Arabidopsis Cytochrome P450 *cyp83B1* Mutations Activate the Tryptophan Biosynthetic Pathway

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ABSTRACT

In plants, the tryptophan biosynthetic pathway provides a number of important secondary metabolites including the growth regulator indole-3-acetic acid (IAA) and indole glucosinolate defense compounds. Genes encoding tryptophan pathway enzymes are transcriptionally induced by a variety of stress signals, presumably to increase the production of both tryptophan and secondary metabolites during defense responses. To understand the mechanism of transcriptional induction, we isolated altered tryptophan regulation (*atr*) mutants in *Arabidopsis thaliana* with activated transcription of tryptophan genes. One *atr* complementation group consisted of mutations in the cytochrome P450 gene *CYP83B1*. Mutant plants had constitutively activated expression of the *ATR1* Myb factor gene, which was identified as a positive regulator of tryptophan genes via the *atr* mutant screen. *cyp83B1* mutants were previously characterized as having defects in IAA homeostasis due to perturbation of secondary tryptophan metabolism. Our findings indicate that the upregulation of tryptophan pathway genes might also contribute to the overaccumulation of IAA in mutant plants. Moreover, we show that *cyp83B1* mutants have lesion-mimic phenotypes, suggesting that multiple stress pathways are activated by loss of CYP83B1 function.

IN plants the tryptophan biosynthetic pathway leads to the synthesis of the amino acid tryptophan and a number of important secondary metabolites including the growth regulator indole-3-acetic acid (IAA) and indole glucosinolate defense compounds (Radwanski and Last 1995). These metabolites differ in abundance, spatial distribution, developmental control, and environmental responsiveness, implying that tryptophan primary and secondary metabolic pathways are highly regulated.

The first committed enzyme in tryptophan biosynthesis is anthranilate synthase (AS). This enzyme provides a key point of control for the flow of metabolites through the pathway. AS consists of an α-subunit that contains the catalytic activity and a β-subunit that provides a glutamine amidotransferase activity. AS is the target of feedback inhibition by free tryptophan, which binds to an allosteric site on the α-subunit to downregulate the enzyme when tryptophan is abundant. In Arabidopsis, the α-subunit of AS is encoded by two somewhat divergent genes, *ASA1* and *ASA2* (Niyogi and Fink 1992). *ASA1* can be transcriptionally induced by several stress signals including bacterial pathogen infection and amino acid starvation, whereas *ASA2* is not inducible (Niyogi and Fink 1992; Zhao and Last 1996; Zhao *et al.* 1998). Arabidopsis also encodes three AS β-subunit genes, *ASB1*, *ASB2*, and *ASB3* (Niyogi *et al.* 1993). *ASB* transcription is induced by the same signals that upregulate *ASA1* expression.

Arabidopsis mutants with *a*ltered *t*ryptophan *r*egulation (*atr*) have been used to elucidate how the tryptophan pathway is controlled. The *atr* mutants were isolated as plants that are resistant to tryptophan feedback inhibition induced either by high levels of exogenous tryptophan or by a toxic analogue of tryptophan (Bender and Fink 1998). The *atr1D* mutation, which creates a dominant overexpression allele of a Myb transcription factor ATR1, was isolated from this screen. The *atr1D* mutation increases *ASA1* expression specifically in the hypocotyl of seedlings. This mutation is also likely to perturb the expression of other tryptophan metabolism genes because overexpression of the *ASA1* gene alone is not sufficient to confer the *atr1D* resistance phenotype (Niyogi 1993). The *ATR1* transcription unit consists of three short open reading frames (ORFs) upstream of the main *ATR1*-encoding ORF. The *atr1D* mutation creates a premature stop codon in the first upstream ORF and causes an increase in the steady-state message levels of the *ATR1* transcript. The mutation is presumed to act primarily by improving the translation of the *ATR1* ORF, which then has the secondary effect of stabilizing the *ATR1* messenger RNA. The deregulated phenotypes of the *atr1D* mutation can be phenocopied by expressing the *ATR1* ORF, in the absence of the short upstream ORFs, from a strong constitutive promoter on a transgene (J. Bender, unpublished observations). Thus,

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high levels of the ATR1 Myb transcription factor can positively regulate tryptophan genes and confer tryptophan feedback resistance. However, the physiological function of ATR1 remains to be characterized.

Another class of mutations recovered from the *atr* screen was the *atr4* complementation group. We report here that the *atr4* locus is the *CYP83B1* cytochrome P450 gene. The CYP83B1 enzyme has been shown to catalyze the N-hydroxylation of indole-3-acetaldoxime (IAOx), which is an intermediate step in the biosynthesis of indole glucosinolates (Bak *et al.* 2001; Hansen *et al.* 2001). Loss-of-function mutations in *CYP83B1* confer adventitious root development from hypocotyl tissue, a phenotype diagnostic of increased IAA responses, and biochemical analysis indicates that *cyp83B1* mutant plants have elevated levels of IAA (Delarue *et al.* 1998; Barlier *et al.* 2000). Thus, *cyp83B1* mutations have been postulated not only to block the synthesis of indole glucosinolate defense compounds, but also to redirect the flow of indolic compounds toward IAA synthesis (Bak *et al.* 2001).

We show that the *cyp83B1* mutants have upregulation of the ATR1 Myb factor, which is the likely cause of their *atr* phenotypes. Double mutants combining *cyp83B1* and the dominant *atr1D* allele yield plants with enhanced adventitious root production. This observation suggests that increased tryptophan gene expression, stimulated by increased levels of the ATR1 Myb factor, contributes to the high IAA phenotype. We also show that *cyp83B1* mutants display a number of morphological and gene regulation phenotypes diagnostic of constitutively upregulated stress responses. Thus, beyond the immediate perturbation of indole glucosinolate biosynthesis, *cyp83B1* mutations confer an array of pleiotropic effects.

