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Auxin Synthesized by the YUCCA Flavin Monooxygenases Is Essential for Embryogenesis and Leaf Formation in *Arabidopsis* [W]

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Auxin plays a key role in embryogenesis and seedling development, but the auxin sources for the two processes are not defined. Here, we demonstrate that auxin synthesized by the YUCCA (YUC) flavin monooxygenases is essential for the establishment of the basal body region during embryogenesis and the formation of embryonic and postembryonic organs. Both *YUC1* and *YUC4* are expressed in discrete groups of cells throughout embryogenesis, and their expression patterns overlap with those of *YUC10* and *YUC11* during embryogenesis. The quadruple mutants of *yuc1 yuc4 yuc10 yuc11* fail to develop a hypocotyl and a root meristem, a phenotype similar to those of *mp* and *tir1 afb1 afb2 afb3* auxin signaling mutants. We further show that *YUC* genes play an essential role in the formation of rosette leaves by analyzing combinations of *yuc* mutants and the polar auxin transport mutants *pin1* and *aux1*. Disruption of *YUC1*, *YUC4*, or *PIN1* alone does not abolish leaf formation, but the triple mutant *yuc1 yuc4 pin1* fails to form leaves and flowers. Furthermore, disruption of auxin influx carrier *AUX1* in the quadruple mutant *yuc1 yuc2 yuc4 yuc6*, but not in wild-type background, phenocopies *yuc1 yuc4 pin1*, demonstrating that auxin influx is required for plant leaf and flower development. Our data demonstrate that auxin synthesized by the YUC flavin monooxygenases is an essential auxin source for *Arabidopsis thaliana* embryogenesis and postembryonic organ formation.

INTRODUCTION

Auxin has been shown to play an essential role in *Arabidopsis thaliana* embryogenesis. Genetic screens for mutants with embryo pattern defects have led to the identification of *monopteros* (*mp*) (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998) and *bodenlos* (*bdl*) (Hamann et al., 1999, 2002), two mutants that fail to develop a hypocotyl and embryonic roots. *MP* encodes the auxin response factor 5 (ARF5), which belongs to the ARF family, with 23 members in the *Arabidopsis* genome (Guilfoyle et al., 1998; Okushima et al., 2005). BDL (IAA12) is one of the 29 AUX/IAA transcription factors in *Arabidopsis* (Kim et al., 1997; Reed, 2001; Liscum and Reed, 2002). It was shown that BDL physically interacts with MP, thus preventing MP from forming homodimers or heterodimers with other ARFs (Weijers et al., 2005a, 2005b). In the presence of auxin, BDL is brought to the SCF^{TIR1}-containing protein degradation machinery mediated by auxin binding directly to the auxin receptor TIR1 (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007). Auxin-induced degradation of BDL allows MP to form ARF-ARF dimers that can then activate transcription necessary for plant development. The studies on *mp* and *bdl* revealed that auxin is necessary for the establishment of the apical-basal axis and the formation of embryonic organs. Recent studies on auxin signaling and polar auxin transport further demonstrate that auxin is required for *Arabidopsis* embryogenesis. The auxin receptor quadruple mutant *tir1 afb1 afb2 afb3* also displays phenotypes similar to *mp* or *bdl* (Dharmasiri et al., 2005b). Disruption of auxin efflux during embryogenesis by simultaneously inactivating multiple PIN genes affects the formation of the apical-basal axis during embryogenesis (Friml et al., 2003). Although much is known regarding the roles of auxin signaling and transport in embryogenesis, genes regulating auxin biosynthesis during embryogenesis are not known. De novo auxin biosynthesis is likely to play a critical role in embryogenesis because previous physiological studies have shown that auxin biosynthesis is dynamic during embryogenesis (Fischer-Iglesias et al., 2001; Ribnicky et al., 2002).

Auxin is also known to play a role in leaf development. It has been clearly demonstrated that auxin plays a critical role in phyllotaxis of leaf formation (Reinhardt et al., 2003). Most of the information on the control of leaf formation by auxin comes from analysis of auxin transport mutants and the effects of local application of auxin (Benkova et al., 2003; Reinhardt et al., 2003). However, the sources of auxin and the locations of auxin production during leaf development are not defined.

We previously showed that the YUCCA (YUC) family of flavin monooxygenases are key enzymes in Trp-dependent auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006). Overexpression of *YUC* genes leads to long hypocotyls and epinastic cotyledons (Zhao et al., 2001), characteristic auxin overproduction phenotypes observed in iaaM overexpression lines (Romano et al., 1995), *superroot1* (Boerjan et al., 1995), and other auxin overproduction mutants (Delarue et al., 1998). There are 11 *YUC* genes in the *Arabidopsis* genome (Zhao et al., 2001; Cheng et al., 2006). Inactivation of *YUC1* and *YUC4* leads to dramatic defects

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in floral and vascular development (Cheng et al., 2006). Phenotypes of *yuc1 yuc4* double mutants are rescued by expressing the bacterial auxin biosynthetic gene iaaM under the control of the *YUC1* promoter (Cheng et al., 2006). Deactivation of *YUC2* and *YUC6* in *yuc1 yuc4* further exacerbates the floral and vascular defects of this double mutant, demonstrating that the *YUC* genes have overlapping functions and are essential for auxin biosynthesis during flower and vascular development (Cheng et al., 2006). Here, we show that *YUC1* and *YUC4* show distinct and overlapping expression patterns during embryogenesis and seedling development, but the *yuc1 yuc4* double mutants do not exhibit any obvious defects in embryogenesis and the formation of leaves. We show that inactivation of *YUC10* and *YUC11* in the *yuc1 yuc4* background leads to phenotypes similar to those of strong *mp* mutants, indicating that auxin synthesized by the YUC flavin monooxygenases is a required auxin source for embryogenesis. Furthermore, we demonstrate that auxin synthesized by the YUC flavin monooxygenases is also a critical auxin source for leaf development. Together with our previous findings that *YUC* genes are essential for flower and vascular development, our data show that the *YUC* genes are necessary for many developmental processes from embryogenesis to seedling development to flower development.

