A Link between Ethylene and Auxin Uncovered by the Characterization of Two Root-Specific Ethylene-Insensitive Mutants in Arabidopsis

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The plant hormone ethylene participates in the regulation of a variety of developmental processes and serves as a key mediator of plant responses to biotic and abiotic stress factors. The diversity of ethylene functions is achieved, at least in part, by combinatorial interactions with other hormonal signals. Here, we show that ethylene-triggered inhibition of root growth, one of the classical effects of ethylene in *Arabidopsis thaliana* seedlings, is mediated by the action of the *WEAK ETHYLENE INSENSITIVE2/ANTHRANILATE SYNTHASE α1* (*WEI2/ASA1*) and *WEI7/ANTHRANILATE SYNTHASE β1* (*ASB1*) genes that encode α- and β-subunits of a rate-limiting enzyme of Trp biosynthesis, anthranilate synthase. Upregulation of *WEI2/ASA1* and *WEI7/ASB1* by ethylene results in the accumulation of auxin in the tip of primary root, whereas loss-of-function mutations in these genes prevent the ethylene-mediated auxin increase. Furthermore, *wei2* and *wei7* suppress the high-auxin phenotypes of *superroot1* (*sur1*) and *sur2*, two auxin-overproducing mutants, suggesting that the roles of *WEI2* and *WEI7* in the regulation of auxin biosynthesis are not restricted to the ethylene response. Together, these findings reveal that *ASA1* and *ASB1* are key elements in the regulation of auxin production and an unexpected node of interaction between ethylene responses and auxin biosynthesis in Arabidopsis. This study provides a mechanistic explanation for the root-specific ethylene insensitivity of *wei2* and *wei7*, illustrating how interactions between hormones can be used to achieve response specificity.

INTRODUCTION

Plant survival depends largely on the ability of these sessile organisms to sense environmental changes, integrate external signals with their own developmental programs, and produce appropriate responses. Many of the diverse internal and external cues converge on the regulation of a handful of plant hormones that, by interacting in a combinatorial manner, generate an appropriate set of responses among the multitude of possible outputs (Bennett et al., 2005). Ethylene and auxin are known to regulate several of the same processes (Davies, 1995), such as root elongation (Swarup et al., 2002; Alonso et al., 2003b), differential growth in the hypocotyls (Lehman et al., 1996; Harper et al., 2000), and root hair formation and elongation (Pitts et al., 1998; Rahman et al., 2002) in *Arabidopsis thaliana* and organ abscission in several other plant species (Brown, 1997). The molecular mechanisms by which ethylene and auxin interact to regulate these processes, agonistically in some cases and antagonistically in others, remain largely unknown.

In the past 15 years, the focus of the majority of hormonal studies in plants has been on elucidating individual biosynthetic, signaling, and response pathways (McCourt, 1999). In fact, many of the molecular components of the ethylene and auxin cascades have been identified (Wang et al., 2002; Cohen et al., 2003; Dharmasiri and Estelle, 2004). Ethylene is produced from Met by a series of well-defined enzymatic activities, in which the conversion of *S*-adenosyl-Met to 1-aminocyclopropane-1-carboxylic acid (ACC) by a family of ACC synthases (ACS) is the first committed step and a key regulatory point (Wang et al., 2002). Detailed expression studies of all individual *ACS* gene family members have provided a comprehensive picture of when and where ethylene is produced (Tsuchisaka and Theologis, 2004). Once ethylene is synthesized, it is perceived by a family of receptors that possess sequence similarity with bacterial two-component His kinases. Ethylene binding results in the inactivation of the receptors and of the receptor-interacting Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a negative regulator of the pathway. In the presence of ethylene, CTR1 loses its ability to repress a positive component of the pathway, the membrane protein ETHYLENE INSENSITIVE2 (EIN2). By an unknown mechanism, activation of EIN2 leads to the stabilization of the transcription factor EIN3, which, in turn, triggers a transcriptional cascade that involves hundreds of genes and is collectively referred to as the ethylene response (Alonso and Stepanova, 2004).

The genetic dissection of the ethylene response is discovering some of the mechanisms that plants use to achieve specificity upon activation of a common signaling pathway. Several distinct branches of the ethylene response that represent different aspects of this hormone's action have been identified. Interestingly, activation of some of these branches depends on interaction with other hormonal pathways. For example,

ethylene-mediated induction of the transcription factor gene *ETHYLENE RESPONSE FACTOR1* (*ERF1*), an *ETHYLENE RESPONSE ELEMENT BINDING PROTEIN* (*EREBP*) family member, results in the transcriptional activation of defense-related genes, a process that requires intact jasmonate signaling and response (Lorenzo et al., 2003). Regulation of a member of the *N-ACETYLTRANSFERASE* gene family, *HOOKLESS1*, by ethylene controls the levels of specific AUXIN RESPONSE FACTOR proteins (ARFs) in the apical part of the hypocotyls, resulting in well-defined patterns of differential growth in these tissues (Li et al., 2004). Finally, ethylene is also known to inhibit cell elongation through the activation of *ETHYLENE RESPONSE DNA BINDING FACTOR1* (*EDF1*) to *EDF4*, members of the *EREBP* family of transcription factors (Alonso et al., 2003a).

In contrast with the ethylene pathway, for most of the genes involved in auxin biosynthesis in plants, the exact spatial location and mechanisms of regulation of indoleacetic acid (IAA) production remain a mystery (Cohen et al., 2003). Unlike ethylene gas, auxin requires a complex transport apparatus to move across the plant. Regulation of specific and well-characterized auxin influx (AUX1 and AUX1-like) and efflux (PINs) carriers is critical to maintain the levels and, more importantly, gradients of this essential hormone (Swarup and Bennett, 2003). Auxin-mediated changes in gene expression are initiated by the direct binding of the hormone to TIR1, an F-box protein that targets members of the AUX/IAA family of transcriptional repressors to ubiquitin-mediated degradation (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). AUX/IAA proteins do not bind DNA directly; rather, they regulate transcription by interacting with the DNA binding ARF proteins. In a very simplistic model, auxin triggers the degradation of AUX/IAA proteins, thus relieving their negative effect on ARFs and allowing the latter to initiate the auxin response (Leyser, 2002; Liscum and Reed, 2002). Interestingly, among the many putative ARF targets known to be regulated by IAA, there are several *ACS* genes (Abel et al., 1995; Tsuchisaka and Theologis, 2004). These findings not only provide a mechanistic explanation for the long-known effect of auxin on ethylene production but also reveal a molecular link between these two hormones.

Perhaps one of the most intriguing examples of the intricate relationship between ethylene and auxin comes from the genetic analysis of various Arabidopsis mutants affected in their responses to auxin or ethylene. Mutants with deficiencies in auxin transport, signaling, or response show significantly lower ethylene sensitivity in root tissues, implying that ethylene signaling and/or response are dependent on the status of the auxin pathway (Swarup et al., 2002; Alonso et al., 2003b; Larsen and Cancel, 2003). On the other hand, mutants with defects in ethylene responses display a normal or nearly normal reaction to exogenous IAA (Hobbie, 1998; Collett et al., 2000), suggesting that ethylene responsiveness is dispensable for the normal operation of the auxin pathway.

To further investigate the molecular mechanisms that govern ethylene–auxin crosstalk in Arabidopsis, we set out to identify mutants affected in the points of interaction between these two hormones. Here, we report the cloning and functional characterization of *WEAK ETHYLENE INSENSITIVE2* (*WEI2*) and *WEI7* and present evidence to suggest that these two genes represent a new link between ethylene responses and auxin biosynthesis.

WEI2 and *WEI7* encode α- and β-subunits of a rate-limiting enzyme of Trp biosynthesis, anthranilate synthase. Using a combination of molecular and genetic approaches, we show that by stimulating the transcription of *WEI2* and *WEI7*, ethylene triggers the accelerated production of Trp and, ultimately, auxin in roots of Arabidopsis seedlings. Furthermore, we demonstrate that these genes are required to maintain high auxin biosynthetic rates in auxin-overproducing mutants, suggesting a broader regulatory role of WEI2 and WEI7 in auxin production.

RESULTS

wei2 and *wei7* Are Root-Specific Ethylene-Insensitive Mutants That Act Downstream of *CTR1*

To identify mutants defective in the components of the ethylene–auxin crosstalk, we searched for plants with phenotypes intermediate between those of the classical ethylene and auxin mutants. Two mutants, *wei2* and *wei7*, were found to possess root-specific ethylene insensitivity (see below), a feature that would group them with the classical auxin mutants. However, unlike auxin mutants, *wei2* and *wei7* do not display any obvious auxin defects (see below), a feature that would group them with the classical ethylene mutants.

The first allele of *WEI2*, *wei2-1*, was isolated previously as described by Alonso et al. (2003b). Three independent mutant alleles of *WEI7* (*wei7-1*, *wei7-2*, and *wei7-4*) were identified from a classical triple-response screen of T-DNA–tagged lines (see Methods). Complementation analysis between *wei2* and the three *wei7* alleles established that the *wei7* mutations affected a locus genetically distinct from and unlinked to *wei2* (data not shown). All three *wei7* alleles were phenotypically indistinguishable (Figure 1A); therefore, only one allele, *wei7-4*, was used for most of the analysis described below.

