The Arabidopsis IDD14, IDD15, and IDD16 Cooperatively Regulate Lateral Organ Morphogenesis and Gravitropism by Promoting Auxin Biosynthesis and Transport

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Abstract

The plant hormone auxin plays a critical role in regulating various aspects of plant growth and development, and the spatial accumulation of auxin within organs, which is primarily attributable to local auxin biosynthesis and polar transport, is largely responsible for lateral organ morphogenesis and the establishment of plant architecture. Here, we show that three Arabidopsis INDETERMINATE DOMAIN (IDD) transcription factors, IDD14, IDD15, and IDD16, cooperatively regulate auxin biosynthesis and transport and thus aerial organ morphogenesis and gravitropic responses. Gain-of-function of each IDD gene in Arabidopsis results in small and transversally down-curled leaves, whereas loss-of-function of these IDD genes causes pleiotropic phenotypes in aerial organs and defects in gravitropic responses, including altered leaf shape, flower development, fertility, and plant architecture. Further analyses indicate that these IDD genes regulate spatial auxin accumulation by directly targeting YUCCAS (YUC5), TRYPHTOPHAN AMINOTRANSFERASE of ARABIDOPSIS1 (TAA1), and PIN-FORMED1 (PIN1) to promote auxin biosynthesis and transport. Moreover, mutation or ectopic expression of YUC suppresses the organ morphogenetic phenotype and partially restores the gravitropic responses in gain- or loss-of-function idd mutants, respectively. Taken together, our results reveal that a subfamily of IDD transcription factors plays a critical role in the regulation of spatial auxin accumulation, thereby controlling organ morphogenesis and gravitropic responses in plants.

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Introduction

Auxin is a key plant hormone that plays critical roles in the regulation of plant growth and development. A combination of physiological, genetic, biochemical, and molecular studies has greatly enriched our understanding of auxin biosynthesis, transport, and signal transduction [1–3]. Increasing evidence indicates that auxin is essential for nearly all developmental processes, including gametogenesis, embryogenesis, lateral organ formation and patterning, branching, and tropic responses [4,5]. It is generally believed that most auxin-mediated developmental events are highly dependent on the differential accumulation of auxin within plant organs (auxin gradients), which are mainly attributable to both local auxin biosynthesis and the intercellular polar transport of auxin [1,5].

Direct evidence that local auxin biosynthesis is involved in the regulation of plant organogenesis comes from studies of several genes in the auxin biosynthetic pathway of Arabidopsis, including YUCCA (YUC) and TRYPHTOPHAN AMINOTRANSFERASE of ARABIDOPSIS1 (TAA1). YUCs encode the flavin monoxygenases that catalyze a key step in converting tryptophan into IAA, a main auxin in plants [6]. Overexpression of YUC genes in Arabidopsis substantially elevates the endogenous IAA level and causes distinct phenotypes such as epinastic cotyledons, elongated hypocotyls, and narrow and curly leaves [6–8]. Although a yuc single mutant in Arabidopsis does not show an obvious phenotype, the mutation of multiple YUC genes leads to a diversity of auxin-related phenotypes, including reduced apical dominance, wrinkled leaves, simple venation, and abnormal flower development, demonstrating that YUC-modulated local auxin biosynthesis is critical for plant morphogenesis and architecture formation [9,10]. Consistently, such developmental defects in yuc mutants can be rescued by local expression of auxM, a bacterial auxin biosynthetic gene, but not by the application of exogenous auxin [9]. The expressions of YUC genes are overlapping and spatiotemporally regulated in various organs [9,10], suggesting that YUCs function redundant and cooperatively in different organs. Further studies demonstrate that TAA1 and its homologs function in auxin biosynthesis in response to environmental and developmental signals in Arabidopsis. The taal plant has a decreased level of endogenous IAA and displays defects in shade avoidance and root-specific ethylene sensitivity, and the simultaneous mutation of TAA1 and its close homologs (TAR1 and TAR2) results in phenotypes that are obviously auxin-related, such as reduced gravitropic response of
Author Summary

