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ORIGINAL ARTICLE

The *Arabidopsis* P450 protein CYP82C2 modulates jasmonate-induced root growth inhibition, defense gene expression and indole glucosinolate biosynthesis

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Jasmonic acid (JA) is a fatty acid-derived signaling molecule that regulates a broad range of plant defense responses against herbivores and some microbial pathogens. Molecular genetic studies have established that JA also performs a critical role in several aspects of plant development. Here, we describe the characterization of the *Arabidopsis* mutant *jasmonic acid-hypersensitive1-1* (*jah1-1*), which is defective in several aspects of JA responses. Although the mutant exhibits increased sensitivity to JA in root growth inhibition, it shows decreased expression of JA-inducible defense genes and reduced resistance to the necrotrophic fungus *Botrytis cinerea*. Gene cloning studies indicate that these defects are caused by a mutation in the cytochrome P450 protein CYP82C2. We provide evidence showing that the compromised resistance of the *jah1-1* mutant to *B. cinerea* is accompanied by decreased expression of JA-induced defense genes and reduced accumulation of JA-induced indole glucosinolates (IGs). Conversely, the enhanced resistance to *B. cinerea* in *CYP82C2*-overexpressing plants is accompanied by increased expression of JA-induced defense genes and elevated levels of JA-induced IGs. We demonstrate that CYP82C2 affects JA-induced accumulation of the IG biosynthetic precursor tryptophan (Trp), but not the JA-induced IAA or pathogen-induced camalexin. Together, our results support a hypothesis that CYP82C2 may act in the metabolism of Trp-derived secondary metabolites under conditions in which JA levels are elevated. The *jah1-1* mutant should thus be important in future studies toward understanding the mechanisms underlying the complexity of JA-mediated differential responses, which are important for plants to adapt their growth to the ever-changing environments.

***Keywords*:** jasmonic acid, root growth inhibition, defense response, indole glucosinolates, tryptophan
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Introduction

The jasmonate family of oxylipins, including jasmonic acid (JA), methyl JA (MeJA) and other bioactive derivatives (collectively referred to here as JAs) are important signaling molecules in the plant kingdom. JAs have a well-established role in regulating defense responses against herbivore attack and pathogen infection. In addition to defense, JAs also regulate many aspects of plant growth and development [1-5].

It has long been recognized that the application of exogenous JAs inhibits root growth [6], and this action has been employed as the most prominent genetic screen to identify mutants with defective JA responses in the reference species, *Arabidopsis thaliana* [2, 4, 7]. Mutant-based molecular genetic studies have identified several important components of JA signaling. The *coronatine insensitive1* (*coi1*) mutant is fully insensitive to JA in both root growth inhibition and defense gene expression [8]. *COI1* encodes an F-box protein showing active E3

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ligase activity [9, 10]. Very recently, COI1 was identified as a JA receptor [11]. Compared with *coi1*, the *jasmonate-resistant1* (*jar1*) [7] and *jasmonate-insensitive1* (*jin1*) [12, 13] mutants exhibit relatively weak phenotypes in the JA-induced inhibition of root growth. *JAR1* encodes an enzyme that has JA adenylation activity to form JA-amino acid conjugates, especially JA-isoleucine (JA-Ile), demonstrating that JA-Ile is the primary jasmonate signal [14-16]. *JIN1* encodes a nuclear-localized basic helix-loop-helix-type transcription factor known as AtMYC2 [13], which acts as both an activator and a repressor to regulate diverse aspects of JA-mediated gene expression [17]. A family of *Arabidopsis* proteins named JAZ (jasmonate ZIM domain) was identified as targets of the SCF^{COI1} ubiquitin ligase complex in JA signaling [18, 19]. The JAZ family proteins represent a molecular link between SCF^{COI1}-mediated protein degradation and transcriptional activation of JA responses [18].

An intact JA signaling pathway is required for plant resistance against the infection of necrotrophic pathogens, such as *B. cinerea* and *Erwinia carotovora* [20]. It was believed that the regulatory role of JA in plant resistance to pathogen infections is possibly achieved through activating the expression of a number of defense-related genes, including genes encoding proteins with antimicrobial properties. Both JA treatment and pathogen infection induce the expression of the plant defensin gene *PDF1.2* [21, 22] and the basic thionin gene *Thi2.1* [23]. *Arabidopsis* mutants defective in JA production or signaling are blocked in pathogen-induced expression of *PDF1.2* and *Thi2.1* and are more susceptible to different fungal pathogens [24, 25]. Conversely, an *Arabidopsis* mutant with increased endogenous JA levels showed constitutive expression of *Thi2.1* and *PDF1.2* and enhanced resistance to powdery mildew infections [26].

Accumulating evidence has indicated that JA also influences the production of various defense-related metabolites, including glucosinolates, which are nitrogen- and sulfur-containing secondary metabolites with a wide range of biological functions [27-29]. On tissue damage, glucosinolates are hydrolyzed by a thioglucosidase called myrosinase to form a series of biologically active compounds such as isothiocyanates, thiocyanates and nitriles [30-32]. Many of these products have been implicated as being important for plant defenses against pathogens and herbivores. Several studies with different plant species indicated that JA induces the production of tryptophan (Trp)-derived indole glucosinolates (IGs), but not aromatic and aliphatic glucosinolates [27, 33-36]. It was generally believed that the JA-induced accumulation of IGs is regulated at the transcriptional level. However, the regulatory interactions between defense signaling and IG metabolism remain largely unknown.

Here, we describe the characterization of the *Arabidopsis jasmonic acid-hypersensitive1-1* (*jah1-1*) mutant that shows altered responses to JA in root growth inhibition and defense gene expression. We show that *CYP82C2* encodes a predicted cytochrome P450 protein. The results that we report support the hypothesis that CYP82C2 acts to alter the metabolism of Trp-derived secondary metabolites under conditions in which JA levels are elevated.

Results

The jah1-1 mutant is hypersensitive to JA in root growth inhibition

A T-DNA-mutagenized population of *Arabidopsis* [37] was screened for plants showing altered responses to relatively low concentrations of JA using the widely applied root growth inhibition assay. A mutant line was identified because it developed short roots on medium containing 10 μM JA (Figure 1A). In the absence of JA, primary root length of the mutant seedlings was comparable to that of wild-type plants. However, in the presence of a range of concentrations of JA, roots of the mutant plants were generally shorter than those of wild-type plants (Figure 1A and 1B). For example, under our growth conditions, roots of 8-day-old wild-type seedlings were 30.6 ± 2.6 mm in length, and reduced by 45% to 16.9 ± 0.9 mm when grown on medium containing 10 μM JA. In contrast, roots of the mutant seedlings were 29.8 ± 2.8 mm in length and were reduced by 70% to a length of 9.0 ± 1.9 mm when grown on medium containing 10 μM JA. These results indicate that the mutant line is more sensitive than wild type to the inhibitory effect of exogenous JA on root growth. The specificity of *jah1-1* in the JA signaling pathway was addressed by testing the sensitivity of *jah1-1* to several other hormones including ABA, ethylene and auxin, and also to abiotic stress reagents including NaCl and mannitol. As shown in Supplementary information, Figure S1, no significant differences in seedling development were observed in these assays between *jah1-1* and wild type, suggesting that the *jah1-1* mutant defines a specific regulator of JA response.

jah1-1 is defective in JA-induced gene expression

Increased sensitivity of *jah1-1* to JA-mediated inhibition of root growth prompted us to investigate whether JA-induced defense gene expression was affected in this mutant. In *Arabidopsis*, JA is believed to regulate the expression of two distinct sets of defense-related genes by different mechanisms [13, 38]. One group of these genes, including the plant defensin gene *PDF1.2* [21]

and the thionin gene *Thi2.1* [39], is associated with plant defense to pathogen infections. The other group, including *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) [40] and *LIPOXYGENASE2* (*LOX2*) genes [41], is believed to be associated with wound responses. The levels of JA-induced expression of *PDF1.2* and *Thi2.1* genes were substantially reduced in *jah1-1* (Figure 1C). In contrast, JA-induced expression of the wound-responsive *LOX2* and *VSP1* genes was largely unaffected (Figure 1C). These results indicate that the *jah1-1* mutation mainly affects JA-induced expression of pathogen defense-related genes.