MATERIALS AND METHODS

Plant growth conditions: For all assays conducted with seedlings, surface-sterilized seeds were plated on plant nutrient 0.5% sucrose (PNS) medium (Haughn and Somerville 1986) with 0.75% agar in 100 × 100 × 15-mm square petri plates. Plates were sealed with Parafilm and grown under glass plates in a Percival CU-32L tissue culture incubator at 20° under continuous illumination. Glass plates were used to screen out ultraviolet (UV) light, which can contribute to the breakdown of chemical supplements, without blocking visible light. Adult plants were grown in Scott's Metromix 360 soilless medium at ~22° under continuous illumination. For all experiments except spontaneous lesion formation (see below), light intensity was ~100 μE m^{-2} sec^{-1}.

Mutant isolation and positional cloning: The *atr4-2* allele was isolated from a previously described screen for mutants that are resistant to feedback inhibition by high levels of exogenous tryptophan (Bender and Fink 1998). This screen exploits the Columbia *trp1-100* mutant, which carries a missense mutation in the *PAT1* gene encoding the second enzyme in the tryptophan pathway (Rose *et al.* 1997). The *trp1-100* strain is weakly blue fluorescent under UV light due to accumulation of anthranilate compounds. However, when *trp1-100* is grown on medium containing 100 μM tryptophan, the fluorescence is suppressed, presumably due to feedback inhibition of AS activity and reduction in anthranilate production. The *atr4-2* allele was thus isolated by screening for ethyl methane sulfonate (EMS)-induced mutants in the *trp1-100* background whose fluorescence persists on high tryptophan medium. The *atr4-2* allele was subsequently segregated away from the *trp1-100* mutation by crosses with wild-type Columbia. The *atr4-1* allele was isolated from a screen of EMS-mutagenized Columbia seedlings for mutants that are resistant to the toxic tryptophan analogue 5-methyl-tryptophan (5MT; Bender and Fink 1998). In this assay, seeds are germinated on PNS medium supplemented with 15 μM 5MT and scored at 10–14 days for root length.

The two *atr4* mutations were shown to be allelic by complementation crosses: the F_1 progeny of the *atr4-1* × *atr4-2* cross were resistant to 5MT and displayed *atr4* morphological defects such as adventitious root formation. The *atr4-2* allele was mapped to chromosome 4 with standard cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel 1993) and simple sequence length polymorphism (Bell and Ecker 1994) methods by crossing with the polymorphic strain Landsberg *erecta* (L*er*). To localize the mutation, a second mapping cross was performed with a derivative of the Ler strain that carries three visible marker mutations along chromosome 4: *bp1-1*, *cer2-2*, and *ap2-1* (strain CS8 from the Arabidopsis Biological Resource Center). Segregation of the *atr4-2* morphology *vs.* the visible marker mutations in the progeny of this cross placed *atr4-2* between *cer2-2* and *ap2-1*, on the lower arm of the chromosome. A total of 419 plants with recombination breakpoints between *cer2-2* and *ap2-1*, and thus near the *atr4-2* locus, were used for fine-structure mapping. This analysis showed complete linkage to the g8300 cosmid-mapping marker (http://www.arabidopsis.org/maps/CAPS_Chr4.html). A contig of wild-type Columbia genomic clones spanning this region was isolated by hybridization from a λ plaque library (Bender and Fink 1998), and fragments containing predicted ORFs were subcloned into the plant transformation vector pBIN19 (Bevan 1984) to see if they could complement the *atr4-2* mutation when introduced by *Agrobacterium tumefaciens*-mediated *in planta* transformation (Clough and Bent 1998). This analysis showed that the *atr4-2* morphological defects and 5MT resistance phenotype were complemented by a 5-kb *Eco*RI fragment containing the *CYP83B1* gene. The mutant was also complemented by a cDNA of *CYP83B1* driven from the cauliflower mosaic virus 35S promoter of transformation vector pBICaMV (Hull *et al.* 2000). The cDNA was isolated by hybridization screening of a Ler seedling cDNA library (Minet *et al.* 1992). The *CYP83B1* Columbia cDNA sequence has been reported as GenBank accession no. D78598 (Mizutani *et al.* 1998) and the Columbia genomic sequence lies on bacterial artificial chromosome F3L17 (GenBank accession no. AL080283).

The *CYP83B1* coding region was amplified by PCR from genomic DNA of both *atr4* alleles. Products from two independent PCR reactions were cloned and sequenced for each allele. Both mutant alleles create a restriction site change, which allows PCR-based detection.

The *atr4-1* mutation destroys one of two *Msp*I sites in the 384-bp region amplified with the primers ATR4-A 5′-ATCTAC CAGCAGAAACGTCC-3′ and ATR4-B 5′-GTGTCGCAAGTTT CAGACCG-3′; the *atr4-2* mutation destroys one of six *Hae*III sites in the 711-bp region amplified with the primers ATR4-A and ATR4-C 5′-AAGACCAACCTTTCTCCATC-3′.

RNA gel blot analysis: Total RNA was prepared from whole seedlings grown aseptically on PNS medium under glass plates for 10 days postgermination before RNA extraction. RNA gel blot analysis was performed by formaldehyde gel electrophore-

sis, transfer to nylon membranes, and hybridization with radiolabeled probes as previously described (Melquist *et al.* 1999). Probes were cDNA fragments from the indicated genes. A β-tubulin probe (*TUB*) was used as a control to correct for loading differences. The *ASA1*, *CYP79B2*, *CYP83B1*, *PDF1.2*, and *TUB* probes were full-length cDNAs. The *CYP79B2* probe has ~80% nucleic acid identity with the related gene *CYP79B3*, and therefore should not significantly cross-hybridize under high stringency conditions. The *PR1* probe was an internal cDNA fragment (Rogers and Ausubel 1997). The *ATR1* probe was a cDNA fragment extending from the 5′ end of the transcript to an internal *Eco*RI site. Band intensities were quantitated using Fuji Phosphoimager and MACBAS 2.2 software. All results were reproduced in two or three independent experiments.