RESULTS

YUC Genes Display Distinct Expression Patterns during Embryogenesis

To test whether the *YUC* genes play a role in synthesizing auxin during embryogenesis, we first analyzed the expression patterns of *YUC1* during embryogenesis by RNA in situ hybridization. *YUC1* expression became obvious in globular stages of embryo but with expression concentrated in the apical region (Figure 1A). Later, the expression of *YUC1* was mainly restricted to the cotyledons and the apical meristem (Figures 1B and 1C). In mature embryos, *YUC1* expression was mainly detected at the apex (Figure 1D). Interestingly, the expression domain of *YUC1* was relatively broad during early stages of embryogenesis and became more restricted to discrete groups of cells in mature embryos. The in situ data with the *YUC1* sense probe are shown in Supplemental Figure 1 online.

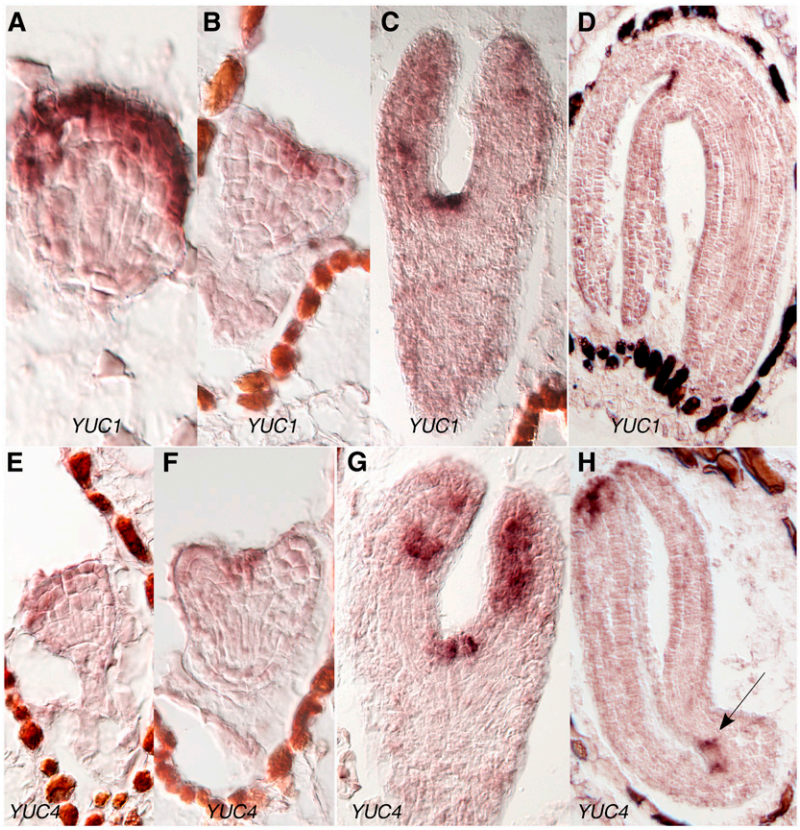

Figure 1. Analysis of the Expression Patterns of *YUC1* and *YUC4* during Embryogenesis by RNA in Situ Hybridization.

(A) to **(D)** *YUC1* expression patterns. Note that *YUC1* is mainly expressed in the first three cell layers in the apical region in the globular-stage embryos **(A)**. At heart stage, the highest expression is at the meristem region **(B)**.
(E) to **(H)** *YUC4* expression patterns. The arrow points to the likely sites for incipient true leaves.

YUC4 was also expressed at various stages of embryogenesis (Figures 1E to 1H). The *YUC4* expression patterns were very similar to those of *YUC1* throughout embryogenesis, suggesting that *YUC1* and *YUC4* have overlapping functions during embryogenesis just like their redundant roles in flower and vascular development. Unlike *YUC1*, *YUC4* was also expressed at the apical region of the cotyledons in the mature embryo (Figure 1H). The in situ data with *YUC4* sense probe are shown in Supplemental Figure 1 online.

The fact that *yuc1 yuc4* double mutants did not display any obvious defects in embryogenesis suggests that other *YUC* genes may have overlapping functions with *YUC1* and *YUC4* during embryogenesis. Analysis of the public microarray data (Zimmermann et al., 2004) indicates that *YUC10*, which is closely related to *YUC1* and *YUC4* in a phylogenetic tree (Cheng et al., 2006), is highly expressed in siliques (Figure 2A). We further analyzed the expression patterns of *YUC10* and its closest homolog *YUC11* during embryogenesis by RNA in situ hybridization and found that their expression overlapped with those of *YUC1* and *YUC4* (Figures 2B to 2E). The data of sense probes are shown in Supplemental Figure 1 online.

Multiple *YUC* Genes Contribute to the Formation of the Apical-Basal Axis and Embryonic Organ Formation

Overexpression of either *YUC10* or *YUC11* in *Arabidopsis* led to auxin overproduction phenotypes similar to those of *YUC1* overexpression lines (data not shown), suggesting that *YUC10* and *YUC11* probably also play a role in auxin biosynthesis and have overlapping functions with *YUC1* and *YUC4* during embryogenesis. The *yuc1 yuc4 yuc10 yuc11* quadruple mutants had remarkable developmental defects. Seedlings of the quadruple mutants did not have hypocotyls or roots (Figure 3A). Most of the *yuc1 yuc4 yuc10 yuc11* seedlings only had one cotyledon with little vascular tissue (Figures 3B to 3E). The defects of *yuc1 yuc4 yuc10 yuc11* occurred as early as the globular stages of the embryo (Figures 3F and 3G). The quadruple mutants lacked a hypophysis (Figures 3F and 3G, arrows), cells that later develop into a root meristem in wild-type plants. The central cells did not elongate and the embryos failed to develop hypocotyls (Figures 3F and 3G). A prominent feature of the *yuc1 yuc4 yuc10 yuc11* embryo was that the basal body region was defective and no primary roots were developed (Figure 3A).