In the classical triple-response assay (Guzman and Ecker, 1990), *wei2-1* and *wei7-4* mutants display root-specific ethylene insensitivity (Alonso et al., 2003b) (Figure 1). Detailed phenotypic analysis of the mutants revealed that they are moderately insensitive to a wide range of concentrations (0.2 to 10 μM) of the ethylene precursor ACC in roots but show a wild-type response in hypocotyls (Alonso et al., 2003b) (Figure 1). In addition to root-specific ethylene insensitivity, all alleles of *wei7* show somewhat shorter hypocotyls compared with wild-type Columbia (Col-0) or *wei2-1* (Figure 1A).

The auxin response of *wei2-1* and *wei7-4* mutants was also examined. At every concentration of exogenous IAA tested (0.1 to 10 μM), the growth responses of roots and hypocotyls of the mutants were indistinguishable from those of wild-type plants (Figure 1C). In soil-grown adults, the phenotype of the *wei2-1* and *wei7-4* mutant lines was similar to that of the wild type, except that under low-light conditions both mutants were somewhat pale (data not shown). Backcrosses of the *wei2-1* and *wei7-4* mutants with wild-type Col-0 revealed the recessive nature of these mutations (Alonso et al., 2003b) (data not shown). To test where in the ethylene signaling pathway *wei2* and *wei7* act, double mutants were constructed with the constitutive ethylene mutant *ctr1-1*. In etiolated seedlings, both *wei2-1* and *wei7-4*
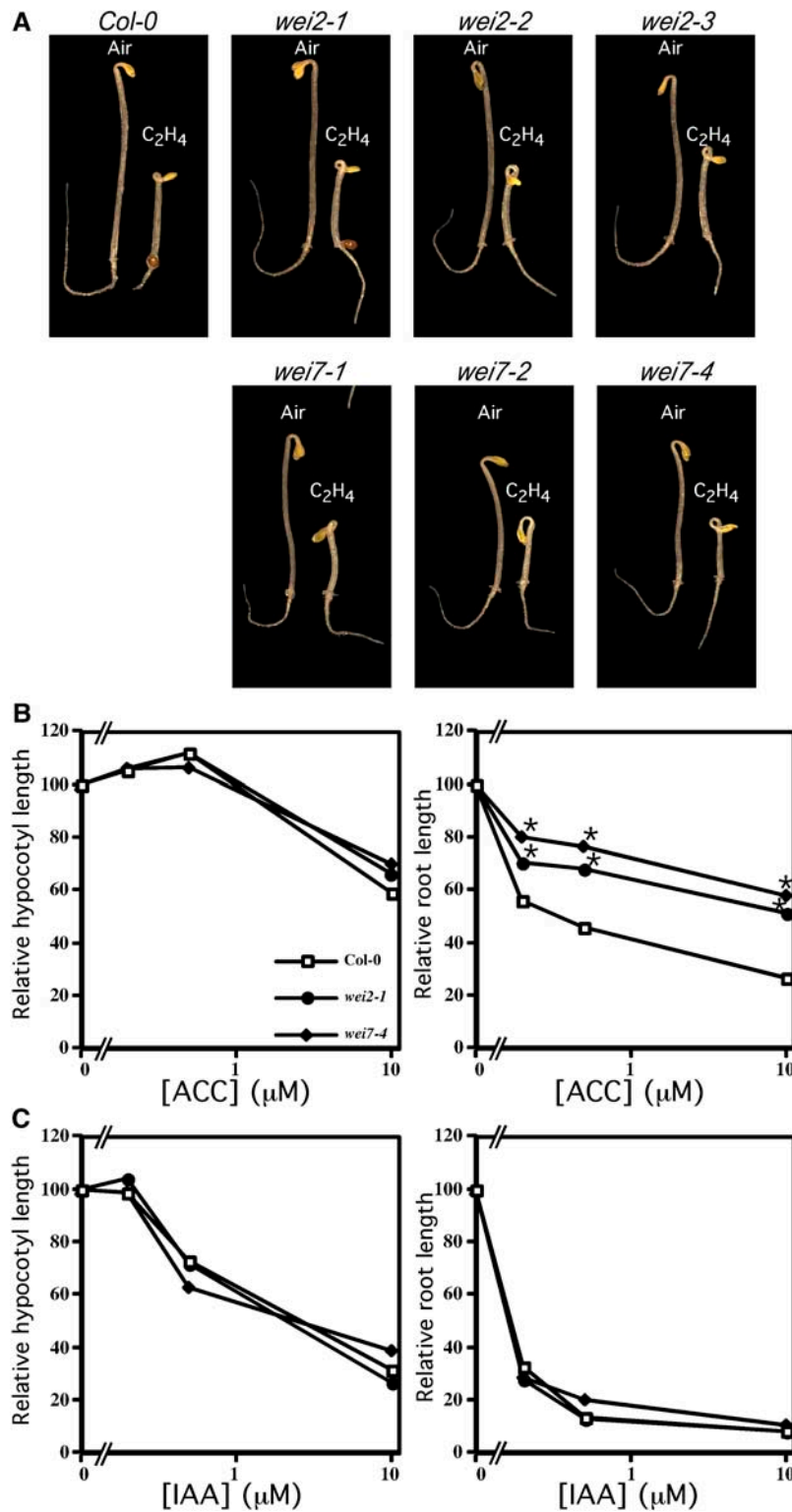
Figure 1. *wei2* and *wei7* Are Root-Specific Ethylene-Insensitive Mutants.

(A) Phenotypes of 3-d-old etiolated Col-0, *wei2-1*, *wei2-2*, *wei2-3*, *wei7-1*, *wei7-2*, and *wei7-4* seedlings grown on AT plates in the presence of hydrocarbon-free air or air supplemented with 10 ppm ethylene.

(B) and **(C)** Dose–response curves of hypocotyls (left) and roots (right) of 3-d-old etiolated Col-0, *wei2-1*, and *wei7-4* seedlings grown in AT medium
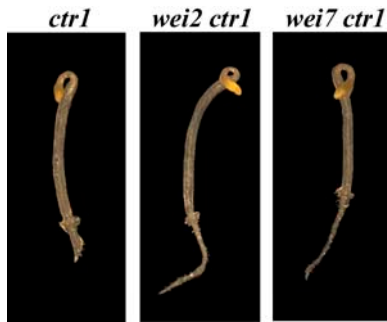
Figure 2. *WEI2* and *WEI7* Function Downstream of *CTR1* in the Ethylene Signaling Pathway.

Phenotypes of 3-d-old etiolated *ctr1-1*, *wei2-1 ctr1-1*, and *wei7-4 ctr1-1* seedlings grown in unsupplemented AT medium in the presence of hydrocarbon-free air are shown.

suppressed the short-root phenotype of *ctr1-1* (Alonso et al., 2003b) (Figure 2), suggesting that *WEI2* and *WEI7* function at the level or downstream of *CTR1*. In adults, the rosette morphology of the *wei2 ctr1* and *wei7 ctr1* double mutants was indistinguishable from that of the *ctr1* single mutant (data not shown), further supporting the root-specific role of *WEI2* and *WEI7* in the ethylene response.

Cloning of *wei2* and *wei7*

wei2-1 has been mapped to the top arm of chromosome 5 between the simple sequence length polymorphism (SSLP) markers nga225 and nga249 (Alonso et al., 2003b). Using a new mapping population of ~1000 F2 plants from a cross to Landsberg *erecta* (L*er*), the *wei2-1* mutation was fine-mapped to a 274-kb region between the SSLP markers nga225 (1.507 Mb, 13 recombinants) and K18J17-1 (1.780 Mb, 2 recombinants). Three internal markers (see Methods), MJJ3-3 (1.696 Mb), MJJ3-1 (1.726 Mb), and MJJ3-7 (1.739 Mb), showed zero recombinants. A candidate gene approach was next used to identify the gene responsible for the *wei2* mutant phenotype. Individual T-DNA lines from the Salk collection (Alonso et al., 2003a) with insertions in the candidate genes in the *WEI2* region were tested in the triple response assay. One line, Salk_017444, showed root-specific ethylene insensitivity (Figure 1A), a phenotype indistinguishable from that of the *wei2* mutant. This line, referred to as *wei2-2*, harbors an insertion in the seventh intron of *ANTHRANILATE SYNTHASE α* (*ASA1*; *At5g05730*), which encodes an α-subunit of AS1, an enzyme that catalyzes the first committed step of Trp biosynthesis, the conversion of chorismate to anthranilate (Radwanski and Last, 1995). Previously, hypomorphic alleles of *ASA1*, *trp5-2/wvc1* and *tir7*, were identified in genetic screens for defective root waving patterns and for resistance to auxin transport inhibitors, respectively (Rutherford et al., 1998; Ljung

et al., 2005). Two paralogs of *ASA1* exist in Arabidopsis, *At2g29690* (*ASA2*) and *At3g55870* (*ASA3*) (Niyogi and Fink, 1992; Arabidopsis Genome Initiative, 2000). Sequencing of the *ASA1* gene in the original *wei2-1* mutant (Alonso et al., 2003b) identified a G-to-A transition at the splice junction of the sixth intron and is predicted to yield a premature termination of the protein. A third allele of *WEI2*, *wei2-3* (Figure 1A), was identified from an ethyl methanesulfonate–mutagenized Col-0 population and found to contain a C-to-T transition at nucleotide +31 of *ASA1*, resulting in a nonsense mutation (Gln-11 to stop) after 10 amino acids (Figure 3A).

