolyzable cutin polymer and nonhydrolyzable fraction, named cutan. Our studies revealed that leaf cutin was altered in the *cyp86a2-1* plants. However, a protocol for isolation of *Arabidopsis* leaf cutin in amounts needed for complete cutin monomer analysis has yet to be developed. Therefore, the more abundant cutin of *Arabidopsis* inflorescence stem was examined. Table I shows that the most abundant cutin monomer in wild-type *Arabidopsis* stem was hexadecane-1,16-dioic acid (49.3%) followed by 10,16-dihydroxy hexadecanoic acid (16.4%), octadecane-1,18-dioic acid (11.8%), 7-hydroxy hexadecane-1,16-dioic acid (11.3%), 10(9)-hydroxy heptadecanoic acid (3.9%), 16-hydroxy hexadecanoic acid (3.2%), and 9-hydroxy pentadecanoic acid (2.6%). In *cyp86a2-1*, the total cutin monomers per surface area were reduced by ~70% compared with that in the wild-type plants. Except for octadecadien-1,18-dioic acid, all monomers were significantly reduced in *cyp86a2* (31–78% less than the



**Figure 7** *cyp86a2-1* displays altered cuticle membrane ultrastructure. Ultrastructure of the wild-type *Col-gl* (A, C) and the *cyp86a2-1* mutant (B, D) cuticle membrane from an epidermal cell (A, B) and substomatal chamber mesophyll cell (C, D). The cuticle (arrowhead) and cell wall (CW) are shown. Note the lower electron density of the *cyp86a2-1* cuticle membrane, which was found in all samples examined, compared with the dense, compact cuticle in the wild-type plant. Bar equals 200 nm.

**Table I** Stem cutin monomer amount ( $\mu\text{g}/\text{dm}^2 \pm \text{s.d.}$ ) and percent cutin monomers ( $\% \pm \text{s.d.}$ )

Cutin monomer	Columbia		<i>att1-1</i>	
	Amount	Percent	Amount	Percent
9-Hydroxy pentadecanoic acid	1.3 ± 0.4	2.6 ± 0.3	0.7 ± 0.1	4.3 ± 1.0
10(9)-Hydroxy heptadecanoic acid	1.9 ± 1.1	3.9 ± 2.3	0.7 ± 0.3	4.3 ± 1.9
16-Hydroxy hexadecanoic acid	1.6 ± 0.3	3.2 ± 0.2	1.1 ± 0.3	6.4 ± 1.6
10,16(9,16)-Dihydroxy hexadecanoic acid	8.1 ± 2.6	16.4 ± 3.5	3.5 ± 1.1	21.3 ± 5.3
Hexadecane-1,16-dioic acid	24.1 ± 3.5	49.3 ± 1.2	5.4 ± 0.1	33.6 ± 2.6
7(8)-Hydroxy hexadecane-1,16-dioic	5.5 ± 0.7	11.3 ± 0.6	2.2 ± 0.3	13.7 ± 2.7
Octadecane-1,18-dioic acid	5.8 ± 1.1	11.8 ± 0.3	1.8 ± 0.1	11.1 ± 1.5
Octadecadien-1,18-dioic acid	0.7 ± 0.1	1.4 ± 0.1	0.8 ± 0.1	5.3 ± 0.7
Total	49.1 ± 8.2		16.1 ± 1.1	

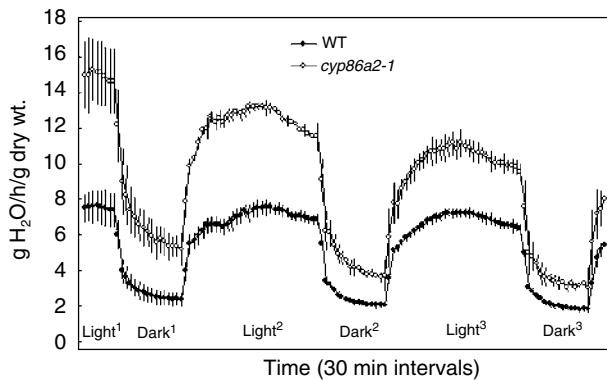
Cuticle was enzymatically isolated from the first three internodes of the main stem of 35-day-old plants (three replicates). Each replicate includes 12–14 individual plants pooled together. Isomers are listed in parentheses. Position of the two double bonds in octadecadien-1,18-dioic acid was not determined.

wild type). The results indicated a broad effect of *cyp86a2-1* on cutin biosynthesis. The ratio of cutin/cutan largely remained unchanged, suggesting that both cutin and cutan were proportionally reduced in *cyp86a2-1* (Table II).