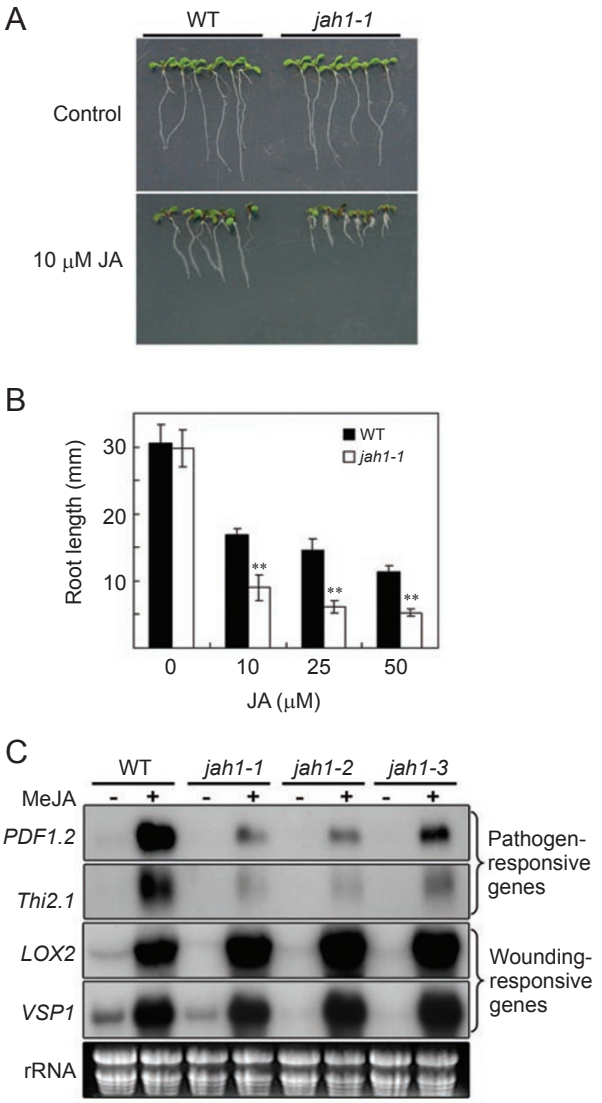

Figure 1 Phenotypes of *jah1* mutants. **(A)** Root growth of 8-day-old wild-type (WT) and *jah1-1* seedlings grown in the absence (Control) or presence of 10 μM JA. **(B)** Root length of 8-day-old WT and *jah1-1* seedlings grown on medium containing indicated concentrations of JA. Data show the mean ± SD of three independent experiments. Asterisks denote Student's *t*-test significance compared with wild type (**$P < 0.01$). **(C)** RNA gel blot analysis of JA-inducible genes (*PDF1.2*, *Thi2.1*, *LOX2* and *VSP1*) in the indicated genotypes. Seedlings (2 weeks old) were treated with water (−) or 50 μM MeJA (+) for 6 h and tissues were collected for RNA extraction. Total RNA (20 μg) was loaded per lane, and blots were hybridized with the indicated probes. rRNA serves as a loading control.

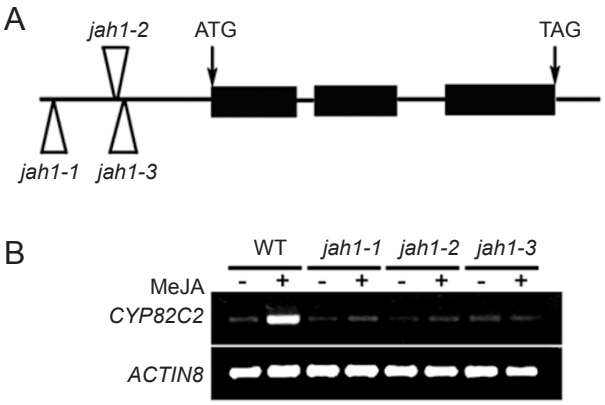

Figure 2 Identification of the *CYP82C2* gene. **(A)** A diagram of the *CYP82C2* gene structure. Black boxes indicate the coding sequence, open boxes indicate the 5′- and 3′-untranslated regions, and lines between boxes indicate introns. Positions of the T-DNA insertions in different alleles of the *jah1* mutant are indicated. **(B)** *CYP82C2* expression in different alleles of the *jah1* mutant analyzed by RT-PCR. Seedlings (2 weeks old) were treated with water (−) or 50 μM MeJA (+) for 48 h and tissues were collected for RNA extraction. Amplification of the *ACTIN8* gene served as a control.

The JA-response phenotype of jah1-1 results from a defect in CYP82C2

Genetic analysis indicated that *jah1-1* defines a single recessive mutation in a nuclear gene. The T-DNA-inserted genomic region was isolated by thermal asymmetric interlaced-PCR (TAIL-PCR), and sequence analysis revealed that the T-DNA was inserted at 900 bp upstream of the translation start codon of the *CYP82C2* gene (At4g31970), which encodes cytochrome P450 monooxygenase (Figure 2A). As shown in the RT-PCR assays, the T-DNA insertion in *jah1-1* led to significant reduction of *CYP82C2* expression (Figure 2B).

A 4.0 kb genomic DNA fragment containing the entire *CYP82C2* coding region and a 1.5 kb upstream sequence was introduced into the *jah1-1* background. The results showed that the *CYP82C2* genomic DNA complemented the *jah1-1* mutant in JA-mediated root growth inhibition, defense gene expression and defense response against *B. cinerea* infection (Supplementary information, Figure S2).

Further confirmation that *CYP82C2* was responsible for the *jah1-1* mutant phenotype came from analy-

ses of two independent T-DNA insertion lines named SALK_128974 and SALK_024364. As shown in Figure 2A, SALK_128974 and SALK_024364 contain T-DNAs inserted at 627 bp and 537 bp, respectively, upstream of the translation start codon of C*YP82C2*. These T-DNA insertions significantly reduced the expression levels of *CYP82C2* (Figure 2B). Similar to *jah1-1* plants, homozygous SALK_128974 and SALK_024364 plants showed a JA-hypersensitive phenotype in root growth inhibition (Supplementary information, Figure S3) and they were therefore designated as *jah1-2* and *jah1-3*, respectively. As expected, JA-induced expression levels of *PDF1.2* and *Thi2.1* genes were also significantly reduced in *jah1-2* and *jah1-3* (Figure 1C). Genetic analyses showed that *jah1-1*, *jah1-2* and *jah1-3* cannot complement each other, indicating that these mutations are allelic (Supplementary information, Figure S3). Collectively, these results showed that *CYP82C2* was responsible for the *jah1-1* mutant phenotype.

CYP82C2 contains three exons and encodes a putative protein of 524 amino acids (Figure 2A). Sequence analysis revealed that CYP82C2 contains all the highly conserved domains, characteristic for the eukaryotic P450 proteins [42]: the N-terminal proline-rich domain involved in membrane anchoring, the WXXXR domain, the substrate recognition site (SRS) regions, the EXXR region and the heme-binding domain (Figure 3A). In the *Arabidopsis* genome, *CYP82C2* is a member of a cluster of three *CYP82C* genes, comprising *CYP82C2*, *CYP82C3* (At4g31950) and *CYP82C4* (At4g31940). The deduced amino acid sequence of CYP82C2 was almost similar to CYP82C4 (89% identity) and CYP82C3 (88% identity, Figure 3B). Notably, the most significant difference among the three CYP82C members was that CYP82C3 contained a deletion of 11 amino acids, which occurs immediately after SRS1 region and preceding WXXXR domain (Figure 3A). Considering that the deletion removed a large portion of the C-helix, which is potentially important for substrate binding [42], CYP82C3 may show altered biochemical activity compared to CYP82C2 or CYP82C4. However, little information is available concerning the biochemical function of the CYP82C subfamily.