WEI7 was mapped to the top arm of chromosome 1. Using a population of 129 individual F2 plants obtained from crosses of all three *wei7* alleles to L*er*, the *WEI7* gene was mapped to a 2.6-Mb region between the SSLP markers F9H16-1 (17.296 Mb, 18 recombinants) and nga248 (9.887 Mb, 7 recombinants). This region contains five putative *ANTHRANILATE SYNTHASE β* (*ASB*) subunit genes (*At1g24807*, *At1g24909*, *At1g25083*, *At1g25155*, and *At1g25220*) whose protein products are thought to function as heterodimers with ASA subunits in the biosynthesis of Trp (Radwanski and Last, 1995). Because of the strong phenotypic similarity between the *wei2* and *wei7* mutants (Figures 1 and 2; see below), the *ASB* genes were considered as possible *WEI7* candidates. Of the five *ASB* genes in the region, four are identical (with sequence identity expanding beyond the coding regions) and are likely to be functionally redundant. Therefore, we decided to first focus on the most divergent *ASB* gene in the region, *ASB1* (*At1g25220*). Because the three *wei7* mutants came from an activation-tagged T-DNA collection, we attempted to amplify T-DNA junction sequences from the three *wei7* mutants using a combination of gene-specific and T-DNA border-specific primers. In the process of testing gene-specific primers, we discovered that *wei7-1* lacks ~1.6 kb of the 5′ end of the *ASB1* gene, including the first 156 bp of the open reading frame, whereas in *wei7-4*, the entire chromosomal region that contains *ASB1* in wild-type plants is deleted. Upon sequencing of the remaining *wei7-2* allele, a G-to-A transition at position 1569 in the sixth exon that results in a Gly-177–to–Glu amino acid substitution was found (Figure 3B). This Gly is highly conserved not only in all six ASB isoforms of Arabidopsis but also in all of the ASB proteins examined, including bacterial orthologs (Figure 3C; data not shown). Based on these results, we conclude that *wei2* and *wei7* are loss-of-function versions of the *ASA1* and *ASB1* genes that encode α- and β-subunits of anthranilate synthase, a rate-limiting Trp biosynthetic enzyme.

wei2 and *wei7* Can Be Rescued by Anthranilate, Trp, or Auxin

To confirm that the *wei2* and *wei7* mutant phenotypes are, in fact, caused by a defect in Trp biosynthesis, we attempted to

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Figure 1. (continued).

supplemented with 0, 0.2, 0.5, or 10 μM ACC **(B)** or with 0, 0.1, 1, or 10 μM IAA **(C)**. Relative organ length (expressed as a percentage of the length observed in unsupplemented medium) is plotted on the *y* axis, and hormone concentrations are plotted on the *x* axis on a logarithmic scale. Asterisks indicate significant difference (P < 0.0001 in a two-way analysis of variance) between the wild-type and mutant responses at a particular concentration of ACC.
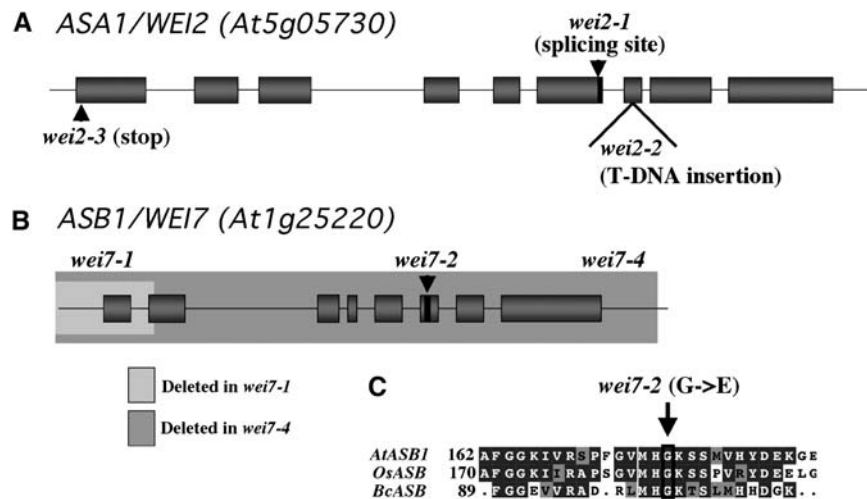
Figure 3. Schemes of the *WEI2* and *WEI7* Genes.

(A) and **(B)** The exon–intron structures of *WEI2* **(A)** and *WEI7* **(B)** are shown by boxes versus lines, respectively. The positions and types of mutations are as indicated.

(C) Alignment of a WEI7 protein fragment of Arabidopsis with the corresponding regions of ASB from *Oryza sativa* (*OsASB*) and *Bacillus cereus* (*BcASB*). The conserved Gly-177 that is mutated to Glu in the *wei7-2* mutant is marked by an arrow.

complement the mutations by supplying exogenous Trp or anthranilate to the mutant seedlings. As expected, both 10 μM anthranilate and 10 μM Trp completely restored the ethylene sensitivity of *wei2-1* and *wei7-4* roots to wild-type levels (Figures 4A and 4B). Interestingly, anthranilate or Trp supplementation of the growth medium also corrected the hypocotyl elongation defect of *wei7* (data not shown), implying that the hypocotyl phenotype of this mutant is also a consequence of Trp deficiency.

Because Trp and the intermediates of its biosynthesis can serve as precursors to auxin (Bartel, 1997) and we and others have previously found that auxin mutants possess root-specific ethylene insensitivity (Swarup et al., 2002; Alonso et al., 2003b; Larsen and Cancel, 2003), we reasoned that a defect in a rate-limiting step of Trp biosynthesis may translate into a defect in the biosynthesis of IAA. To examine the possibility that the ethylene insensitivity of *wei2* and *wei7* roots arises from reduced levels of endogenous auxin, we tested the ability of low doses of exogenous IAA to rescue the mutant phenotypes. IAA at 10 nM does not have significant inhibitory effect on root growth in wild-type Col-0 seedlings (Rahman et al., 2001) (Figure 4C). When applied to the growth medium along with 10 μM of the ethylene precursor ACC, 10 nM IAA was able to partially restore the reduced ethylene response of the *wei2* and *wei7* seedlings (Figure 4C). These results suggest that the phenotypic defect of *wei2* and *wei7* is caused by a reduction in the levels of endogenous IAA.

WEI2 and *WEI7* Show Ethylene-Inducible Expression in Root Tips

To address the question of the role of *WEI2* and *WEI7* in the ethylene response, we examined the expression of these genes using transcriptional reporter fusions with β-*GLUCURONIDASE*

(*GUS*). *ASA1-GUS* and *ASB1-GUS* reporters contain 2.3- and 2.0-kb promoter fragments of *WEI2* and *WEI7*, respectively, fused with *GUS* (Niyogi, 1993) (see Methods). Both constructs were introduced into Col-0 and *ein2-5* mutant plants, and their expression was examined in the roots of 3-d-old etiolated seedlings grown in the absence or presence of the ethylene gas. In air, GUS activities driven by the *ASA1* and *ASB1* promoters were found to be colocalized in root tips, with Col-0 seedlings showing somewhat stronger GUS staining than *ein2-5* (Figure 5). Ethylene treatment resulted in a significant induction of the *ASA1-GUS* and *ASB1-GUS* reporters in the Col-0 background but not in the ethylene-insensitive mutant *ein2-5* (Figure 5). GUS activity was also found in young cotyledons; however, no significant increase was observed in these tissues after ethylene treatment (data not shown). These findings suggest that ethylene can induce the transcription of *WEI2* and *WEI7* in roots of etiolated seedlings and provide a possible explanation for the root-specific ethylene resistance of the *wei2* and *wei7* mutants.

In Roots, Ethylene Induces *DR5-GUS* Expression in a *WEI2*- and *WEI7*-Dependent Manner

Because WEI2 (ASA1) and WEI7 (ASB1) catalyze a rate-limiting step of Trp biosynthesis and Trp and its biosynthetic intermediates serve as precursors to IAA (Radwanski and Last, 1995), it is plausible that the transcriptional induction of *WEI2* and *WEI7* may result in higher rates of Trp and, ultimately, auxin biosynthesis. To test the ability of ethylene to induce auxin levels, the expression patterns of the synthetic auxin reporter *DR5-GUS* were examined in air-grown versus ethylene-grown Col-0 and *ein2-5* mutant plants. *DR5-GUS* activity has been found to correlate well with endogenous auxin levels in roots (Casimiro et al., 2001; Benkova et al., 2003). In air, roots of 3-d-old etiolated
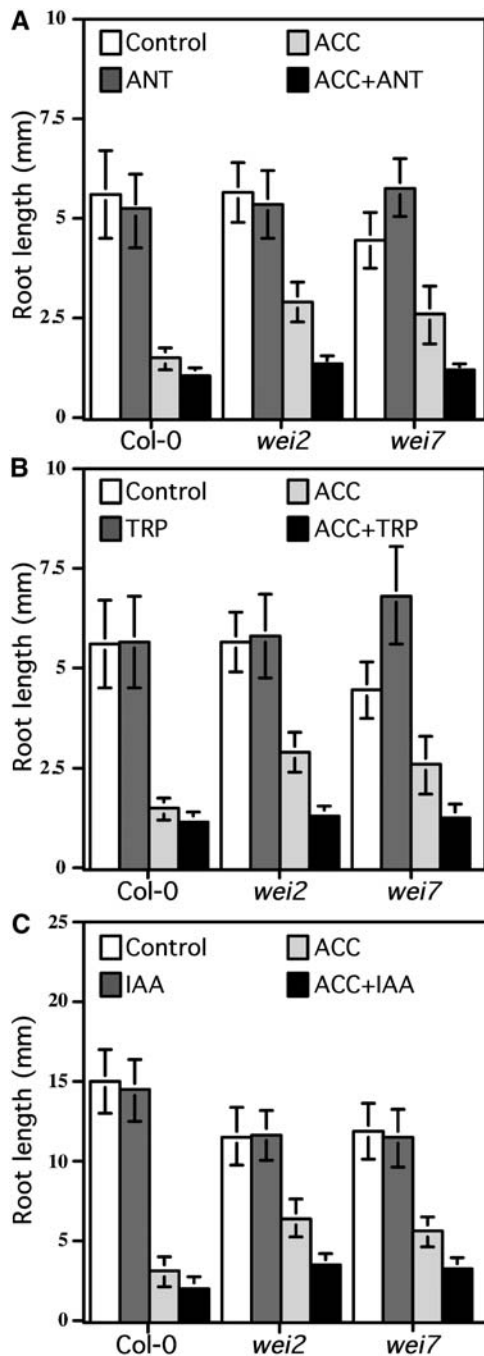
Figure 4. Rescue of the Root Defect of *wei2* and *wei7* by Anthranilate, Trp, and IAA.