Auxin is a key plant hormone and the spatial accumulation of auxin is essential for lateral organ morphogenesis and gravitropic responses in higher plants. However, the various mechanisms through which spatial auxin accumulation is regulated remain to be fully elucidated. Here, we identify a gain-of-function mutant of Arabidopsis IDD14 that exhibits small and transversally down-curved leaves. Further characterization of both gain- and loss-of-function mutants in IDD14 and its close homologs, IDD15 and IDD16, reveals that these three IDD transcription factors function redundantly and cooperatively in the regulation of multiple aspects of lateral organ morphogenesis and gravitropic responses. We further demonstrate that these IDD transcription factors influence the spatial accumulation of auxin by directly targeting auxin biosynthetic and transport genes to activate their expression. These findings identify a subfamily of IDD transcription factors that coordinates spatial auxin gradients and thus directs lateral organ morphogenesis and gravitropic responses in plants.

results

cuf1-D/idd14-1D Displays Pleiotropic Leaf Phenotypes

To gain insight into how lateral organ morphogenesis is controlled in plants, we screened for mutants with altered aerial organ morphology in a transgenic Arabidopsis population harboring a T-DNA activation-tagging plasmid (pSKI015) [49]. A semi-dominant mutant was identified by its smaller and dramatically down-curved leaves, and thus designated as cuf1-D and subsequently as idd14-1D (see below) (Figure 1A). To examine the genetic nature of cuf1-D, we backcrossed cuf1-D with wild type (WT) plants. All F1 plants exhibited an intermediate phenotype as cuf1-D/+; and F2 plants displayed a phenotypic segregation of WT:cuf1-D+:cuf1-D as 1:2:1 (82:173:87, P = 0.89, \(X^2\)-test), in which all WT plants were BASTA sensitive. This observation suggests that cuf1-D results from a semi-dominant mutation of a single gene that is likely to co-segregate with a T-DNA insertion event. The most striking phenotype in cuf1-D was the leaf size and shape. Detailed quantification showed that the average areas of fully-expanded leaf blades in heterozygous and homozygous cuf1-D were only about 70% and 47% of that in WT, respectively (Figure 1B). The leaves of cuf1-D were also dramatically curled downward in a transverse direction. The leaf transverse curvature (TC) index in WT was about 0.88, whereas the leaf TC index in heterozygous and homozygous cuf1-D reached about 0.29 and 0.36, respectively (Figure 1C). Moreover, the lamina of a mature WT rosette leaf displayed an elliptical shape with a leaf length/width index at about 1.5, while the heterozygous and homozygous cuf1-D leaves were comparatively narrow and their leaf indices were about 1.9 (Figure 1D). In addition to the morphological changes in leaves, cuf1-D was also late flowering and dwarfed (Figure 1A, Table S1). These observations demonstrate that the dominant mutation in cuf1-D has pleiotropic effects on lateral organ development and plant architecture.
CUF1 Encodes the INDETERMINATE DOMAIN 14 Transcription Factor

Since cuf1-D is a single gene mutation that is likely to cosegregate with a T-DNA insertion event, we amplified the genomic DNA adjacent to the left border of the T-DNA by thermal asymmetric interlaced PCR (TAIL-PCR). The sequencing analysis indicated that a T-DNA was inserted in the intergenic region between At1g68120 and At1g68130 (Figure 2A). Genotyping analysis showed that the T-DNA insertion co-segregated with the leaf phenotype in heterozygous and homozygous cuf1-D mutants (Figure 2B), suggesting that cuf1-D was caused by the T-DNA insertion event. Because cuf1-D was a dominant mutant, we monitored the transcripts of genes flanking the T-DNA insertion using semi-quantitative reverse transcription PCR (RT-PCR) analysis. Compared to those in the WT, the transcripts of At1g68130 (IDD14) were dramatically elevated, while At1g68140 mRNA levels were slightly decreased in cuf1-D (Figure 2C). To determine whether the elevated levels of IDD14 transcripts are responsible for the cuf1-D phenotype, we introduced a p35S:anti-IDD14 construct into cuf1-D and a p35S::IDD14 construct into WT plants, respectively. Transgenic plants overexpressing IDD14 fully recapitulated the phenotype of cuf1-D, while the expression level of At1g68140 in these plants was comparable with that in WT plants (Figure 2D, S1B). Moreover, introduction of p35S:anti-IDD14 into cuf1-D restored the cuf1-D to WT morphology (Figure 2D). These results demonstrate that cuf1-D results from the ectopic expression of IDD14, and accordingly, the cuf1-D was thus re-designated as idd14-1D.