*cyp86a2* mutants showed greater sensitivity to dehydration. Detached leaves from the *cyp86a2-1* mutant wilted much faster than did wild-type leaves, indicating a higher epidermal permeability of *cyp86a2-1* to water vapor than that

of wild-type (data not shown). Whole-plant in-pot transpiration analysis showed that *cyp86a2-1* plants had higher transpiration rates than did wild-type plants in both light and dark environments (Figure 8). The *cyp86a2-1* mutant had normal stomatal opening and closing responses to photoperiod. However, *cyp86a2-1* had ~2-fold higher water loss rate relative to wild-type both in the light and dark, when stomata were closed, indicating that a higher permeability of cuticle





**Figure 8** *cyp86a2-1* has a higher transpiration rate. Whole flowering plant in-pot transpiration rates were measured for consecutive 16 h light and 8 h dark photoperiods. Error bars indicate standard deviation.

rather than stomatal pore was the primary cause for the increase in the transpiration rate.

### The *wax2* mutant does not show enhanced *avrPto-luc* expression

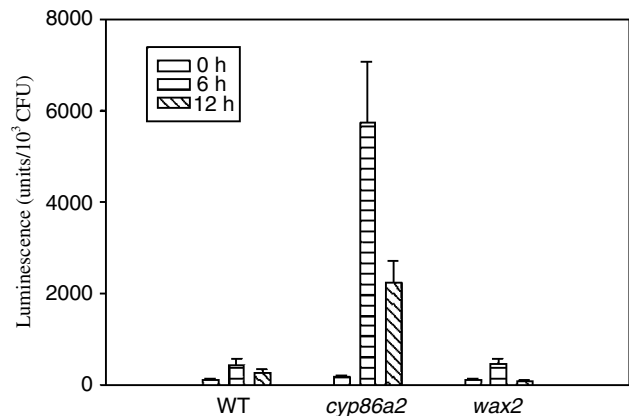
The defects of *cyp86a2* cuticle membrane prompted us to test the potential role of cuticle structure in bacterial type III gene expression. Bacterial *avrPto-luc* expression was examined in another *Arabidopsis* mutant, *wax2*. Like *att1*, *wax2* also shows a thick but translucent cuticle membrane (Chen *et al*, 2003). However, *wax2* only supported normal *avrPto-luc* expression similar to the wild-type plants (Figure 9), indicating that the enhanced type III gene induction was specific to *cyp86a2* mutants.

To further determine the role of cuticle in *avrPto* induction, we constructed *avrPto-gfp* and examined its expression from bacteria in the intercellular spaces and at the leaf surface. *P. syringae* pv. *phaseolicola* bacteria expressed *avrPto-gfp* only in the intercellular spaces, primarily in the substomatal chamber, whereas no expression was detected at the leaf surface of either wild-type or *cyp86a2* plants (Figure 10). The *avrPto-gfp* expression in the intercellular spaces was much stronger in *cyp86a2-1* than in wild-type plants.

## Discussion

The work described here assigns two important biological functions to CYP86A2: normal development of cuticle and repressing the *in planta* expression of bacterial type III genes *avrPto* and *hrpL*. The latter is a master regulatory gene for *P. syringae* genes containing the *hrp* box. At least when dip-inoculated, the *cyp86a2-1* mutant displays enhanced disease severity to the virulent bacteria *P. syringae* pv. *tomato*, indicating a positive role of CYP86A2 in disease resistance. CYP86A2 belongs to a family of fatty acid hydroxylases and is involved in the biosynthesis of extracellular lipids, as indicated by the severely reduced cutin content in *att1* plants.