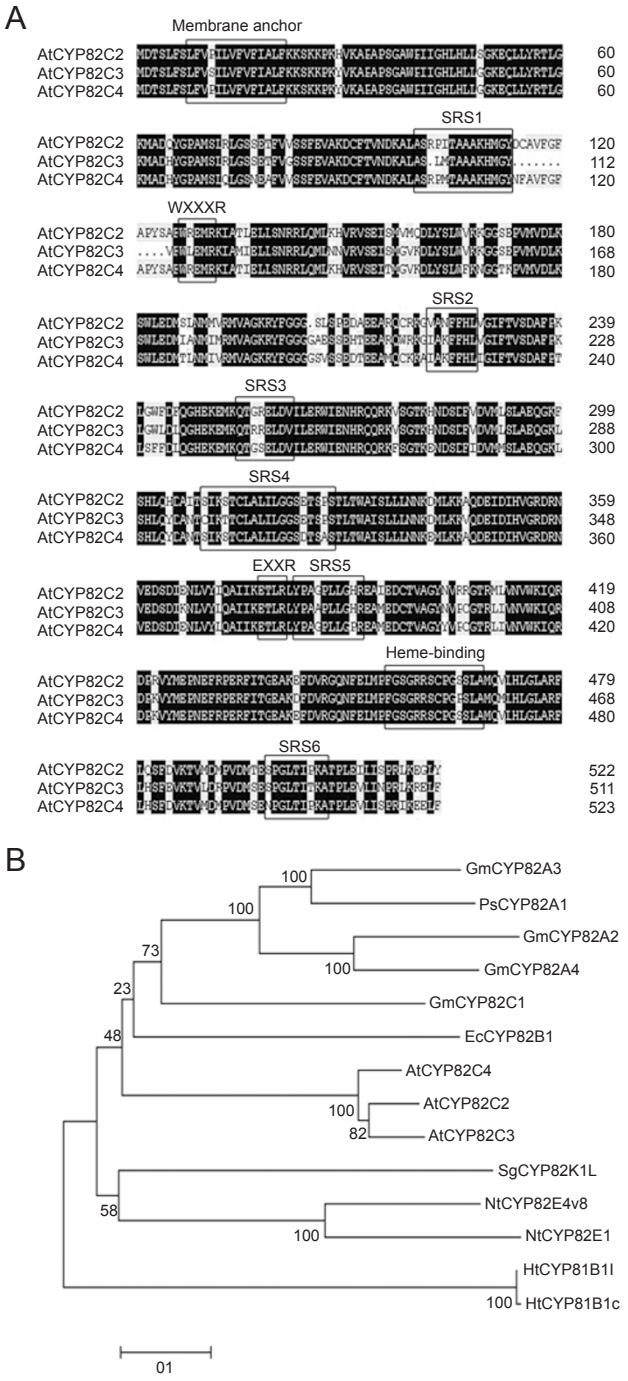

Figure 3 *CYP82C2* encodes the cytochrome P450 CYP82C2. **(A)** Sequence alignment of CYP82C2 with CYP82C3 and CYP82C4. Sequence alignment was done by DNAMAN4.0 (Lynnon Biosoft). Black backgrounds are identical residues; gray backgrounds are similar ones; dotted lines indicate gaps. Framed regions indicate locations of conserved domains of microsomal P450s, including the membrane anchor region, the WXXXR domain, the substrate recognition site (SRS) regions, the EXXR region and the heme-binding domain. **(B)** Phylogenetic analysis of CYP82C2 and the selected stress-inducible P450 proteins from *Glycine max* (Gm), *Pisum sativum* (Ps), *Eschscholzia californica* (Ec), *Arabidopsis thaliana* (At), *Stylosanthes guianensis* (Sg) and *Nicotiana tabacum* (Nt) plants. A neighbor-joining tree construction was performed by the MEGA4 program (http://www.megasoftware.net/). Topological robustness was assessed by bootstrap analysis with 500 replicates. The scale bar is an indicator of genetic distance based on branch length.

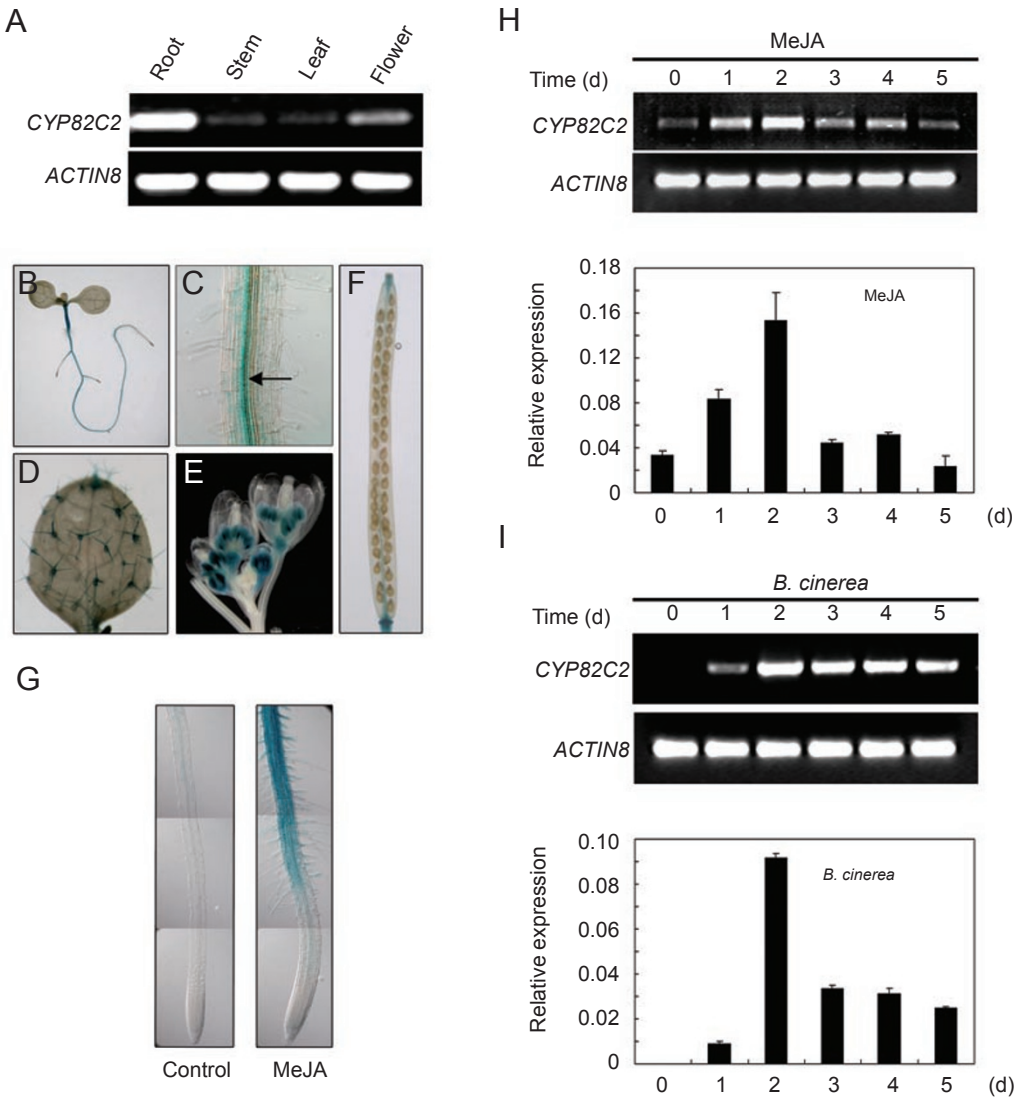

Figure 4 Temporal and spatial patterns of *CYP82C2* expression. **(A)** RT-PCR analysis of *CYP82C2* expression in different tissues. Total RNA was isolated from roots, stems, leaves and flowers of wild-type (WT) plants. Amplification of the *ACTIN8* gene serves as a control. **(B)** *CYP82C2:GUS* expression in 6-day-old seedling. **(C)** *CYP82C2:GUS* expression in root vascular tissues of 6-day-old seedling. **(D)** *CYP82C2:GUS* expression in leaf of 14-day-old seedling. **(E)** *CYP82C2:GUS* is richly expressed in anthers of open flowers. **(F)** *CYP82C2:GUS* expression in silique. **(G)** MeJA-induced *CYP82C2:GUS* expression in the root. *CYP82C2:GUS* seedlings (4 days old) were exposed to 50 μM MeJA or water (Control) for 2 days and stained overnight. **(H)** MeJA-induced *CYP82C2* expression analyzed by RT-PCR (top) and qRT-PCR (bottom) assays. WT plants (2 weeks old) were treated with 50 μM MeJA for the indicated time periods. **(I)** *B. cinerea*-induced *CYP82C2* expression analyzed by RT-PCR (top) and qRT-PCR (bottom) assays. WT (4 weeks old) plants grown in the soil were inoculated with a 5-μl droplet of *B. cinerea* spore suspension at a density of 10^5 spores per ml for the indicated time periods. **(H** and **I)** The transcript levels of *CYP82C2* are normalized based on the expression of *ACTIN8*. Data are mean ± SD of three independent experiments.