In Arabidopsis, the IDD14 transcription factor is phylogenetically sub-grouped with two close homologs: IDD15 (SGR5) and IDD16, to form a small subfamily that is distinct from the other IDD family members (Figure S1A) [34,39]. IDD14, IDD15 and IDD16 share 52%–62% amino acid identity, and their ID domains are highly conserved with 89%–95% amino acid identity (Figure 2E). To investigate the possible redundancy of IDD15 and IDD16 with IDD14, we generated transgenic Arabidopsis plants overexpressing IDD15 or IDD16, respectively. The ectopic expression of either IDD15 or IDD16 resulted in a similar leaf phenotype as observed in idd14-1D (Figure 2F, S1B), suggesting that the three IDD members may have redundant function during plant development.

The Differential and Overlapping Expression of IDD14, IDD15, and IDD16 in Multiple Organs

To explore the functions of this IDD subfamily, we examined the tissue-specific expression patterns of these genes in multiple organs of transgenic plants harboring a pIDD::GUS (β-Glucuronidase) construct. As shown in Figure 3A–3C, IDD14 was mainly...
expressed in cotyledons and the vasculature of rosette leaves, and a weak level of expression was observed in hypocotyls and floral organs. However, the GUS signal was undetectable in roots and inflorescence stems.

IDD15 was highly expressed in petioles, hypocotyls, roots, floral organs, and especially in inflorescence stems. In inflorescence stems, GUS staining was mainly present in the cortex, endodermis and vasculature tissues (Figure 3A–3C). IDD16 was highly expressed in leaves, hypocotyls, roots, vasculature of cotyledons, floral organs, and in the endodermis and vasculature of inflorescence stems (Figure 3A–3C). RNA in situ

Figure 2. Molecular characterization of CUF1/IDD14. (A) Scheme of the genomic region flanking T-DNA insertion in cuff1-D. Genes are shown as thick arrows and intergenic regions are shown as lines. The orientation of T-DNA left border (LB), right border (RB), and the four CaMV 35S enhancers (4×35S) are indicated. (B) Linkage analysis of the T-DNA and cuff1-D phenotype. The 954-bp genomic fragments in WT and cuff1-D/+ plants were amplified with primers in the genomic region flanking the T-DNA insertion site, and the 894-bp fragments in cuff1-D/+ and cuff1-D were amplified with an LB primer and a downstream genomic primer. (C) Expression analysis of the genes flanking the T-DNA in WT and cuff1-D plants. ACTIN2 (ACT2) was used as an internal control. Note that transcripts of At1g68130 (IDD14) are highly elevated in cuff1-D. (D) Morphology of 25-days-old WT, cuff1-D carrying p35S::anti-IDD14, and p35S::IDD14 plants. The scale bar represents 2 cm. (E) Alignment of the ID domains in IDD14, IDD15, and IDD16. ZF1-ZF4 represents the four C2H2-type zinc finger motifs. The arrowheads indicate the conserved cysteine and histidine residues. (F) Morphology of 4-week-old transgenic plants overexpressing IDD15 or IDD16. The scale bar represents 1 cm.

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hybridization assayed in the inflorescence stems validated the expressions of the three *IDD* genes detected by the GUS reporter (Figure 3D). Additionally, consistent with the previous finding that *IDD* members act as transcription factors [38,39], an *IDD14*-GFP fusion protein in transgenic plants carrying p35S::*IDD14*-GFP, which recapitulated the phenotype of *idd14-1D*, was found to be localized in nuclei (Figure S2A, S2B).