Plant strains: The *axr1-3* mutant was obtained from the Arabidopsis Biological Resource Center (CS3075). The *atr4-1 axr1-3* double mutant was constructed by screening for F_2 progeny of the cross that were resistant to 150 nm 2,4-D, a phenotype diagnostic of the homozygous *axr1-3* mutation (Estelle and Somerville 1987). The *axr1-3* homozygous individuals were then screened for the *atr4-1* mutation using the PCR genotype assay described above. The *atr4-1 NahG* strain was made by screening progeny of the cross for the kanamycin resistance marker on the *NahG* transgene and the *atr4-1* genotype. The *NahG* transgenic strain in the Ler background (Bowling *et al.* 1994) was a gift of X. Dong, Duke University. To eliminate potential phenotypic contributions from the *erecta* mutation in this background, plants were also scored for a wild-type *ERECTA* phenotype. The *atr4-1 atr1D* double mutant was constructed by screening progeny of the cross with PCR genotype assays for both *atr4-1* and *atr1D* (Bender and Fink 1998). Because this double mutant is sterile, it was propagated by segregation from an *atr1D/atr1D* homozygous *atr4-1/ATR4* heterozygous parent. The *cpr5-2* mutant (Bowling *et al.* 1997) was obtained from the Arabidopsis Biological Resource Center (CS3770) and the *rty* mutant was obtained as a gift from J. Celenza, Boston University.

Assays for *cyp83B1* phenotypes: Spontaneous lesion formation was scored by growing seedlings aseptically on PNS medium for 20 days postgermination under glass plates and low light (~25 μE m^{-2} sec^{-1}) conditions. For each strain tested, a population of 48 seedlings was inspected for the presence of leaf lesions; if none of the plants in the population displayed lesions, the strain was scored as negative, and if any of the plants in the population displayed lesions, the strain was scored as positive. Under these conditions, both *cyp83B1* and *cpr5* mutant populations consisted of ~75% seedlings with no lesions and 25% seedlings with one or two lesions. Adventitious root formation was scored by growing seedlings on PNS medium under glass plates and observing the phenotype at 14 days postgermination. 5MT resistance was scored by growing seedlings on PNS medium supplemented with 15 μm 5MT under glass plates and measuring root growth at 10 days postgermination.

RESULTS

Isolation and characterization of *cyp83B1* mutants: The *atr4-1* and *atr4-2* mutations in the *CYP83B1* gene were originally isolated from EMS-mutagenized seedlings of the Columbia strain by screening for resistance to feedback inhibition induced by high levels of exogenous tryptophan or a toxic tryptophan analogue 5MT (see materials and methods). 5MT presumably acts by feedback inhibiting the AS catalytic α-subunit without substituting for the nutritional role of true tryptophan. One mechanism for 5MT resistance is creation of feedback resistance mutations in the AS α-subunit gene, *ASA1* (Niyogi 1993; Kreps *et al.* 1996; Li and Last 1996). Another mechanism of resistance is transcriptional upregulation of *ASA1* and other tryptophan metabolism genes. For example, the dominant *atr1D* mutant carries an overexpression mutation in the *ATR1* Myb transcription factor gene that leads to activation of *ASA1* expression specifically in hypocotyl tissue (Bender and Fink 1998). Under 5MT resistance screening conditions, wild-type Arabidopsis seedlings fail to develop roots whereas *atr* mutants display partial or full root development (Figure 1A).

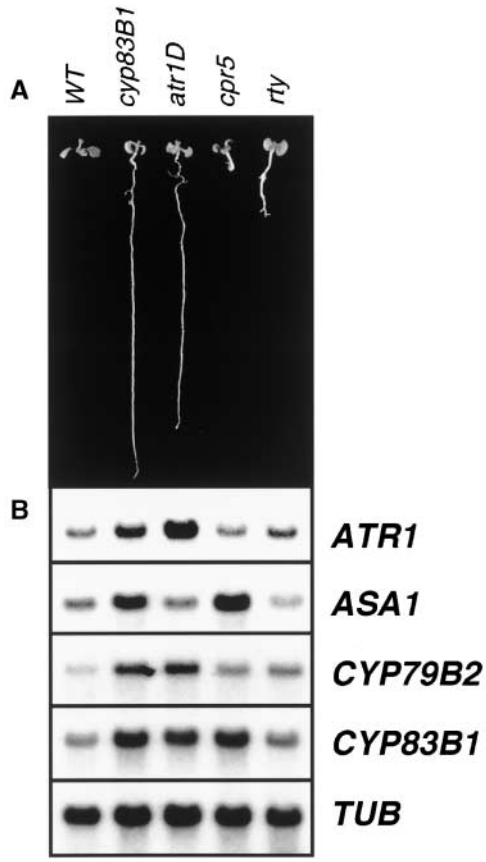

Figure 1.—Altered tryptophan regulation phenotypes of the *cyp83B1* mutant. (A) 5MT resistance. (B) RNA gel blot assays.

Unlike the previously characterized *atr1D* mutant, the *atr4* mutants displayed a number of morphological abnormalities including the development of adventitious roots from the hypocotyl, epinastic (curled under) leaves, elongated petioles, formation of spontaneous necrotic lesions, delayed time to flowering, dark green pigmentation, reduced size, and reduced viability relative to the parental strain (Figures 2 and 4; data not shown). The *atr4-1* allele had morphological phenotypes stronger than those of the *atr4-2* allele and was therefore used for all subsequent analyses unless otherwise specified.

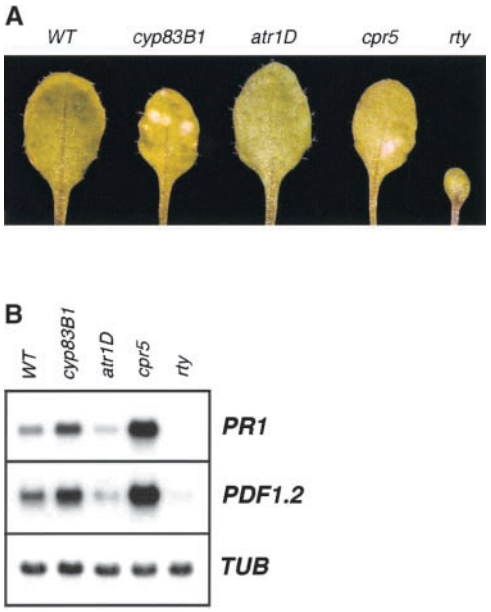

FIGURE 2.—Pathogen response phenotypes of the *cyp83B1* mutant. (A) Spontaneous lesion formation. (B) RNA gel blot assays.