Cutin monomers are synthesized intracellularly through multiple hydroxylation and epoxidation reactions catalyzed by cytochrome P450-dependent enzymes (Kolattukudy, 2001). These hydroxyl fatty acids are then exported and polymerized extracellularly. Several genes encoding cytochrome P450 monooxygenases that catalyze internal and



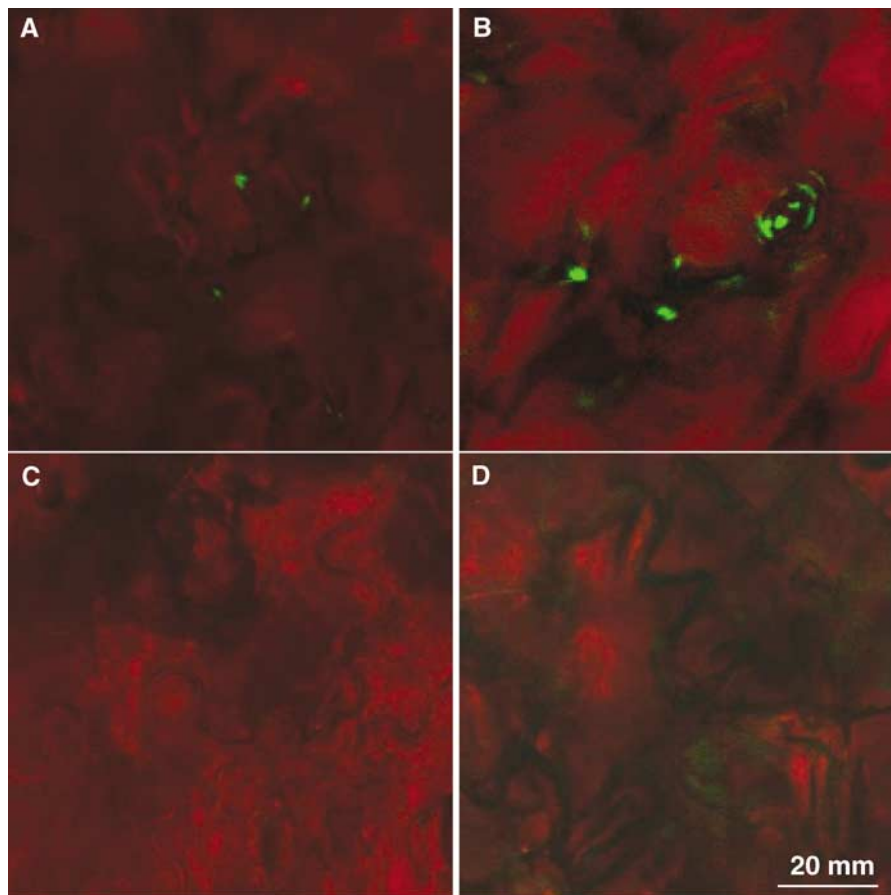
**Figure 9** *wax2* mutant shows normal induction of *avrPto-luc*. Col-0 (WT), *cyp86a2-2*, and *wax2* plants were infiltrated with *P. syringae* pv. *phaseolicola* (*avrPto-luc*), and the luciferase activity was measured at 0, 6, and 12 h after inoculation. Bars indicate standard errors.

$\omega$ -hydroxylation of fatty acids have been isolated, and their *in vitro* enzymatic activities demonstrated (Pinot *et al*, 1992, 1998; Beneviste *et al*, 1998; Cabello-Hurtado *et al*, 1998; Tijet *et al*, 1998). However, no cytochrome P450 monooxygenase genes have been demonstrated to function in cutin biosynthesis *in vivo*.