CYP82C2 expression is upregulated by MeJA treatment and B. cinerea infection

The expression pattern of *CYP82C2* was determined by RT-PCR analysis. *CYP82C2* was shown to be expressed in all examined tissue types including roots, stems, leaves and flowers (Figure 4A). The highest expression level of *CYP82C2* was found in roots, followed by flowers, with relatively low expression in stems and leaves. A DNA fragment covering the 1.5 kb promoter region of *CYP82C2* was fused with the glucuronidase (GUS) reporter gene and transformed into wild-type plants. Representative transgenic plants named

CYP82C2:GUS were used to follow the promoter activity of *CYP82C2* at different developmental stages. In 6-day-old seedlings, GUS activity was detected predominantly in roots and hypocotyls, and weakly in cotyledons (Figure 4B). Closer observation of 6-day-old seedlings revealed that GUS staining in roots was mainly focused in the vascular tissues (Figure 4C). In leaves of 14-day-old plants, GUS activity was detected in trichomes (Figure 4D). In flowers, GUS activity was mainly observed in the anthers (Figure 4E). In nearly mature siliques, GUS was barely detectable in seeds, but richly expressed in the pedicel-silique junctions (Figure 4F).

To examine whether the expression of *CYP82C2* was JA inducible, the *CYP82C2:GUS* transgenic plants were treated with MeJA and the GUS activity was monitored. *CYP82C2:GUS* expression in the root was significantly increased following MeJA treatment (Figure 4G). To study the induction kinetics of MeJA on *CYP82C2* expression, 2-week-old wild-type seedlings were treated with MeJA and the accumulation of *CYP82C2* transcripts was measured with RT-PCR and quantitative RT-PCR (qRT-PCR) assays at different times. The *CYP82C2* transcripts started to increase at 1 day after MeJA treatment and reached a maximum at 2 days (Figure 4H). The transcript level started to drop after 3 days. A similar induction pattern of *CYP82C2* expression was observed when 4-week-old soil-grown plants were infected with the necrotrophic pathogen *B. cinerea* (Figure 4I). These results suggest that *CYP82C2* possibly plays a role in MeJA-mediated defense responses against pathogen infection.

The MeJA-induced expression of CYP82C2 is independent of COI1

To study whether the JA-induced expression of *CYP82C2* requires the function of COI1, a central regulator of jasmonate-mediated processes in *Arabidopsis* [9], we compared the MeJA-induced *CYP82C2* expression in the wild type and the *coi1-1* mutant plants [9]. Interestingly, qRT-PCR analysis showed that the *coi1-1* mutation did not significantly affect the expression of *CYP82C2* in the absence or presence of MeJA (Figure 5A), indicating that MeJA induced the expression of *CYP82C2* in a COI1-independent manner. We also generated a double mutant between *jah1-1* and *coi1-2*, which is insensitive to JA, but partially fertile and able to produce a small quantity of seeds [43]. The *jah1-1/coi1-2* double mutant was compared with *jah1-1*, *coi1-2* and wild type for JA-induced root growth inhibition. In the absence of JA, 8-day-old seedlings of the four genotypes showed comparable root length. In the presence of JA, however, the root length of the double mutant showed high similarity to that of the *coi1-2* mutant, i.e., insensitive to JA-

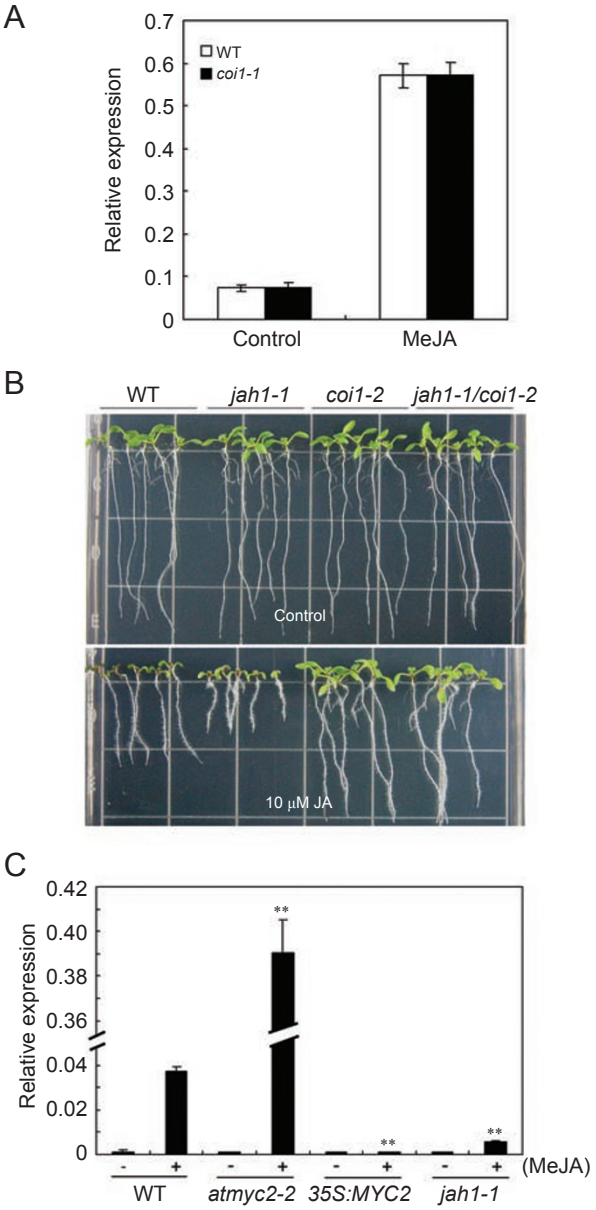

Figure 5 MeJA-induced expression of *CYP82C2* in *coi1-1* and *atmyc2-2*. **(A)** qRT-PCR analysis of MeJA-induced *CYP82C2* expression in wild type (WT) and *coi1-1*. Seedlings (2 weeks old) were treated without (Control) or with 50 μM MeJA for 48 h. The transcript levels of the *CYP82C2* were normalized to the *ACTIN8* expression. Data are mean ± SD of three independent experiments. Student's *t*-test was performed and did not detect a significant difference compared with WT plants ($P > 0.05$). **(B)** WT (8 days old), *jah1-1*, *coi1-2* and *jah1-1/coi1-2* (from left to right) seedlings grown on medium without (Control) or with 10 μM JA. **(C)** qRT-PCR analysis of MeJA-induced *CYP82C2* expression in WT, *atmyc2-2*, *35S:MYC2* and *jah1-1*. Seedlings (2 weeks old) were treated with water (−) or 50 μM MeJA (+) for 48 h. The transcript levels of *CYP82C2* were normalized based on the expression of *ACTIN8*. Data are mean ± SD of three independent experiments. Asterisks denote Student's *t*-test significance compared with wild type (**$P < 0.01$).

mediated inhibition, which was in contrast to the JA-hypersensitive phenotype of *jah1-1* (Figure 5B). This observation further indicated that the root growth phenotype of *jah1-1* was specific to JA.