For comparison, we examined other mutants with *atr4*-related morphological phenotypes for whether they were also resistant to 5MT. The *rty* or *sur1* mutant has a strong adventitious root phenotype and accumulation of increased free IAA (BOERJAN *et al.* 1995; KING *et al.* 1995). This mutant displayed weak resistance to 5MT (Figure 1A), although the effect was probably underestimated because *rty/sur1* has stunted root development even in the absence of 5MT. The necrotic lesion phenotype (Figure 2A) suggested that *atr4* might have constitutive upregulation of pathogen response genes. Indeed, RNA gel blot analysis revealed that both the *PR1* and *PDF1.2* pathogen-responsive genes (REYMOND and FARMER 1998) were upregulated in the mutant (Figure 2B). The *cpr5* mutant (BOWLING *et al.* 1997) constitutively activates a number of pathogen defense pathways and forms spontaneous necrotic lesions in the absence of pathogen challenge (Figure 2). However, this mutant was not resistant to 5MT (Figure 1A). Conversely, *rty/sur1* did not display any necrotic lesions despite weak 5MT resistance. Together these results suggest that the *atr4* mutant has a unique profile of gene expression changes that cause 5MT resistance, adventitious roots, and lesion-mimic phenotypes.

Cloning of *cyp83B1* mutants: The *atr4* mutant locus was cloned on the basis of its map position (see MATERIALS AND METHODS). Standard mapping analysis placed the mutant locus on the lower arm of chromosome 4. Fine-structure mapping narrowed down the mutant region to an interval that contained several genes including the cytochrome P450 gene *CYP83B1*. This gene was shown to be the site of the *atr4* lesions by two methods. First, the mutant alleles were sequenced, and both were found to have missense mutations relative to the wild-type Col sequence. The stronger *atr4-1* allele corresponded to *R438W* (CGG to TGG), which alters a highly conserved residue in the heme-binding domain. The weaker *atr4-2* allele corresponded to *A291V* (GCC to GTC), which affects an unconserved position. Second, the *atr4-2* mutant phenotypes were completely complemented either by a wild-type genomic clone carrying only the *CYP83B1* gene or by a *CYP83B1* cDNA driven by the strong constitutive cauliflower mosaic virus 35S promoter. Furthermore, two other groups have reported *cyp83B1* mutations with similar morphological defects to the *atr4* isolates. The *rnt1-1* mutant was isolated by reverse genetic screening for T-DNA insertions into cytochrome P450 genes (WINKLER *et al.* 1998), and the *sur2* mutant was isolated from a *En-1* insertional mutant collection on the basis of its adventitious root phenotype and cloned via the *En-1* tag (BARLIER *et al.* 2000). The phenotypes of the stronger *atr4-1* missense mutant allele are similar to those described for both insertional mutant alleles, suggesting that *atr4-1* is a strong loss-of-function or even a null allele.

***ATR1* is upregulated in the *cyp83B1* mutant:** To determine whether the 5MT resistance phenotype of *cyp83B1* correlates with activation of *ASA1* expression, the transcription of *ASA1* was monitored by RNA gel blot analysis of seedling tissues. This analysis revealed activation of *ASA1* steady-state transcript levels in whole seedlings for *cyp83B1* mutant *vs.* the wild-type parent strain (Figure 1B). These results were similar to those previously determined for the *atr1D* Myb overexpression mutant (BENDER and FINK 1998), although the whole-plant activation of *ASA1* expression was higher in *cyp83B1* than in *atr1D* (Figure 1B). The increased level of *ASA1* expression might reflect input from elevated pathogen response signaling, as observed in the *cpr5* mutant. Taken together, these results indicated that the *atr* phenotypes of *cyp83B1* might be due to the upregulated tryptophan biosynthetic pathway and suggested the involvement of ATR1.

To investigate this possibility, we monitored steady-state levels of *ATR1* transcripts by RNA gel blot analysis. *ATR1* steady-state transcript levels were elevated in the *cyp83B1* mutant relative to wild-type *CYP83B1* plants, although not to as high an extent as in *atr1D* (Figure 1B). The *rty* mutant, which displayed weak 5MT resistance, also displayed weak activation of *ATR1* expression. In contrast, the *cpr5* mutant, which displayed no 5MT resistance, displayed no activation of *ATR1* expression. These results show a correlation between increased *ATR1* steady-state transcript levels and 5MT resistance.

The highly related Arabidopsis cytochrome P450 enzymes CYP79B2 and CYP79B3 were recently shown to convert tryptophan to IAOx, a precursor for both IAA and indole glucosinolate synthesis (HULL *et al.* 2000). Furthermore, overexpression of the *CYP79B2* gene from the cauliflower mosaic virus 35S promoter on a trans-

gene rendered wild-type Columbia seedlings weakly resistant to 5MT. This resistance is thought to be due to CYP79B2-mediated conversion of 5MT to nontoxic secondary metabolites. *CYP79B2* is thus a candidate target gene for upregulation leading to 5MT resistance in *atr1D* and *cyp83B1* mutants. To test this possibility, we monitored *CYP79B2* steady-state transcript levels in wild-type and mutant strains by RNA gel blot analysis (Figure 1B). This analysis showed that *CYP79B2* transcripts were indeed elevated in *atr1D* and *cyp83B1* relative to wild-type Columbia. Therefore, *CYP79B2* could contribute to 5MT resistance in these mutants.

We also used RNA gel blot analysis to determine whether *CYP83B1* steady-state message levels were altered in either *atr1D* or *cyp83B1* mutants relative to a wild-type control. *CYP83B1* expression was increased in both of these mutants as well as in the *cpr5* mutant (Figure 1B). However, because the *CYP83B1* gene does not encode functional protein in the *cyp83B1* mutant background, it is unlikely that its expression levels contribute significantly to the 5MT resistance phenotype. Interestingly, we found no change in *CYP83B1* expression levels in the *rty* mutant background. This result contrasts with a previous report that *CYP83B1* is upregulated in the *sur1-3* allele (Barlier *et al.* 2000). This difference might reflect a difference in alleles, a difference in the stage at which RNA was harvested, or a difference in growth conditions between the two experiments.

