A New Gene for Auxin Synthesis

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There is much interest in understanding the pathways that trigger biosynthesis of the plant hormone auxin. In this issue, Stepanova et al. (2008) and Tao et al. (2008) reveal that a small family of tryptophan aminotransferases catalyze formation of indole-3-pyruvic acid (IPA) from L-tryptophan (L-Trp), the first step in a pathway for auxin biosynthesis.

Hormones are produced at specific locations and are then released and transported to target cells where they affect cellular processes at very low concentrations. Plants also use hormones as signaling molecules, but in contrast to animals, which have lymphatic and cardiovascular systems, plants have a more difficult time moving hormones around. Plant cells are encased by a rigid wall that provides stability but restricts fluid transport to the plant’s own vascular system, which consists of sieve tubes to move sugars from the leaves to the roots and xylem to move water and soluble minerals from the roots to the leaves. Processes as diverse as flowering, fruit development, leaf formation, stem growth, longevity, and cell death are controlled through tight regulation of the amount and distribution of plant hormones. To achieve this, plants modify the availability of substrates used for hormone biosynthesis or the amount or activity of enzymes that catalyze these biosynthetic reactions. Plants also inactivate hormones by conjugating or modifying them and fine-tune local hormone concentrations by regulating their transport into or out of cells (Teale et al., 2006). However, due to the complexity in plant hormone regulation it has been difficult to attribute particular regulatory mechanisms to specific physiological responses. The findings of Stepanova et al. (2008) and Tao et al. (2008) in this issue represent significant progress toward understanding how the regulation of biosynthesis of indole-3-acetic acid (IAA), the most abundant naturally occurring auxin, can affect well-defined steps in development.

IAA is made either by de novo synthesis or by release from conjugates (Bartel, 1997). IAA is chemically similar to the amino acid tryptophan, although more than 1000 times less abundant, and it can be synthesized by many functionally redundant biochemical pathways operating in parallel. Both plants and certain plant pathogens can synthesize IAA from tryptophan. The rate-limiting N-hydroxylation of tryptophan to N-hydroxylyrptamine, a precursor of IAA, is catalyzed by flavin monooxygenases. These enzymes are essential for many processes in plants, including the establishment of the basal body during embryogenesis and the formation of embryonic and postembryonic organs and vascular tissue (Cheng et al., 2006). IAA biosynthesis occurs in rapidly dividing and growing tissues, especially shoots. Directed polar transport of IAA to other tissues, far away from the site of synthesis, is performed by a group of membrane proteins called PINs. Although the biochemical function of PIN proteins remains elusive, genetic analysis has convincingly demonstrated (Paponov et al., 2005) that they establish, through their polar localization, highly specific patterns of auxin distribution throughout the plant body and play multiple roles in plant growth and development (Gälweiler et al., 1998; Billou et al., 2005; Teale et al., 2006). It should be noted that not all plant cells respond to IAA. However, those cells that respond to IAA do so at very specific times and at specific locations. Hence, cells may reduce their sensitivity to IAA when it is no longer needed.

Stepanova et al. performed a genetic screen in the model plant Arabidopsis thaliana to isolate mutants impaired in tissue-specific responses to ethylene (a gaseous plant hormone). Characterization of the weak ethylene insensitive8 (wei8) mutant identified a small family of genes required for tissue-specific responses to ethylene. Biochemical studies indicated that this gene encodes a tryptophan aminotransferase, which has been named tryptophan aminotransferase...
in Arabidopsis (TAA1) (Figure 1). The authors showed that WEI8 functions in an essential, yet genetically uncharacterized, indole-3-pyruvic acid (IPA) branch of the auxin biosynthetic pathway. Mutant and expression analyses of members of the WEI8 gene family revealed a link between tissue-specific ethylene effects and local auxin production. Importantly, Stepanova et al. also showed that the role of the WEI8 gene family is not limited to modulating the response to ethylene but is also critical for maintenance of the root stem cell niche, flower development, and embryonic patterning.

As often occurs in science, a seemingly unrelated study performed by Tao et al. (2008) to understand why plants grow taller to avoid shade identified the same branch of the auxin biosynthetic pathway. Plants are sessile and to stay in tune with their environment, they constantly adjust their growth and development through the action of a limited set of phytohormones, including IAA. An example of such a response is the shade avoidance syndrome. It is triggered by a reduction in the ratio of red to far-red (R/FR) light, which is a feature of shady environments. Shade avoidance syndrome provides an early warning of shading and induces specific developmental responses. As such, plants sense a low R/FR ratio, plants very rapidly increase their height at the expense of leaf development. In the longer term, low R/FR exposure leads to early flowering, making shade avoidance syndrome an agronomically important trait that conditions simulating shade rapidly induce both the rate of IAA biosynthesis and the amount of free IAA in wild-type seedlings. In contrast, sav3 seedlings contain reduced IAA levels in white light and are unable to adapt cellular IAA concentrations in response to shade. It appears that the main source of new auxin is the leaves, where TAA1 is highly expressed. From the leaves, auxin is then transported to sites of cell elongation, such as hypocotyls.

Plants are intimately tied to their environment and have evolved a network of sophisticated mechanisms to deal with fluctuating local conditions (Teale et al., 2008). To respond optimally, plants monitor their ambient biotic (e.g., pathogens) and abiotic (e.g., light, temperature, nutrient supply) environments. Ultimate success depends on a plant’s ability to translate these environmental signals into specific cellular responses, fine-tuning their growth and development. The reports by Stepanova et al. and Tao et al. provide evidence that internal and external cues such as ethylene and light quality can modulate activity of a tryptophan-dependent auxin biosynthesis pathway, thus changing local auxin levels and triggering specific developmental responses. As often is the case, these studies raise more questions than they answer. For example, how many functional pools of IAA exist in plants and are they all sensitive to environmental changes? What is the molecular mechanism by which light regulates activity of TAA1?

Tao et al. isolated a series of mutants in Arabidopsis that were unable to elongate under low R/FR light and showed that inactivation of the SHADE AVOIDANCE3 (SAV3) gene is responsible for the mutant phenotype. SAV3, like WEI8, encodes the tryptophan aminotransferase TAA1 (Figure 1). Importantly, the authors also demonstrated that conditions simulating shade rapidly induce both the rate of IAA biosynthesis and the amount of free IAA in wild-type seedlings. In contrast, sav3 seedlings contain reduced IAA levels in white light and are unable to adapt cellular IAA concentrations in response to shade. It appears that the main source of new auxin is the leaves, where TAA1 is highly expressed. From the leaves, auxin is then transported to sites of cell elongation, such as hypocotyls.

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As TAA1 does not appear to catalyze the rate-limiting step of IAA biosynthesis, what enzyme does? And do the various growth responses displayed by plants require similar levels of free IAA? Clearly, data from both groups demonstrate that the tryptophan aminotransferases are key enzymes for the indole pyruvic acid route of auxin production and are critical for generating robust auxin gradients in response to environmental and developmental cues.