To date, genetic studies have suggested three proteins involved in the biosynthesis of cutin. These include CYP86A8 (Wellesen *et al*, 2001), WAX2, a lipid desaturase-like metabolic enzyme (Chen *et al*, 2003), and LACS2 (long-chain acyl-CoA synthetase; Schnurr *et al*, 2004). Mutations in CYP86A8 and WAX2 lead to postgenital organ fusion, a phenotype known for plants expressing a fungal cutinase (Sieber *et al*, 2000). However, cutin compositions in these mutants remain unknown. Consequently, the role of these proteins in cutin biosynthesis remains to be established. Our results demonstrate that CYP86A2 is required for the accumulation of the majority of cutin monomers and normal cuticle membrane development *in vivo*, suggesting that CYP86A2 is a major cutin biosynthetic enzyme. Unlike other mutants, *cyp86a2* mutants are morphologically normal and do not show postgenital fusions. The cuticle membrane of *cyp86a2* mutants has a 'puffy' appearance, which may reflect reduced polyester bonding between cutin monomers, a consequence of reduced fatty acid hydroxylation.

The finding that *cyp86a2* mutants have much greater water permeability is highly significant. One of the major functions of the cuticle is to prevent water loss. However, the relative contribution of the wax layer and the cuticle membrane has been unclear. A causal relationship between wax load and dehydration tolerance has not been convincingly established (Jenks, 2002). Our results show that the cuticle membrane plays a large role in preventing water loss by reducing the permeability to water vapor.

CYP86A2 may have multiple functions, a condition that might explain the reduction of all cutin monomers of the *cyp86a2-1* mutant. A comparable P450, CYP94A5, was shown in *Nicotiana tabacum* to both hydroxylate the terminal methyl group of 9,10-epoxy octadecanoic acid and then oxidize the hydroxy end group to a carboxyl *in vitro* (Le Bouquin *et al*, 2001). The *Arabidopsis* CYP86A family



**Figure 10** Induction of *avrPto-gfp* in the intercellular spaces. *avrPto-gfp* expression in the intercellular spaces (A, B) or at the leaf surface (C, D) of the wild-type (A, C) and *cyp86a2-2* (B, D) plants. Leaves were inoculated with *P. syringae* pv. *phaseolicola* (*avrPto-gfp*) for 6 h before being examined under a confocal microscope. The images are representative of multiple plants in two experiments.

contains five members, CYP86A1, CYP86A2, CYP86A4, CYP86A7, and CYP86A8 (*LCR*). The *cyp86a2* mutants are not completely abolished in cuticle development, suggesting that other family members may account for the synthesis of the remaining cutin.

While the biochemical function of CYP86A2 readily explains the defects in cutin composition and cuticle ultrastructure in the *cyp86a2* mutants, it remains to be elucidated how the mutants enable a stronger type III gene induction in the bacterium. The enhanced *avrPto-luc* gene induction occurs in *cyp86a2* but not the *wax2* mutants. Furthermore, the *avrPto-luc* expression does not occur epiphytically in either wild-type or *cyp86a2* plants. These results indicate that the alteration of cuticle membrane structure, by itself, does stimulate type III gene expression. *CYP86A2* must repress bacterial type III gene expression from the intercellular spaces. This appeared puzzling at first, because cuticle is known as a surface structure. However, our findings that cells in the substomatal chambers are covered with a continuous cuticle membrane and that this cuticle membrane is also altered in *cyp86a2* plants argue for a functional relevance of cutin in type III gene expression. The substomatal chamber cuticle membrane may repress bacterial type III gene expression, either by blocking the access of a positive host factor or active repression of bacterial gene expression. The less compact structure of the *cyp86a2* substomatal chamber cuticle membrane may lead to the leakage of this host factor or enable better

attachment of the bacterial cell to the host cell in the intercellular spaces, which in turn stimulates type III gene expression.