**IDD14, IDD15, and IDD16 Cooperatively Regulate Lateral Organ Morphogenesis and Gravitropic Responses**

To gain further insight into the functions of this *IDD* subfamily, we obtained the T-DNA insertion mutant *idd14-1* (CS367164) and *idd15-5* (Salk_087765) from the Arabidopsis Biological Resource Center (ABRC), in which the T-DNA is inserted in an exon of *IDD14* or *IDD15*. Semi-quantitative RT-PCR analyses indicated that the transcripts of *IDD14* or *IDD15* were undetectable in *idd14-1* or *idd15-5*, respectively (Figure 4A, 4B). As no *idd16* mutant is publicly available, we generated *IDD16*-RNAi transgenic plants with an *IDD16*-specific cDNA fragment (Figure 4A). Semi-quantitative RT-PCR analysis indicated that *IDD16* transcripts were dramatically reduced in transgenic *IDD16-RNAi* plants (Figure 4B). We then generated double and triple mutants of the three *IDD* genes, which allowed us to closely examine their differential and redundant functions during plant growth and development.

We first examined the aerial organ morphogenesis in these *idd* mutants. As shown in Figure 4C and 4D, none of the *idd* single mutants exhibited any obvious organ phenotype. However, the leaves of *idd14-1* *IDD16-RNAi* and *idd triple mutants were not only downward-curved in the longitudinal direction, but were also more rotund when compared to WT leaves. Furthermore, *idd14-1* *IDD16-RNAi* and the *idd* triple mutant had enlarged floral organs and infertile siliques. Careful examination showed that the infertile siliques resulted from the asynchronous elongation of stamen filaments and styles, and thus had poorly pollinated stigmas (Figure 4C, 4E). Manual pollination of styles in these mutants resulted in the development of normal siliques. In contrast to these dramatic phenotypes, the *idd15-5* *IDD16-RNAi* plants had only slightly curled leaves (Figure 4C, 4D). These observations imply that *IDD14* and *IDD16* have redundant roles in directing leaf and floral organ morphogenesis.

Consistent with the previous finding that *idd15* displays increased angles between inflorescence stems and siliques [40], we noticed that the orientation angles of both branches and siliques were obviously increased in *idd15-5* (Figure 4C). This phenotype was further enhanced in the *idd15-5* *IDD16-RNAi* and *idd triple mutants, but not in *idd14-1* *idd15-5* plants (Figure 4C, 5A), indicating that *IDD13* and *IDD16* act cooperatively to control siliques and branch orientation. As *idd15* has a reduced gravitropic response in inflorescence stems [39–41], it is likely that the altered orientation of branches and siliques is related to the gravitropism defect in the *idd* mutants. To test this, we investigated the gravitropic responses in gain- and loss-of-function of *idd* plants. As expected, *idd15-5* inflorescence stems exhibited an obviously reduced gravitropic response, and this phenotype was greatly enhanced in *idd15-5* *IDD16-RNAi* and the *idd* triple mutant.
IDD Modulates Auxin Biosynthesis and Transport

A

B

C

D

E
Figure 4. Pleiotropic organ phenotypes in loss-of-function *idd* mutants. (A) Schematic illustration of the *idd14-1*, *idd15-5*, and the *IDD16-RNAi* construct. A 248-bp specific *IDD16* cDNA fragment was used for construction of p35S::IDD16-RNAi. (B) Semi-quantitative RT-PCR analysis of *IDD* genes in WT and *idd* mutants. The transcripts of *IDD14*, *IDD15*, and *IDD16* in the *idd* triple mutant are shown as representatives. (C) 36-day-old plants of *idd* single, double, and triple mutants. Arrows indicate the infertile siliques. The arrowheads show the increased angles between the inflorescence stems and branches. The scale bar represents 2 cm. (D) Morphology of sixth leaves in 25-day-old *idd* mutants. The longitudinal curvature (LC) index and leaf index were determined from at least 10 leaves in each genotype. The data are shown as mean values ± one SD (Student’s t-test, *p* < 0.05 and **p** < 0.001). (E) Enlarged floral organs and infertile siliques in the *idd* triple mutant. The arrow indicates the stigma lacking pollen. The arrowheads show the unfertilized ovules. doi:10.1371/journal.pgen.1003759.g004