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Rapid Synthesis of Auxin via a New Tryptophan-Dependent Pathway Is Required for Shade Avoidance in Plants

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SUMMARY

Plants grown at high densities perceive a decrease in the red to far-red (R:FR) ratio of incoming light, resulting from absorption of red light by canopy leaves and reflection of far-red light from neighboring plants. These changes in light quality trigger a series of responses known collectively as the shade avoidance syndrome. During shade avoidance, stems elongate at the expense of leaf and storage organ expansion, branching is inhibited, and flowering is accelerated. We identified several loci in Arabidopsis, mutations in which lead to plants defective in multiple shade avoidance responses. Here we describe TAA1, an aminotransferase, and show that TAA1 catalyzes the formation of indole-3-pyruvic acid (IPA) from L-tryptophan (L-Trp), the first step in a previously proposed, but uncharacterized, auxin biosynthetic pathway. This pathway is rapidly deployed to synthesize auxin at the high levels required to initiate the multiple changes in body plan associated with shade avoidance.

INTRODUCTION

The mechanisms by which organisms alter their growth and development in response to changes in their ambient environment are largely unknown. Plants exhibit an enormous array of phenotypic plasticity because most plant organs do not arise until after the seed germinates, allowing organ size and shape to be optimized to the local environment. Because they are sessile and photosynthetic, plants are especially attuned to their light environment. Light influences every developmental transition from seed germination and seedling emergence to flowering. For shade-intolerant plants, such as Arabidopsis thaliana, a reduction in the R:FR ratio of incoming radiation, which is caused by absorption of red light and reflection of far-red radiation by canopy leaves, signals the proximity of neighboring plants and triggers the shade avoidance syndrome (SAS). A common phenotype of the SAS is re-allocation of energy resources from storage organs to stems and petioles so that the plant outgrows its competitors. Other responses induced by reduction in R:FR ratio include increased leaf angle, accelerated leaf senescence and reduced deposition of fixed carbon to storage organs (Ballare, 1999). In response to prolonged shade, reproductive development is accelerated, potentially leading to decreased biomass and seed yield (Franklin and Whitelam, 2005). As such, the SAS, a strategy of major adaptive significance to plants growing in natural ecosystems, can significantly impact yield in high-density plantings typical of modern agriculture (Ballare et al., 1997; Izaguirre et al., 2006).

Changes in light quality are perceived by the phytochromes, a family of R/FR photoreceptors. Arabidopsis has 5 phytochromes, PHYA-PHYE. PHYB is the major phytochrome in light-grown plants and plays a predominant role in the SAS (Ballare, 1999). phyB mutants display a constitutive shade-avoiding phenotype that is characterized by long hypocotyls and petioles, reduced chlorophyll content, early flowering...
(Reed et al., 1993), and a reduced response to low R:FR (Halliday et al., 1994).

The events following photoreceptor excitation by changes in light quality are poorly understood. Analysis of the Arabidopsis transcriptome following transfer of plants to simulated shade (low R:FR) revealed a large number of shade-induced, early response genes (Devlin et al., 2003; Salter et al., 2003; Sessa et al., 2005). mRNA levels of several transcription factor genes increase within a few minutes of exposure to low R:FR light, and falls very rapidly after transfer from low to high R:FR light. A negative regulatory gene is also rapidly induced by low R:FR (Sessa et al., 2005), suggesting that there is a gas-and-brake mechanism that ensures that plants do not have an exaggerated response to shade.

Genes encoding metabolic enzymes or signaling components of several phytohormones are also among the early response genes, implicating a role for plant hormones in the SAS (Devlin et al., 2003). Brassinosteroids (BRs), auxin, ethylene and gibberellins appear to be involved in the SAS as mutants that are defective in the metabolism or signaling of these hormones either have reduced elongation growth in response to shade or can suppress the constitutive shade-avoiding phenotype of phyB (Hisamatsu et al., 2005; Kanyuka et al., 2003; Kim et al., 1998; Kurepin et al., 2007; Luccioni et al., 2002; Morelli and Ruberti, 2002; Neff et al., 1999; Peng and Harberd, 1997; Pierik et al., 2004). The role of auxin in the SAS has been explored most extensively. Several studies have shown auxin transport is required (Kanyuka et al., 2003; Morelli and Ruberti, 2000; Steindler et al., 1999). In addition, low R:FR induces the expression of many known auxin-responsive genes and it also arrests the growth of leaf primordia through auxin-induced cytokinin metabolism (Kurepin et al., 2007). These results suggest that a functional auxin signaling pathway is required to have a shade avoidance response. Recently, Kurepin et al. showed that prolonged growth in the shade resulted in changes in levels of indole-3-acetic acid (IAA, an endogenous auxin) and other hormones, suggesting that light quality also influences auxin homeostasis.

Despite the ecological and economic impact of the SAS, little is known about the underlying mechanisms linking photoperception to changes in physiology and development. Here we describe a genetic screen in Arabidopsis for mutants unable to elongate in simulated shade light (sav mutants for shade avoidance). We identified the defective gene in the sav3 mutant and show that the wild-type locus encodes a protein, TAA1, with a C-terminal alliinase/aminotransferase domain. We present multiple lines of evidence that indicate a role for TAA1 in a previously proposed, but genetically and biochemically undefined, IAA biosynthetic pathway from L-Trp. We show that within 1 hr after transferring seedlings from white light to shade, the levels of free IAA increase in wild-type (WT) owing to an increase in the rate of IAA biosynthesis; in contrast, IAA levels are reduced in mutant seedlings lacking the TAA1 protein and there is no significant change in IAA levels in response to shade. Our results suggest that certain growth responses require a higher threshold of available IAA. In addition, as other IAA biosynthetic pathways (Zhao et al., 2001) cannot compensate for the loss of the TAA1-dependent pathway, there may exist multiple functional pools of IAA in Arabidopsis.

RESULTS

Identification of Arabidopsis sav Mutants

To identify Arabidopsis genes that are involved in the SAS, we performed a forward genetic screen for seedlings that did not elongate after transfer from continuous white light (Wc) to simulated shade light. Details of the screen are outlined in Figures S1A and S1B (available online). Seedlings that appeared similar to WT in Wc but had shorter hypocotyls than WT in shade were identified as shade avoidance (sav) mutants. To eliminate mutants with light-independent elongation defects, we germinated the mutants in the dark and grew them for 4 days, conditions under which wild-type hypocotyls become long. Of the 47 lines, 30 were significantly shorter than WT in the dark and we reasoned that they were likely to contain mutations in components of the cellular machinery required for elongation growth. To test this idea, we identified the defective gene in the sav2 mutant as TUB4 (At5g44340), which encodes a β-tubulin isoform (Snustad et al., 1992).