***ATR1* expression is inducible:** To elucidate signals that might trigger the accumulation of the *ATR1* transcript, we carried out induction experiments on wild-type plants using a variety of exogenously applied plant signaling molecules (Figure 3A). This analysis showed that several treatments, including methyl jasmonate, brassinolide, abscisic acid, and a cytokinin (6-benzylaminopurine), upregulated *ATR1*. Other treatments such as ACC, IAA, and salicylic acid decreased *ATR1* expression. Consistent with a direct relationship between upregulation of *ATR1* and 5MT resistance, addition of methyl jasmonate to 5MT medium was able to phenocopy an *atr* resistance phenotype in wild-type seedlings (Figure 3B). Thus, the *ATR1* gene is regulated by multiple signaling pathways, one or more of which might be perturbed in the *cyp83B1* mutant to yield activation of *ATR1* and 5MT resistance.

The *atr1D* mutant enhances the adventitious root formation of *cyp83B1*: The *atr1D* mutation is thought to improve the translation of the ATR1 protein (Bender and Fink 1998). This mutation might thus have a stronger effect on ATR1 expression than the *cyp83B1* mutation alone, especially if the *cyp83B1* mutant effect is primarily at the level of transcription initiation (see discussion). We constructed a double mutant from these two alleles to determine whether *atr1D* could enhance *cyp83B1* phenotypes. We found that the double mutant displayed markedly enhanced adventitious root formation (Figure 4A). Like the strong adventitious root mutant *rty/sur1* (Boerjan *et al.* 1995; King *et al.* 1995), the *cyp83B1 atr1D* double mutant was sterile. The *ATR1* steady-state message levels were also increased in the double mutant to approximately the same extent as in the *atr1D* single mutant (Figure 4B). These results suggest that increased deregulation of the genes controlled by *ATR1*, such as *ASA1* and *CYP79B2* (Figure 1B), contributes to the adventitious root phenotype in the *cyp83B1* mutant background. This phenotype is correlated with increased levels of free IAA (Boerjan *et al.* 1995; Delarue *et al.* 1998). Therefore, IAA accumulation in *cyp83B1* might reflect both the repartitioning of metabolites between indole glucosinolate and IAA biosynthesis (Bak *et al.* 2001) and an increased level of precursor compounds stimulated by ATR1.

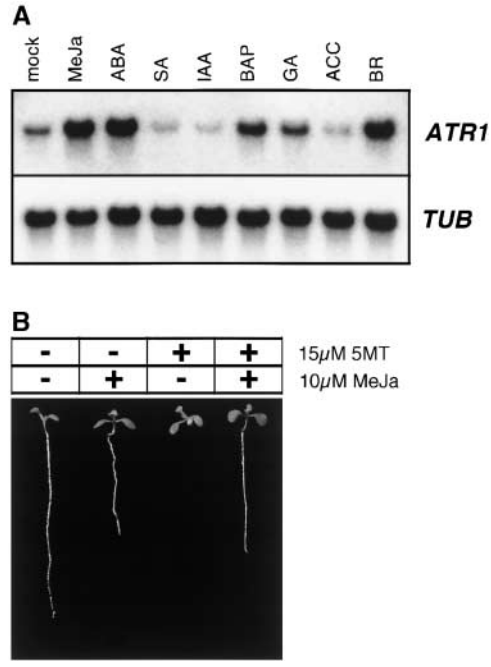

Figure 3.—*ATR1* steady-state message levels can accumulate in response to various hormones and trigger 5MT resistance in wild-type seedlings. (A) RNA gel blot assays. Wild-type Columbia seedlings were grown aseptically on PNS medium under glass plates for 10 days postgermination and then transferred to liquid PNS medium for a 6-hr induction before RNA extraction. The following concentrations of hormones were used: 20 μM methyl jasmonate (MeJA), 20 μM abscisic acid (ABA), 500 μM salicylic acid (SA), 20 μM IAA, 20 μM 6-benzylaminopurine (BAP), 20 μM gibberellic acid A_3 (GA), 20 μM 1-aminocyclopropane 1-carboxylic acid (ACC), and 1 μM brassinolide (BR). (B) An inducer of *ATR1* can elicit 5MT resistance in wild-type seedlings. Wild-type Columbia seedlings are shown after being grown for 10 days postgermination under glass plates on PNS medium containing no supplement, 15 μM 5MT, 10 μM MeJA, or both.

***AXR1* is necessary for *cyp83B1* 5MT resistance:** To further dissect the relationship between morphological defects and 5MT resistance in the *cyp83B1* mutant, we constructed a double mutant with a block in IAA signal-

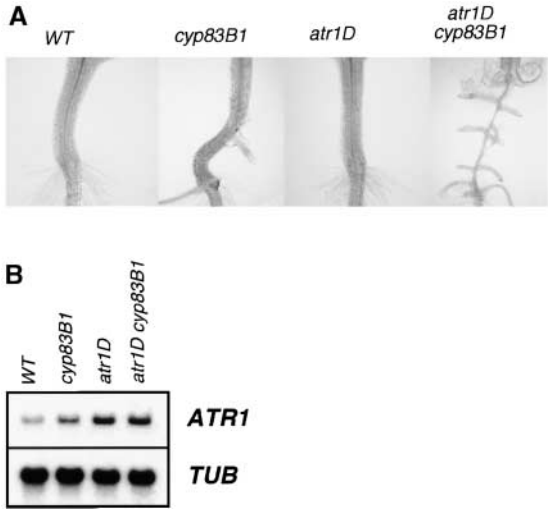

FIGURE 4.—The *atr1D* mutation enhances *cyp83B1* adventitious roots. (A) Adventitious root development. (B) RNA gel blot assays.