We analyzed seedlings from a single plant that was *yuc1 yuc10 yuc11* homozygous and *yuc4* heterozygous to determine whether the *mp*-like phenotypes are completely penetrant. Among the 369 seedlings analyzed, 73 plants displayed *mp*-like phenotypes and had the *yuc1 yuc4 yuc10 yuc11* genotype. Because we only observed 20% of the progenies that had *mp*-like phenotypes, we hypothesized that the *mp*-like phenotypes of *yuc1 yuc4 yuc10 yuc11* may not be 100% penetrant or some of the quadruple mutants died during embryogenesis. We then genotyped 96 plants that were from a single *yuc1*−/− *yuc4*+/− *yuc10*−/− *yuc11*−/− plant and did not display obvious *mp*-like phenotypes. One of the plants among the 96 plants was identified as *yuc1 yuc4 yuc10 yuc11*, indicating that occasionally the quadruple mutants could escape from the *mp*-like phenotypes.

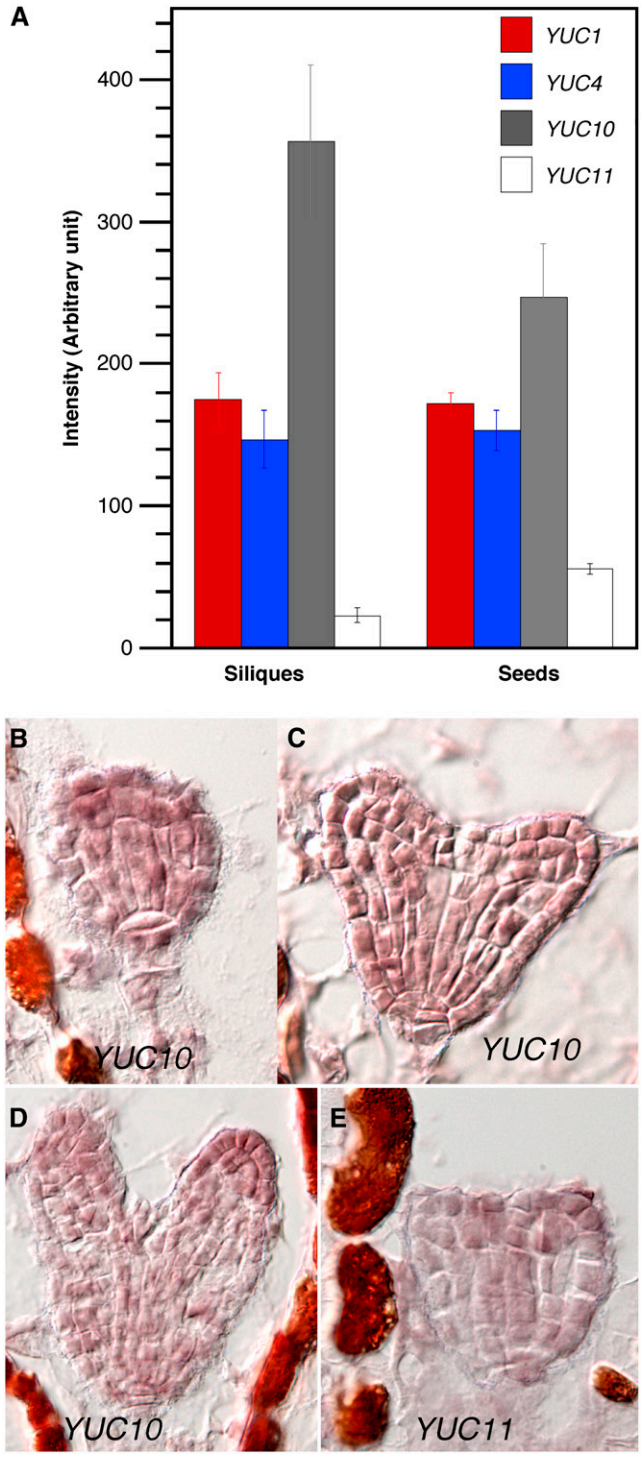

Figure 2. Analysis of the Expression Profile of *YUC10* and *YUC11*.

(A) Expression profiles in siliques and seeds. Data were from the public microarray data sets (https://www.genevestigator.ethz.ch).
(B) to **(D)** RNA in situ hybridization of *YUC10*.
(E) RNA in situ hybridization of *YUC11*.

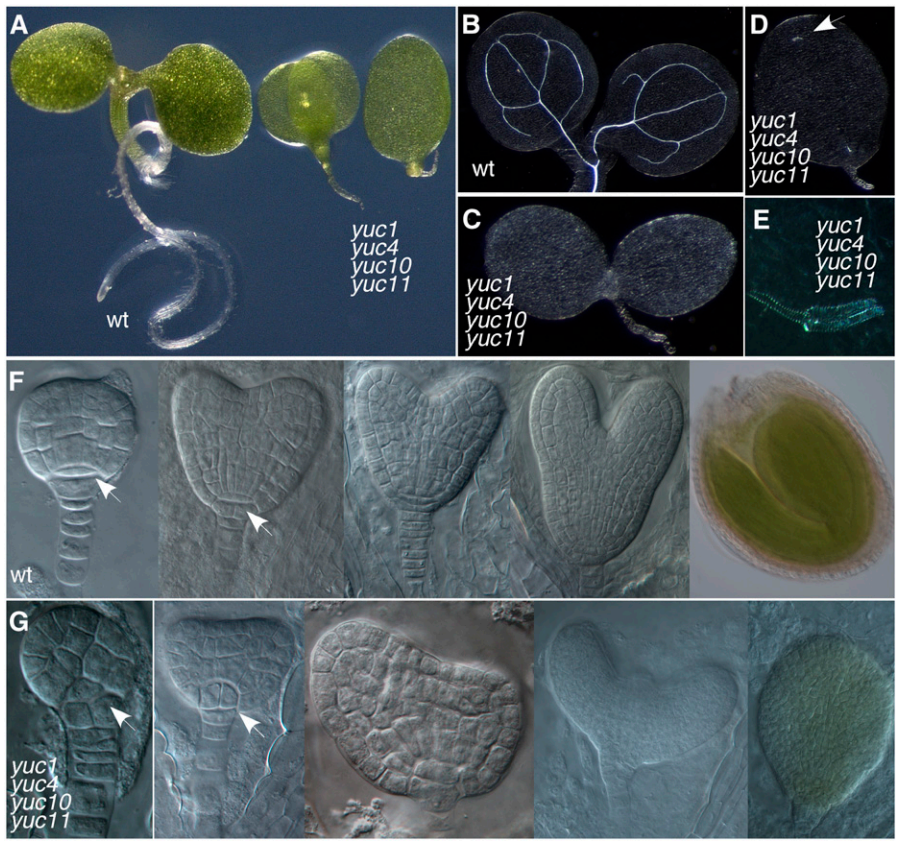

Figure 3. Regulation of *Arabidopsis* Embryogenesis by *YUC* Genes.

