The Arabidopsis ATR1 Myb Transcription Factor Controls Indolic Glucosinolate Homeostasis

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Plants derive a number of important secondary metabolites from the amino acid tryptophan (Trp), including the growth regulator indole-3-acetic acid (IAA) and defense compounds against pathogens and herbivores. In previous work, we found that a dominant overexpression allele of the Arabidopsis (Arabidopsis thaliana) Myb transcription factor ATR1, atr1D, activates expression of a Trp synthesis gene as well as the Trp-metabolizing genes CYP79B2, CYP79B3, and CYP83B1, which encode enzymes implicated in production of IAA and indolic glucosinolate (IG) antiherbivore compounds. Here, we show that ATR1 overexpression confers elevated levels of IAA and IGs. In addition, we show that an atr1 loss-of-function mutation impairs expression of IG synthesis genes and confers reduced IG levels. Furthermore, the atr1-defective mutation suppresses Trp gene dysregulation in a cyp83B1 mutant background. Together, this work implicates ATR1 as a key homeostatic regulator of Trp metabolism and suggests that ATR1 can be manipulated to coordinately control the suite of enzymes that synthesize IGS.

In plants, the Trp pathway provides precursors for a variety of important secondary metabolites. For example, indole-3-acetic acid (IAA), a central regulator of cell division and elongation, is derived via several metabolic routes from Trp pathway compounds (Barter et al., 2001; Ljung et al., 2002). One route of IAA synthesis elucidated in Arabidopsis (Arabidopsis thaliana) involves the conversion of Trp to an indole-3-acetaldoxime (IAOx) intermediate by a pair of functionally redundant cytochrome P450 enzymes, CYP79B2 and CYP79B3 (Zhao et al., 2002; Fig. 1). IAOx is then converted to IAA, likely via indole-3-acetonitrile and/or indole-3-acetaldehyde.

In Brassicas, including Arabidopsis, an important class of Trp secondary metabolites is indolic glucosinolate (IG) defense compounds. Upon tissue damage, such as during insect or herbivore attack, IGs and other glucosinolates are metabolized by myrosinases into biologically active nitrile, isothiocyanate, or thiosulfinate forms that give Brassicas their distinctive mustard flavor (Wittstock and Halkier, 2002). In the human diet, glucosinolate-derived isothiocyanates act as anticancer compounds by inducing carcinogen-detoxifying enzymes (Talalay and Fahey, 2001).

In Arabidopsis, IG synthesis involves the CYP79B2/CYP79B3-catalyzed conversion of Trp to IAOx (Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002). IAOx is then converted by the cytochrome P450 enzyme CYP83B1 to the next intermediate in the IG pathway, which is proposed to be 1-acetyl-2-indolylthione (Bak et al., 2001; Hansen et al., 2001a). Thus, IAOx lies at a metabolic branch point between the synthesis of IAA and IGs (Fig. 1). Plants that overexpress CYP79B2 from the cauliflower mosaic virus (CaMV) 35S promoter display elevated levels of both IAA and IGs (Zhao et al., 2002). Conversely, Arabidopsis cyp79B2 cyp79B3 double mutants are strongly deficient in IGs and partially deficient in IAA, suggesting that IAOx produced by CYP79B2 and CYP79B3 is the sole source of IGs, but that other pathways exist for IAA production (Zhao et al., 2002). The cyp83B1 mutant is also deficient in IGs, but to the extent found in cyp79B2 cyp79B3 plants (Bak et al., 2001). Apparently, the CYP83A1 gene, whose primary role is in the production of Met-derived aliphatic glucosinolates, can compensate partially for the loss of CYP83B1 activity (Bak and Feyereisen, 2001; Bak et al., 2001; Hemm et al., 2003; Naur et al., 2003). Consistent with the position of CYP83B1 at a branch point between IG and IAA synthesis (Fig. 1), cyp83B1 mutants have elevated levels of IAA and display a number of high-IAA phenotypes, including adventitious roots along the hypocotyl (Delarue et al., 1998; Barlier et al., 2000; Bak et al., 2001; Smolen and Bender, 2002). Mutation of the SURI gene, which encodes the C-S lyase enzyme downstream from CYP83B1 in the glucosinolate pathway, also causes elevated IAA levels and adventitious rooting, further demonstrating that