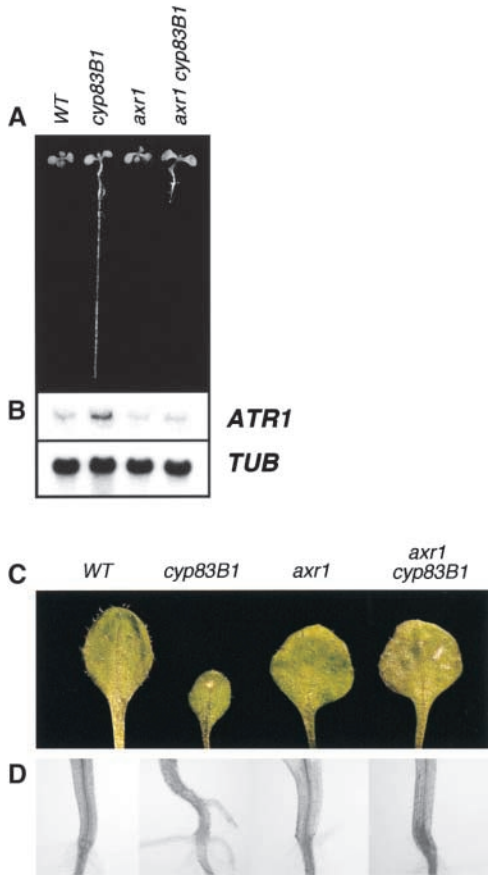

FIGURE 5.—AXR1 is necessary for 5MT resistance of *cyp83B1* seedlings. (A) 5MT resistance. (B) RNA gel blot assays. (C) Spontaneous lesion formation. (D) Adventitious root development.

ing using the *axr1* mutation. *axr1* was isolated by its resistance to exogenous auxin and confers a bushy plant morphology (ESTELLE and SOMERVILLE 1987). The *AXR1* gene encodes a subunit of an E1 enzyme that activates the small protein RUB1 prior to its conjugation to target substrate proteins (DEL POZO *et al.* 1998). The emerging view is that AXR1 might contribute to the RUB1 regulation of an SCF ubiquitin ligase complex that controls the protein turnover of repressors of IAA-responsive genes (LEYSER 1998). By this model, in *axr1* mutant strains the SCF complex would be inactive, IAA repressor proteins would accumulate to high levels, and IAA-responsive genes would remain quiescent, accounting for the IAA-resistant phenotype.

Double mutant *axr1 cyp83B1* plants lacked adventitious root formation (Figure 5) and displayed the *axr1* bushy morphology (data not shown). The double mutants also lacked upregulation of *ATR1* and 5MT resistance (Figure 5). These results suggest that AXR1 function is required for the signal that triggers *ATR1* activation in the *cyp83B1* mutant background. However, the formation of spontaneous necrotic lesions was not affected in *axr1 cyp83B1* plants. Therefore, AXR1 function is not required for this aspect of the *cyp83B1* phenotype.

***NahG* expression does not block *cyp83B1* 5MT resistance:** The increased expression of the *PR1* gene in *cyp83B1* mutants is diagnostic of increased bacterial pathogen response signaling. Components of this response are mediated by elevated levels of salicylic acid (REYMOND and FARMER 1998). In fact, *PR1* activation can be phenocopied by application of exogenous salicylic acid to uninfected plants (PENNINCKX *et al.* 1996). Salicylic acid levels can be modulated *in planta* by expression of the bacterial NahG salicylate hydroxylase enzyme (GAFFNEY *et al.* 1993; BOWLING *et al.* 1994). This enzyme acts by depleting salicylic acid through conversion into an inactive catechol.

To test whether salicylic acid might be involved in the 5MT resistance of *cyp83B1* plants, we constructed a *NahG cyp83B1* strain. In this strain the upregulation of *ATR1* and 5MT resistance were unimpaired (Figure 6). The strain also displayed unimpaired adventitious root formation. However, the *NahG cyp83B1* strain was suppressed for the formation of spontaneous necrotic lesions and displayed downregulation of *PR1* steady-state transcript levels. These results suggest that tryptophan pathway responses are distinct from pathogen responses in the *cyp83B1* mutant.

DISCUSSION

The tryptophan pathway in plants provides tryptophan for protein synthesis, IAA for regulation of development, and indole glucosinolate defense compounds. Genes in the pathway are transcriptionally responsive to a variety of biotic and abiotic stresses that cause increased demand for these metabolites (RADWANSKI and LAST 1995; ZHAO and LAST 1996; ZHAO *et al.* 1998). The

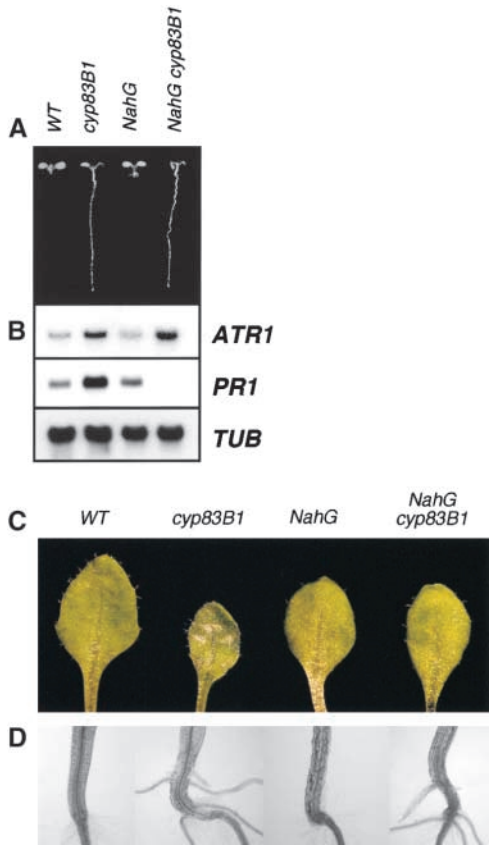

FIGURE 6.—*NahG* expression does not block 5MT resistance of *cyp83B1* seedlings. (A) 5MT resistance. (B) RNA gel blot assays. (C) Spontaneous lesion formation. (D) Adventitious root development.