We also tested whether the MeJA-induced expression of the *CYP82C2* gene was regulated by AtMYC2, an important regulator of a wide range of JA-mediated gene expression [13, 17, 38]. As shown in Figure 5C, the MeJA-induced expression levels of *CYP82C2* were considerably increased in the *atmyc2-2* mutant that disrupts *AtMYC2* expression, while decreased in *35S:MYC2*, an *AtMYC2*-overexpressor [38]. These results indicate that the JA-induced expression of *CYP82C2* is negatively regulated by AtMYC2.

CYP82C2 plays an important role in plant resistance to B. cinerea infection

To investigate the physiological role of *CYP82C2* further, we generated transgenic *Arabidopsis* plants overexpressing the *CYP82C2* cDNA driven by the cauliflower mosaic virus 35S promoter. Overexpression of *CYP82C2* in transgenic lines was confirmed by RNA gel blot analysis (data not shown). All the *CYP82C2* overexpression lines showed similar JA-related phenotypes; thus, only the results obtained with one representative line, named *OE37,* are discussed below.

Under normal growth conditions, the overall growth rate and morphology of *OE37* plants were similar to those of wild-type and *jah1-1* plants (Figure 6A). However, in the presence of JA, roots of *OE37* were longer than those of wild type (Figure 6A), suggesting that, in contrast to *jah1-1*, *OE37* is less sensitive to JA-mediated inhibition of root growth. We next compared the expression levels of JA-induced *PDF1.2* and *Thi2.1* genes among the three genotypes using RNA gel blot analysis. In response to MeJA treatment, a significantly elevated level of these mRNAs was observed in *OE37* plants (Figure 6B). In contrast, *jah1-1* plants showed decreased expression levels of MeJA-induced *PDF1.2* and *Thi2.1* genes (Figure 6B), which is consistent with the results described earlier (Figure 1C).

OE37 and *jah1-1* were then compared with wild-type plants for their response to pathogen infection.

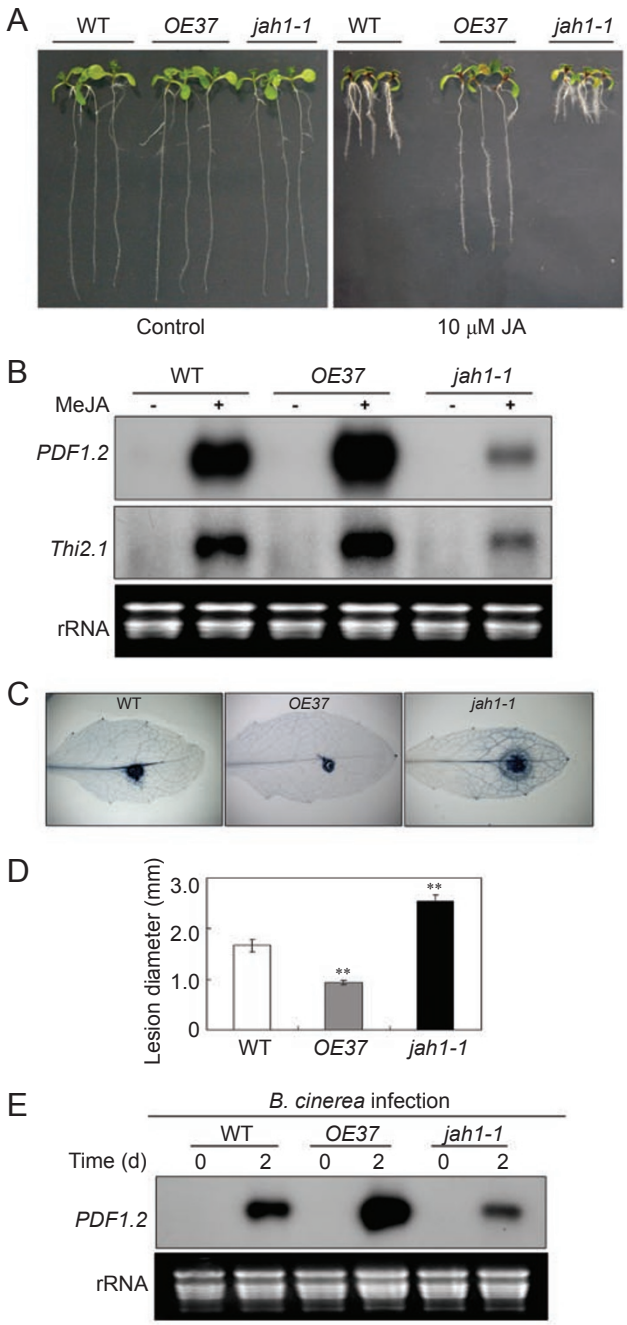

Figure 6 *CYP82C2* plays an important role in plant resistance against *B. cinerea* infection. **(A)** *35S:CYP82C2* (*OE37*) plants are less sensitive to JA in root growth inhibition. Shown are 8-day-old seedlings of the indicated genotypes grown on medium without (Control) or with 10 μM JA. **(B)** *35S:CYP82C2* (*OE37*) plants show increased MeJA-induced defense gene expression. Seedlings (2 weeks old) of the indicated genotypes were treated with water (−) or 50 μM MeJA (+) for 6 h and MeJA-induced expression of *PDF1.2* and *Thi2.1* was analyzed by RNA gel blot tissues. **(C)** Hyphae growth revealed by lactophenol-trypan blue staining. Representative leaves of the indicated genotypes are shown. **(D)** Lesion size generated by *B. cinerea* was measured 5 days after inoculation. Data are mean ± SD of three independent experiments. Asterisks denote Student's *t*-test significance compared with wild type (**$P < 0.01$). **(E)** RNA gel blot analysis of *PDF1.2* expression in the indicated genotypes after *B. cinerea* infection. **(B** and **E)** Total RNA (20 μg) was loaded per lane and rRNA serves as a loading control. **(C**-**E)**, 4-week-old plants were inoculated with spore suspensions of *B. cinerea* as described in Experimental procedures, disease symptoms were investigated 5 days after inoculation.

Soil-grown plants (4 weeks old) were challenged with *B. cinerea* and the infection progress was monitored. At 5 days after inoculation, while wild-type plants produced restricted necrotic lesions, *jah1-1* plants produced spreading lesions that were heavily colonized by the fungal hyphae (Figure 6C). The average diameter of the necrotic lesions of *B. cinerea*-infected *jah1-1* plants was significantly larger than that of the infected wild-type plants (Figure 6D). These results indicate that *jah1-1* plants are more susceptible to *B. cinerea* than wild-type plants. In contrast, *OE37* plants showed an apparent reduction in infection symptoms compared with wild-type plants, when assessed by trypan blue staining of the infected leaves and by measuring the size of the necrotic lesions (Figure 6C and 6D), indicating that *OE37* plants were more resistant to *B. cinerea*. Consistent with the performance of the three genotypes on pathogen infection, the *jah1-1* plants infected by *B. cinerea* showed significantly reduced expression levels of *PDF1.2,* whereas *OE37* plants showed increased expression levels of this gene (Figure 6E). Together, these data demonstrate that *CYP82C2* is important for plant resistance to *B. cinerea*, and that overexpression of *CYP82C2* was sufficient to increase plant resistance to this pathogen.