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disrupting the conversion of IAOx into IGs causes increased flux of IAOx into IAA (Mikkelsen et al., 2004). In wild-type Arabidopsis, the arrangement of Trp secondary metabolites derived from a common intermediate followed by unique committed steps directed to the production of IAA and IGs offers the flexibility to up-regulate both metabolites coordinately, or each metabolite separately, depending on which enzymes in the pathway are induced. In addition, Trp synthesis needs to be coordinated with Trp secondary metabolism to respond to changes in the demand for Trp. To understand control of Trp metabolism, we previously screened for Arabidopsis altered Trp regulation (atr) mutants with increased resistance to a toxic Trp analog, 5-methyl-Trp (5MT). 5MT feedback inhibits Trp synthesis without substituting for the nutritional role of Trp, resulting in growth inhibition. A potential mechanism of 5MT resistance is up-regulation anthranilate synthase (AS), the target enzyme for Trp/5MT feedback inhibition. Another mechanism of 5MT resistance is to increase its conversion into non-toxic secondary metabolites. For example, overexpression of CYP79B2 confers 5MT resistance (Hull et al., 2000). Similarly, overexpression of the YUCCA flavin monooxygenase-related Trp secondary metabolism enzyme confers 5MT resistance (Zhao et al., 2001). Conversely, the cyp79B2 cyp79B3 double mutant displays increased sensitivity to 5MT (Zhao et al., 2002). Thus, 5MT resistance serves to monitor the overall rate of Trp metabolism.

Our screen for 5MT-resistant mutants recovered atr1D, a dominant overexpression allele of the Myb transcription factor ATR1 (Bender and Fink, 1998), also known as AtMyb34 (Stracke et al., 2001). The atr1D mutant displays elevated expression of CYP79B2, CYP79B3, and CYP83B1, accounting for the 5MT-resistant phenotype (Smolen and Bender, 2002; Smolen et al., 2002). The atr1D mutant also displays tissuespecific up-regulation of the Trp synthesis gene ASA1 (Bender and Fink, 1998). These results suggest that ATR1 acts either directly or indirectly as a positive regulator of Trp secondary metabolism and Trp synthesis genes.

ATR1 expression has the potential to be controlled at the translational as well as the transcriptional level because the ATR1 transcript contains three short open reading frames (ORFs) upstream and out of frame with the Myb-encoding ORF (Bender and Fink, 1998). The atr1D overexpression mutation creates a premature stop codon in the first of the upstream ORFs, and confers increased steady-state levels of ATR1 transcripts. Given the nature of the atr1D mutation, the increased transcript levels are likely to arise by improved message stability caused by improved translation through the main ATR1 ORF. ATR1 message levels in wild-type plants can be modulated either up or down by treatment with a variety of plant signaling molecules (Smolen and Bender, 2002). However, it is currently unknown whether translational inhibition
by the upstream ORFs contributes to regulation of ATR1 expression.

Another complementation group of 5MT-resistant mutants recovered from the atr screen was atr4, corresponding to loss-of-function mutations in the CYP83B1 IG synthesis gene (Smolen and Bender, 2002). The cyp83B1 mutants contain elevated levels of ATR1, CYP79B2, and CYP83B1 transcripts. This finding led us to hypothesize that the IG deficiency conferred by the cyp83B1 mutation signals the activation of ATR1.

Here, we further dissect the role of ATR1 in controlling Trp secondary metabolism using both ATR1 overexpression and loss-of-function strains. We show that ATR1 overexpression confers modest elevation of IAA levels but strong elevation of IG levels, consistent with the coordinate up-regulation of CYP79B2/CYP79B3 and CYP83B1. Thus, ATR1 acts as a strong positive regulator of IG synthesis. In addition, we show that ATR1 overexpression phenotypes require CYP79B2 and CYP79B3, indicating that ATR1 regulates Trp secondary metabolism via the IAOx intermediate. By contrast, a loss-of-function atr1 mutant is impaired for expression of IG synthesis genes in adult leaves and has a corresponding decrease in IG levels. Furthermore, the loss-of-function atr1 mutant is impaired in 5MT resistance and in up-regulation of Trp synthesis genes induced by the cyp83B1 mutation. These results demonstrate a physiological role for ATR1 in Trp gene control in response to IG deficiency cues.

RESULTS
ATR1 Overexpression Coordinate Up-Regulates Trp Gene Expression

To achieve robust overexpression of ATR1 without translational inhibition conferred by upstream out-of-frame ORFs in the ATR1 transcript (Bender and Fink, 1998), we generated transgenic plants where the CaMV 35S promoter drives expression of an ATR1 cDNA carrying only the ATR1 ORF, with the upstream ORFs deleted. Transformants were resistant to 5MT but did not display any obvious morphological abnormalities (Fig. 2). A representative single-copy insert line of 35S-ATR1 was selected for further analysis.