(A) Developmental defects of *yuc1 yuc4 yuc10 yuc11* at seedling stages. The seedling at the left is the wild type, and the two seedlings at the right are *yuc1 yuc4 yuc10 yuc11*. Note that the mutant lacks a hypocotyl and a root.
(B) to **(E)** Vascular defects in *yuc1 yuc4 yuc10 yuc11*.
(B) The wild type.
(C) to **(E)** *yuc1 yuc4 yuc10 yuc11*. The arrow points to a short and discontinuous vein. The vascular tissue in **(E)** was from the seedling of **(D)**.
(F) Various stages of wild-type embryos.
(G) The corresponding stages of embryos of *yuc1 yuc4 yuc10 yuc11*. The arrows point to where the hypophysis should be.

YUC Genes Display Distinct Expression Patterns during Seedling Growth

To investigate whether the *YUC* genes play a role in providing auxin for seedling growth and leaf development, we analyzed the patterns of β-glucuronidase (GUS) staining of *YUC1* promoter GUS lines and *YUC4* promoter GUS lines. For *YUC1-GUS* lines, the GUS staining was restricted to the shoot apex and no staining was observed in the cotyledons (Figure 4A, top panel). In the shoot apex, cells flanking the apical meristem showed GUS staining, but the strongest GUS staining was located at the base of young leaves and stipules (Figures 4A and 4B). There was no staining in the apical meristem (Figure 4A, bottom panel). Further analysis of sections of the *YUC1-GUS* seedlings indicate that *YUC1* is not expressed in the apical meristem (data not shown).

GUS staining of *YUC4-GUS* was also mainly restricted to the shoot apex (Figures 4C and 4D, top panels). Like *YUC1-GUS*, GUS staining was observed in the cells surrounding the apical meristem in *YUC4-GUS* lines (Figure 4C, bottom panel). Unlike *YUC1-GUS*, weak staining was observed at the tips of cotyledons (Figures 4C and 4D, top panels). In true leaves, GUS staining was observed initially throughout the young leaf primordia, and later the staining was concentrated at the basal and apical regions of leaves. Strong GUS staining was also observed in stipules (Figures 4C and 4D).

The Expression of the Auxin Reporter DR5-GUS Overlaps with *YUC* Expression Domains during Seedling Development

In wild-type *Arabidopsis* seedlings, *DR5-GUS* mainly expressed at newly developed leaves, the tip of cotyledons, and root tips (Figure 4E). At the apex, *DR5-GUS* was mainly expressed in young leaves, stipules, and later in vascular tissues (Figure 4E, bottom panel). The *DR5-GUS* patterns in wild-type plants overlapped significantly with the expression patterns of *YUC1* and *YUC4* in the shoot apex (Figure 4). Disruption of *YUC1* and *YUC4*

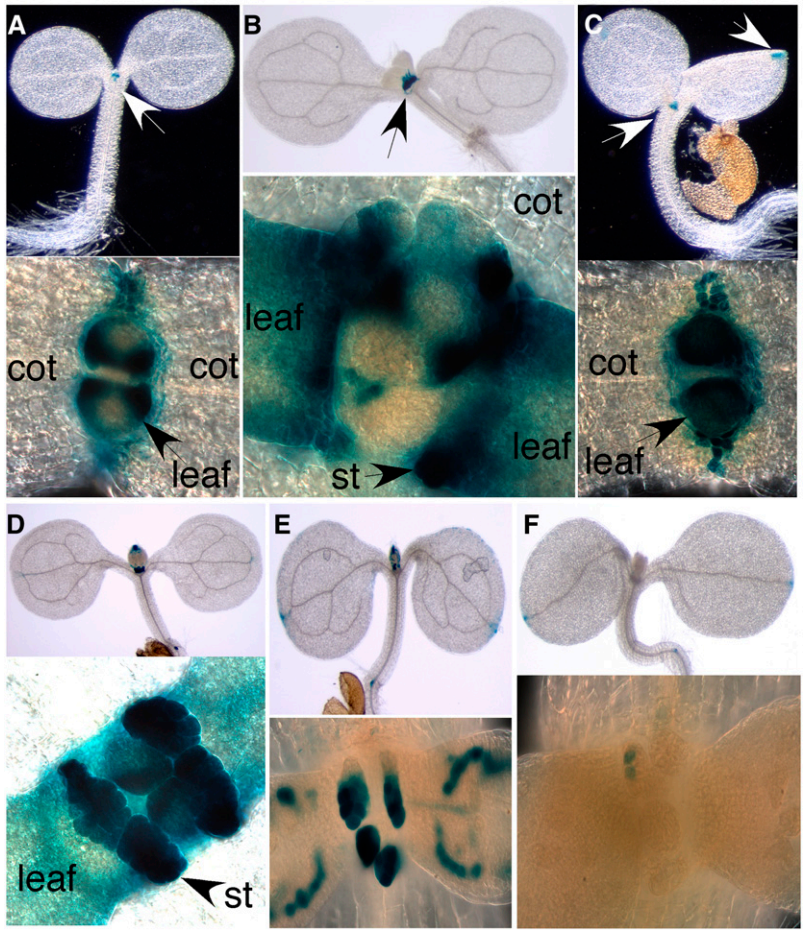

Figure 4. Expression Patterns of *YUC1* and *YUC4* during Seedling Development and Effects of *yuc1 yuc4* on Auxin Reporter DR5-GUS Expression.