Seventeen lines exhibited no or very minor phenotypes in the dark, while being significantly shorter than WT in simulated shade. Through map-based cloning, we identified the SAV1 gene as DWF4, which encodes a C-22 hydroxylase involved in brassinosteroid biosynthesis; sav1 is predicted to be a weak allele of DWF4. SAV3 was defined by 3 alleles and we thus focused our studies on the analysis of this locus.

sav3 Seedlings Are Defective in Multiple, but Not All, Shade Avoidance Responses

sav3 mutants exhibit shorter hypocotyls than WT when grown in simulated shade (Figures 1A and 1B) and partially suppress the constitutive shade avoidance phenotype of a phyB null mutant (Figure S2A). The SAS is a complex syndrome, involving rapid changes in gene expression, elongation of petioles, changes in leaf shape and angle, and accelerated flowering, in addition to elongation of the primary stem. Figure 1D shows that sav3-2 (a null allele, see below) seedlings have shorter petioles and larger leaf area than WT Col-0 plants when grown in shade. Further, when subjected to supplementary FR, sav3-1 plants were shorter and had reduced leaf hyponasty as compared to WT (Figures 1C, 1D, and S2B). Our results demonstrate that the sav3-1 mutant fails to induce SAS in a controlled environment typically used to detect PHYB-mediated SAS responses in light-grown plants. However, sav3 mutants flowered at the same time as WT when grown in simulated shade (data not shown), suggesting that the light quality-controlled flowering time pathway operates independently of other shade-regulated pathways (Cerdan and Chory, 2003).

SAV3 Encodes a Protein with a Predicted Alliinase C-Terminal/Aminotransferase Domain

We identified SAV3 by map-based cloning (Lukowicz et al., 2000). SAV3 encodes a protein with an alliinase C-terminal/aminotransferase class I and II domain (At1g70560, Figure 2A). Because this locus was also identified in a different mutant screen (Stepanova et al., 2008) and we describe its biochemical function below, we renamed the gene, TAA1, for IRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS and call the protein,
TAA1. The mutant alleles retain the sav3 nomenclature. Alliinase is a pyridoxal phosphate (PLP) enzyme that catalyzes the production of the characteristic flavor molecule of onion, garlic and other related alliums (Kuettner et al., 2002a, 2002b). sav3-1 contains a G to A point mutation at the splice junction of the fourth intron, resulting in elevated levels of a longer TAA1 transcript and a predicted C-terminal truncation in the TAA1 protein (Figure S1C), which may explain the slightly different response of this allele in some experiments (e.g., Figure 3A). sav3-2 contains a G to A mutation in the second exon, converting Trp39 to a stop codon, and does not accumulate TAA1 protein (data not shown). sav3-2 is presumed to be a null allele. sav3-3 harbors a G to A mutation in the fourth exon, which converts Gly250 to Ser. Defects in the three alleles were rescued by a genomic copy of TAA1 expressed under the control of the CaMV 35S promoter (data not shown). A PLP cofactor is required for the enzymatic activity of alliinase. We found that recombinant TAA1 apoprotein expressed in E. coli binds to PLP in vitro. To test whether PLP is also required for the function of TAA1 and that TAA1 is likely to function as an enzyme.

sav3 Mutants Have Reduced Auxin Levels and a Diminished Auxin Response

Given sav3’s pleiotropic phenotype, its predicted protein structure and knowing that several plant hormones are involved in the SAS, we reasoned that TAA1 might be involved in the biosynthesis or metabolism of auxin, which can be derived from L-Trp. Given the SAS, we reasoned that TAA1 might be involved in the biosynthesis or metabolism of auxin, which can be derived from L-Trp. To assess this putative role, we tested the responses of sav3 mutants to various concentrations of picloram (Sorin et al., 2005). Under Wc, the responses of sav3 mutants to high concentrations of picloram were similar to that of WT. Under simulated shade, we found that high concentrations of picloram fully rescued the short hypocotyl phenotype of sav3 (Figure 3A). Picloram did not rescue sav1-1. To examine the ability of sav3 to respond to increases in endogenous auxin, we tested the hypocotyl elongation response of sav3 mutants to high temperature treatment, conditions known to increase free auxin levels (Gray et al., 1998). As shown in Figure S2C, sav3 mutants were defective in high temperature induced hypocotyl elongation, whereas sav1 had a response similar to WT. These results suggest that TAA1 is involved in auxin homeostasis.

To investigate whether endogenous auxin levels were altered in sav3, we measured free IAA levels in 7-day-old WT and sav3 seedlings grown in Wc. sav3 seedlings had about 60% of wild-type levels of auxin. To assess the influence of light on auxin levels, WT and sav3 seedlings were grown in Wc and then transferred to shade for 1 hr. As shown in Figure 3B, simulated shade treatment increased free IAA levels significantly in WT, but to a much lesser extent in sav3-2, suggesting that TAA1 is involved in shade-induced auxin production.

To assess the source of the shade-induced increase in free IAA, we performed a deuterium dioxide feeding experiment to
measure the IAA biosynthesis rate. As shown in Figure 3C, the rate of IAA biosynthesis in Wc is similar in WT and sav3 mutants. However, after 2 hr of shade treatment, an increase in IAA biosynthesis rate was detected in WT but not sav3, (indicated by the higher ratio of the deuterium labeled vs. unlabelled IAA). These results show that TAA1 is directly involved in shade-induced de novo IAA biosynthesis.

To better understand the molecular consequences of reduced auxin levels, we interrogated the transcriptome of WT and sav3-2 seedlings before and after 1 hr of simulated shade using the Affymetrix ATH1 array (data submitted to GEO, GSE9816). RNA was prepared from whole seedlings of WT, sav3-2 and sav1-1 grown in simulated Wc for 7 days and either left in Wc or transferred to simulated shade (R:FR = 0.7) for 1 hr. sav1

Figure 2. TAA1 Encodes an Enzyme with an Alliinase C-Terminal/Aminotransferase Domain
(A) Protein alignment of Arabidopsis TAA1 family. The alliinase C-terminal domain is marked by a blue line. K217 is the PLP binding site.
(B) Cytoplasmic localization of TAA1-YFP in root meristem cells. TAA1-YFP was expressed stably in sav3-1 under the control of the CaMV 35S promoter. The YFP fusion protein was visualized using a Leica confocal microscope.
(C) Complementation test using TAA1 genomic DNA fragment (TAA1) or mutant forms of TAA1 (K217G, K217R, or an N-terminal truncation containing the first 130 aa). At least three independent transgenic lines were used for characterization of TAA1 localization and phenotypes. Mean values of more than 12 seedlings are shown; error bars represent SEM.
was included as a control for specificity. Comparisons of genotypes and treatments were performed using the RankProd package from Bioconductor (Hong et al., 2006). At a 5% false discovery rate, the levels of 80 transcripts were increased in the WT following shade treatment. The expression patterns of these genes are shown in Figure 4A. The majority of genes that were upregulated by shade in WT plants had reduced expression in shade-treated sav3 mutants, although there were some notable exceptions, e.g., the known shade-upregulated genes, PAR1, HFR1, and ATHB2 (Figure S3A). The expression of the shade upregulated genes in sav1 was similar to WT. We carried out a similar analysis to identify genes that were differentially expressed in shade-treated WT and sav3. 66 genes were found to be expressed at significantly lower levels in sav3 compared to WT, of which 36 were among the list of 80 genes identified as shade upregulated in WT. We performed coresponse analysis of these 36 genes using data from Genevestigator V2 (https://www.genevestigator.ethz.ch/at/, Figure 4 B). We found that most of these genes were also upregulated by IAA, but not other hormones, indicating that the sav3 mutation specifically affects the induction of auxin-responsive genes.