Col-0, *wei2-1*, and *wei7-4* seedlings were grown for 3 d in the dark in AT versus AT plus 10 μM ACC, with or without supplementation with 10 μM anthranilate (ANT) **(A)**, 10 μM Trp **(B)**, or 10 nM IAA **(C)**. Average and SD of ≥20 seedlings are shown.

seedlings show localized *DR5-GUS* expression in the quiescent zone and columella cells of root tips (Sabatini et al., 1999). Remarkably, upon ethylene exposure of wild-type plants, GUS staining increased dramatically (Figures 6A and 6B), spreading to the surrounding cells and into the root vasculature, which suggests an ethylene-mediated accumulation of IAA in root tips. Not surprisingly, in the ethylene-resistant mutant *ein2-5*, ethylene treatment failed to enhance auxin reporter staining (Figures 6A and 6B).

To examine the role of *WEI2* and *WEI7* in the ethylene-mediated induction of *DR5-GUS*, the reporter was introduced into the *wei2* and *wei7* mutant backgrounds. In roots of air-grown mutant seedlings, basal *GUS* expression levels were reduced compared with those of wild-type plants (Figures 6A and 6B), suggesting lower basal levels of auxin in the mutants. This observation is consistent with the recent finding that another mutant allele of *WEI2*, *tir7*, possesses reduced auxin levels in root tips (Ljung et al., 2005). Remarkably, ethylene-mediated induction of the *DR5-GUS* reporter was also reduced significantly in the *wei2* and *wei7* backgrounds (Figures 6A and 6B). These results indicate that the function of *WEI2* and *WEI7* is required for the ethylene-triggered boost of *DR5-GUS* expression in root tips and suggest the involvement of *WEI2* and *WEI7* in the regulation of auxin biosynthesis in response to ethylene. In contrast with the effects of ethylene in roots, ethylene treatment had no effect on *DR5-GUS* levels in the hypocotyls of wild-type, *wei2*, or *wei7* plants (Figures 6A and 6B). As expected, *ein2-5* plants showed no changes in *DR5-GUS* activity in response to ethylene, although constitutively higher levels of the reporter were observed in both hypocotyls and roots.

wei2 and *wei7* Suppress the High-Auxin Phenotypes of Auxin-Overproducing Mutants

To evaluate the role of *WEI2* and *WEI7* in the biosynthesis of IAA, double mutants between *wei2* or *wei7* and auxin-overproducing
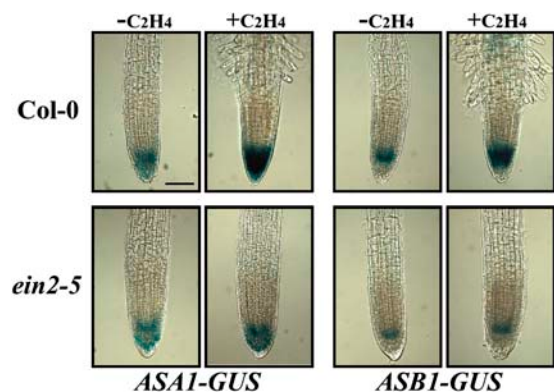

Figure 5. *WEI2* and *WEI7* Show Ethylene-Inducible Expression in Root Tips of Etiolated Seedlings.

GUS activity of the transcriptional reporters *ASA1-GUS* and *ASB1-GUS* was analyzed in Col-0 and *ein2-5* seedlings grown in AT medium for 3 d in the dark in the presence of hydrocarbon-free air or air supplemented with 10 ppm ethylene. Plants were stained for GUS for 1 h, and representative seedlings were photographed. Bar = 0.1 mm.
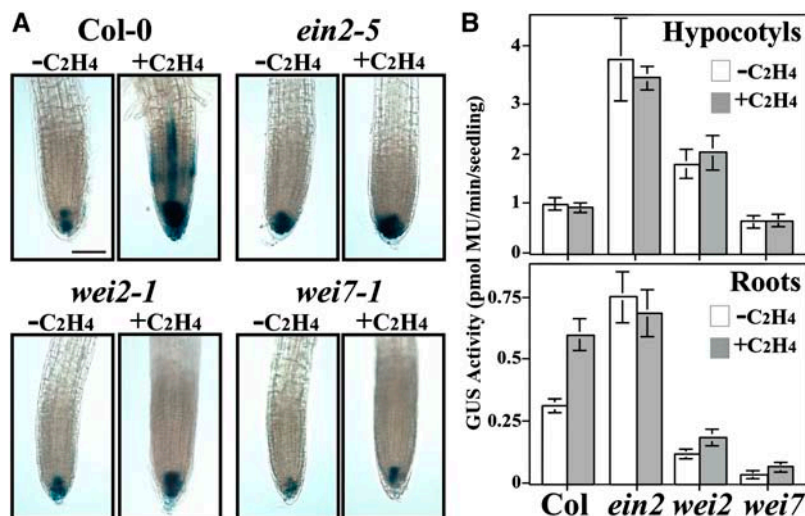
Figure 6. Ethylene Stimulates the Expression of the Synthetic Auxin Reporter *DR5-GUS* in Root Tips of Wild-Type Etiolated Seedlings.

GUS activity was monitored in roots of Col-0, *ein2-5*, *wei2-1*, and *wei7-4* seedlings grown in AT medium for 3 d in the dark in the presence of hydrocarbon-free air or air supplemented with 10 ppm ethylene.

(A) Plants were stained for GUS overnight, and representative seedlings were photographed. Bar = 0.1 mm.

(B) Seedlings were dissected into hypocotyls and roots, and MUG assay was performed. Pools of 100 to 140 seedlings per genotype per treatment per experiment were analyzed. Averages and SD values of three independent experiments are shown. The ethylene effects in Col versus *wei2* or *wei7* were significantly different (P < 0.05 by analysis of variance). MU, 4-methylumbelliferone.

mutants *superroot1* (*sur1*) (*rty1*, *hls3*) or *sur2* were constructed. *sur1* and *sur2* are loss-of-function mutants defective in a branch of indole-glucosinolate biosynthesis that diverts part of the IAA precursor indole-3-acetaldoxime from the auxin biosynthetic pathway into the production of glucosinolates (Winkler et al., 1998; Barlier et al., 2000; Mikkelsen et al., 2004). When grown in the light, *sur1* and *sur2* mutants develop curly epinastic cotyledons and a large number of adventitious roots as a result of the high levels of endogenous auxin produced (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996; Winkler et al., 1998; Barlier et al., 2000). Adventitious root formation from the hypocotyl tissues is usually accompanied by peeling and disintegration of hypocotyl cells (Boerjan et al., 1995; King et al., 1995; Barlier et al., 2000). Adult *sur1* and *sur2* plants show a dramatic reduction of rosette size, delayed flowering, and, in the case of *sur1*, complete sterility (Boerjan et al., 1995; King et al., 1995). Remarkably, *wei2* and *wei7* were able to suppress the high-auxin phenotypes of both *sur1* and *sur2* (Figure 7). The suppression of the defects of *sur2* was nearly complete: *wei2 sur2* and *wei7 sur2* were indistinguishable from the single *wei2* and *wei7* mutants in both seedlings and adults, with the exception of somewhat smaller rosette size of the double mutants grown in soil (Figures 7A and 7B). In *wei2 sur1* and *wei7 sur1* seedlings, the epinasty of the cotyledons, adventitious root formation, and hypocotyl cell disintegration were much less dramatic than in the *sur1* single mutant (Figure 7A). Importantly, unlike *sur1*, the *wei2 sur1* adult plants were fertile and produced viable seeds (Figures 7B and 7C).

The ability of *wei2* and *wei7* to block the auxin-overproducing effects of *sur1* and *sur2* suggests an important role of *WEI2* and *WEI7* in the general biosynthesis of IAA, at least in situations in which the auxin biosynthetic pathway is hyperactive, such as in auxin-overproducing mutants.

DISCUSSION

Cloning and characterization of two ethylene response mutants, *wei2* and *wei7*, strongly imply the involvement of *WEI2* and *WEI7* in the regulation of auxin biosynthesis by ethylene and provide a mechanistic explanation for this interaction. We have identified one of the steps in the biosynthesis of IAA that is regulated by ethylene (conversion of chorismate into anthranilate), determined specific isoforms of the enzymes involved in this regulation (ASA1 and ASB1), and established the mode of regulation (transcriptional induction of the *WEI2/ASA1* and *WEI7/ASB1* genes by the ethylene gas). Furthermore, the conditional auxin deficiency of the *wei2* and *wei7* mutants suggests a role for these genes in the control of auxin biosynthesis under specific developmental or environmental circumstances. These data indicate the importance of a site-specific regulation of auxin biosynthetic genes for the wild-type response of roots to ethylene.