(A) GUS staining of a 3-d-old light-grown *YUC1-GUS* seedling. Staining was restricted to the shoot apex without any staining in cotyledons (cot) (top panel). In the apex, strong GUS staining was located at the basal parts of the true leaves (bottom panel).
(B) GUS staining of a 5-d-old *YUC1-GUS* seedling. Staining was observed in stipules (st).
(C) GUS staining of a 3-d-old *YUC4-GUS* seedling. Staining was located at cotyledon tips and the shoot apex (top panel).
(D) GUS staining of a 5-d-old *YUC4-GUS* seedling. Note the strong staining in the stipules (bottom panel).
(E) Expression of the auxin reporter DR5-GUS. GUS activities were observed at the cotyledon tip and the shoot apex. At the shoot apex, DR5-GUS is expressed in stipules and vascular tissue (bottom panel).
(F) GUS staining of DR5-GUS in the *yuc1 yuc4* background. Inactivation of *YUC1* and *YUC4* leads to a dramatic decrease of DR5-GUS expression in the apex without affecting the staining at the cotyledon tip.

greatly decreased *DR5-GUS* level in the apex without affecting *DR5-GUS* at cotyledon tips (Figure 4F) and in roots (data not shown).

PIN1 and *YUC* Genes Synergistically Control Leaf Development

The *YUC* expression pattern and DR5-GUS analysis suggest that *YUC* genes may play a role in leaf development. However, the *yuc1 yuc4* double mutants did not show obvious defects in the formation of leaves in terms of the number and position of the leaves, although *yuc1 yuc4* leaves had vascular defects. We hypothesized that other *YUC* genes may compensate for *yuc1 yuc4* during leaf initiation and elongation. The *yuc1 yuc2 yuc4 yuc6* quadruple mutants have severe defects in vascular and flower defects, but the number of leaves formed in the quadruple mutants appeared to be similar to the wild type. So far, all of the *yuc* mutant combinations we have constructed either do not have defects in the formation of leaves or have embryonic defects, making it difficult to assess the roles of *YUC* genes in normal leaf development.

We hypothesized that the roles of *YUC* genes in leaf development may also be masked by polar auxin transport. To test this hypothesis, we constructed *yuc1 yuc4 pin1* triple mutants. Single *pin1* mutations or double *yuc1 yuc4* mutations only partially disrupt auxin efflux and biosynthesis, respectively, because there

are other *PIN*s and *YUC*s in the *Arabidopsis* genome with overlapping functions (Galweiler et al., 1998; Cheng et al., 2006). Both *yuc1 yuc4* and *pin1* had defects in forming normal flowers and vascular tissues (Galweiler et al., 1998; Cheng et al., 2006), but they had leaves and other lateral organs (Figure 5A). The *yuc1 yuc4 pin1* triple mutants failed to form true leaves (Figure 5A), a phenotype not observed in either *pin1* or *yuc1 yuc4* alone, demonstrating the synergistic effects between *yuc1 yuc4* and *pin1*. Electron micrographs showed that the shoot apex of *yuc1 yuc4 pin1* was not flat; instead, we observed a dozen or so stipule-like structures with three or four cells (Figures 5B and 5C). The triple mutant *yuc1 yuc4 pin1* eventually formed *pin*-like inflorescences without producing any flowers or cauline leaves (Figure 5D).

Analysis of the interactions between *yuc1 yuc4* double mutants and the weak mutant *pin1-5* (Bennett et al., 1995; Sohlberg et al., 2006) further supports that *PIN1* and *YUC1/YUC4* have synergistic interactions. The *pin1-5* allele never forms pin-like inflorescences and is fertile (Figure 5E). When *pin1-5* was combined with *yuc1*, the *yuc1 pin1-5* double mutants produced pin-like inflorescences (Figure 5E). The *yuc4 pin1-5* double mutants displayed stronger phenotypes than *yuc1 pin1-5* double mutants (Figure 5E). The *yuc1 yuc4 pin1-5* triple mutants produced pin-like inflorescences with a stature smaller than either *yuc1 pin1-5* or *yuc4 pin1-5* (Figure 5E). Our data indicate that auxin synthesized by the YUC flavin monooxygenases is a critical auxin source required for leaf and flower development.

yuc1 yuc4 Double Mutants Treated with the Auxin Transport Inhibitor Naphthylphthalamic Acid Phenocopy *yuc1 yuc4 pin1* Triple Mutants

Treatment of wild-type *Arabidopsis* plants with the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) leads to the formation of pin-like inflorescences (Okada et al., 1991) but does not inhibit leaf formation (Figure 6A). When *yuc1 yuc4* was grown on

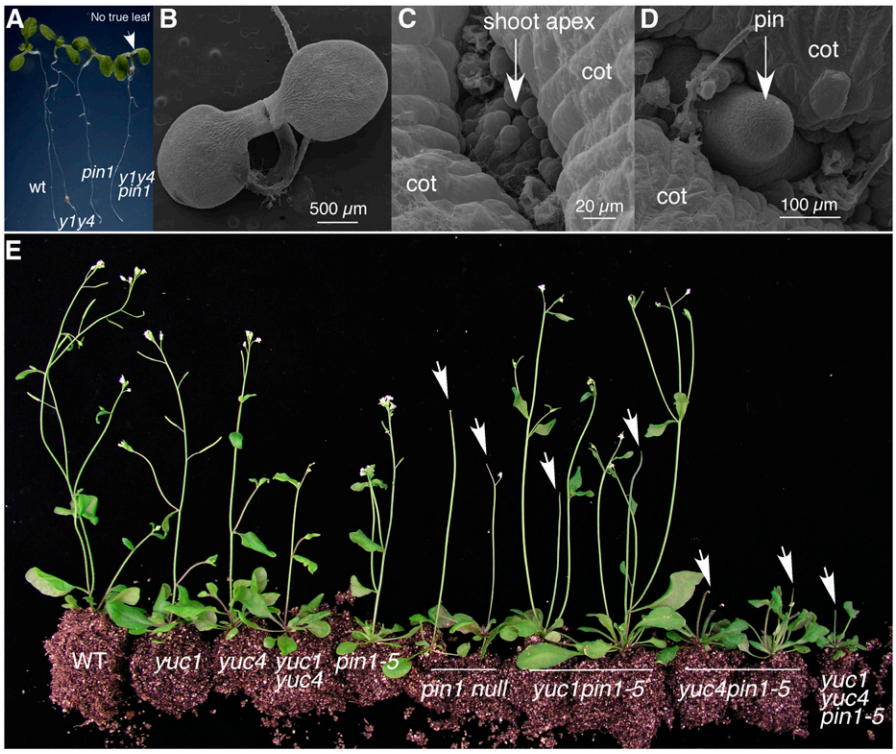

Figure 5. Synergistic Interactions between *yuc1 yuc4* and *pin1* Mutants during Leaf Development.