In RNA gel-blot analysis, 35S-ATR1 displayed elevated accumulation of ATR1 and Trp gene transcripts, including two Trp synthesis genes, ASA1 and TSB1, and three Trp secondary metabolism genes, CYP79B2, CYP79B3, and CYP83B1 (Figs. 1 and 2A). ASA1 encodes the catalytic α-subunit of AS, the first committed enzyme of the Trp pathway, and TSB1 encodes the Trp synthase β-subunit activity that converts indole to Trp (Fig. 1). The up-regulation of these genes in 35S-ATR1 was stronger than that detected in the atr1D strain. In fact, ASA1 and TSB1 were not obviously up-regulated in atr1D at the whole-seedling RNA level, presumably due to tissue-specific regulation of these genes (Bender and Fink, 1998). By contrast, the three Trp secondary metabolism CYP genes displayed clear up-regulation in both atr1D and 35S-ATR1, suggesting that these genes are major targets for activation. 35S-ATR1 did not alter regulation of CYP79F1, which encodes a cytochrome P450 important for the synthesis of short-chain aliphatic glucosinolates derived from Met (Hansen et al., 2001b; Reintanz et al., 2001; Fig. 2A).

ATR1 Overexpression Confers Elevated IAA and IG Levels

Because atr1D and 35S-ATR1 coordinately increased the expression of the CYP79B2, CYP79B3, and CYP83B1
genes implicated in the synthesis of both IAA and IGs (Fig. 1), we measured the levels of these Trp secondary metabolites in whole seedlings of the ATR1 overexpression strains. Both strains had elevated levels of free IAA relative to wild-type Columbia (Col), with a greater accumulation in atr1D than in 35S-ATR1 (Table I). The free IAA levels in atr1D seedlings were comparable to those previously measured in a strain that overexpresses CYP79B2 from the 35S promoter (Zhao et al., 2002). However, in contrast with the 35S-CYP79B2 strain, atr1D does not display a conspicuous elongated hypocotyl or leaf epinasty (curling under) phenotype (Smolen and Bender, 2002; data not shown). This difference could reflect different spatial patterns of IAA accumulation between these two strains.

Both 35S-ATR1 and atr1D also had elevated levels of IGs in whole seedlings and adult leaves (Fig. 3). Strikingly, atr1D conferred approximately 10-fold increased IG levels relative to wild type in both tissue sources. By contrast, levels of glucosinolates derived from Met were not elevated in the ATR1 overexpression strains (data not shown). Therefore, ATR1 overexpression specifically alters the flux of Trp-derived secondary metabolites, consistent with the transcriptional effects of ATR1 overexpression being limited to genes involved in Trp metabolism. Data are presented for the major IG species, indolyl-3-methyl glucosinolate (I3M; Fig. 3), but more modest increases in two I3M-derived IGs were also observed in the ATR1 overexpression strains (data not shown). A similar pattern of strong I3M elevation but more modest elevation in I3M-derived IGs was previously observed in 35S-CYP79B2 transgenic strains (Mikkelsen et al., 2000; Zhao et al., 2002; data not shown). These patterns suggest that the enzymes involved in I3M modifications may be limiting.

Despite the stronger effect on overall transcript levels of Trp genes (Fig. 2A), 35S-ATR1 was less effective than atr1D in modulating secondary metabolite levels (Fig. 3; Table I). This discrepancy likely reflects the difference in ATR1 expression patterns for the heterologous 35S promoter versus the endogenous ATR1 promoter.

To determine whether the high levels of IGs synthesized in atr1D are derived exclusively from the CYP79B2/CYP79B3 pathway (Fig. 1), we constructed an atr1D cyp79B2 cyp79B3 triple mutant and assayed it for IG levels. This analysis showed that the triple mutant had severely reduced IG levels, similar to the cyp79B2 cyp79B3 double mutant (Fig. 3). Thus, atr1D stimulates IG synthesis through activation of CYP79B2 and CYP79B3. In addition, the cyp79B2 cyp79B3 atr1D triple mutant showed the same supersensitivity to 5 μM 5MT as the cyp79B2 cyp79B3 double mutant (Zhao et al., 2002; data not shown), suggesting that the 5MT resistance phenotype conferred by atr1D is mediated by CYP79B2 and CYP79B3.

### Table I. Quantification of free IAA in ATR1 overexpression mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Free IAA a</th>
</tr>
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<tbody>
<tr>
<td>Col</td>
<td>5.3 ± 0.33</td>
</tr>
<tr>
<td>35S-ATR1</td>
<td>7.7 ± 0.88</td>
</tr>
<tr>
<td>atr1D</td>
<td>11.0 ± 2.1</td>
</tr>
</tbody>
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aFree IAA levels were determined on seedlings grown as described in “Materials and Methods.” Values are given in nanograms per gram fresh weight of tissue, presented as the mean ± se from three independent samples.