CYP82C2 affects MeJA-induced Trp accumulation

The CYP82 family of P450 proteins is only present in *Arabidopsis* and other dicot plants, but is absent in the rice genome [44]. Members of the CYP82 family (Figure 3B) are reported to be highly stress responsive in

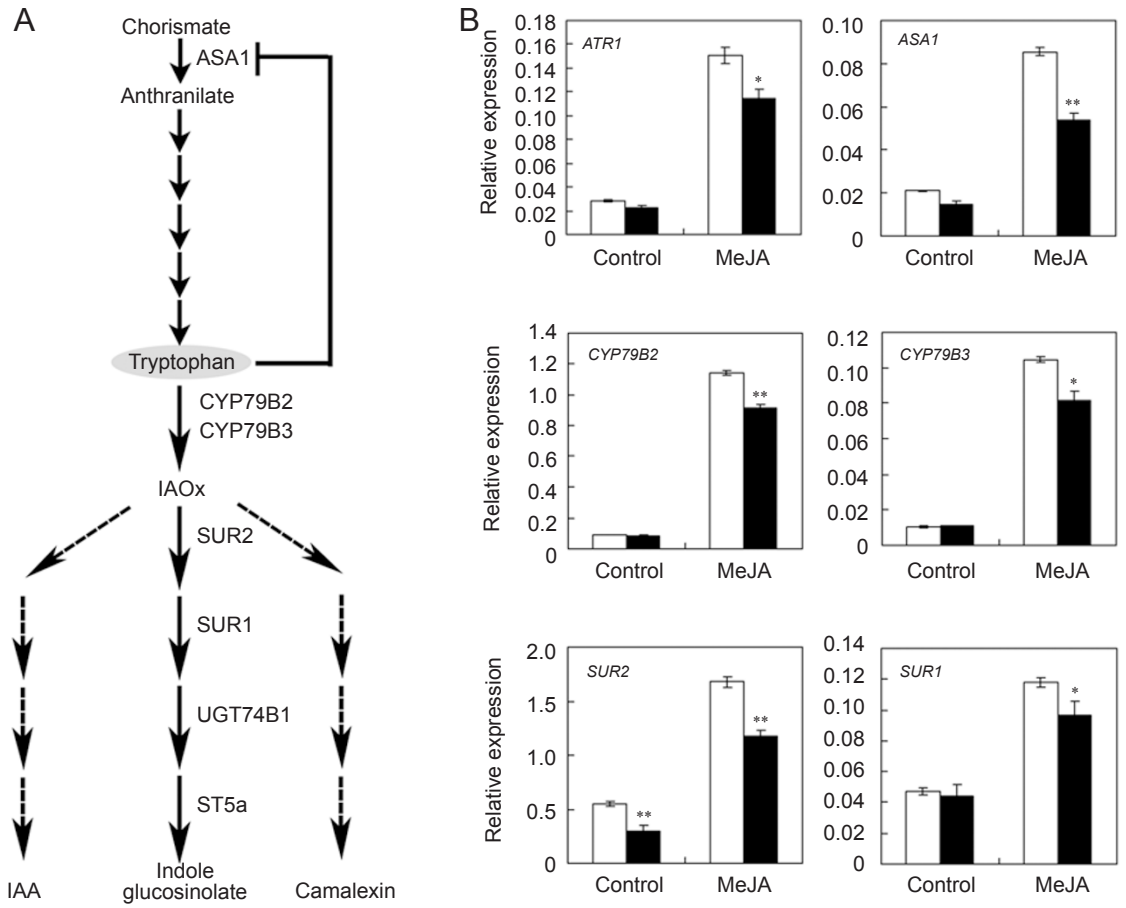

Figure 7 MeJA-induced expression of Trp-pathway genes in wild type and *jah1-1*. **(A)** Trp metabolism in *Arabidopsis*. The pathway for Trp biosynthesis and its subsequent conversion into IGs, IAA or camalexin via IAOx is shown, with key enzymatic steps discussed in the text. Trp feedback inhibition of AS activity is indicated by a line with a perpendicular bar at *ASA1*. The dashed arrow indicates that the number of biosynthetic steps in the IAA and camalexin pathways is unknown. **(B)** qRT-PCR analyses of MeJA-regulated expression of *ATR1*, *ASA1*, *CYP79B2*, *CYP79B3*, *SUR2* and *SUR1* in WT and *jah1-1*. Seedlings (2 weeks old) were treated without (Control) or with 50 μM MeJA for 6 h. The transcript levels of the indicated genes were normalized to the *ACTIN8* expression. Data are mean ± SD of three independent experiments. Asterisks denote Student's *t*-test significance between wild type (white bar) and *jah1-1* (black bar) (**P* < 0.05, ***P* < 0.01).

pea (*Pisum sativum*), tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*) [45-48]. These, together with our finding that the *CYP82C2* gene plays a role in plant resistance to *B. cinerea,* led us to a hypothesis that *CYP82C2* may be involved in the production of species-specific secondary metabolites that are important for disease resistance. *Arabidopsis* plants derive a number of important secondary metabolites from the amino acid Trp, including the growth regulator IAA and defense compounds such as IGs and camalexin (Figure 7A). To test whether *CYP82C2* may affect the Trp pathway, we compared MeJA-induced expression levels of several genes involved in Trp synthesis or metabolism between wild-type and *jah1-1* plants. As shown in Figure 7B, the MeJA-induced expression levels of the Trp synthesis gene *ASA1*, which encodes the α-subunit of anthranilate synthase (AS, Figure 7A) [49], were significantly reduced in *jah1-1* compared to the levels in the wild type (Figure 7B). Similarly, the MeJA-induced expression levels of the Trp-metabolizing genes *CYP79B2* and *CYP79B3* (Figure 7A) [50], were also significantly reduced in *jah1-1* as compared with wild type (Figure 7B).

Next, we measured Trp accumulation in wild type, *jah1-1* and the *CYP82C2*-overexpressor *OE37*. In the absence of MeJA, Trp levels were similar among the three genotypes (Figure 8A). As expected, MeJA treatment led to significant increase of Trp accumulation in wild type, and the MeJA-mediated induction of Trp levels was impaired by the *jah1-1* mutation (Figure 8A). Surprisingly, however, the MeJA-mediated induction of Trp levels was more severely impaired in *OE37* than shown in the *jah1-1* mutant (Figure 8A), suggesting an increased Trp-metabolizing flux in the *CYP82C2* overexpressor.

CYP82C2 affects MeJA-induced biosynthesis of IGs, but not of IAA and camalexin

In *Arabidopsis*, CYP79B2 and CYP79B3 specifically catalyze the conversion of Trp to IAOx (indole-3-acetaldoxime), which is then directed into the biosynthesis of IGs, camalexin or IAA (Figure 7A) [51, 52]. Our finding that *CYP82C2* affects JA-induced Trp accumulation prompted us to determine whether *CYP82C2* played a role in the biosynthesis of IGs, camalexin or IAA. Interestingly, we found that the MeJA-induced expression levels of several IG biosynthesis-related genes including *ATR1* [53], *SUPERROOT2* (*SUR2*) [54, 55] and *SUPERROOT1* (*SUR1*) [56] were reduced in the *jah1-1* mutant, as compared with wild type (Figure 7B), suggesting that *CYP82C2* may affect MeJA-induced biosynthesis of IGs. To test this, we treated 14-day-old plants with MeJA and measured the levels of three major IGs including indol-3-ylmethyl glucosinolate, 4-methoxy-indol-3-