Role of *WEI2* and *WEI7* in the Ethylene Response

Two root-specific ethylene response mutants, *wei2* and *wei7*, with no obvious auxin defects are described. Cloning of these mutants revealed that *WEI2* and *WEI7* code for an α- and β-subunit of anthranilate synthase, an enzyme that catalyzes a rate-limiting step in Trp biosynthesis. Trp and its precursors serve as substrates in the biosynthesis of auxin, suggesting that the ethylene defects of the *wei2* and *wei7* mutants arise from a
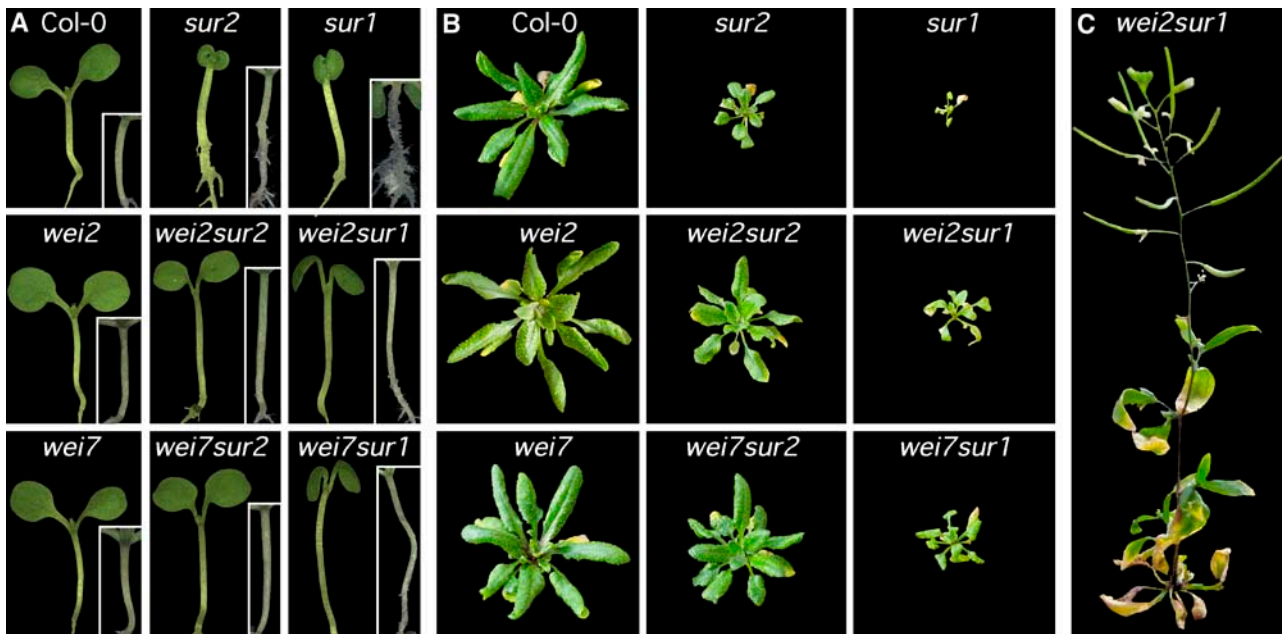
Figure 7. *wei2* and *wei7* Suppress the High-Auxin Phenotypes of *sur1* and *sur2*.

(A) and **(B)** Col-0, *wei2-1*, *wei7-4*, *sur1*, *sur2*, *wei2-1 sur1*, *wei7-4 sur1*, *wei2-1 sur2*, and *wei7-4 sur2* were germinated on horizontal AT plates in the light for 5 d (large insets) or for 7 d followed by 4 d on vertical AT plates (small insets) **(A)** or grown in soil for 4 weeks **(B)** and then photographed.
(C) Double mutant *wei2-1 sur1* plants were grown in soil for 8 weeks and then photographed.

decrease in the production of this essential hormone. Comple-mentation of the ethylene resistance by low levels of auxin has been used previously to explain the ethylene defects of *aux1* and *eir1* (Rahman et al., 2001). Similarly, we showed that the ethylene insensitivity of *wei2* and *wei7* could be corrected by supplying low levels of IAA in the growth medium. Although a decline in auxin production could explain the observed *wei2* and *wei7* phenotypes, it was surprising to discover that this decrease was caused by mutations in an early step of the Trp biosynthetic pathway. In fact, no alteration in the levels of active auxins was detected in previous studies of strong Trp-deficient mutants, such as *trp2* and *trp3* (Last et al., 1991; Normanly et al., 1993; Radwanski et al., 1996). Interestingly, these conditional Trp auxotrophs show normal or nearly normal responses to ethylene (A.N. Stepanova and J.M. Alonso, unpublished data), further implying the presence of a selective mechanism that limits the effects of the *wei2* and *wei7* mutations to specific pools of Trp.

Alternatively, the lack of ethylene phenotypes in *trp2* and *trp3* suggests that ethylene induces IAA biosynthesis via a Trp-independent route that branches from the Trp biosynthetic pathway downstream of anthranilate synthase, explaining the ethylene insensitivity of *wei2* and *wei7*, but upstream of the TRP2- and TRP3-catalyzed steps (Bartel, 1997). Although pos-sible, this is a less likely scenario in light of the ability of Trp to rescue the ethylene defects of *wei2* and *wei7*. The presence of additional α and β anthranilate synthase genes in the Arabidopsis genome (Arabidopsis Genome Initiative, 2000) likely accounts for the lack of general Trp or auxin defects in *wei2* and *wei7*. Restricted patterns of expression of *WEI2* and *WEI7*, on the other

hand, could explain the specificity of the mutant phenotypes. Indeed, we show that the patterns of expression of *WEI2* and *WEI7* in etiolated seedlings are highly overlapping. Furthermore, the ethylene phenotype of the *wei2 wei7* double mutant is indistinguishable from that of the single mutants (A.N. Stepanova and J.M. Alonso, unpublished data), supporting the idea that these two specific subunits work together in the ethylene re-sponse. The expression of *WEI2* (Niyogi, 1993) and *WEI7* in young cotyledons and root tips, tissues that are capable of synthesizing auxin (Ljung et al., 2001), is consistent with their proposed role in auxin biosynthesis. Finally, the strong induction of *WEI2* and *WEI7* expression by ethylene in root tips provides a plausible explanation for the selective effect of these mutations on the ethylene response.

Ethylene Stimulates the Accumulation of IAA in Roots through the Activation of *WEI2* and *WEI7*

A *DR5-GUS* construct that consists of a synthetic auxin-responsive promoter fused to the *GUS* reporter gene (Ulmasov et al., 1997) was used to infer local levels of endogenous auxin in roots of etiolated seedlings. Although this reporter of the auxin response does not directly monitor auxin levels and there are examples in the literature in which DR5 activity and auxin levels do not correlate (Ljung et al., 2004), in Arabidopsis roots there seems to be a strong correlation between the two (Casimiro et al., 2001). In fact, many studies relied on *DR5-GUS* as an indirect indicator of auxin levels and distribution (Sabatini et al., 1999; Benkova et al., 2003; Boonsirichai et al., 2003; Friml et al., 2003;

Bao et al., 2004). The advantage of using *DR5-GUS* over the direct quantification of auxin levels in root tips is that fewer manipulations of the experimental system are required with the former approach. This becomes a critical issue when dealing with the effects of a gaseous stress-inducible compound such as ethylene, in which rapid sampling after treatment is crucial.

In root tips, the pattern of expression of *DR5-GUS* greatly overlaps with that of *WEI2/ASA1* and *WEI7/ASB1*. Furthermore, ethylene treatment results in a dramatic induction of *DR5-GUS*, *WEI2/ASA1*, and *WEI7/ASB1* in these root tissues. The correlation between auxin accumulation in root tips upon ethylene treatment and the increase in the expression of *WEI2* and *WEI7* in these same tissues suggests a connection between these two phenomena. A causal relationship between the increase in *WEI2* and *WEI7* activity and auxin accumulation was established by examining the effects of the mutations in these Trp biosynthetic genes on *DR5-GUS* activity in response to ethylene. In the absence of ethylene, the expression of *DR5-GUS* in root tips of *wei2* and *wei7* was reduced compared with that in wild-type seedlings, with the effect of *wei7* being much more dramatic than that of *wei2* (Figure 6). In neither mutant, however, was this decrease in *DR5-GUS* accompanied by any obvious auxin phenotypes, suggesting that the action of other anthranilate synthase α and β isoforms maintains sufficient basal levels of IAA to support normal root development in both *wei2* and *wei7*. By contrast, the ethylene effect on *DR5-GUS* expression is likely to be largely mediated through *WEI2* and *WEI7*, as indicated by the significant level of suppression of the reporter induction in the presence of ethylene in *wei2* and *wei7* (Figure 6).