An atr1 Loss-of-Function Mutant Displays Reduced Expression of IG Synthesis Genes and IG Accumulation

Overexpression of the ATR1 transcription factor, either in atr1D or 35S-ATR1 plants, could lead to fortuitous activation of Trp metabolism genes. We therefore isolated a loss-of-function allele in the ATR1 gene, atr1-2, to validate the role of ATR1 as a physiological regulator of the Trp pathway. The atr1-2 mutant was isolated in the Wassilewskija (Ws) strain background as a T-DNA insertion into the first intron of the gene, which lies midway through the conserved Myb DNA-binding domain sequences (Bender and Fink, 1998). The insertion allele disrupted expression of normal-length ATR1 transcripts and instead produced low levels of aberrantly sized transcripts (Figs. 2 and 4).
The *atr1-2* mutant plants had no obvious morphological abnormalities. Nor was there a significant effect on the steady-state transcript levels of Trp genes in seedling tissues (Fig. 2A). However, adult leaves of *atr1-2* mutant plants contained reduced transcript levels of CYP79B2, CYP79B3, and CYP83B1 relative to parental *Ws* (Fig. 4). These results suggest that the *atr1-2* mutation impairs optimal expression of the IG synthesis genes during the development of the plant. Correspondingly, IG measurements showed approximately 20% reduced IG accumulation in *atr1-2* adult leaves (Fig. 5). Steady-state transcript levels of the primary Trp synthesis genes ASA1 and TSB1 were not significantly different between wild-type and *atr1-2* adult leaves (Fig. 4), consistent with only modest effects on these genes in ATR1 overexpression strains (Fig. 2A).

In addition, *atr1-2* mutant seedlings were supersensitive to 5MT, showing completely impaired root growth at a 5 μM concentration where wild-type roots are only partially inhibited (Fig. 2B). The 5MT supersensitivity phenotype is similar to that previously observed for the *cyp79B2* *cyp79B3* double mutant (Zhao et al., 2002).

**An *atr1* Loss-of-Function Mutation Suppresses Trp Secondary Metabolism Dysregulation Caused by the *cyp83B1* Mutation**

In previous work, we found that the *cyp83B1* IG-deficient mutant accumulates increased levels of ATR1, CYP79B2, and mutant *cyp83B1* transcripts and displays 5MT resistance (Smolen and Bender, 2002). To understand whether the CYP gene up-regulation and 5MT resistance are dependent on ATR1, we constructed an *atr1-2* *cyp83B1* double mutant and tested it for these phenotypes. In RNA-blot analysis, the *atr1-2* mutation suppressed elevated expression of Trp genes caused by the *cyp83B1* mutation: The ASA1 and TSB1 Trp synthesis genes were suppressed to wild-type levels by *atr1-2*, but the CYP79B2, CYP79B3, and CYP83B1 IG synthesis genes were only partially suppressed to levels intermediate between wild type and the *cyp83B1* single mutant (Fig. 6A). Thus, ATR1 has a partial contribution to the induction of the IG synthesis genes in *cyp83B1*. Nonetheless, the *atr1-2* mutation was sufficient to completely suppress *cyp83B1* 5MT resistance: The *atr1-2* *cyp83B1* double mutant was sensitive to 5MT at both 5 and 15 μM (Fig. 6B), similar to the *atr1-2* single mutant (Fig. 2B).

In previous work, we determined that ATR1 contributes to the high-IAA phenotypes of the *cyp83B1* mutant by showing that a *cyp83B1 atr1D* double mutant enhances the adventitious root formation phenotype of the *cyp83B1* single mutant (Smolen and Bender, 2002). Conversely, the *atr1-2 cyp83B1* double mutant partially suppressed the *cyp83B1* adventitious rooting phenotype (Fig. 7), consistent with a decrease but not a complete loss of flux through CYP79B2 and CYP79B3 into IAA synthesis. Adult double-mutant plants also displayed increased size and vigor relative to the *cyp83B1* single mutant (data not shown).

The adventitious root formation and the 5MT resistance of the *cyp83B1* mutant were completely suppressed in a triple mutant with *cyp79B2* and *cyp79B3* (Fig. 7; data not shown). These results are consistent with specific activation of the CYP79B2/CYP79B3 pathway accounting for elevated Trp secondary metabolism in the *cyp83B1* mutant.
The cyp79B2 cyp79B3 IG-Deficient Mutant Displays Elevated Expression of ATR1 and IG Synthesis Genes