It is also possible that certain lipids may repress type III gene expression from the intercellular spaces, thus serving to discourage bacterial infection. These lipids could be either cutin monomers or other fatty acids related to cutin monomers that are synthesized by CYP86A2. Consistent with a role of extracellular lipids in plant–pathogen interaction, lipid transfer protein genes are often induced during defense responses. A recent report showed that the apoplast lipid transfer protein DIR1 is required for systemic acquired resistance (Maldonado *et al*, 2002), although the interacting lipids have yet to be identified. Interestingly, both *DIR1* and *ATT1* transcripts are downregulated following *P. syringae* inoculation, suggesting a functional link between the two genes. While a role of fatty acids in bacterial type III gene regulation has not been reported, free fatty acids derived from cutin are known to function as a signal for plant fungal pathogens (Woloshuk and Kolattukudy, 1986; Gilbert *et al*, 1996; Dickman *et al*, 2003). In support of this hypothesis, we have tested a variety of commercial fatty acids and found that certain cutin-related fatty acid species are capable of repressing *avrPto-luc* but not *trp-luc* expression (Y Xiao and JM Zhou, unpublished results). A sensitive assay for leaf cutin and other extracellular lipids remains to be developed to determine if these fatty acid species are responsible for



type III gene repression *in planta*. Regardless of the nature of the host factor involved, the predicted enzymatic function of CYP86A2 and the reduction of cutin in *cyp86a2* mutants strongly suggest that lipids are host factors modulating bacterial gene expression in the intercellular spaces.

In summary, our results demonstrate that CYP86A2 plays a major role in cutin biosynthesis and is required for normal development of cuticle membrane. CYP86A2 negatively modulates the expression of *P. syringae* type III genes. The predicted biochemical function of CYP86A2 raises the possibility that plant lipids may act as host factors modulating bacterial virulence gene expression.

## Materials and methods

### Bacterial strains and plants

The bacterial strains used in this study were *P. syringae* pv. *phaseolicola* NPS3121 (Lindgren *et al*, 1986) and *P. syringae* pv. *tomato* DC3000 (Davis *et al*, 1991). Reporter gene constructs were made as follows: for *avrPto-luc*, the *luc* gene was PCR-amplified from pSP-*luc* (Promega, Madison, WI) with a forward primer (5'-AGAATTCGGATCCGAAGACGCCAAAAACATAAAG-3') and the T7 primer. The PCR product was cloned into pGEM7Z (Promega, Madison, WI) between *EcoRI* and *XbaI* for sequencing confirmation. The resulting plasmid was digested with *XbaI* and *BamHI*, and the *luc* fragment was inserted into the corresponding sites of pPTE6 (Ronald *et al*, 1992) to replace the *avrPto* open reading frame (ORF). This gave rise to *avrPto-luc*. For the *hrpL-luc* construct, the *hrpL* promoter was PCR-amplified from DC3000 genomic DNA with a forward primer (5'-AGGAATTCTGTGCAGATCAGCATTGGGAAG-3') and a reverse primer (5'-TTGGATCCCATGGGCTTACCCTGATTTAGTG-3') and inserted into the *BamHI* and *EcoRI* sites of *avrPto-luc* to replace the *avrPto* promoter. To construct the *trp-luc* construct, *luc* was first excised from pSP-*luc* with *XbaI* and *BglII* and inserted between the *XbaI* and *BamHI* sites of pBluscript SK (-) (Stratagene, La Jolla, CA) to create pBS-*luc*. A *HindIII* fragment containing the *Salmonella typhimurium trp* promoter (Gage *et al*, 1996) was inserted into the *HindIII* site of pBS-*luc* to create pBS-*trp-luc*. The *trp-luc* cassette was then excised with *XbaI* and *KpnI* and inserted between the *XbaI* and *KpnI* sites of pPTE6 (Ronald *et al*, 1992). For *avrPto-gfp* construct, the ORF of *gfp* was amplified from pCKGFP S65C containing *gfp* (Reichel *et al*, 1996) with the following primers: 5'-TTGGATCCGGTAAAGGAGAAGAAGCTTTTC-3', and 5'-TTCTAGATTATTTGTATAGTTCATCCATG-3'. The PCR product was digested with *BamHI* and *XbaI* and replaced the *luc* gene in *avrPto-luc*.

All *Arabidopsis thaliana* ecotypes (Col-0, Col-gl, and No-0) and EMS-mutagenized M<sub>2</sub> seeds (Col-gl background) were obtained from Lehle Seeds (Tucson, AZ). Plants were grown in growth chambers or greenhouse at 20°C at night and 22°C during the day with a 10 h/day photoperiod.