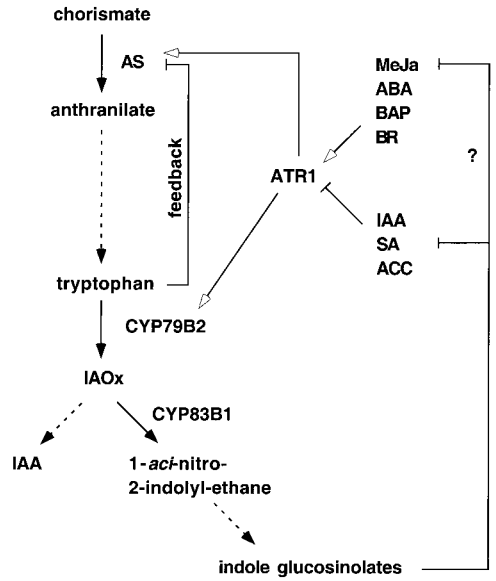

FIGURE 7.—The tryptophan pathway in Arabidopsis. Dashed lines with solid arrowheads indicate multistep pathways. Lines with open arrowheads indicate positive regulation and lines with perpendicular bars indicate negative regulation. Abbreviations for signaling molecules are as described in Figure 3.

atr screen in Arabidopsis was designed to elucidate the signals that converge on tryptophan pathway genes to control their expression. The screen has identified mutants that are resistant to high levels of exogenous tryptophan and toxic tryptophan analogues due to deregulation of the pathway.

In this work we show that loss-of-function alleles in the cytochrome P450 gene *CYP83B1* are *atr* mutants of the *atr4* complementation group. Loss-of-function *cyp83B1* mutants were previously reported as *sur2* mutants isolated on the basis of the adventitious root phenotype (DELARUE *et al.* 1998). In addition, the *rnt1-1* allele of *cyp83B1* was identified by reverse genetic screening for T-DNA insertions in cytochrome P450 genes (WINKLER *et al.* 1998). Because the adventitious root phenotype suggests a perturbation in IAA levels, work on both the *sur2* and *rnt1-1* mutations has focused on IAA metabolism. For example, *sur2* alleles of *cyp83B1* have been reported to have elevated levels of free IAA (DELARUE *et al.* 1998; BARLIER *et al.* 2000). Furthermore, the *CYP83B1* enzyme has been shown *in vitro* to catalyze the conversion of IAOx to 1-*aci*-nitro-2-indolyl-ethane, a precursor for indole glucosinolates (BAK *et al.* 2001; HANSEN *et al.* 2001). Putting these observations together, the emerging view is that *cyp83B1* mutants accumulate IAA because the block in the indole glucosinolate pathway causes an accumulation of IAOx, which is redirected into the IAA synthesis branch of tryptophan secondary metabolism (BAK *et al.* 2001; Figure 7).

Here we have focused on the *atr* phenotypes of *cyp83B1* mutants. One of the genes that is upregulated in *cyp83B1* is the *ATR1* Myb transcription factor-encoding gene (Figure 1). ATR1 was originally identified as a putative positive regulator of tryptophan genes via an overexpression allele, *atr1D* (BENDER and FINK 1998). Thus, a simple explanation for the *cyp83B1 atr* phenotypes is that they are also caused by the overexpression of *ATR1*. Consistent with this model, when *ATR1* upregulation is blocked in the *axr1 cyp83B1* double mutant, 5MT resistance is also blocked (Figure 5). However, because many genes are deregulated in *cyp83B1* (Figures 1 and 2), it remains possible that the *atr* phenotypes are due to other factors in addition to ATR1. We are currently pursuing various strategies to isolate loss-of-function *atr1* alleles. If such mutants are viable, we can use double mutant analysis with *cyp83B1* to determine whether 5MT resistance and perhaps other *cyp83B1* phenotypes are ATR1 dependent.

In the *atr1D* and *cyp83B1* mutants, *ASA1* and *CYP79B2* steady-state transcript levels are upregulated (BENDER and FINK 1998; Figure 1). Increased expression of both these genes is consistent with the mutant tryptophan feedback resistance phenotypes. Increased expression of *ASA1* would create additional targets for tryptophan feedback inhibition, thus diluting the effects of high tryptophan or 5MT. Increased expression of *CYP79B2*

would allow increased conversion of tryptophan or 5MT to noninhibitory secondary metabolites (Hull *et al.* 2000). Thus, even though overexpression of *ASA1* alone is not sufficient to confer 5MT resistance (Niyogi 1993), and overexpression of *CYP79B2* alone confers only weak resistance (Hull *et al.* 2000), the combined deregulation of both genes could account for the stronger resistance displayed in strains that overexpress *ATR1*. ATR1 could also have additional target genes that contribute toward tryptophan feedback resistance.

The upregulation of *ASA1* and *CYP79B2* is also consistent with our observation that the *atr1D cyp83B1* double mutant has exaggerated adventitious root formation (Figure 4). *ASA1* is a key point of control for the flow of metabolites through the tryptophan pathway (Figure 7). CYP79B2 produces IAOx (Hull *et al.* 2000), which presumably lies at the branchpoint between IAA and indole glucosinolate biosynthesis (Bak *et al.* 2001). Therefore, stimulation of *ASA1* and *CYP79B2* transcript levels by *atr1D* combined with the *cyp83B1* block in the indole glucosinolate branch of the pathway would be predicted to increase the flow of tryptophan metabolites toward IAA (Figure 7). Interestingly, the characteristic phenotype of *sur2* alleles of *cyp83B1* was previously shown to be suppressed when plants were grown in the presence of exogenous IAA (Barlier *et al.* 2000). Taken together with our observation that exogenous IAA suppresses *ATR1* expression (Figure 3), a possible explanation for this result is that downregulation of *ATR1* blocks activation of genes like *ASA1* and *CYP79B2* that contribute to the adventitious root phenotype.