To validate the microarray results, we selected two genes (IAA19 and IAA29), whose expression was upregulated by shade in WT, but not in sav3-2. Using quantitative PCR, we showed that the shade-induced induction of these two genes was reduced in sav3-1, sav3-2, and sav3-3 (Figure 4C). Moreover, this reduction could be rescued by treating sav3 mutants with 1 μM of IAA (Figure S3B). These data support the hypothesis that TAA1 is involved directly in auxin biosynthesis. Additionally, since TAA1 is required for shade-induced gene expression as early as 1 hr after transfer to shade, we conclude that changes in auxin levels are required for the primary responses of the SAS.

**TAA1 Has a Localized and Dynamic Expression Pattern**

To further explore the connection between auxin and TAA1, we examined the expression pattern of TAA1. Transgenic lines expressing a TAA1-GUS fusion protein under the control of a 2Kb TAA1 promoter were generated. In 5-day-old seedlings, expression of TAA1 in the shoot was observed mainly in the emerging young leaves, at the leaf margin and in the vasculature. In roots, it was expressed in the quiescent center and in the vasculature of root tips (Figure 5A; data not shown).

During embryogenesis, the expression pattern of TAA1 changes dynamically. Using in situ hybridization, TAA1 mRNA accumulation was first detected at the 32 to 64-cell stage of embryogenesis. Initially, TAA1 was expressed strongly in the most apical 3–4 cells of the epidermis and was weakly expressed in the cells that give rise to the vasculature of the hypocotyl. By the heart stage of embryogenesis, TAA1 was strongly expressed in the developing vasculature and was detected in the derivatives of the hypophyseal cell that gives rise to the quiescent center of the root. TAA1 was also expressed in the apical epidermal layer (Figure 5B). At the torpedo stage of embryogenesis, TAA1 expression was detected in the developing vasculature of the root, hypocotyl and cotyledons, as well as in the L1 layer of the presumptive shoot apical meristem and the adaxial epidermis.
of the developing cotyledons (Figure 5C). In 5-day-old seedlings, TAA1 expression was maintained in the L1 of the shoot apical meristem and was detected in the developing vasculature of leaf primordia (data not shown).

Of interest, the expression pattern of SAV3 in the shoot was similar to that of DR5::GUS, an artificial auxin reporter gene construct, whose expression is thought to reflect the levels of free auxin (Aloni et al., 2003; Cheng et al., 2006; Mallory et al., 2005; Sabatini et al., 1999). Although sav3 is defective in shade-induced hypocotyl elongation, we observed little expression of SAV3 in hypocotyls of 5-day-old seedlings. We found that shade treatment did not alter the expression pattern of TAA1 (Figure 5A), rather it reduced the expression of TAA1 after 2 hrs of shade treatment (Figure S4 A), suggesting a possible feedback regulation on TAA1 expression by shade. DR5::GUS expression levels increased in cotyledons after 8 hr of shade treatment.

Figure 4. Global Expression Analysis of sav3 Implicates a Role for TAA1 in Auxin Response
(A) Expression pattern of shade-induced genes.
(B) Coresponse analysis of TAA1-dependent, shade upregulated genes. Expression data of each gene were normalized and medium-centered using Cluster and visualized by Treeview (http://rana.lbl.gov/EisenSoftware.htm). Green and red represent lower- and higher-expression levels as compared to the median value, respectively.
(C) Quantification of IAA19 and IAA29 expression using quantitative RT-PCR. Relative expression level as compared to a reference gene (At2G39960) is shown. Mean values from three replicates are shown. Error bars represent SEM.
treatment, but we detected no GUS activity in hypocotyls at this time point. To investigate the site of auxin accumulation in Wc and following shade treatment, we dissected hypocotyls from other aerial tissues in WT harboring a DR5::GUS reporter. We found that after 4 hrs of shade treatment, GUS activity increased in both hypocotyls and the other aerial tissues (Figure 5D). However, in the presence of 5 μM NPA, an auxin transport inhibitor, the increases of GUS activity caused by shade were not observed in hypocotyls, while increased GUS activity was still observed in the other aerial tissues. This indicates that the main source of new auxin is in leaves, where TAA1 is highly expressed; auxin is then transported to sites of elongation growth, such as hypocotyls.

**Figure 5. TAA1 Expression Is Dynamic**

(A) TAA1 is expressed predominantly in the leaf margins. Expression patterns of P_{TAA1}:TAA1-GUS and DR5::GUS are shown. Five-day-old seedlings were treated with Wc or shade for 8 hr.

(B) and (C) In situ hybridization results show TAA1 is expressed during the heart (B) and torpedo (C) stages of embryogenesis.

(D) Shade-induced increase in DR5-GUS expression is dependent on TAA1 expression in leaves and functional auxin transport. Five-day old seedlings were pretreated with 5 μM of NPA by submerging roots in NPA solution for 30 min and then subjected to Wc or shade for 4 hrs. Relative GUS activity was calculated by normalizing to the amount of total protein measured by Bradford assay. Mean values from three replicates are shown; error bars represent SEM. T-test assuming equal variance was carried out for Wc and shade-treated sample pairs. Comparing Wc and shade treated samples, only NPA-treated hypocotyls show no significant difference (using p < 0.05 as cut-off).

**TAA1 Is an L-Trp Aminotransferase Involved in IAA Biosynthesis**

Because TAA1 is annotated as containing an aminotransferase domain and sav3 mutants have reduced auxin, we investigated whether L-Trp, an IAA biosynthetic precursor, serves as an in vitro substrate of TAA1. Several Trp-dependent auxin biosynthetic pathways have been proposed in *Arabidopsis*: the indole-3-acetamide (IAM) pathway, the tryptamine pathway, the indole-3-acetaldoxime (IAOx) pathway and the indole-3-pyruvic acid (IPA) pathway (Figure 6A). While there is evidence for the IAOx and tryptamine pathways, the IPA pathway remains conjecture. Since PLP-utilizing enzymes can catalyze a variety of reactions including transaminations, racemization, decarboxylation, and
side-chain eliminations or replacements (Aitken and Kirsch, 2005; Dunathan, 1966), we tested whether TAA1 could catalyze the formation of IPA from L-Trp. Using bacterial-expressed recombinant TAA1 protein, we found that, when supplied with sodium pyruvate or α-ketoglutarate as cosubstrates for PLP-dependent transamination reactions, TAA1 produced IPA from L-Trp but not from D-Trp. The production of IPA was confirmed using LC/MS (Figure 6B). This result suggests that TAA1 is involved in IPA-dependent auxin biosynthesis.