### Luminescence and fluorescence imaging assay

For luminescence imaging, plants were inoculated with *P. syringae* pv. *phaseolicola* carrying a *luc* construct at 10<sup>8</sup> CFU/ml, and leaves were detached at the indicated times, sprayed with a solution containing 1 mM luciferin and 0.01% Triton X-100, and kept in the dark for 5 min before imaging by using a cooled CCD (Roper Scientific, Trenton, NJ). The relative LUC activity was normalized to leaf bacterial numbers. Each data point represents an average of 3–4 replicates.

For assaying the bacterial expression of *avrPto-gfp* in the intercellular spaces, plants were infiltrated with 5 × 10<sup>8</sup> CFU/ml *P. syringae* pv. *phaseolicola* containing *avrPto-gfp*. For assaying bacterial expression of *avrPto-gfp* at the leaf surface, leaves were coated with the same bacteria suspension in the presence of 0.02% silwet L-77. Leaves were then examined for green fluorescence under a confocal microscope 6 h after inoculation.

### Mutant screening and cloning of ATT1

Col-gl M<sub>2</sub> plants were grown in the greenhouse for 5 weeks. Fully expanded leaves were hand-inoculated with *P. s. pv. phaseolicola* (*avrPto-luc*) at 4 × 10<sup>8</sup> CFU/ml. The luciferase activity was exam-

ined 8 h after injection by CCD imaging. Plants showing high luminescence were verified in the M<sub>3</sub> generation.

For genetic mapping, the *att1-1* mutant was crossed with wild-type No-0 plants. The resulting F<sub>1</sub> plants were self-pollinated to produce F<sub>2</sub> populations. F<sub>2</sub> plants were screened for individuals with the *att1* phenotype. Genomic DNA from these plants was extracted and analyzed with SSLP markers spanning the five chromosomes. For fine mapping, a single nucleotide polymorphism (SNP) was developed for every 5 kb of the chromosome by comparing a 1 kb PCR-amplified genomic DNA fragment from No-0 with that of Col-0 in the database. DNA fragments spanning the SNPs were then PCR-amplified from putative recombinant plants and sequenced to determine the heterozygosity of the marker.

### Disease and bacterial growth assay

Bacterial culture was grown in King's B medium (King *et al*, 1954) with appropriate antibiotics, and the inoculum was prepared in sterile water. Plants (5–6 weeks old) were dipped in a suspension containing 10<sup>8</sup> CFU/ml bacteria and 0.04% silwet L-77 (OSI, Danbury, CT), and leaf bacterial number was determined by plating bacteria on King's B agar plates containing appropriate antibiotics.

### Transpiration assay

For whole-plant transpiration measurements, 3-inch pots containing one 35-day-old plant were enclosed in polyethylene terephthalate film and bound under the rosette to prevent water loss except through the exposed plant surfaces. Allowing 8 h for plant transpiration rates to stabilize, transpiration rates of three wild-type and three *cyp86a2-1* replicates were monitored instantaneously using six electronic balances and WinWedge data acquisition software (TalTech, Philadelphia, PA). To increase water loss amounts to improve measurement accuracy, two plants placed on one balance constituted one replicate. Measurements were performed in complete darkness (when stomata were assumed to be generally closed) and in the light (when stomata were assumed to be generally open). A 400 W metal halide lamp provided illumination (120 mol m<sup>-2</sup> s). Temperature during the study ranged from 22 to 24°C, and RH was between 25 and 35%.

### Agrobacterium-mediated transformation

An 8 kb *PvuII* genomic DNA fragment containing At4g00360 was subcloned from the BAC clone F5110, blunt-ended by Klenow fill-in, and inserted between the *HindIII* and *EcoRI* sites (also blunt-ended by Klenow fill-in) of the binary vector pBI121 (Clontech, Palo Alto, CA). *att1-1* inflorescences were dipped with *Agrobacterium tumefaciens* strain LBA4404 carrying the transgene construct as described (<http://plantpath.wisc.edu/%7Eaef/protocol.html>).