Expression of *ATR1* can be induced by exogenous application of several signaling compounds including methyl jasmonate, brassinolide, abscisic acid, and cytokinin (Figure 3). Thus, one or more of these compounds is likely to be elevated in the *cyp83B1* mutant, counterbalancing potential negative effects from IAA and salicylic acid (see below). Consistent with the possibility that the mutant might have elevated levels of methyl jasmonate, methyl jasmonate-responsive genes including *PDF1.2* (Figure 2) and *LOX2* (data not shown) are upregulated in the mutant. However, these methyl jasmonate-responsive genes are also upregulated in the *cpr5* mutant (Bowling *et al.* 1997), and yet this strain does not display activation of *ATR1* or 5MT resistance (Figure 1). Perhaps in *cpr5*, signals such as methyl jasmonate that act positively on *ATR1* expression are overwhelmed by other signals, such as salicylic acid (Bowling *et al.* 1997; Figures 2 and 3), that act negatively on *ATR1* expression.

It remains unknown whether the increase in *ATR1* steady-state message levels in *cyp83B1* reflects activation of transcription initiation, increased transcript stability, or both. The *ATR1* transcript includes three short upstream ORFs (Bender and Fink 1998). The *atr1D* overexpression allele creates a premature termination codon in the first and longest of these ORFs. The simplest interpretation of this observation is that the first upstream ORF inhibits translation of the downstream *ATR1* ORF and that the *atr1D* mutation disrupts this translational inhibition, leading to improved *ATR1* message stability. Thus, the *atr1D* mutation is predicted to have a strong positive effect on the levels of ATR1 protein. In contrast, signaling molecules such as methyl jasmonate that increase *ATR1* steady-state message levels (Figure 3) might act solely at the level of transcription initiation, without a mechanism to override the translational repression of the upstream ORFs. *ATR1* transcriptional activation alone would thus be predicted to yield only a modest increase in ATR1 protein levels. This scenario would explain our observation that the *atr1D cyp83B1* double mutants display an exaggerated adventitious root phenotype (Figure 4), whereas growing the *cyp83B1* mutant on medium supplemented with 20 μM methyl jasmonate does not confer this phenotype (data not shown).

The *axr1* mutation, which was previously isolated on the basis of its resistance to high levels of exogenous auxin, blocks upregulation of *ATR1* and 5MT resistance in *cyp83B1* plants (Figure 5). One interpretation of this result is that the activation of *ATR1* is mediated by IAA signaling. However, two observations argue against this view. First, exogenous IAA downregulates *ATR1* (Figure 3). Second, the *rty/sur1* mutant has stronger IAA responses than does the *cyp83B1/sur2* mutant (Boerjan *et al.* 1995; Delarue *et al.* 1998), and yet it has only modest upregulation of *ATR1* and weak 5MT resistance (Figure 1). An alternative possibility is that *axr1* affects more than just IAA sensing. Because the AXR1 protein is involved in RUB1 post-translational modifications (Leyser 1998), it could have multiple target substrates, some of which control the signals that activate *ATR1*. Loss of AXR1 function does not block spontaneous lesion formation in the *cyp83B1* mutant (Figure 5), suggesting that AXR1-mediated signaling is not required for this phenotype.

Because the *cyp83B1* mutant forms spontaneous necrotic lesions (Figure 2), it can be considered a "lesion-mimic" mutant (Alvarez 2000). Lesion formation is usually induced by the hypersensitive response (HR) to pathogen infection. During this response, tissue around the site of infection undergoes rapid cell death to block the spread of the pathogen and releases a signal to prime systemic acquired resistance (SAR) against subsequent pathogen infection. The salicylic acid-responsive gene *PR1* is upregulated locally around HR lesions and globally in SAR (Carr *et al.* 1987; Reymond and Farmer 1998). Thus, a possible explanation for the lesion-mimic phenotypes and *PR1* upregulation of *cyp83B1* is that the mutant has elevated levels of salicylic acid. Consistent with this possibility, expression of the *NahG* salicylate hydroxylase enzyme blocks lesion formation and *PR1* activation (Figure 6). In fact, *PR1* expression is suppressed well below the normal basal level in the *NahG*

cyp83B1 strain. This reduced expression might represent the effects of other signaling pathways that are perturbed in *cyp83B1* and that act contrary to the stimulatory effects of salicylic acid on *PR1* expression.

If salicylic acid is elevated in *cyp83B1*, it presumably does not block *ATR1* activation (Figure 3) because of its levels, tissue distribution, or competing signals from other pathways (Figure 7). It is interesting to note that when salicylic acid is depleted by *NahG* in the *cyp83B1* background, *ATR1* expression is elevated to a greater degree than in *cyp83B1* (Figure 6). This observation suggests that there actually is partial suppression of *ATR1* due to elevated salicylic acid in the *cyp83B1* mutant, but that this suppression is antagonized by other activating signals. Blocking lesion-mimic phenotypes with *NahG* does not affect either 5MT resistance or adventitious root formation (Figure 6). Thus, salicylic acid is unlikely to be involved in the activation of these phenotypes.

Although the *cyp83B1* mutation affects a single biosynthetic enzyme, it has multiple pleiotropic effects. The perturbation of IAA levels and the formation of adventitious roots in mutant plants can be explained by a primary disruption in tryptophan secondary metabolism (Figure 7). However, other mutant phenotypes, including activation of *ATR1* and 5MT resistance or activation of *PR1* and lesion formation, are not consistent with elevated IAA as the cause. As discussed above, *ATR1* expression is suppressed by exogenous IAA. Furthermore, exogenous IAA does not activate *PR1* expression in wild-type plants (data not shown), and the high IAA mutant *rty/sur1* does not display activation of *PR1* or obvious lesion formation (Figure 2). Instead, the loss of CYP83B1 function might activate non-IAA signaling pathways as a secondary consequence of the mutation. For example, the reduction of indole glucosinolate defense compounds in the *cyp83B1* mutant (Bak *et al.* 2001; Figure 7) might upregulate other defense pathways such as those involving methyl jasmonate (accounting for the activation of *ATR1* and *PDF1.2*) and salicylic acid (accounting for the activation of *PR1*). A promising direction for unraveling these complex signaling interactions is the isolation of second-site suppressor mutations that block some or all of the *cyp83B1* mutant phenotypes.

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