These results convincingly demonstrate that *WEI2* and *WEI7* function is required for the ethylene-mediated induction of *DR5-GUS* and, presumably, auxin accumulation in root tips. Although alterations in auxin transport and/or sensitivity could also explain the ethylene insensitivity of *wei2* and *wei7* as well as the effect of these mutations on *DR5-GUS* expression, several results argue against this possibility. First, *wei2* and *wei7* show no detectable alterations in their response to gravity or exogenous auxins, phenotypes typically found in mutants with altered auxin transport or sensitivity (Dolan, 1998; Chen et al., 2002). Moreover, the biochemical function of *WEI2* and *WEI7* as key elements in the biosynthesis of Trp, an auxin precursor, together with the suppression of the high-auxin phenotypes of the auxin overproducers *sur1* and *sur2* by *wei2* or *wei7*, and the recent finding that *tir7*, another mutant allele of *WEI2*, has reduced levels and biosynthetic rates of auxin in root tips (Ljung et al., 2005), strongly support the auxin biosynthetic hypothesis. On the other hand, the root-specific ethylene insensitivity of *wei2* and *wei7* could be the result of the reduction in the basal levels of IAA in these tissues, rather than an effect of these mutations on ethylene-mediated IAA accumulation. The similarity in the degree of ethylene insensitivity of *wei2* and *wei7*, despite their significant differences in basal *DR5-GUS* levels, argues in favor of the ethylene-mediated IAA accumulation hypothesis, although, at this time, we cannot rule out the basal level theory or a combination of both.

It is noteworthy that in both hypocotyls and roots the levels of the *DR5-GUS* reporter were significantly higher in the *ein2-5* mutant than in wild-type seedlings. Although the mechanism

by which the *ein2-5* mutation affects *DR5-GUS* expression is currently unknown, this phenomenon further illustrates the complexity of interactions between ethylene and auxin.

The Role of *WEI2* and *WEI7* in Regulating Auxin Levels Is Not Restricted to the Ethylene Response

Loss of function of *WEI2* or *WEI7* had no obvious auxin phenotypes beyond the root-specific reduction in ethylene sensitivity. Lack of general auxin deficiency phenotypes in *wei2* and *wei7* is the likely result of functional redundancy and compensatory mechanisms operating in the auxin biosynthetic pathway, the phenomenon that has largely prevented the identification of auxin-deficient mutants (Cohen et al., 2003). Conversely, the ethylene-insensitive phenotypes of *wei2* and *wei7* suggest the existence of specialization among auxin biosynthetic gene family members; here, ethylene-inducible isoforms of anthranilate synthase α and β have been identified. Basal levels of expression of *WEI2* and *WEI7* in the *ein2-5* mutant background (Figure 5), however, indicate that the functions of these genes are not restricted to the response to ethylene. To test their role in general
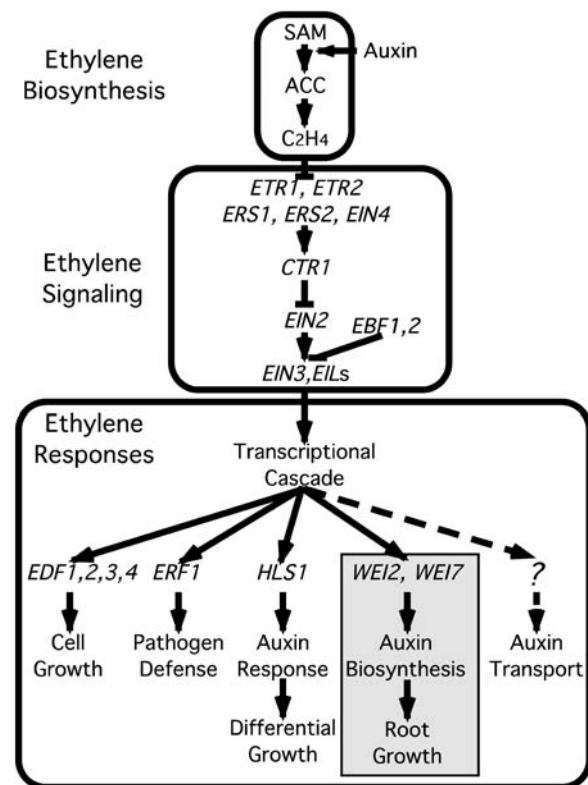

Figure 8. Schemes of the Ethylene Biosynthetic, Signaling, and Response Pathways.

Key elements of the pathways are displayed in a linear manner and are connected by arrows. A previously established link between auxin and ethylene biosynthesis is indicated. The new branch in the ethylene response pathway that connects ethylene with auxin biosynthesis is highlighted by a gray box. SAM, *S*-adenosyl-Met.

auxin biosynthesis, we examined their function in situations of high auxin demand.

Overexpression of a flavin monooxygenase, *YUCCA1*, its paralogs, or either of the two cytochrome P450 genes *CYP79B2* and *CYP79B3* results in plants with higher auxin content (Hull et al., 2000; Zhao et al., 2001, 2002). Similarly, the loss-of-function mutants *sur1* (a *C-S lyase*) and *sur2* (*CYP83B1*) also synthesize increased levels of IAA and show typical high-auxin phenotypes (King et al., 1995; Winkler and Feldmann, 1998; Winkler et al., 1998; Barlier et al., 2000; Mikkelsen et al., 2004) (Figure 7). Double mutants between *wei2* or *wei7* and each of the four auxin overproducers (*yucca1*, *CYP79B2ox*, *sur2*, and *sur1*) were generated (A.N. Stepanova and J.M. Alonso, unpublished data) (Figure 7). *wei2* and *wei7* suppressed the high-auxin phenotypes of all four auxin-overproducing mutants. Because *yucca1* and *CYP79B2ox* are transgenic lines that over-express the corresponding wild-type genes, we tested the levels of the transgenes in the double mutants. Surprisingly, double homozygotes *wei2 yucca1*, *wei7 yucca1*, *wei2 CYP79B2ox*, and *wei7 CYP79B2ox* showed strong reductions in transgene levels compared with the parental *yucca1* and *CYP79B2ox* lines, whereas no such phenomenon was observed in crosses to the wild type or other mutants (data not shown). Therefore, although *wei2* and *wei7* clearly suppress *yucca1* and *CYP79B2ox* pheno-typically, the mechanism by which they do so is unclear. More conclusive results were obtained with the loss-of-function mutants *sur1* and *sur2*. All auxin phenotypes were either partially (in the case of *sur1*) or nearly completely (in *sur2*) suppressed in the double mutants (Figure 7). Remarkably, *wei2 sur1* double mutants were viable, flowered, and produced healthy seeds, illustrating the strong requirement for *WEI2* function in auxin biosynthesis in the *sur1* background. Therefore, we conclude that *WEI2* and *WEI7* functions are not restricted to their role in the ethylene response but are also important for general auxin biosynthesis. Although under normal conditions other anthranilate synthase family members can efficiently compensate for the loss of *WEI2* or *WEI7* functions, the role of *WEI2/WEI7* in auxin biosynthesis could be revealed in the presence of the *sur1* and *sur2* mutations.

Together, the results presented here suggest a mechanistic model in which ethylene stimulates auxin biosynthesis in roots by inducing the transcription of *WEI2*, *WEI7*, and possibly other auxin biosynthetic genes (Figure 8). As a consequence of activating this biosynthetic pathway, ethylene triggers the accumulation of auxin, which in turn is required for some of the morphological effects of ethylene in roots. This model does not exclude the possibility that other mechanisms, such as ethylene-mediated regulation of auxin transport or sensitivity, or post-transcriptional induction of auxin biosynthetic enzymes, also play a relevant role in the response to ethylene. The discovery of the new branch of the ethylene response (i.e., ethylene-triggered activation of auxin biosynthesis) presents a significant contribution to our current view of the mode of action of this hormone. Together with the role of *HLS1* in differential growth through the regulation of the auxin response (Li et al., 2004), *ERF1* in the response to pathogens through the interaction with the jasmonic acid pathway (Lorenzo et al., 2003), and *EDF1* to *EDF4* in general growth (Alonso et al., 2003a), a new picture of the complex net of

interactions, which we simplistically refer to as the ethylene response, is emerging (Figure 8). It is easy to imagine how, by differentially activating *WEI2* and *WEI7*, *HLS1*, or *ERF1* in a tissue–, developmental stage–, or environmental conditions–specific manner, ethylene can promote a large diversity of responses.

The proposed model of ethylene-induced auxin biosynthesis could also explain the compressed root waving phenotype of a previously described mutant, *wvc1* (Rutherford et al., 1998), another hypomorphic allele of *WEI2/ASA1*. The lack of clear changes in the levels of total Trp or IAA in extracts of whole mutant plants left the authors with no obvious hypothesis to explain this phenotype. The discovery by Buer and colleagues (2003) that ethylene regulates root waving, together with our findings that *WEI2/ASA1* loss of function results in a root-specific ethylene insensitivity, provide a likely explanation for the original phenotype described for *wvc1*.

METHODS

Arabidopsis Strains and Growth Conditions

All of the mutant alleles used in this study are in the Col-0 background of *Arabidopsis thaliana*. The *DR5-GUS* reporter line was generously provided by T. Guilfoyle, *yucca1* and *CYP79B2* overexpression mutants were a kind gift of Y. Zhao, and *sur1* and *sur2* were obtained from the ABRC (*rty1-1*, CS8156, and a *CYP83B1* T-DNA line Salk_028573, respectively). *wei2-1* (Alonso et al., 2003b) and *wei2-3* were derived from two independent ethyl methanesulfonate–mutagenized populations. *wei2-2* corresponds to the Salk_017444 line. *wei7-1* was obtained from the Detlef Weigel activation-tagged collection (ABRC), whereas *wei7-2* and *wei7-4* were identified from the Chris Somerville collection of activation-tagged lines (ABRC). All new mutants were backcrossed to Col-0 at least twice before phenotypic analyses were performed.