The activation of ATR1 in the cyp83B1 mutant background suggests that IG starvation might trigger ATR1-mediated activation of IG synthesis genes. As another test of this hypothesis, ATR1 and Trp secondary metabolism gene expression patterns were assayed by RNA gel blot in the cyp79B2 cyp79B3 double mutant versus each single mutant and wild-type Col. The Col cyp79B2 and cyp79B3 alleles used for this analysis are T-DNA insertion mutants that produce detectable, but altered, transcripts (Zhao et al., 2002). In the cyp79B2 mutant, with an insertion in the second exon of the gene, the message is slightly longer and the steady-state levels are reduced relative to the wild-type CYP79B2 message. In the cyp79B3 mutant, with an insertion in the single central intron of the gene, there are two shorter messages with reduced steady-state levels relative to the wild-type CYP79B3 message. ATR1 and all three Trp secondary metabolism genes, CYP79B2, CYP79B3, and CYP83B1, displayed elevated steady-state message levels in the cyp79B2 cyp79B3 double mutant relative to the single mutant or wild-type strains (Fig. 8). This pattern is most clearly seen in the ATR1- and CYP83B1-probed samples, where the mutations do not disrupt the target transcripts. In the case of the CYP79B2- and CYP79B3-probed samples, the pattern is more complex because the cyp79B2 and cyp79B3 gene activation phenotypes, while apparent, are counteracted by the messenger-stabilizing effects of the insertional disruption. A similar pattern of activation of CYP79B2 and CYP79B3 transcription was found in the cyp79B2 cyp79B3 double mutant constructed in the Ws background (Zhao et al., 2002; data not shown).

DISCUSSION

Plants depend on a variety of metabolites to defend against pathogen and herbivore attack. In Brassicas, glucosinolates, including Trp-derived IGs, are a major class of antitherbivore compounds. Trp also gives rise to other metabolites, including IAA, which have biological roles distinct from IGs. The diversity of Trp secondary metabolism calls for a regulatory network with the potential to modulate each metabolite either coordinately or independently, depending on particular developmental and environmental cues. Understanding this regulatory network will aid in designing metabolic engineering strategies aimed at modifying production of Trp-derived compounds. Here, we show that the Arabidopsis Myb transcription factor ATR1, identified as a positive regulator of Trp genes (Bender and Fink, 1998; Smolen and Bender, 2002), is a key controller of IG homeostasis and also contributes to IAA homeostasis.

Because expression of the ATR1 gene is elevated in the cyp83B1 mutant background, we previously speculated that this transcription factor might respond to a deficiency of compounds in the IG pathway (Smolen and Bender, 2002). In this view, the IG deficiency-induced up-regulation of ATR1 would lead to up-regulation of IG synthesis genes, ultimately restoring IG pools to the appropriate levels. Consistent with this model, ATR1 and IG synthesis gene transcript levels are up-regulated in the cyp79B2 cyp79B3 double mutant (Fig. 8), which is strongly deficient in IGs (Zhao et al., 2002; Fig. 3).

Recently, the enzyme defective in the sur1/rty high-IAA Arabidopsis mutant has been shown to be the C-S lyase enzyme in the synthesis of both IGs and aliphatic Met-derived glucosinolates (Mikkelsen et al., 2004). In the IG pathway, SUR1 acts downstream from CYP83B1 (Fig. 1), using as its substrate S-(indolylacetoxy)hydroxymethyl)-L-Cys. The sur1/rty mutant is thus devoid of all glucosinolates, including IGs, but accumulates this...
intermediate compound. In previous work, we determined that the sur1/rtv mutant has only slightly elevated levels of ATR1, CYP79B2, and CYP83B1 transcripts relative to wild type (Smolen and Bender, 2002), in contrast with the stronger elevation of these transcripts in the cyp79B2 cyp79B3 double mutant (Fig. 8) and the cyp83B1 mutant (Smolen and Bender, 2002; Fig. 6A). These data suggest that the activation of ATR1 and the IG synthesis genes in cyp79B2 cyp79B3 and cyp83B1 occurs in response to reduced levels of S-(indolylacetohydroximolyl)-L-Cys rather than downstream compounds in the IG pathway. The activation is unlikely to be due to a deficiency in the proposed CYP83B1 product 1-aci-nitro-2-indolyl-ethane because this compound is a highly reactive nucleophile that will not stably accumulate (Hansen et al., 2001a). Regulation based on levels of the SUR1 substrate might reflect its accumulation relative to downstream intermediates and the diversity of IG end products.