(A) The triple mutant *yuc1 yuc4 pin1* did not make a leaf. Seedlings shown are 7 d old and grown on 0.5× Murashige and Skoog media. Arrow points to the apex of *yuc1 yuc4 pin1*. The *pin1* mutant was a null mutant caused by a T-DNA insertion. From left to right: wild type, *yuc1 yuc4*, *pin1*, and *yuc1 yuc4 pin1*.

(B) to **(D)** Electron micrographs of *yuc1 yuc4 pin1*.

(B) Seven-day-old *yuc1 yuc4 pin1*. Note that no true leaves were visible at the shoot apex.

(C) A close-up of the shoot apex of a *yuc1 yuc4 pin1* seedling.

(D) Pin-like structure in 2-week-old *yuc1 yuc4 pin1*.

(E) Genetic interactions between *yuc1 yuc4* and *pin1-5*, a weak allele of *pin1*. From left to right: wild type, *yuc1*, *yuc4*, *yuc1 yuc4* double mutants, *pin1-5*, two plants of *pin1* null, two plants of *yuc1 pin1-5* double mutants, two plants of *yuc4 pin1-5* double mutants, and *yuc1 yuc4 pin1-5* triple mutants. Note that *pin1-5* never forms pin-like inflorescences, but *yuc1 pin1-5* or *yuc4 pin1-5* generated pin-like inflorescences. The *yuc1 yuc4 pin1-5* triple mutants had stronger phenotypes than *pin1* null. Arrows point to pin-like inflorescences.

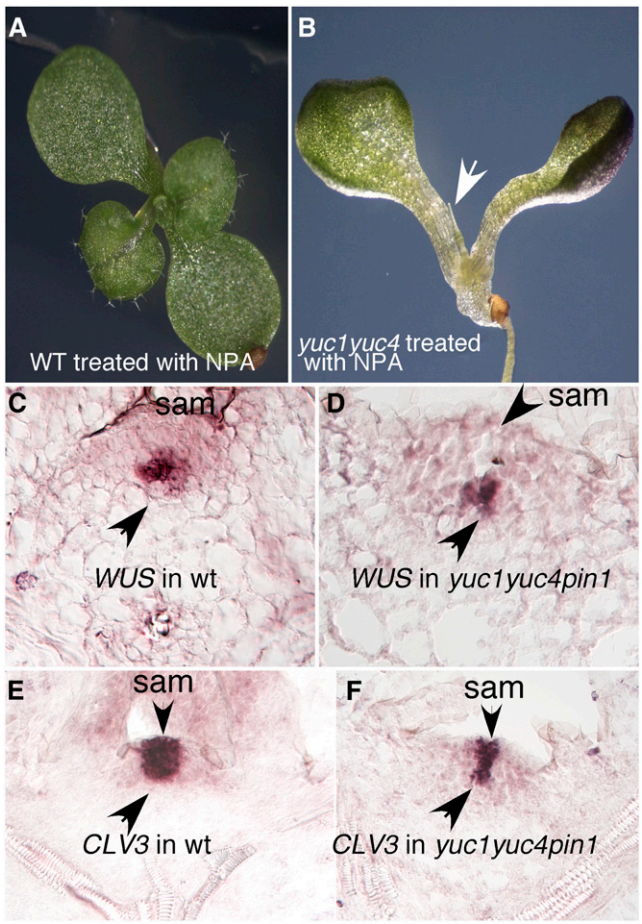

Figure 6. Analysis of Expression Patterns of Apical Meristem Markers in *yuc1 yuc4 pin1* and the Effects of NPA on *yuc1 yuc4* Development.

(A) Wild-type plants grown on 25 μM NPA for 12 d.
(B) *yuc1 yuc4* seedlings grown on NPA for 12 d. Arrow points to a pin-like structure.
(C) Expression patterns of *WUS* in wild-type shoot apical meristem (sam). *WUS* was expressed in a small group of cells several layers away from the epidermis.
(D) *WUS* expression in *yuc1 yuc4 pin1*. Note the similar patterns in the wild type and the mutant.
(E) and **(F)** *CLV3* expression in the shoot apical meristem in the wild type **(E)** and *yuc1 yuc4 pin1* **(F)**. The expression patterns were analyzed by RNA in situ hybridization using 8-d-old light-grown seedlings.

NPA-containing media, no true leaves were formed (Figure 6B). The observation that *yuc1 yuc4 pin1* phenotypes can be phenocopied by treating *yuc1 yuc4* with NPA further supports the conclusion that auxin synthesized by the YUCs contributes to leaf development (Figures 6A and 6B). NPA treatment also led to a further decrease of DR5-GUS expression in the apex of *yuc1 yuc4* (see Supplemental Figure 2 online).

The Apical Meristem in *yuc1 yuc4 pin1* Triple Mutants Is Not Collapsed

We analyzed whether the failure to form true leaves in *yuc1 yuc4 pin1* was caused by a collapse of the apical meristem. We chose to analyze the meristem markers *CLV3* and *WUS* by RNA in situ hybridization (Fletcher et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001). In the wild type, *WUS* mRNA was detected in a small group of cells in the center of the shoot apical meristem (Figure 6C), while *CLV3* expression was restricted to a small zone of cells at the meristem apex, including cells in the L1 and L2 layers (Figure 6E). In *yuc1 yuc4 pin1* triple mutants, both *CLV3* and *WUS* displayed expression patterns similar to those of the wild type, suggesting that *yuc1 yuc4 pin1* still had an apical meristem (Figures 6D and 6F). Therefore, the *yuc1 yuc4 pin1* triple mutants mainly failed to differentiate the cells flanking the meristem into leaves and flowers.