For the dose–response experiments, seeds were surface-sterilized for 5 to 10 min in 50% bleach plus 0.005% Triton, washed three times with sterile water, resuspended in melted, precooled 0.7% low melting point agarose in water, and plated on Arabidopsis (AT) medium (1× MS salts [Gibco-BRL, Cleveland, OH], pH 6.0, 1% sucrose, and 0.8% agar) supplemented with the indicated concentrations of ACC, Trp, anthranilate, and/or IAA. Plates with seeds were cold-treated at 4°C for 3 d, exposed to light at room temperature for 2 h to improve germination, then wrapped with aluminum foil and incubated at 22°C for ∼70 h in the dark. A minimum of 20 seedlings were scored per mutant per hormone concentration by pulling them out of the growth medium, stretching them flat on the surface of another agar plate, scanning the images, and then quantifying root and hypocotyl lengths using a combination of the Image Processing Tools (ReinderGraphics, Ashville, NC) and Adobe Photoshop (Adobe Systems, Mountain View, CA) software. For the triple response assay, surface-sterilized seeds were germinated in the dark on unsupplemented AT medium in the presence of 10 ppm ethylene versus hydrocarbon-free air. For propagation, dark-grown seedlings were exposed to light for an additional 2 to 4 d, transferred to prewetted soil (1:1 mix of MetroMix-200 [Scotts-Sierra, Marysville, OH] and Fafard germinating mix [Conrad Fafard, Agawam, MA]), and grown to maturity at 22°C under a 16-h-light/8-h-dark cycle.

Cloning of *wei2* and *wei7*

For the genetic mapping of *wei2* and *wei7*, the mutants were crossed to L*er*. Ethylene-insensitive individuals were selected in the F2 generation

and propagated in soil, and their phenotypes were retested in F3. DNAs were isolated from F3 seedlings as described (Doyle and Doyle, 1987). For mapping, classical as well as novel SSLP markers (Bell and Ecker, 1994) divergent between Col-0 and L*er* Arabidopsis accessions were used (see Results). The following new SSLP markers were designed (forward, reverse): top of chromosome 5 markers MJJ3-3 (1.696 Mb, Col > Ler), 5′-CTCGTATAGGTACCTATCACC-3′ and 5′-CACACACGCA-TTGTAAGACAC-3′; MJJ3-1 (1.726 Mb, Col ≪ Ler), 5′-CACCGTCCT-ATTCCAAATGCAG-3′ and 5′-GGAAACTAATGCATCCATAGTG-3′; MJJ3-7 (1.739 Mb, Col ≫ Ler), 5′-CAAGAACGTGCTTTGATACGC-3′ and 5′-GCAAAATGAGTATCCTTAACAAGG-3′; K18J17-1 (1.780 Mb, Col ≪ Ler), 5′-CCGGTATTTGTATGGTTCGG-3′ and 5′-GCAATAAGAGTGATT-CCACCAAGC-3′; and a top of chromosome 1 marker F9H16-1 (7.297 Mb, Col ≫ Ler), 5′-GGTGAGATACTGAGATTATCCTTG-3′ and 5′-GATT-CTATTTTGCTTGGCGTATGTG-3′. PCR amplification was performed for 40 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. Sequencing of the mutant alleles of *wei2* and *wei7* was done using a BigDye Termi-nator version 3.1 kit (ABI Prism; PE-Applied Biosystems, Sunnyvale, CA).

Plant Transformation

The *ASA1-GUS* construct that contains an ∼2.3-kb *Xba*I-*Eag*I promoter fragment of *WEI2* fused to *GUS* in pBI101.1 was provided by J. Bender and K. Niogi. The *ASB1-GUS* construct was generated by PCR-amplifying an ∼2-kb promoter region of *WEI7* using Vent polymerase (New England Biolabs, Beverly, MA) and gene-specific primers ASB1-p5′ (5′-TTCGGGCAGAGATCGCAGAGC-3′) and ASB1-p3′:BamHI (5′-AGC-AAAGGATCCTGATTTAATTCCAAAAGAGAGG-3′), digesting the product with *Sal*I-*Bam*HI, and inserting it upstream of *GUS* into the *Sal*I-*Bam*HI–cut pBI101.1. The two reporters were transformed into *Agrobacterium tumefaciens* and introduced into Col-0 plants using the floral dip method (Clough and Bent, 1998). Primary transformants (T1) were selected on AT plates supplemented with 100 μg/mL kanamycin. Lines that segregated 3:1 for kanamycin resistance in T2 were propagated, and plants homo-zygous for the reporter were identified in T3.

GUS Staining

Tissues were harvested and fixed in ice-cold 90% acetone, washed once with the rinse buffer [50 mM NaPO₄ buffer, pH 7.0, 0.5 mM K₃Fe(CN)₆, and 0.5 mM K₄Fe(CN)₆], and then vacuum-infiltrated and stained for the indicated times in staining buffer [50 mM NaPO₄ buffer, pH 7.0, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 1 mg/mL cyclohexylammonium salt]. Staining solution was then replaced with 15% ethanol to stop the reaction, and individual representative seedlings were photographed.

4-Methylumbelliferyl β-D-Glucuronide Hydrate Assay

Surface-sterilized seeds were germinated for 3 d in the dark on horizontal AT plates in the presence of hydrocarbon-free air or air supplemented with 10 ppm ethylene. One hundred to 140 seedlings per genotype per treatment were then quickly pooled out of the media and laid flat in rows on a moist Nitex 03-100/47 membrane (Sefar America, Depew, NY) resting on the surface of a fresh AT plate (for air-grown seedlings) or an AT plate supplemented with 10 μM ACC (for ethylene-grown seedlings). Roots and hypocotyls were quickly dissected out with a scalpel using a dissecting microscope and frozen separately in microfuge tubes. Tissues were then ground in 100 μL of GUS extraction buffer (150 mM NaPO₄ buffer, pH 7.0, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.1% Sarcosyl, and 140 μM phenylmethylsulfonyl fluoride] using 1-mm glass beads and a Silamat S5 (Ivoclar Vivadent, Amherst, NY) shaker. Extracts were cleared by centrifugation, and 10-μL aliquots of each lysate were incubated at 37°C for 1 h and 20 min in 130 μL of MUG assay

buffer (GUS extraction buffer supplemented with 1 mM 4-methylumbelli-feryl β-D-glucuronide hydrate [MUG] [Sigma-Aldrich, St. Louis, MO]). Reactions were run in duplicate in a 96-well format. A 10-μL aliquot of each reaction was combined with 190 μL of stop buffer (200 mM Na₂CO₃), and fluorescence was measured on opaque 96-well plates in a Fluo-Star (BMG LabTechnologies, Durham, NC) fluorometer using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Readings were plotted on a standard curve generated with a series of 4-methyl-umbelliferone sodium salt (Sigma-Aldrich) standards (0 to 50 μM range) and converted to picomoles of 4-methylumbelliferone per minute per seedling.

Double Mutant Analysis

To construct double mutants, the respective single mutants were crossed to each other, potential double mutants were chosen in the F2 generation and propagated, and their progeny were phenotypically and/or genotyp-ically analyzed in F3.

GUS reporters (*ASA1-GUS*, *ASB1-GUS*, and *DR5-GUS*) were intro-duced into different mutant backgrounds exclusively by crossing to eliminate any possible chromosomal position differential effect. To select for the double mutants, plants phenotypically homozygous both for the *wei2*, *wei7*, or *ein2* mutation (i.e., in F3, 100% of seedlings showed ethylene insensitivity) and for the reporter construct (i.e., in F3, 100% of seedlings showed kanamycin resistance and uniform staining for GUS) were chosen.

To isolate *wei2 ctr1* and *wei7 ctr1* double mutants, seedlings homo-zygous for *ctr1* (i.e., showing a constitutive ethylene phenotype in the absence of ethylene) but possessing longer roots than the *ctr1* single mutant were selected on unsupplemented AT plates from the segregating F2 populations of crosses *wei2* × *ctr1* and *wei7* × *ctr1*, respectively. Phenotypes of the air-grown double mutants, confirmed in F3, were morphologically indistinguishable from those of ethylene-grown *wei2* or *wei7* mutant seedlings.

To identify double mutants between *wei2* or *wei7* and auxin-overproducing mutants, the following strategy was used. In a cross be-tween *wei2* (or *wei7*) and the *sur1* heterozygote (because *sur1* homozy-gotes are lethal), F1 plants were propagated individually, and their progeny were tested for segregation of the *sur1* phenotype. Those F2 populations that segregated for *sur1* were plated on AT plates supplemented with 10 μM ACC and grown for 3 d in the dark followed by 3 to 5 d in the light. Double mutants were identified as plants that developed long roots in ACC (i.e., showed ethylene insensitivity) and epinastic cotyledons in the light (i.e., had a *sur1* mutation). In a cross between *wei2* (or *wei7*) and *sur2*, F2 plants that possessed epinastic cotyledons in the light (i.e., were homozygous for *sur2*) were propagated and retested in F3 in the presence of 10 μM ACC. Approximately two-thirds of the *sur2* homozygotes segregated out *wei2* (*wei7*)-like plants (i.e., seedlings with ethylene-insensitive roots and broad cotyledons). These seedlings were selected and propagated as potential double mutants. The homozygosity of the *sur2* locus was confirmed genotypically using the following primer combina-tions: cyp83B1-F (5′-GAGACTCTTGACCCTAACCGC-3′) with cyp83B1-R (5′-GCGAGTCCAGTCATGACGTCC-3′) to detect the wild-type allele, and cyp83B1-F with JMLB1 (5′-GGCAATCAGCTGTTGCCCGTCTCAC-TGGTG-3′) to detect the mutant allele. The phenotypes of Col-0, *wei2*, *wei7*, *sur1*, *sur2*, and four double mutant combinations were evaluated after 5 d of growth in the light on horizontal unsupplemented AT plates to score cotyledon morphology and after 7 d of growth in the light on horizontal AT plates followed by another 4 d in the light on vertical AT plates to score adventitious root formation.