### Northern blot analysis

Plants were vacuum-infiltrated with bacteria, and leaves were sampled at indicated times for RNA isolation. Total RNA (10 µg/lane) was fractionated with formaldehyde gel, transferred to a nitrocellulose membrane, and hybridized to <sup>32</sup>P-radiolabeled At4g00360 genomic DNA fragment.

### Transmission electron microscopy

Mid-blade sections of fully expanded leaves were dissected into 1 × 1 mm squares and immediately placed in 100 mM sodium cacodylate buffer (pH 7.0) containing 2.5% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde. Vacuum was applied until samples were fully submerged. Tissues were then fixed for 3 h at room temperature, washed three times with 100 mM sodium cacodylate buffer (pH 7.0), and postfixed in 100 mM sodium cacodylate buffer (pH 7.0) containing 1% (w/v) osmium tetroxide overnight. Samples were subsequently washed with 100 mM sodium cacodylate buffer (pH 7.0), dehydrated in an acetone series (30–100% by 10% steps, 10 min each step), and infiltrated with Spurr's (Spurr, 1969) epoxy resin (1:2, 1:1, 2:1 resin:acetone, and pure resin for 4 h, overnight, 3 h, and 5 h, respectively). Infiltrated tissues were placed in molds, and the resin was cured at 60°C for 2 days.

Embedded materials were thin-sectioned using an ultramicrotome (Leica Inc., Deerfield, IL). Sections were collected onto 200-mesh copper grids, stained with 2% (w/v) uranyl acetate for 15 min, and rinsed for 30 min, before being viewed and photographed with a transmission electron microscope (FEI, Hillsboro, OR).

**Table II** Cutin and cutan as a percent (%) of total weight of dried, dewaxed cuticle membrane

	Col-0	<i>cyp86a2-1</i>
% cutin	35.8 ± 2.5	41.8 ± 5.0
% cutan	64.2 ± 2.5	58.2 ± 5.0

### Cutin monomer analysis

The cuticle was enzymatically isolated as per Schönherr and Riederer (1986) from first to third internodes of main stems of 35-day-old plants grown under greenhouse conditions as stated previously. Stem areas were measured, and then stem segments from 12–14 individual plants were pooled per sample. The cuticle was refluxed in chloroform:methanol (1:1) for 24 h and then depolymerized with 14% BF<sub>3</sub> in anhydrous methanol (Sigma) to produce methyl esters, which were then extracted with diethyl ether as per Riederer and Schönherr (1986). Extracts were derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide and incubated for 30 min at 70°C. After derivatization, samples were analyzed with a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with a flame ionization detector. The GC was equipped with a 12 m, 0.2 mm HP-1 capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of 80°C, and then increased at 15°C/min to 200°C and at 2°C/min to 280°C. Quantification was based on flame ionization detector peak areas and the internal standard eicosane added prior to depolymerization. Specific correction factors were developed from multilevel calibration curves (as for wax analysis; Chen *et al*, 2003) developed from external standards hexadecanoic acid (Aldrich), hexadecane-1,16-dioic acid (Aldrich), and 16-hydroxy hexadecanoic acid (Sigma). All values shown in tables represent the average of three replicate samples. Selected subsamples were analyzed in a GC-mass spectrometer (FinniganMAT/Thermospray Corporation, San Jose, CA). Percentage of cutin and cutan (nonsaponable fraction) was determined by weighing cuticle before and after depolymerization (Table II).

Cutin monomer composition was also analyzed using whole-organ samples. Results of three replicates were statistically equivalent ( $P < 0.05$ ) to the results obtained from enzymatically isolated cuticles, as determined by the Student's *t*-test. Palmitate and stearate had amounts 4- to 8-fold greater in whole than the enzymatically isolated samples, and were likely derived in large part from internal lipid pools. For this reason, we did not include these acid compounds in the cutin monomer profiles presented.

All cutin monomers were identified from electron impact mass spectra of the methyl ester TMS derivatives on the basis of the published spectra (Eglinton and Hunneman, 1968; Holloway, 1982), retention indexes (Holloway, 1984), and retention times of the external standards.