Inactivating *AUX1* in *yuc1 yuc2 yuc4 yuc6* Mutants Phenocopies *yuc1 yuc4 pin1* Triple Mutants

Polar auxin transport has been explained by the Chemiosmotic Hypothesis of auxin transport in which directional auxin transport is mediated by specific auxin influx and efflux carriers (Rubery and Sheldrake, 1974; Raven, 1975). Both passive diffusion and carrier-mediated influx are proposed for auxin influx, whereas auxin efflux totally depends on efflux carriers. The AUX1 family proteins have been shown to be influx carriers (Bennett et al., 1996; Swarup et al., 2001; Yang et al., 2006). Previous studies have shown that AUX1 plays an important role in root development (Swarup et al., 2001), but the contributions of AUX1 to leaf and flower development are not as defined. We hypothesized that AUX1 and the YUCs may mask each other's functions during leaf development.

To test this hypothesis, we introduced *aux1* mutation into various *yuc* mutant combinations. The quintuple mutants of *yuc1 yuc2 yuc4 yuc6 aux1* had fewer leaves than either *yuc1 yuc2 yuc4 yuc6* quadruple mutants or *aux1* single mutants (Figures 7A to 7D). The overall developmental defects of the quintuple mutants *yuc1 yuc2 yuc4 yuc6 aux1* were very similar to those of *yuc1 yuc4 pin1* (Figure 5). The fact that *aux1* and *pin1* displayed similar phenotypes in the *yuc* mutant background clearly demonstrates that both auxin influx and efflux are very important to leaf development. Our data also indicate that auxin synthesized by the YUCs is a necessary auxin source for leaf development.

DISCUSSION

YUC Genes Are Essential for Embryogenesis

We have shown that simultaneous disruption of four *YUC* genes (*YUC1*, *YUC4*, *YUC10*, and *YUC11*) led to seedlings without a hypocotyl and a root meristem. The phenotypes of *yuc1 yuc4 yuc10 yuc11* quadruple mutants resembled the strong alleles of *mp* (Berleth and Jurgens, 1993), *bdl* (Hamann et al., 2002), and *tir1 afb1 afb2 afb3* auxin receptor mutants (Dharmasiri et al., 2005b), which are defective in auxin signaling. Similarities of embryo defects between *yuc1 yuc4 yuc10 yuc11* and *pin1 pin3 pin4 pin7* mutants (Friml et al., 2003) are also remarkable, indicating that auxin synthesized by YUC flavin monooxygenases is a critical auxin source for embryogenesis.

YUC1 and *YUC4* were mainly expressed in the apical region of globular or heart stages of embryos with little expression in the

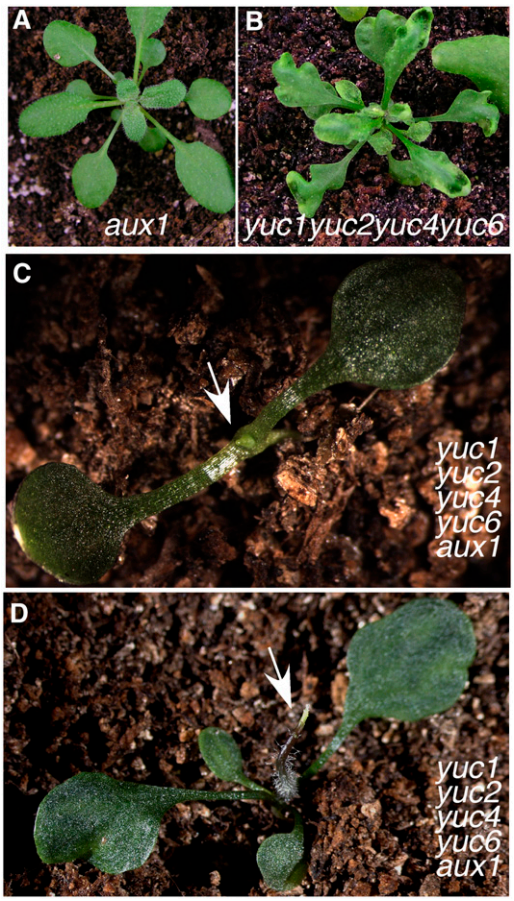

Figure 7. A Role for AUX1 in Leaf and Flower Development.

(A) Adult *aux1*.
(B) Adult *yuc1 yuc2 yuc4 yuc6*.
(C) and **(D)** *yuc1 yuc2 yuc4 yuc6 aux1*.
Note that both *aux1* and *yuc1 yuc2 yuc4 yuc6* developed leaves, whereas the *yuc1 yuc4 yuc6 aux1* quintuple mutants developed fewer leaves and showed phenotypes similar to *yuc1 yuc4 pin1*.

hypophysis, yet the main defects in the *yuc1 yuc4 yuc10 yuc11* quadruple mutants were in hypocotyl and root meristem. We envision two mechanisms for how auxin synthesized in the apical region can regulate the development of hypophysis and root meristem. Our results suggest that auxin synthesized by the YUCs may be transported to the hypophysis to regulate the root specification. This hypothesis is consistent with the observations that there is a DR5-GFP maximum at the hypophysis and there is a PIN1 polarity switch during embryogenesis (Friml et al., 2003). However, it is also known that MP and BDL are not expressed in the hypophysis (Weijers et al., 2006), yet both *mp* and *bdl* mutants fail to form a root meristem (Hardtke and Berleth, 1998; Hamann et al., 2002). An alternative mechanism for controlling root meristem development by *YUC* genes during embryogenesis is to degrade BDL and to activate MP by YUC-mediated auxin synthesis in the apical region. Once BDL is degraded and MP is activated to initiate transcription, an unidentified MP-dependant signal is sent to the hypophysis to specify its development. We should point out that these two mechanisms are not mutually exclusive. Nevertheless, the elucidation of the roles of *YUC* genes in embryogenesis and the *YUC* expression patterns provides a foundation for further analyzing the mechanisms by which auxin controls embryogenesis.