Figure 5. Silique orientation and gravitropic response in gain-and loss-of-function *idd* mutants. (A) Silique orientation in the *idd* single, double, and triple mutants. The angles between siliques and inflorescence stems were measured in 45-day-old WT and *idd* mutant plants. The data are shown as mean values ± one SD (Student's t-test, **p** < 0.01 and ***p** < 0.001). (B) Gravitropic responses of inflorescence stems in *idd14-1D* and loss-of-function *idd* mutants. The degree of curvature was determined from at least 18 inflorescence stems in each genotype after gravistimulation at the indicated times. The data are shown as mean values ± one SD (Student's t-test, *p* < 0.05 and **p** < 0.01). doi:10.1371/journal.pgen.1003759.g005

**IDD Modulates Auxin Biosynthesis and Transport**

Previously studies have suggested that *IDD14* and *IDD15* are involved in the regulation of starch metabolism [38,40]. However, the narrow, epinastic leaves in gain-of-function *IDD* mutant or transgenic plants seem to resemble, to some extent, those observed in auxin overproduction mutants or transgenic plants, such as *yucD* or *p335:FUCs* transgenic plants [6,7,9]. By contrast, the rotund and curly rosette leaves, abnormal floral phenotype, and gravitropism defect in the loss-of-function *idd* mutants are also documented in the mutants defective in auxin biosynthesis or transport [2,9–11]. This led us speculate that auxin accumulation or signaling may also be involved in *IDD*-mediated organ development and/or gravitropic response. To test this, we used the *idd14-1D* and *idd* triple mutant plants as representatives of gain- and loss-of-function *idd* mutants for our further analysis. We first examined auxin accumulation in their leaf, inflorescence stem, and root, by monitoring the expression of DR5::GUS or DR5::GFP, a widely used auxin gradient reporter [50]. On day 3 after leaf initiation, an obvious GUS signal was observed in WT leaf distal tips. A stronger and spatially-expanded GUS signal was observed in *idd14-1D* leaf tips. No obvious GUS signal, and thus no localized auxin maxima, was observed in the leaf tips of *idd* triple mutant plants (Figure 6A). Similar differential GUS patterns were subsequently observed in 5-day-old expanding and 15-day-old expanded leaves (Figure 6A). In inflorescence stems, the GUS signal was mainly observed in vascular tissues in WT, whereas strong GUS staining was present in the cortex and endodermis tissues in *idd14-1D* and weak GUS expression without a tissue-specific pattern was observed in the *idd* triple mutant (Figure 6B). The increased or decreased auxin accumulation was also found in the meristem regions of primary roots in *idd14-1D* and the *idd* triple mutant, respectively (Figure 6C). Further quantification of the GUS activity in these organs confirmed the variations of DR5::GUS signals observed in *idd14-1D* and the *idd* triple mutant (Figure 6D). This observation strongly suggests that alteration of *IDD* expression affects auxin accumulation in multiple organs.

Auxin homeostasis and transport are key factors that determine the accumulation of auxin in plant organs [1,2,4]. To assess the contributions of auxin homeostasis and transport to *IDD*-mediated auxin accumulation, we first quantified the endogenous free IAA levels in the *idd14-1D* and *idd* triple mutants. Consistent with enhanced or decreased expression of *DR5::GUS* reporter observed,
Figure 6. Altered auxin accumulation and transport in gain- and loss-of-function *idd* mutants. (A) Auxin accumulation assayed with the *DR5:GUS* reporter in WT, *idd14-1D*, and *idd* triple mutant leaves. The leaves at 3, 5, and 15 days after initiation (from top to bottom panels) were subjected to GUS staining. The scale bars represent 50 μm in the top and middle panels and 5 mm in the bottom panel. (B) Expression of the *DR5:GUS* reporter in inflorescence stems of WT, *idd14-1D*, and *idd* triple mutant plants. The scale bars represent 2 mm in the top panel and 100 μm in the bottom panel. (C) GFP fluorescence signals in the primary roots of WT, *idd14-1D*, and *idd* triple mutant carrying a *DR5:GFP* reporter. The scale bar represents 50 μm. (D) Quantification of the GUS activity in the organs of the WT, *idd14-1D*, and *idd* triple mutant carrying a *DR5:GUS* reporter. Data are from three biological replicates and shown as mean values ± one SD (Student’s t-test, *P*<0.05 and **P**<0.01). (E) Endogenous free IAA levels in WT, *idd14-1D*, and *idd* triple mutant plants. Aerial organs of 15-day-old plants were used for measurement of free IAA levels. Data are from four biological replicates and are shown as mean values ± one SD (Student’s t-test, *P*<0.05 and **P**<0.01). (F) Polar auxin transport capability of WT, *idd14-1D*, and *idd* triple mutant stems. The inflorescence stem segments of 5-week-old plants were used to determine the basipetal IAA transport efficiency and the background acropetal movement. Data are from four biological replicates and shown as mean values ± one SD (Student’s t-test, *P*<0.05 and **P**<0.01).