We further characterized the biochemical properties of TAA1 and found that the optimal temperature for TAA1 catalyzed IPA production was 55°C; the optimal pH was 8.8 (Figure S5A). TAA1 has an apparent Km for L-Trp of 0.29 mM and a Vmax of 12.9 μM/min (Figure 7A). To test the substrate specificity of TAA1, we examined the aminotransferase activity of TAA1 towards other amino acids. In our assays, TAA1 also used L-Phe, Tyr, Leu, Ala, Met and Gln as substrates (Figure S5B). To investigate whether TAA1 uses L-Trp as a substrate in vivo, we examined the susceptibility of sav3 to the toxic Trp analog: 5-methyl tryptophan (5-MT) (Zhao et al., 2001). Enzymes that use Trp as a substrate can metabolize 5-MT; thus, mutations in these enzymes give rise to plants that are hypersensitive to 5-MT. Indeed, when grown on 20 μM 5-MT in Wc, sav3-2 was more susceptible to 5-MT than WT seedlings, suggesting that TAA1 is involved in Trp-dependent auxin biosynthesis in vivo (Figure 7B). A role for TAA1 in auxin biosynthesis was further supported by expressing the bacterial auxin biosynthesis gene, iaaM, under the control of the TAA1 promoter, which should increase auxin levels at sites where TAA1 is expressed (Cheng et al., 2006). We found that expression of iaaM caused a long hypocotyl phenotype in both WT and sav3-2, regardless of the light condition (Figure S4B).

To further examine whether L-Trp is the preferred substrate of TAA1, we performed an in silico docking experiment using the crystal structure obtained from TAA1 crystals soaked in L-Phe.
and cocrystallized with PLP (PDB code: 3bwn) (unpublished data). We found that the structure of TAA1 shares a degree of homology with alliinase from *Allium sativum* (garlic), for which the structure of both the apo form and the ternary complex with the aminoacrylate reaction intermediate covalently bound to the PLP cofactor are available (PDB code: 1lk9, 2hor and 2hox). We computationally tested L-Trp, Phe, Tyr and His covalently tethered to PLP through their respective amino groups as Schiff bases on a model of TAA1 free of the PLP cofactor (Figure 7A). We also examined computationally IPA, L-Trp, Tyr, His, and D-Trp on a model of TAA1 now containing PLP. IPA docking resulted in the best score followed by L-Trp and then L-Phe, L-Tyr, L-His, and D-Trp scored the lowest. Independent of prior knowledge, we also completed a large in silico docking experiment with a small molecule library (http://blaster.docking.org/zinc/choose.shtml). Notably, the best scoring small molecules possessed chemical structures similar to the presumptive TAA1 reaction product IPA (unpublished data). In summary, the in silico computational docking experiments support the hypothesis that L-Trp serves as the preferred physiological substrate of TAA1 while IPA is the expected product.

**DISCUSSION**

**Identification and Biochemical Characterization of TAA1 Supports the Existence of a New Trp-Dependent IAA Biosynthetic Pathway in Higher Plants**

Despite the central role of auxin in plant growth and development, details of how auxin is synthesized continue to puzzle plant biologists. Multiple Trp-dependent pathways and a Trp-independent pathway for the production of IAA have been proposed to function in higher plants (Woodward and Bartel, 2005). Through forward genetics, several auxin overproduction mutants have been identified; however, very few auxin-deficient mutants—which are critical for evaluating the function of each proposed pathway—have been reported. None of the pathways have been characterized completely, either in terms of detecting the proposed biosynthetic intermediates or identifying the
enzymes that catalyze each step. In addition, the specific role of each pathway in planta and how these biosynthetic pathways intersect and are regulated are not known.

Of the multiple pathways proposed for the biosynthesis of IAA, the IPA pathway (L-Trp to IPA, to indole-3-acetaldehyde (IAAld), to IAA) is the least characterized. Mutations in the IPA pathway (L-Trp to IPA, to indole-3-acetaldehyde (IAAld), intersect and are regulated are not known. Each pathway enzymes that catalyze each step. In addition, the specific role of droxytryptamine in the IAOx pathway (Figure 6 A, Zhao et al., 2002) identified a set of related enzymes that convert tryptamine to N-hydroxytryptamine in the IAox pathway (Figure 6 A, Zhao et al., 2002). This double mutant, lacking two cytochrome P450 genes, cyp79b2/cyp79b3, shows a significant reduction in free IAA levels when grown at 26°C, although levels are not significantly different from WT when plants are grown at lower temperatures, conditions in which Arabidopsis normally is found.

We provide multiple lines of evidence that TAA1 is an aminotransferase specifically involved in the formation of IPA from L-Trp in an IAA biosynthetic pathway. sav3 mutants fail to upregulate scores of auxin-inducible genes during shade avoidance, accumulate approximately half the amount of free IAA compared to WT, do not increase IAA biosynthesis rates as WT plants do in response to shade, and are hypersensitive to a toxic Trp analog. Second, TAA1 catalyzes the formation of IPA from L-Trp in vitro. In other studies, we determined the 3-D structure of TAA1 (unpublished data). We used this information to model the active site of TAA1, and show here that L-Trp and IPA dock into the TAA1 active site with the lowest energies and in conformations consistent with the expected enzymatic mechanism for PLP-dependent transamination. Together with quantitative in vitro biochemical assays, these series of computational experiments constitute reliable evidence that L-Trp is the in vivo substrate of TAA1, even though TAA1 and other aminotransferases are known to turnover a number of related uncharged and hydrophobic amino acids with reduced catalytic efficiency (Soto-Urzua et al., 1996).

An IPA-dependent IAA biosynthetic pathway has been characterized in several bacteria (Badenoch-Jones et al., 1982; Kaneshiro et al., 1983). IPA has been identified from several plant species; however, the existence of an IPA-mediated IAA biosynthetic pathway has not been shown, which may be due to the instability of IPA (Tam and Normanly, 1998). The identification of TAA1 as the Trp aminotransferase of the IPA-pathway indicates that this pathway is operative in Arabidopsis. TAA1 has a relatively high apparent Km for L-Trp (0.29 mM), comparable to the activities reported in crude extracts of Phaseolus aureus (0.33mM), in tomato shoots (5 mM), or in bacteria (1.05 to 3.3mM). In Enterobacter cloacae, a Trp aminotransferase has a Km of 3.3 mM for L-Trp and 24 μM for IPA and it was hypothesized that the low affinity of the enzyme for L-Trp and the high affinity for the product may reflect the need to maintain low intracellular IPA levels (Koga et al., 1994). Most enzymes operating under near steady state physiological conditions maintain Km values that approximate the available concentration of substrate. In the case of TAA1, the localized concentration of L-Trp may serve as an exquisitely fine-tuned control point for time and spatially dependent auxin production. Knowledge of the temporal and spatial concentrations of substrates and intermediates of the IPA-dependent IAA biosynthetic pathway together with quantitative descriptions of IAA biosynthetic enzymes should shed light on the regulatory functions of enzymes such as TAA1 in plant development.

TAA1 shares properties reported for Trp aminotransferases measured in crude extracts from multiple organisms. TAA1 can utilize all of the aromatic amino acids as well as Leu, Ala and Met as substrates, as can those from Phaseolus and Azospirillum brasilense (Baca et al., 1994; Truelsen, 1972). However, TAA1’s Km for L-Tyr and L-Phe is 4.96 mM and 9.08 mM (Figure S5C), respectively, while under the same conditions, the Km for L-Trp is 0.29 mM. Thus, L-Trp is likely to be the preferred substrate for TAA1.