ATR1 overexpression primarily activates Trp secondary metabolism genes, including CYP83B1, which encodes the first committed step in IG synthesis relative to Trp synthesis genes like ASA1 and TSB1 (Fig. 2A; Bender and Fink, 1998). This pattern of gene activation is appropriate for a factor dedicated to controlling IG pools. The simplest model is that ATR1 serves as a direct transcriptional activator of both Trp synthesis genes and Trp secondary metabolism genes, with the different degrees of activation determined by the precise control elements for each gene. More complex scenarios are also possible. For example, ATR1 might act directly to activate the Trp secondary metabolism genes, with the more modest up-regulation of the Trp synthesis genes occurring as an indirect response to Trp depletion. However, this model is inconsistent with the observation that the Trp synthesis genes are more strongly activated in 35S-ATR1 than in atr1D (Fig. 2A), even though Trp secondary metabolites accumulate to significantly higher levels in atr1D than in 35S-ATR1 (Fig. 3; Table I).

Some plant Myb factors work in conjunction with other transcription factors to achieve their regulatory functions. For example, the Arabidopsis GL1 Myb partners with the basic helix-loop-helix protein GL3 and the WD repeat protein TTG1 to direct formation of trichomes (leaf hairs; Oppenheimer et al., 1991; Walker et al., 1999; Payne et al., 2000). Similarly, anthocyanin biosynthetic genes in maize and petunia are regulated by Myb factors that partner with basic helix-loop-helix factors (Mol et al., 1998). Our observation that strong overexpression of ATR1 in the 35S-ATR1 strain results in hyperactivation of Trp gene expression (Fig. 2A) suggests either that this Myb factor does not require partner factors or that the levels of partner factors are not limiting.

Consistent with a role for ATR1 in controlling IG homeostasis, the atr1-2 loss-of-function mutation impairs optimal expression of IG synthesis genes and reduces IG levels in the leaves of adult plants (Figs. 4 and 5). The atr1-2 mutation also impairs the up-regulation of Trp gene expression and the formation
of adventitious roots in cyp83B1 seedlings (Figs. 6 and 7). Thus, ATR1 contributes to but is not the only factor involved in transcriptional activation of Trp genes. One possibility is that other closely related Myb factors (Stracke et al., 2001) might be functionally redundant with ATR1, but act over different tissue types or in response to different stimuli to control Trp gene expression. As discussed above, the diversity of compounds generated from the Trp pathway requires such combinatorial regulation of Trp gene expression. Interestingly, although the atr1-2 mutation has no obvious effects on accumulation of Trp gene transcripts in whole seedlings (Fig. 2A), the mutation confers a 5MT-supersensitive phenotype (Fig. 2B) and is able to suppress the cyp83B1 5MT resistance phenotype (Fig. 6B). The most likely explanation for this discrepancy is that atr1-2 impairs Trp gene expression in the specific tissues that mediate the 5MT sensitivity phenotype, such as root tips, and these spatial and/or temporal changes in gene expression are not evident at the whole-seedling level.

In Arabidopsis, Trp-derived IGs and Met-derived glucosinolates are the most abundant glucosinolate species (Wittstock and Halliker, 2002). Two lines of evidence suggest that ATR1 specifically regulates IGs rather than acting as a general glucosinolate control factor. First, ATR1 overexpression strains contain elevated levels of IGs (Fig. 3) but not Met-derived glucosinolates (data not shown). Second, 35S-ATR1 specifically up-regulates IG synthesis genes without a significant effect on levels of CYP79F1-hybridizing transcripts (Fig. 2A). In the short-chain Met-derived glucosinolate pathway, CYP79F1 acts analogously to CYP79B2 and CYP79B3 in the IG pathway (Fig. 1) to convert amino acid-derived precursors into the corresponding aldoximes (Hansen et al., 2001b; Reintanz et al., 2001).

Evidence for differential control of the synthesis of the distinct classes of glucosinolates has been suggested by the identification of quantitative trait loci specific to Met-derived alphatic or indolic glucosinolates (Kliebenstein et al., 2001). Quantitative trait locus DF119L, a significant regulator of IGs in a Landsberg erecta × Cape Verdi Islands (Cvi-0) recombinant inbred population, maps in the vicinity of ATR1 on the lower arm of chromosome 5. However, it is unlikely that DF119L corresponds to a defective ATR1 allele in the Cvi-0 strain because, unlike the atr1-2 mutant (Fig. 2B), Cvi-0 is not supersensitive to 5MT (data not shown). Furthermore, the Cvi-0 allele of ATR1 is expressed and has protein-coding sequences identical to those of the Landsberg erecta ATR1 (J. Bender, unpublished data).