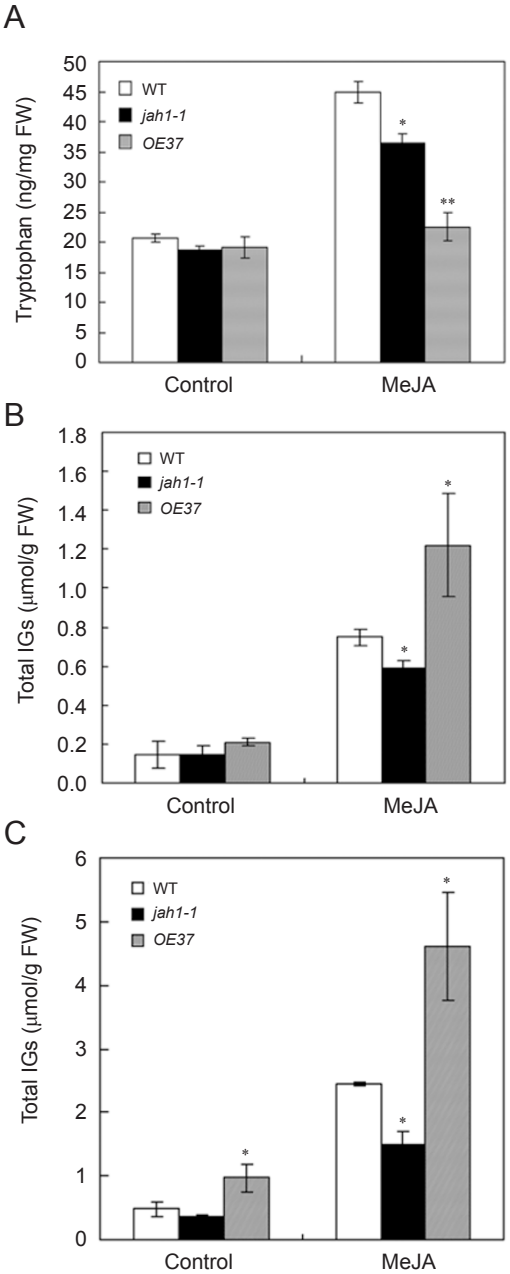

Figure 8 *CYP82C2* affects MeJA-induced accumulation of Trp and IGs. **(A)** *CYP82C2* affects MeJA-induced accumulation of Trp levels. Seedlings (10 days old) of the indicated genotypes were treated without (Control) or with 100 μM MeJA for 2 days and then Trp levels were measured by GC-MS. Values indicate mean ± SEM of three biological replicates. Asterisks indicate the significant difference from the wild type (two-way ANOVA, **P* < 0.05, ***P* < 0.01). **(B** and **C)** *CYP82C2* affects MeJA-induced biosynthesis of IGs. Seedlings (2 weeks old) of the indicated genotypes were treated with water (Control) or 50 μM MeJA for 2 days, and total IGs were extracted from leaves **(B)** and roots **(C)** and measured by HPLC. Values indicate mean ± SD of three biological replicates. Asterisks denote Student's *t*-test significance compared with wild type (**P* < 0.05).

ylmethyl glucosinolate and 1-methoxy-indol-3-ylmethyl glucosinolate (Supplementary information, Figure S4). Figure 8B shows the calculation of the results of the total IGs in leaves. In the absence of MeJA, the basal levels of total IGs were similar among *jah1-1*, *OE37* and wild-type plants. In line with previous findings, MeJA treatment led to significant increase of IG levels in all the three genotypes. However, while the levels of total IGs in *jah1-1* were lower than those in wild type, *OE37* plants accumulated significantly higher levels of total IGs than wild-type plants (Figure 8B).

Considering the fact that the *jah1* mutants are hypersensitive to JA-mediated inhibition of root growth and that *CYP82C2* is preferentially expressed in the root, we further measured the levels of IGs extracted from roots of the same set of plants, as shown in Figure 8B. In the absence of MeJA, roots of *OE37* plants contained significantly higher levels of total IGs than wild-type and *jah1-1* plants (Figure 8C). In response to MeJA treatment, total IGs in *jah1-1* roots were significantly lower than those in wild type, whereas total IGs in *OE37* were significantly higher than those in wild type (Figure 8C). These results indicate that *CYP82C2* affects MeJA-induced biosynthesis of IGs.

Given the potential of a tight link between the biosynthesis of IGs and IAA from the common intermediate IAOx, we measured MeJA-induced free IAA levels in wild type, *jah1-1* and *OE37* plants. Without MeJA treatment, free IAA levels were similar among the three genotypes (Supplementary information, Figure S5). MeJA treatment increased free IAA levels in all of the three genotypes and, again, the MeJA-induced free IAA levels did not exhibit significant difference among wild type, *jah1-1* and *OE37* plants (Supplementary information, Figure S5), indicating that *CYP82C2* does not affect MeJA-induced IAA biosynthesis.

We also measured camalexin production following infection with *Alternaria brassicicola* and found that both *jah1-1* and *OE37* show wild type levels of camalexin accumulation (Supplementary information, Figure S6). In contrast, the *pad3* (*phytoalexin-deficient 3*) mutant [57] produced a smaller amount of camalexin in response to the same pathogen.

Discussion

CYP82C2 differentially modulates JA-induced root growth inhibition and defense gene expression

The inhibitory effect of JA on primary root growth has been widely employed as a genetic assay to identify JA-related mutants in *Arabidopsis*. Most of the JA-insensitive mutants, including *jar1* [7, 24], *jin1* and *jin4* [12] were identified by screening for JA-insensitive root growth in the presence of relatively high concentrations of JA or MeJA (50 to 100 μM). To identify additional components involved in JA signaling, we set up a genetic screen by using relatively low doses of JA (10 μM in this study). The mutant *jah1-1* was identified because it showed stunted root growth in response to low concentrations of JA. Given that the root growth of *jah1-1* was normal in control medium and showed increased sensitivity to the inhibitory effect of JA (Figure 1A and 1B), this mutant was considered to be hypersensitive to JA-mediated inhibition of root growth. In contrast to root growth inhibition, however, *jah1-1* was less sensitive to JA in induction of defense gene expression, because the JA-induced expression levels of defense-related genes, including *PDF1.2* and *Thi2.1*, were decreased in this mutant (Figure 1C). The known JA-related mutants, including *cev1* [26] and *cex1* [58], exhibit stunted root growth without JA and constitutively express JA-induced defense genes. Therefore, the differential regulation of JA-induced root growth inhibition and defense gene expression in *jah1-1* identifies this mutant as being distinct from the known JA-related mutants. It is noteworthy that the *jah1-1* mutation mainly affected JA-induced expression of pathogen defense-related genes, but had little effect on the JA-induced expression of wound-responsive genes including *LOX2* and *VSP1* (Figure 1C). Together, these observations support a hypothesis that JA-induced root growth inhibition and defense response are regulated by different mechanisms. In line with previous findings that not all of the JA responses were mediated through the F-box protein COI1 [59-62], which was recently shown to be a jasmonate receptor [11, 15, 18, 19], we found that MeJA induced the expression of *CYP82C2* in a COI1-independent manner. COI1-independent expression of *CYP82C2* is consistent with its effect on JA-induced IG biosynthesis. It has been demonstrated that the JA-induced production of IGs does not rely completely on COI1, as *coi1-1* plants produce IGs, albeit at lower levels [27, 35, 63]. Together, our characterization of the *jah1* mutant revealed the complexity of JA-mediated responses. Future elucidation of the biochemical function of the CYP82C2 protein should provide evidence to help explain the versatile aspects of the *jah1* mutant phenotype.

CYP82C2 is important for plant resistance to B. cinerea

From the publicly available microarray database, we noticed that the expression of *CYP82C2* was induced by pathogen infections [64]. Our gene expression analyses indicated that while the *jah1-1* plants were defective in JA-induced expression of a group of pathogen defense

genes including *PDF1.2* and *Thi2.1* (Figure 1C), transgenic plants overexpressing *CYP82C2* showed increased expression of these genes (Figure 6B). Given that the expression of *PDF1.2* and *Thi2.1* is associated with plant resistance to pathogens [24-26], together with the fact that the expression of *CYP82C2* itself was induced by both JA treatment and pathogen inoculation (Figure 4G-4I), our results suggest that *CYP82C2* may play a role in plant resistance to pathogen infection. Indeed, pathogen attack assays indicated that while the *jah1-1* mutant plants were more susceptible to the necrotrophic fungus *B. cinerea*, transgenic plants overexpressing *CYP82C2* were more resistant (Figure 6C and 6D). On the basis of these findings, we conclude that the *CYP82C2* gene plays an important role in plant defense responses against the necrotrophic fungus *B. cinerea*.