In bacteria, IPA decarboxylase (IPDC) is believed to be the key enzyme for the IPA-dependent IAA biosynthetic pathway because the other two enzymes, Trp aminotransferase and indole-3-acetaldehyde oxidase, are present in most bacteria including those that are incapable of producing IAA (Koga et al., 1991, 1992, 1994). In Enterobacter cloacae, IPDC catalysis is the rate-limiting step in the production of IAA (Koga et al., 1994). This biosynthetic bottleneck may also be shared in the higher plant pathway as we found that over-expression of TAA1 did not result in an auxin overproduction phenotype; TAA1 over-expression also did not enhance hypocotyl elongation in shade, suggesting that TAA1 is unlikely to be a rate-limiting enzyme in the higher plant IPA-dependent IAA biosynthetic pathway. As such, identification of a plant IPDC is a top priority.

**Auxin Biosynthetic Pathways Are Not Redundant**

The difficulty in genetically dissecting auxin biosynthetic pathways has been attributed to the redundancy of auxin biosynthetic genes and the existence of multiple routes to IAA (Cheng et al., 2006; Woodward and Bartel, 2005). When grown at 22°C in Wc, 7-day old seedlings carrying a null allele of sav3 appear generally similar to WT, even though sav3 mutants contain only 60% of wild-type levels of free auxin. This suggests that under such growth conditions, auxin is not limiting. However, when seedlings are transferred to shade, the rate of IAA biosynthesis increases dramatically in WT, but not in sav3 seedlings. As such, when Arabidopsis encounters shade similar to our conditions, other Trp-dependent IAA biosynthesis pathways, such as the YUCCA-dependent pathway, cannot compensate for loss of the IPA pathway. We noticed that YUC 2,5,8 and 9 were induced by low R:FIR in our array data (Figure S6A). We thus examined the shade phenotypes of the corresponding yuc mutants and a yuc 3,5,7,8,9 quintuple mutant. We did not see any defect in shade induced hypocotyl elongation (Figure S6B), indicating that these YUC genes are not required for the SAS in our conditions.

We offer several alternatives to explain this observation. In the first, IAA biosynthetic enzymes may accumulate in spatially or temporally distinct patterns during development. In support of this, members of the YUCCA gene family (Cheng et al., 2006)
and TAA1 are expressed in discrete and dynamic patterns during development. Further analysis is required to spatially refine the degree of overlap in expression at the cell type level. It is also possible that there are separate pools of IAA in plants and one pool is not available to compensate for the loss of another (Jones et al., 1991). In addition, the subcellular localization of IAA biosynthetic enzymes might lead to localized production, and perhaps a distinct pool, of auxin within a cell. It is important to point out that the complete set of enzymes for a particular pathway from Trp to IAA has not been isolated. Thus, in each cell type, which Trp-dependent pathway(s) is utilized to synthesize IAA remains unknown.

The TAA1 Pathway Can Be Rapidly Deployed to Increase Auxin Levels in Response to Shade

Here we show that mutations in TAA1 alone lead to a dramatic reduction in free IAA levels, suggesting that IPA-dependent IAA biosynthesis is an important pathway for the biosynthesis of free IAA. In addition, TAA1 is required for the rapid increase in auxin levels through de novo IAA biosynthesis upon exposure to shade. This increase in free IAA is a prerequisite for the induction of a large number of auxin-regulated genes and is required for the full implementation of the SAS. Notably, cells with the highest expression levels of TAA1 are distinct from the ones that show maximal elongation growth in response to low R:FR. This is consistent with previous reports in which auxin transport is required for low R:FR induced hypocotyl elongation (Steindler et al., 1999), and for the induced expression of some shade-induced marker genes in stem, the photoreceptive sites in shoots are in cotyledons, not in hypocotyls (Tanaka et al., 2002). Thus, we propose that the low R:FR signal is perceived by phytochrome in cotyledons or leaves where TAA1 is highly expressed. TAA1 then mediates a transient increase in free IAA, which is transported to hypocotyls, leading to the upregulation of auxin-responsive genes involved in elongation growth. In support of this model, we found that an auxin transporter inhibitor, NPA, can block the shade-induced increase of a DR5::GUS reporter in hypocotyls, indicating that shade induced increases in auxin biosynthesis occur in the upper part of the shoot, and auxin is transported to the hypocotyls where it participates in a growth response.

Conclusions

Our data show that TAA1 is a key enzyme required for rapid shade-induced changes in auxin levels. TAA1 is critical for the initiation of and full induction of shade avoidance responses in Arabidopsis. Identification of this enzyme, both in our screen and in the screen described in the accompanying paper by Stepanova et al. (2008) provides evidence that the proposed IPA-dependent IAA biosynthetic pathway operates in higher plants. The TAA1-dependent pathway is a major production route of free IAA in Arabidopsis, and appears to be required to rapidly increase free IAA levels in response to environmental changes. Since IAA can be synthesized from Trp via other known pathways, our results suggest that certain growth responses, such as the SAS, require higher levels of free IAA than other auxin-dependent responses.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

EMS mutagenized M2 seeds of the WT accession, Col-0, were purchased from Lehle seeds (http://www.arabidopsis.com). The screen is outlined in Figure S1A. Seedlings were grown at 22°C. Four light conditions were used: Wc: fluorescent light, 30–50 μE·m⁻²·s⁻¹; simulated Wc: LED light, R: 13 μE·m⁻²·s⁻¹ and Blue (B): 1.23 μE·m⁻²·s⁻¹; simulated shade: shaded Wc light plus LED FR light: 20.2 μE·m⁻²·s⁻¹ (R:FR ratio: 0.7); supplementary FR light: greenhouse light supplemented with FR filter covered incandescent light (R:FR ratio: 0.24 for +FR and 0.68 for −FR). Details of this and other experiments are described in the Supplemental Data. Quantitative measurements of hypocotyls, petioles, and leaf area were performed on scanned images of seedlings using scion image software (http://www.scioncorp.com/). For all measurements, at least 12 seedlings were used per treatment or genotype. In all figures, error bars represent standard error. Construction of plasmids for complementation, TAA1 protein localization, and expression studies are described in detail in the Supplement. At least three lines were used for characterization. GUS staining was performed using 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (Gold Biotechnology) as described (Jefferson et al., 1987).

Map-Based Cloning

Mutants were crossed to Ler and the segregating F2 seedlings were screened for short hypocotyls in shade. The Monsanto Arabidopsis Polymorphism and Ler Sequence Collections (http://www.arabidopsis.org/browse/Cereon/index.jsp) were used for designing SSLP, CAPS and dCAPS markers.

Gene Expression Profiling

Total RNA was extracted using Trizol (Invitrogen) and Biotin labeled using One-cycle target labeling kit (Affymetrix). Affymetrix ATH1 array was used according to the manufacturer’s guidelines. Scanned arrays were analyzed with Affymetrix MAS 5.0 software and then normalized with gcRMA obtained from bioconductor (http://bioconductor.org/).