The atr1D overexpression mutation confers elevation of IG levels approximately an order of magnitude above wild-type levels (Fig. 3). By contrast, 35S-driven overexpression of ATR1 (Fig. 3), CYP79B2 (Mikkelsen et al., 2000; Zhao et al., 2002), or CYP83B1 (Bak et al., 2001) elevates IG levels only 1.5- to 3.5-fold above wild-type levels. These findings illustrate two key points regarding plant metabolic engineering. First, although 35S-cDNA transgene constructs can produce very high transcript levels (e.g. see the ATR1-probed 35S-ATR1 lane in Fig. 2A), these transcripts might not be produced in the appropriate spatial and temporal patterns to optimally affect the metabolic pathway of interest. For example, manipulation of lignin monomer composition in Arabidopsis by overexpression of the ferrulate-5-hydroxylase gene is much less effective with 35S-driven expression versus expression driven by the promoter of an endogenous phenylpropanoid synthesis gene, even though 35S produces extremely high ferrulate-5-hydroxylase transcript levels (Meyer et al., 1998). Second, overexpression of a positive transcriptional regulator for a suite of genes specific to a pathway is a more effective means of metabolic engineering than manipulation of any single enzyme in the pathway. The potential for specific modulation of IG production via ATR1 in Arabidopsis should translate into the metabolic engineering of glucosinolates in other Brassicas. As our understanding of the role of IGs in both defense against herbivory and human nutrition increases, it may be beneficial to create crops with increased or decreased levels of IGs.

In addition to elevated IGs (Fig. 3), the ATR1 overexpression strains display elevated free IAA (Table 1). The more subtle effect on IAA versus IG levels presumably reflects the metabolism of free IAA as well as the preferential partitioning of IAOx into the IG pathway by up-regulation of CYP83B1 (Figs. 1 and 2A), whose controlling role in IAA homeostasis has been postulated previously (Barlier et al., 2000; Bak et al., 2001). The atr1D and 35S-ATR1 strains do not display obvious high-IAA phenotypes such as elongated hypocotyls, leaf epinasty, or adventitious rooting (Fig. 2B; Smolen and Bender, 2002; data not shown). However, we previously found that the atr1D mutation enhances the adventitious rooting of a cyp83B1 mutant (Smolen and Bender, 2002), consistent with elevated IAA in atr1D. Similarly, we show here that the atr1-2 loss-of-function mutation partially suppresses cyp83B1 adventitious rooting (Fig. 7). Thus, although the primary effect of ATR1 overexpression is activation of IG synthesis, there are also modest effects on IAA synthesis and perhaps other interlinked metabolic pathways. For example, IAOx derived from CYP79B2/CYP79B3 is used for synthesis of the antipathogenic compound camalexin (Glawischnig et al., 2004) as well as IAA and IGs. However, camalexin levels are not elevated in a strain that overexpresses CYP79B2, suggesting that IAOx is not limiting for camalexin synthesis and that regulation occurs at a downstream point specific to the camalexin pathway during pathogen infection (Glawischnig et al., 2004).

The elevated IG levels and enhanced 5MT resistance in the ATR1 overexpression mutant atr1D are dependent on CYP79B2 and CYP79B3 function: Both atr1D phenotypes are completely suppressed by the double cyp79B2 cyp79B3 mutation (Fig. 3; data not shown). Furthermore, the high-IAA adventitious rooting
phenotype of cyp83B1, which can be modulated upward by atr1D (Smolen and Bender, 2002) or downward by atr1-2 (Fig. 7), is completely suppressed by the double cyp79B2 cyp79B3 mutation (Fig. 7). These results show that ATR1 regulation is specifically mediated through CYP79B2/CYP79B3-catalyzed IAOx synthesis without coactivation of other Trp secondary metabolism pathways.

In this regard, studies on the Arabidopsis yucca IAA overproduction mutant indicate that IAA can be significantly elevated independently from IG production. Overexpression of the YUCCA flavin monoxygenase-related protein via insertion of a strong transcriptional enhancer confers high-IAA phenotypes by activating a CYP79B2/CYP79B3-independent pathway for IAA synthesis (Zhao et al., 2001, 2002). However, the yucca overexpression mutation does not confer elevated levels of IGs (Zhao et al., 2002). Thus, the specificity of atr1D for CYP79B2/CYP79B3 plus CYP83B1-mediated Trp metabolism is consistent with a primary role for ATR1 as a regulator of IG homeostasis.

MATERIALS AND METHODS

Plant Strains

The 3SS-ATR1 cDNA construct was made by inserting a Landsberg erecta ATRI cDNA (Bender and Fink, 1998) extending from an SnaBI site that lies 55 bp upstream of the ATR1 translational start codon through the end of the 3SS transcript into the pBIcMaV 35S expression/plant transformation vector (Hull et al., 2000). This construct lacks the three ORFs that lie upstream of the ATR1 ORF. The 3SS-ATR1 construct was transformed into the Col strain by Agrobacterium tumefaciens-mediated plant transformation (Dough and Bent, 1998). The representative single-copy insert line used in this study was identified by segregation and Southern-blot analysis. Additional lines displayed similar 5MT resistance phenotypes.