CYP82C2 affects MeJA-induced synthesis of Trp and IGs

Metabolic profiling of the Trp-pathway compounds revealed that the expression level of *CYP82C2* affects MeJA-induced accumulation of Trp (Figure 8A), which is then converted into IAOx. As a key metabolic branch point, IAOx can be directed to the biosynthesis of IGs, camalexin or IAA, in a highly regulated manner (Figure 7A) [51]. Our data revealed an effect of *CYP82C2* on the MeJA-induced biosynthesis of IGs, as evidenced by the reduction of IG levels in the *jah1-1* mutant and the increase of IG levels in *CYP82C2*-overexpressing plants (Figure 8B and 8C). The effect of *CYP82C2* on IG metabolism was further supported by analysis of its expression pattern (Figure 4B-4E). *CYP82C2* was expressed in all examined tissues, with the highest level being found in roots. The expression pattern of *CYP82C2* was consistent with the known fact that IGs are most prominent in the root and with the root growth phenotype of *jah1-1* seedlings in JA-containing medium. Furthermore, the expression of *CYP82C2* was induced by MeJA treatment and *B. cinerea* infection, which was consistent with previous reports that IG biosynthesis can be induced by JAs in several *Brassica* species [27, 33, 34]. However, a direct biochemical function of the CYP82C2 protein in IG biosynthesis remains to be determined.

It has been proposed that IAOx, the direct Trp-metabolizing product by the *CYP79B2* and *CYP79B3* genes, plays a central role in the biosynthesis of IGs, camalexin or IAA (Figure 7A). It has been shown that post-oxime blockage of the IG biosynthetic pathway leads to increased IAA biosynthesis. For example, the *sur1* [65], *sur2* [54] and *ugt74B1* [66] mutants gained their high-auxin phenotype by blocking post aldoxime enzymes in the glucosinolate pathway. On the other hand, mutational blockage of enzymes acting upstream of IAOx leads to a low-auxin phenotype. For example, the *cyp79B2cyp79B3* double mutant produced less IGs and decreased levels of IAA in a temperature-dependent manner [50]. In this context, determination of whether free IAA levels were altered in *jah1-1* was important in order to understand the possible site of action of the *CYP82C2* gene in the metabolic pathways leading to indolic compounds. However, we did not find any significant difference in free IAA levels among wild-type, *jah1-1* or *OE37* seedlings with or without of MeJA treatment (Supplementary information, Figure S5). We also measured camalexin levels in response to *A. brassicicola* infection and did not find significant difference among the three genotypes (Supplementary information, Figure S6). Taken together, our metabolic profiling of the Trp-pathway compounds indicate that CYP82C2 affects the MeJA-induced production of Trp and IGs, but not the MeJA-induced production of IAA and pathogen-induced camalexin. We propose that CYP82C2 might act in a step involved in converting Trp to IGs under conditions in which JA levels are increased. This hypothesis is consistent with our finding that the MeJA-induced production of Trp was significantly reduced in the CYP82C2 overexpressing line, in which the Trp-metabolizing flux is increased. Future experiments that include measuring JA-induced IAOx should enable us to define CYP82C2 activity as being downstream or upstream of IAOx. Under this scenario, the reduction of MeJA-induced Trp production in the *jah1-1* could be explained by Trp feedback control mechanisms. Trp biosynthesis in *Escherichia coli* is found to be regulated by three distinct negative feedback control mechanisms, namely, transcriptional repression, transcriptional attenuation and feedback enzyme inhibition [67]. In *Arabidopsis*, it has been well documented that Trp may inhibit the activity of AS through feedback mechanism by binding to an allosteric site on the catalytic α-subunit of AS [68, 69]. In this way, although the blockage in the Trp-metabolizing pathway may lead to increased Trp content in *jah1-1*, this increase in Trp accumulation might induce a feedback inhibition of AS activity and, eventually, lead to a reduced Trp production. However, relatively little is known about the transcript regulation of AS from plants. We found that the MeJA-induced expression of *ASA1*, which encodes the α-subunit of AS [69], was downregulated in *jah1-1* (Figure 7B), suggesting that AS may also be regulated at the transcriptional level in *Arabidopsis*.

Materials and Methods

Biological materials and growth conditions

All *A. thaliana* lines used were in the Columbia (Col-0) background. The *jah1-2* (SALK_128974) and *jah1-3* (SALK_024364)

mutants were obtained from the *Arabidopsis* Biological Resources Center. A homozygous T-DNA insertion line (SALK_083483, designated as *atmyc2-2*), which disrupted the expression of *AtMYC2*, was described previously [38]. The *coi1-1* [8], *coi1-2* [11] and *pad3* [57] mutants were previously described. *Arabidopsis* plants were grown as described [70]. Growth and spore harvesting of the fungus *B. cinerea* and *A. brassicicola* was done as described [71].

Isolation of the jah1-1 mutant

Seeds of a T-DNA mutagenized population of *Arabidopsis* [37] were germinated on a medium containing 10 μM JA (Sigma). Plates were placed vertically in a growth chamber for 8 to 10 days and seedlings with shorter roots were selected as putative jah mutants. Seeds from the putative mutants were retested on MS medium with 10 μM JA by measuring root elongation. The *jah1-1* was one of the identified mutants showing elevated responses to JA in root growth. Further characterization of the mutant was performed with a homozygous line obtained from three successive backcrosses of the original mutant with the wild-type parental plant Col-0.

Cloning of the CYP82C2 gene

The *CYP82C2* gene was identified with TAIL-PCR assay [72]. Three T-DNA-specific primers (LexA2, LexA4 and LexA5) and an arbitrary degenerate primer (AD3) were used for the primary, secondary and tertiary reactions, respectively. To determine the location of the T-DNA insertions, the amplified fragments from the tertiary PCR were sequenced using LexA5 as a primer. Homozygous T-DNA insertion lines for *jah1-1*, *jah1-2*, *jah1-3* and *atmyc2-2* were identified with diagnostic PCR using gene-specific primers and T-DNA primers (Supplementary information, Table S1). Homozygous *coi1/coi1* plants were selected from the progeny of *coi1/COI1* heterozygous plants as described (Supplementary information, Table S1) [9].

For complementation analysis, a 4.0 kb DNA fragment containing the entire *CYP82C2*/At4g31970 coding region and the 1.5 kb upstream sequence was amplified from wild-type plant (Col-0) by PCR and cloned into the *Kpn*I/*Xba*I sites of pCAMBIA1300 to generate the *jah1-comp*. construct. A 1.5 kb promoter for *CYP82C2* was PCR amplified and cloned into the *Hind*III/*Bam*HI sites of pCAMBIA1391Z to generate the *CYP82C2:GUS* construct. The coding sequences of *CYP82C2* cDNA was amplified by PCR and cloned into the *Bam*HI/*Sac*I sites of pCanG-HA under the control of the 35S promoter to generate the *35S:CYP82C2* construct. Similarly, we also generated a *35S:MYC2* construct. Primers used for PCR amplification are listed in Supplementary information, Table S1. The above constructs were then electroporated into *Agrobacterium tumefaciens* strain GV3101 as described [73], which were used for transformation of *Arabidopsis* plants by vacuum infiltration.

Gene expression assays

Total RNA was prepared by a guanidine thiocyanate extraction method. RNA gel blot, RT-PCR and qRT-PCR analyses were performed as described previously [70]. Gene-specific primers are listed in Supplementary information, Table S1.

Histochemical staining for GUS activity in transgenic plants was performed as described [74].

Induction treatments

Plants (2 weeks old) growing in Petri dishes were sprayed with 50 μM MeJA (Sigma), and the controls were sprayed with water. Soil-grown plants (4 weeks old) were sprayed with 1×10^5 *B.cinerea* spores per ml. Tissues were then harvested at indicated time intervals for RNA extraction.

Fungal disease assay

Plants (4 weeks old) were infected with a 5-μl drop of *B. cinerea* (1×10^5 spores per ml) or with buffer as the control and covered with clear plastic to maintain high humidity. At least 15 plants per genotype were inoculated in each experiment. Infection and development of the fungus were visualized by lactophenol-trypan blue staining as described [75].

Measurement of Trp, IAA and camalexin levels

Measurement of Trp, IAA and camalexin levels was described in Supplementary information, Data S1.

HPLC analysis of glucosinolates

Seedlings (2 weeks old) were treated with 50 μM MeJA solution for 2 days and IGs were extracted from leaves or roots. Analysis of IGs by HPLC was performed as previously described [76].

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)