TAA1 Activity Assays

The 100 μl assay mixture contained 50mM L-Trp, 50mM sodium pyruvate, 100 μM PLP and 30 μg of purified TAA1 in reaction buffer (50 mM KH₂PO₄/K₂HPO₄ [pH 8.5]). The reaction was incubated at 25°C for 3 hr and then stopped by acidifying with 3M phosphoric acid to pH 3, followed by extraction with equal volume of ethyl acetate (3×). The supernatant was dried and the pellet was resuspended in 30 μl of methanol. The ethyl acetate layer was collected, dried and re-suspended in 30 μl of methanol. The methanol solubilized extracts were analyzed by liquid chromatography (LC) on an Agilent 1100 HPLC using a chiralcel OD-RH column (0.46 cm I.D. × 15 cm) (Daicel Chemical Ind., LTD) at a flow rate of 0.5 ml/min, coupled to an electrospray ionization (ESI) XCT ion trap mass spectrometer XCT ion trap (Agilent) run in the negative-ion mode. Eluants were mixed with 20 mM ammonium acetate in 65% acetonitrile and re-suspended in 30 l of methanol. The methanol solubilized extracts were analyzed by liquid chromatography (LC) on an Agilent 1100 HPLC using a chiralcel OD-RH column (0.46 cm I.D. × 15 cm) (Daicel Chemical Ind., LTD) at a flow rate of 0.5 ml/min, coupled to an electrospray ionization (ESI) XCT ion trap mass spectrometer XCT ion trap (Agilent) run in the negative-ion mode. Eluants were mixed with 20 mM ammonium acetate in 65% acetonitrile (100 μl/min) prior to injection in the mass spectrometer. A linear gradient of acetonitrile/0.1% formic acid (1:70%) in water/0.1% formic acid was used for column elution. The negative ion-ESI mass spectrum of IPA standards behaved as expected with a m/z = 202.2 (M – H⁻).

Biochemical characterization of TAA1 was performed using a borate buffer assay (Matheron and Moore, 1973). For 100 μl of reaction, 0.5 μg of TAA1 was used. The reaction was performed at 55°C for 5 min (2 min for kinetics). Km and Vmax were determined by Graphpad Prism 5 software using non-linear regression for Michaelis-Menten equation.

Quantification of IAA

Seven-day old, Wc-grown Col-0 and sax3-2 seedlings were used. For quantification of free IAA, they were treated with or without simulated shade for 1 hour and the aerial part of seedlings was weighed and collected. For IAA biosynthesis rate measurements, seedlings were pretreated with 1/2 MS containing 30% ²H₂O for 30 min and then subjected to Wc or shade for 2 hr. Aerial part of seedlings was collected. The measurements were performed as described (Jung et al., 2005). For calculation of the relative synthesis rate of IAA, enrichment is expressed as the ratio of deuterium labeled IAA.
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ACCESSION NUMBERS
The GEO accession number for the microarray sequence data deposited and
reported in this paper is GSE9816.

SUPPLEMENTAL DATA
Supplemental Data include Supplemental Experimental Procedures, six fig-
ures, and Supplemental References and can be found with this article online
at http://www.cell.com/cgi/content/full/133/1/164/DC1/.

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Supplemental Data

Rapid Synthesis of Auxin
via a New Tryptophan-Dependent Pathway

Is Required for Shade Avoidance in Plants

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Supplemental Experimental Procedures

Detailed Growth Conditions

For the greenhouse experiment described in Figure 1, seedlings were grown for 4 weeks on a greenhouse bench without supplemental lighting (peak photosynthetically active radiation at midday was 1200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Seedlings were then aligned in front of banks of incandescent lamps covered with either opaque screens (-FR treatment) or FR filters (+FR treatment). Plants were irradiated with FR from 10:00 to 19:00 every day. The R:FR ratios, measured with a Skye SKR 100/SKR 110 radiometer pointed to the light sources (Skye Instruments), were 0.24 and 0.68 for +FR and -FR treatments, respectively (Izaguirre et al., 2006). The drop in R:FR caused by FR supplementation was equivalent to the effect of neighbor proximity in a canopy of leaf-area index = 0.5. For responses to picloram (Sigma), seedlings were grown on \( \frac{1}{2} \) MS supplemented with varying amounts of picloram for 3 days under Wc. The plates were then either left in Wc or transferred to simulated shade for 3 days before hypocotyl measurements were made. For microarray experiments, seedlings were grown under simulated white light condition (R: 13 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \); B: 1.23 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \); (R:FR ratio of 1.1)) for 7 days and were then
treated with simulated white light or simulated shade for 1 hour. Whole seedlings were collected. For 5-MT sensitivity tests, seedlings were grown for 9 days on ½ MS supplemented with 20 \( \mu \)M of 5-MT (Sigma) in Wc.

**Protein Sequence Alignment**

Protein sequence alignment was carried out using clustalW program ([http://www.ebi.ac.uk/Tools/clustalw2/index.html](http://www.ebi.ac.uk/Tools/clustalw2/index.html)) and visualized as box shade alignment ([http://www.ch.embnet.org/software/BOX_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

**Constructs**

For complementation experiments, genomic TAA1 DNA, including 2 Kb of upstream sequence and 800 bp of downstream sequence, was PCR-amplified from Col-0 genomic DNA and cloned into the pJHA212K vector using EcoRI and PstI sites (Yoo et al., 2005). For expression pattern analysis of TAA1, the uidA gene was first cloned into pJHA212K using Sall/BamHI; the 2 Kb promoter of TAA1 was PCR-amplified and cloned in pJHA212K-GUS using KpnI/SacI; finally the genomic DNA of TAA1 and the 800bp DNA downstream region of TAA1 was PCR-amplified and inserted using the Sall site. For TAA1 localization test, TAA1 cDNA was first amplified from a cDNA library from Col-0 and was then cloned into a modified pPZP212 vector with YFP (Chen et al., 2005). For complementation tests with the mutant form of TAA1, cDNA of TAA1 was cloned into a modified pPZP212 vector with 3X Flag tag (Wang et al., 2005). This clone was then used as a template for DpnI-directed mutagenesis to generate the indicated mutation using QuikChange® Site-Directed Mutagenesis Kit from Stratagene.

**Quantification of free IAA and IAA biosynthesis rate**

Col-0 and sav3-2 seedlings were grown under Wc for 7 days after germination. They were then treated with or without simulated shade for one hour and the aerial parts of seedlings were pooled, weighed and frozen in liquid nitrogen for quantification of free IAA content. Four
replicates were analyzed for samples without shade treatment and three replicates were analyzed for samples with shade treatment. The frozen samples (15 mg of plant tissue (fresh weight)) was homogenized in 0.5 ml 50 mM Na-phosphate buffer pH 7.0 containing 0.02% diethyldithiocarbamic acid (antioxidant) and 500 pg $^{13}$C$_6$-IAA internal standard, using the Retsch vibration mill (Retsch GmbH & Co. KG) and a 3 mm tungsten carbide bead at a frequency of 30 Hz for 2 min. The pH was adjusted to 2.7 and the sample was then purified by solid phase extraction on a 500 mg Isolute C$_8$-EC column (International Sorbent Technology) conditioned with 2 ml methanol and 2 ml 1% acetic acid. The column was washed with 2 ml 10% methanol in 1% acetic acid, eluted with 2 ml 70% methanol in 1% acetic acid and the sample was evaporated to dryness. The sample was dissolved in 0.2 ml 2-propanol and 1 ml dichloromethane, and IAA was methylated by adding 5 μl 2 M trimethylsilyl-diazomethane in hexane (Aldrich). The sample was then left at room temperature for 30 min. 5 μl of 2 M acetic acid in hexane was added to destroy excess diazomethane and the sample was evaporated to dryness. The sample was then trimethyl-silylated and analyzed by gas chromatography-selected reaction monitoring–mass spectrometry as described (Edlund et al., 1995).