Genetic Interactions between *YUC*s and Polar Auxin Transport

Previous work shows that both *pin1* mutants and *yuc1 yuc4* double mutants produce fewer flowers than the wild type and that the flowers in *pin1* or *yuc1 yuc4* are defective and completely sterile (Galweiler et al., 1998; Tobena-Santamaria et al., 2002; Cheng et al., 2006), but it is not clear whether the *pin* mutants and *yuc* mutants have epistatic interactions. The fact that *yuc1 yuc4 pin1* triple mutants displayed phenotypes (Figure 5) that are not seen in either *pin1* or *yuc1 yuc4* alone demonstrates that polar auxin transport and YUC-mediated auxin biosynthesis have strong genetic interactions. This conclusion is further supported by the findings that *aux1* and *yuc* mutants also synergistically enhance each other (Figure 7). *Arabidopsis* organogenesis probably requires that the founder cells for an organ primordium must have a threshold of auxin. The auxin threshold hypothesis is also consistent with our previous observations that the double, triple, and quadruple *yuc* mutants displayed increasingly severe defects in vascular development and overall plant architecture (Cheng et al., 2006).

Because both auxin transport and auxin biosynthesis are redundantly regulated and the *pin1* and *yuc1 yuc4* mutants only partially disrupt auxin transport and biosynthesis, respectively, it is more difficult to interpret the synergistic interactions between *yuc* and transport mutants. The simplest interpretation for the synergistic interactions is that in the *yuc1 yuc4* double mutant background, auxin levels are low and there is not sufficient auxin to be transported to the founder cells of leaf primordia. Another explanation is that auxin in the founder cells of leaf primordia comes from auxin transported from surrounding cells and from de novo synthesis by the YUCs in the founder cells as well. The latter explanation is consistent with the observations that YUC genes are expressed at the incipient sites of new leaves and emerging leaf primordia. The two interpretations are not mutually exclusive because it is likely both auxin levels and the locations of auxin production are important for the establishment of an auxin gradient for leaf formation. Regardless of which mechanisms are involved, our data have clearly shown that auxin produced by the *YUC* genes is an essential auxin source for leaf and flower development.

Auxin Influx by AUX1 Plays a Key Role in Leaf and Flower Development

The role of AUX1 in root development and root gravitropic responses is well established (Bennett et al., 1996; Swarup et al., 2001). However, the role of auxin influx mediated by AUX1 in leaf and flower development received much less attention compared with PIN-mediated auxin efflux, partly because disruption of *AUX1* causes no obvious developmental defects in the shoot, although AUX1 is highly expressed in the shoot

(https://www.genevestigator.ethz.ch). It is argued that auxin influx is probably not regulated because most of the IAA molecules outside of the cell are protonated and potentially can cross the plasma membrane by diffusion. The fact that *aux1* developed pin-like inflorescences in *yuc1 yuc2 yuc4 yuc6* quadruple mutants, but not in wild-type background, demonstrates that the role of AUX1 in plant development is masked by YUC-mediated auxin biosynthesis. Our data have clearly shown that AUX1-mediated auxin influx becomes limiting in the *yuc1 yuc2 yuc4 yuc6* background in terms of providing auxin to the founder cells of leaf primordia.

METHODS

Plant Materials

We obtained the T-DNA insertion mutants from either the ABRC or from Institut National de la Recherche Agronomique (INRA) (Samson et al., 2004). The mutants were genotyped according to the published protocols (Alonso et al., 2003; Samson et al., 2004). Genotyping primers for *yuc1*, *yuc2*, *yuc4*, and *yuc6* were described previously (Cheng et al., 2006). The insertion mutant for *YUC10* (At1g48910) was the FLAG_599G05 line from INRA (Samson et al., 2004). Our DNA sequencing data showed that the T-DNA was inserted 653 bp downstream of the start codon of *YUC10*. Primers for genotyping *yuc10* are 5′-CCTGAATCTCGCCATCGCGAATC-3′, 5′-CCAAAGAGCTTTTCGTCAACTACC-3′, and the T-DNA specific primer FLAG_LB4 5′-CGTGTGCCAGGTGCCCACGGAATAGT-3′.

The T-DNA line for *YUC11* (At1g21430) was the SALK_073485 line from ABRC. The T-DNA was inserted in the second exon of the gene, 822 bp downstream of the start codon. Genotyping primers for *yuc11* were as follows: LP, 5′-TGTCAACTCCCTCACATGCCA-3′; RP, 5′-CAGATCTCCATCATCGACCTGTGT-3′; and JMLB1 as the T-DNA–specific primer.

The *pin1* mutant contains a T-DNA insertion 1945 bp downstream of the ATG site. The mutant was genotyped with the following primers: 5′-ACAACCAGTACGTGGAGAGGG-3′ and 5′-TCATAGACCCAAGAGAATGTAGTAG-3′. The T-DNA insertion in *aux1* was located at 1893 bp from the ATG site. The *aux1* mutant is genotyped using the following primers: 5′-CGATCATCTGGACAAGAGAACATG-3′ and 5′-TCCTCCACCGACTCTTCATTTC-3′.

DR5-GUS and *YUC* promoter GUS lines were described previously (Cheng et al., 2006).

Methods

In situ RNA hybridization and scanning electron microscopy analysis were performed according to the methods described by Dinneny et al. (2004). Vascular visualization was performed using protocols from Cheng et al. (2006). We performed GUS staining as previously described (Cheng et al., 2006).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_175321 (*YUC10*) and NP_173564 (*YUC11*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. RNA in Situ Hybridization Using Sense Probes of *YUC* Genes.

Supplemental Figure 2. Effects of NPA Treatment on DR5-GUS Expression in *yuc1 yuc4* Double Mutants.

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