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Methyl ester monomers identified in this study are listed with retention times, diagnostic fragment ions, and relative abundances. Minor isomers are in parentheses.

*Methyl hexadecanoate*, retention time (RT) 8.305 min, molecular mass (MM) 270; *m/z* 270, 5; *m/z* 241, 2; *m/z* 239, 3; *m/z* 227, 7; *m/z* 87, 60; *m/z* 74, 100.

*Methyl 9-TMS pentadecanoate*, RT 9.658 min, MM 344; *m/z* 329, 4; *m/z* 313, 5; *m/z* 297, 3; *m/z* 259, 4; *m/z* 230, 5; *m/z* 187, 90; *m/z* 75, 32; *m/z* 73, 100.

*Methyl octadecanoate*, RT 10.144 min, MM 298; *m/z* 298, 32; *m/z* 269, 11; *m/z* 267, 10; *m/z* 255, 18; *m/z* 87, 68; *m/z* 74, 100.

*Dimethyl hexadecane-1,16-dioate*, RT 11.597 min, MM 314; *m/z* 283, 32; *m/z* 250, 10; *m/z* 241, 21; *m/z* 222, 3; *m/z* 209, 18; *m/z* 191, 14; *m/z* 112, 32; *m/z* 98, 100; *m/z* 84, 45; *m/z* 74, 56.

*Methyl 16-TMS hexadecanoate*, RT 12.298 min, MM 358; *m/z* 343, 32; *m/z* 327, 5; *m/z* 311, 100; *m/z* 159, 9; *m/z* 146, 10; *m/z* 75, 45.

*Methyl 10(9)-TMS heptadecanoic acid*, RT 13.225 min, MS 372; *m/z* 357, 4; *m/z* 325, 4; *m/z* 273, 68; *m/z* 259, 9; *m/z* 244, 10; *m/z* 215, 5; *m/z* 201, 29; *m/z* 81, 37; *m/z* 75, 50; *m/z* 73, 100; *m/z* 55, 60.

*Dimethyl octadecadiene-1,18-dioate*, RT 13.996 min, MM 338; *m/z* 306, 45; *m/z* 274, 34; *m/z* 264, 5; *m/z* 246, 8; *m/z* 209, 40; *m/z* 94, 50; *m/z* 93, 44; *m/z* 81, 62; *m/z* 67, 100; *m/z* 55, 96.

*Dimethyl octadecane-1,18-dioate*, RT 14.996 min, MM 342; *m/z* 311, 22; *m/z* 269, 18; *m/z* 250, 8; *m/z* 237, 11; *m/z* 219, 10; *m/z* 112, 42; *m/z* 98, 90; *m/z* 74, 54; *m/z* 55, 100.

*Dimethyl 7(8)-TMS hexadecane-1,16-dioate*, RT 15.099 min, MM 402; *m/z* 387, 6; *m/z* 371, 2; *m/z* 355, 3; *m/z* 273, 82; *m/z* 259, 36; *m/z* 245, 35; *m/z* 244, 12; *m/z* 231, 98; *m/z* 230, 5; *m/z* 216, 5; *m/z* 202, 12; *m/z* 73, 100.

*Methyl 10,16(9,16)-bisTMS hexadecanoate*, RT 16.024 min, MM 446; *m/z* 431, 5; *m/z* 415, 3; *m/z* 399, 3; *m/z* 309, 9; *m/z* 289, 2; *m/z* 275, 44; *m/z* 273, 100; *m/z* 259, 3; *m/z* 244, 16; *m/z* 230, 1; *m/z* 73, 63.

Two non-cutin phenolic acids esterified to carbohydrates (Holloway, 1982) were identified.

*Methyl m-coumarate*, RT 7.239 min, MM 250; *m/z* 250, 100; *m/z* 235, 64; *m/z* 219, 34; *m/z* 203, 40; *m/z* 75, 75; *m/z* 73, 39.

*Methyl ferulate*, RT 7.409 min, MM 280; *m/z* 280, 74; *m/z* 265, 45; *m/z* 250, 100; *m/z* 219, 35; *m/z* 191, 8; *m/z* 73, 20.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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