For IAA biosynthesis measurements, Col-0 and sav3-2 seedlings were grown under Wc for 7 days after germination. Seedlings were pretreated with ½ MS containing 30% $^2$H$_2$O for 0.5 hours. They were then treated with Wc or shade for 2 hours and the aerial parts of 10 seedlings were collected and frozen in liquid nitrogen for each sample. Each treatment contains four replicates. Samples were homogenized, extracted and purified as described above. IAA synthesis rates were measured by GC-SRM-MS as described in Ljung et al.2005 (Ljung et al., 2005). For calculation of the relative synthesis rate of IAA, enrichment is expressed as the ratio of deuterium-labeled IAA ($m/z$ 203+204+205) to unlabelled IAA ($m/z$ 202), after correction for natural isotope distribution to $m/z$ 203, 204 and 205. Four replicates were analyzed for all samples.
Supplemental Figure Legends

**Figure S1.** Description of the Mutant Screen and Molecular Characterization of the sav3 Alleles

(A) Schematic diagram of the mutant screen.

(B) Hypocotyl length distribution of wild-type seedlings grown under Wc or simulated shade. Pictures of representative seedlings are also shown.

(C) DNA agarose gel picture of RT-PCR products of TAA1 (nucleotides 501 -1145 of the coding region, TAA1-C), full-length TAA1 (TAA1-F) and ubiquitin control (UBQ) (left panel). In the right panel, relative expression levels of TAA1 transcript were shown. Total RNAs were extracted from 5-day old seedlings grown under Wc. The expression levels of TAA1 were measured using quantitative RT-PCR (qRT-PCR) in triplicates and were normalized against a reference gene (AT2G39960). Error bars represent standard error of means (SEM).

**Figure S2.** Phenotypes of sav3 Mutants

(A) sav3 partially suppresses the phenotype of phyb9, a null mutant of phyB. Hypocotyl elongation in response to shade was quantitatively measured.

(B) Canopy height of sav3-1 grown in the greenhouse with or without supplementary FR light.

(C) Hypocotyl elongation in response to high temperature. Seedlings were grown under Wc at 22°C for 3 days. They were then left at 22°C or transferred to 29°C and allowed to grow for 4 more days. Mean values of at least 12 seedlings are shown and error bars represent SEM.

**Figure S3.** Expression Analysis of TAA1-dependent and Independent Early Response Genes

(A) Expression patterns of TAA1-independent early response genes. Expression values of PAR1, ATHB2, HFR1 and several other TAA1-independent shade-induced genes were obtained from our microarray data. For each gene, median expression value was set to one.
(B) Expression levels of TAA1-dependent early response genes, IAA19 and IAA29, under Wc or shade, treated with or without 1μM IAA. 5-day-old, Wc-grown seedlings were soaked in control (1/2 MS) or 1/2 MS plus 1μM IAA solution and were then treated with simulated Wc or shade for 1 hour. Expression levels were quantified using qRT-PCR in triplicates and normalized by the expression level of the reference gene. Error bars represent SEM.

**Figure S4. Expression Analysis of TAA1 and Rescue with iaaM**

(A) Quantification of TAA1 mRNA levels. 5-day old WT seedlings were treated with Wc or shade for 2 hours and relative expression levels of TAA1 were measured using qRT-PCR and were normalized to the reference gene.

(B) Hypocotyl phenotypes of transgenic lines expressing the bacterial iaaM gene under the control of the TAA1 promoter in sav3-2 background. 5-day old T2 seedlings were treated with Wc or shade for 4 days. Transgenic lines (sav3-2) containing TAA1 cDNA driven by TAA1 promoter and iaaM gene driven by the 35S promoter were included as controls. Mean values of at least 12 seedlings are shown.

Error bars represent SEM.

**Figure S5. Biochemical Characterization of TAA1**

(A) Optimal temperature and pH of TAA1. The borate buffer assay was employed to assay the production of IPA. 100 µl of reaction buffer containing 0.5M borate buffer (pH8.5 or as indicated), 10 µM of PLP, 1mM pyruvate and 1 µg of TAA1 was incubated at 55 °C or indicated temperature for 5 minutes. The production of IPA was monitored by absorption at 330nm.

(B) Aminotransferase activity of TAA1 towards various L- amino acids. 100 µl of reaction mixtures containing 50 mM K2HPO4/KH2PO4 (pH 8.5), 10mM α-ketoglutarate, various amino acids (5mM) and 3µg of TAA1 were incubated at 37°C for 20 min. The reaction was stopped by heat inactivation (95°C for 5 min). Production of L-glutamate was measured using a kit from
Boehringer Mannheim/ R-Biophram, which is based on an L-glutamic acid colorimetric method. The amount of L-glutamate was expressed as absorption at 492nm. The line marks the production of L-glutamate by TAA1 using L-Trp as a substrate. Using this line as a cutoff, TAA1 can use at least 6 out of the 19 amino acids (Phe, Tyr, Trp, Leu, Ala and Met) (Glu is not assayed due to the limit of the assay). Mean values of 3 measurements are shown. Error bars represent SEM.

(C) Km and Vmax of TAA1 to L-Phe and L-Tyr. Initial velocities of TAA1 in buffers with various concentrations of Phe or Tyr (0.125-4 mM) were measured using the borate buffer assay as described above except that 20 mM sodium pyruvate was utilized. Production of β-phenylpyruvate or p-hydroxyphenylpyruvate was measured by absorption at 310 nm. Km and Vmax were determined as described in the Materials and Methods.

**Figure S6.** Analysis of *Yucca* Pathway

(A) A subset of *Yucca* genes are induced by shade. *Yucca* genes are the rate-limiting enzymes for the tryptamine pathway of IAA biosynthesis (Zhao et al., 2001). Relative expression levels of *Yucca* gene family members from the microarray experiment are shown.

(B) Hypocotyl phenotypes of *yucca* mutants in shade. 5-day old seedlings were treated with Wc or shade (R:FR = 0.7) for 4 days before measurement. Mean values of at least 12 seedlings are presented. Error bars represent SEM.

**Supplemental References**


**Procedure of Mutant Screen**

Sterilize EMS mutagenized seeds in ethanol and plate seeds on 1/2 MS medium

Keep at 4 °C for 4 days

Grow seedlings under continuous white light (Wc) for 5 days

Move seedlings to simulated shade (Mixture of red, blue and far red light) and grow for 4 days

Select seedlings with short hypocots

**B**

Hypocotyl Length Distribution of Col-0 seedlings

![Graph showing hypocotyl length distribution](Image)

**C**

Relative Expression Level of TAA1

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**Figure S1**
Figure S2
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