The atr-1 mutant in the Ws strain was identified by screening the Arabidopsis Knockout Facility collection of T-DNA insertional mutants following their guidelines (http://www.biotech.wisc.edu/NewServices/Arabidopsis/Knockouts/Guidelines.asp). The atr-1 insertion was identified using the JI-202 left-border primer (5'-CATTCTATAAACAAGCTCAGG-GACATCTAC-3') and a gene-specific forward primer lying just upstream of the ATR1 transcription unit atr-1A (5'-GATTTAAAGAACGTGTAATCC-CAACCATT-3'). Sequencing revealed that the left-border junction of the atr-1 insertion lies in the first intron 196 bp after the ATG translational start codon of the ATR1 ORF. The 5MT-sensitivity phenotype (Fig. 2B) cosegregated with the atr-1 insertion in a back-cross to wild-type Ws, indicating that this phenotype is linked to the insertion.

For double and triple mutants, the previously described Col atr1D allele (Bender and Fink, 1998), Col atr4-1 allele of cyp83B1 (Smolen and Bender, 2002), and Col cyp79B2 and Col cyp79B3 T-DNA insertions (Zhao et al., 2002) were used. Plants of the desired genotype were identified by molecular markers for each mutation. Because the double mutants of atr1-2 and cyp83B1 (Figs. 6 and 7) are hybrids between Ws and Col, three independent lines were analyzed, with results for a single representative line shown.

RNA Gel-Blot and 5MT Resistance Analysis

Total RNA was prepared from whole seedlings grown asexually on plant nutrient 0.5% Suc (PNS) medium (Haughn and Somerville, 1986), with 0.75% agar sealed with parafilm under continuous illumination of 30 to 45 μmol m⁻² s⁻¹ at 21°C. At 10 d postgermination (Figs. 2A, 6A, and 8), or from rosette leaves of 3-week-old plants grown in soilless medium (Fafard mix no. 2) under continuous illumination of 35 to 50 μmol m⁻² s⁻¹ at 21°C (Fig. 4), as previously described (Smolen and Bender, 2002). For blot analysis, full-length or nearly full-length cDNA probes of ASA1 (At5g05730), ATR1 (At5g05890), CYP79B2 (At4g39950), CYP78B1 (At4g22330), CYP83B1 (At4g31500), TSBI (At1g58410), and β-tubulin (TuB; At5g44340) were used. The CYP79F1 (At1g16410) probe consisted of the first-exon coding sequence of the gene, which is 90% identical to the first exon of CYP79F2 (At1g16400). In each experiment, the TuB probe served as a gel-loading control.

To score 5MT resistance, seedlings were grown asexually on parafilm-sealed PNS medium supplemented with either 5 or 15 μM 5MT under the same conditions described above for seedling RNA preparation, and root growth was assessed at 10 d postgermination on a population of at least 48 seedlings per experiment. If none of the seedlings in the population was able to elongate a root more than 5 mm (failing to reach the bottom of the petri plate), the strain was scored as sensitive to 5MT.

IG and IAA Measurements

For IAA analysis, 10-d postgermination seedlings or 3-week-old rosette leaves were grown as described above for RNA analysis. Desulfoglucosinolates were measured as previously described using HPLC (Zhao et al., 2002). In this method, IGS and Met-derived glucosinolates can be discriminated from each other by their different absorbance properties and retention times and by comparison to purified indolyl-3-methyl desulfoglucosinolate and 4-methyl-sulfinylbutyl desulfoglucosinolate (gift of J. Gershenzon and M. Reichelt). Absolute levels of 4-methylsulfinylbutyl desulfoglucosinolate were not elevated in ATR1 overexpression strains relative to wild-type Col (data not shown). The relative abundance of indolyl-3-methyl desulfoglucosinolate was determined as the ratio of the peak area for this compound to the peak area for 4-methylsulfinylbutyl desulfoglucosinolate, normalizing to the ratio measured in wild-type Col.

For IAA analysis, seeds were plated in 10 mL of top agar (plant nutrient medium without Suc) on PNS in 15 × 150-mm petri plates. For each strain, approximately 1,500 seeds were divided among five plates. Plated seeds were vernalized for 4 to 6 d at 4°C before being moved to a growth chamber, where they were grown at 21°C under continuous illumination (18–30 μE m⁻² s⁻¹) under low-pass yellow filters for 6 d postgermination. IAA was purified by the method described in Chen et al. (1988) with slight modifications that are described in Tam and Normanly (2002). The conditions for gas chromatography–selected ion monitoring–mass spectrometry of IAA are described in Tam and Normanly (2002).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requester.

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