REFERENCES

Abel, S., Nguyen, M.D., Chow, W., and Theologis, A. (1995). ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclo-propane-1-carboxylate synthase in *Arabidopsis thaliana*: Structural characterization, expression in *Escherichia coli*, and expression characteristics in response to auxin. J. Biol. Chem. **270,** 19093–19099; Erratum. J. Biol. Chem. **270,** 26020.

Alonso, J.M., and Stepanova, A.N. (2004). The ethylene signaling pathway. Science **306,** 1513–1515.

Alonso, J.M., et al. (2003a). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301,** 653–657.

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003b). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc. Natl. Acad. Sci. USA **100,** 2992–2997.

Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408,** 796–815.

Bao, F., Shen, J., Brady, S.R., Muday, G.K., Asami, T., and Yang, Z. (2004). Brassinosteroids interact with auxin to promote lateral root development in Arabidopsis. Plant Physiol. **134,** 1624–1631.

Barlier, I., Kowalczyk, M., Marchant, A., Ljung, K., Bhalerao, R., Bennett, M., Sandberg, G., and Bellini, C. (2000). The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proc. Natl. Acad. Sci. USA **97,** 14819–14824.

Bartel, B. (1997). Auxin biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 51–66.

Bell, C., and Ecker, J. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19,** 137–144.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115,** 591–602.

Bennett, M., Bellini, C., and Van Der Straeten, D. (2005). Integrative biology: Dissecting cross-talk between plant signaling pathways. Physiol. Plant. **123,** 109.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Van Onckelen, H., Van Montagu, M., and Inze, D. (1995). Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. Plant Cell **7,** 1405–1419.

Boonsirichai, K., Sedbrook, J.C., Chen, R., Gilroy, S., and Masson, P.H. (2003). ALTERED RESPONSE TO GRAVITY is a peripheral membrane protein that modulates gravity-induced cytoplasmic alkalinization and lateral auxin transport in plant statocytes. Plant Cell **15,** 2612–2625.

Brown, K.M. (1997). Ethylene and abscission. Physiol. Plant. **100,** 567–576.

Buer, C.S., Wasteneys, G.O., and Masle, J. (2003). Ethylene modulates root-wave responses in Arabidopsis. Plant Physiol. **132,** 1085–1096.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P.J., and Bennett, M. (2001). Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell **13,** 843–852.

Chen, R., Guan, C., Boonsirichai, K., and Masson, P.H. (2002). Complex physiological and molecular processes underlying root gravitropism. Plant Mol. Biol. **49,** 305–317.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

Cohen, J.D., Slovin, J.P., and Hendrickson, A.M. (2003). Two genetically discrete pathways convert tryptophan to auxin: More redundancy in auxin biosynthesis. Trends Plant Sci. **8,** 197–199.

Collett, C.E., Harberd, N.P., and Leyser, O. (2000). Hormonal interactions in the control of Arabidopsis hypocotyl elongation. Plant Physiol. **124,** 553–562.

Davies, P.J. (1995). Plant Hormones. (Dordrecht, The Netherlands: Kluwer Academic Publishers).

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature **435,** 441–445.

Dharmasiri, N., and Estelle, M. (2004). Auxin signaling and regulated protein degradation. Trends Plant Sci. **9,** 302–308.

Dolan, L. (1998). Pointing roots in the right direction: The role of auxin transport in response to gravity. Genes Dev. **12,** 2091–2095.

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19,** 11–15.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature **426,** 147–153.

Guzman, P., and Ecker, J. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513–523.

Harper, R.M., Stowe-Evans, E.L., Luesse, D.R., Muto, H., Tatematsu, K., Watahiki, M.K., Yamamoto, K., and Liscum, E. (2000). The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial Arabidopsis tissue. Plant Cell **12,** 757–770.

Hobbie, L.J. (1998). Auxin: Molecular genetic approaches in *Arabidopsis*. Plant Physiol. Biochem. **36,** 91–102.

Hull, A.K., Vij, R., and Celenza, J.L. (2000). Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. Proc. Natl. Acad. Sci. USA **97,** 2379–2384.

Kepinski, S., and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature **435,** 446–451.

King, J., Stimart, D., Fisher, R., and Bleecker, A. (1995). A mutation altering auxin homeostasis and plant morphology in Arabidopsis. Plant Cell **7,** 2023–2037.

Larsen, P.B., and Cancel, J.D. (2003). Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant J. **34,** 709–718.

Last, R.L., Bissinger, P.H., Mahoney, D.J., Radwanski, E.R., and Fink, G.R. (1991). Tryptophan mutants in Arabidopsis: The consequences of duplicated tryptophan synthase beta genes. Plant Cell **3,** 345–358.

Lehman, A., Black, R., and Ecker, J. (1996). *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. Cell **85,** 183–194.

Leyser, O. (2002). Molecular genetics of auxin signaling. Annu. Rev. Plant Biol. **53,** 377–398.

Li, H., Johnson, P., Stepanova, A., Alonso, J.M., and Ecker, J.R. (2004). Convergence of signaling pathways in the control of differential cell growth in Arabidopsis. Dev. Cell **7,** 193–204.

Liscum, E., and Reed, J.W. (2002). Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol. Biol. **49,** 387–400.

Ljung, K., Bhalerao, R.P., and Sandberg, G. (2001). Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. Plant J. **28,** 465–474.

Ljung, K., Hull, A.K., Celenza, J., Yamada, M., Estelle, M., Normanly, J., and Sandberg, G. (2005). Sites and regulation of auxin biosynthesis in Arabidopsis roots. Plant Cell **17,** 1090–1104.

Ljung, K., Sandberg, G., and Moritz, T. (2004). Methods of plant hormone analysis. In Plant Hormones: Biosynthesis, Signal Transduction, Action! P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 671–694.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165–178.

McCourt, P. (1999). Genetic analysis of hormone signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50,** 219–243.

Mikkelsen, M.D., Naur, P., and Halkier, B.A. (2004). Arabidopsis mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. Plant J. **37,** 770–777.

Niyogi, K.K. (1993). Molecular and Genetic Analysis of Anthranilate Synthase in *Arabidopsis thaliana*. PhD dissertation (Boston, MA: Massachusetts Institute of Technology).

Niyogi, K.K., and Fink, G.R. (1992). Two anthranilate synthase genes in Arabidopsis: Defense-related regulation of the tryptophan pathway. Plant Cell **4,** 721–733.

Normanly, J., Cohen, J.D., and Fink, G.R. (1993). *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc. Natl. Acad. Sci. USA **90,** 10355–10359.

Pitts, R.J., Cernac, A., and Estelle, M. (1998). Auxin and ethylene promote root hair elongation in Arabidopsis. Plant J. **16,** 553–560.

Radwanski, E.R., Barczak, A.J., and Last, R.L. (1996). Characterization of tryptophan synthase alpha subunit mutants of *Arabidopsis thaliana*. Mol. Gen. Genet. **253,** 353–361.

Radwanski, E.R., and Last, R.L. (1995). Tryptophan biosynthesis and metabolism: Biochemical and molecular genetics. Plant Cell **7,** 921–934.

Rahman, A., Amakawa, T., Goto, N., and Tsurumi, S. (2001). Auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots. Plant Cell Physiol. **42,** 301–307.

Rahman, A., Hosokawa, S., Oono, Y., Amakawa, T., Goto, N., and Tsurumi, S. (2002). Auxin and ethylene response interactions during Arabidopsis root hair development dissected by auxin influx modulators. Plant Physiol. **130,** 1908–1917.

Rutherford, R., Gallois, P., and Masson, P.H. (1998). Mutations in *Arabidopsis thaliana* genes involved in the tryptophan biosynthesis pathway affect root waving on tilted agar surfaces. Plant J. **16,** 145–154.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell **99,** 463–472.

Swarup, R., and Bennett, M. (2003). Auxin transport: The fountain of life in plants? Dev. Cell **5,** 824–826.

Swarup, R., Parry, G., Graham, N., Allen, T., and Bennett, M. (2002). Auxin cross-talk: Integration of signalling pathways to control plant development. Plant Mol. Biol. **49,** 411–426.

Tsuchisaka, A., and Theologis, A. (2004). Unique and overlapping expression patterns among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol. **136,** 2982–3000.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9,** 1963–1971.

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14** (suppl.), S131–S151.

Winkler, R.G., and Feldmann, K.A. (1998). PCR-Based Identification of T-DNA Insertion Mutants. (Totowa, NY: Humana Press).

Winkler, R.G., Frank, M.R., Galbraith, D.W., Feyereisen, R., and Feldmann, K.A. (1998). Systematic reverse genetics of transfer-DNA-tagged lines of Arabidopsis: Isolation of mutations in the cytochrome P450 gene superfamily. Plant Physiol. **118,** 743–750.

Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291,** 306–309.

Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.R., Normanly, J., Chory, J., and Celenza, J.L. (2002). Trp-dependent auxin biosynthesis in Arabidopsis: Involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. **16,** 3100–3112.