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the endogenous IAA level was increased by about 13% in idd14-1D but decreased by approximately 19% in idd triple mutant plants as compared to that in WT (Figure 6E). This result implied that the IDD genes may affect auxin biosynthesis. We then measured the auxin transport capability of inflorescence stems in idd14-1D and idd triple mutant. As shown in Figure 6F, the basipetal IAA transport efficiency was increased by over 50% in idd14-1D stems but decreased about 18% in idd triple mutant stems when compared with that in WT stem, demonstrating that IDD also modulates the auxin transport process. To further examine whether auxin signaling is affected in gain- and loss-of-function idd mutants, we monitored the expression of the DR5:GUS reporter, IAA5, and IAA29 in response to exogenous IAA treatment in idd14-1D and the idd triple mutant, and observed that the expression of the DR5:GUS reporter, IAA5, and IAA29 was normally induced following application of IAA as that in WT plants (Figure S4A, S4B), suggesting that IDD has no effect on auxin perception or signaling. We also observed that the expression of IDD14, IDD15, and IDD16 transcripts was not modulated by auxin treatment (Figure S4C). Taken together, our results strongly suggest that the three IDD genes are involved in the establishment of auxin gradients through the regulation of auxin biosynthesis and transport.

IDD Directly Activates the Expression of YUC5, TAA1, and PIN1

To identify the genes downstream of IDD, we first carried out a real-time quantitative RT-PCR (qRT-PCR) analysis to examine the transcript abundances of genes known to function in auxin biosynthesis and transport in the idd14-1D and idd triple mutants. Among the YUC and TAA family genes, the transcription of YUC1, YUC2, YUC3, YUC4, YUC5, YUC8, and TAA1 was found to be elevated in idd14-1D, and the transcription levels of YUC2 and YUC5 were decreased in idd triple mutant, when compared to those in WT plants (Figure 7A). Among 14 genes related to auxin transport, the transcription of AUX1, PIN1, ABCB1, ABCB4 and WAG1 were found to be elevated in idd14-1D but reduced in the idd triple mutant compared to those in WT plants. PIN4 and PINOID (PID) transcripts were only elevated in idd14-1D or deceased in the idd triple mutant (Figure 7A). Further, we investigated the expression of 11 genes, which had the apparently differential expression in idd14-1D or the idd triple mutant (with a significance at P<0.01), in the transgenic plants overexpressing IDD15 or IDD16 and the idd15-5-IDD16-RNAi plants. As expected, their differential expressions in gain- and loss-of-function IDD15 and IDD16 plants were much similar to those observed in idd14-1D and the idd triple mutant (Figure S5A), demonstrating that these genes are also downstream of IDD15 and IDD16.

As the IDDs are transcription factors, we speculated that some of the genes differentially expressed in gain- and loss-of-function idd mutants might be directly targeted by IDD. We first identified the genes whose expression was rapidly induced by the activation of IDD, using the chemically-inducible IDD16 transgenic plants. After the transgenic seedlings were treated with the inducer, IDD14 was dramatically induced by 0.5 h, and the expression of YUC5, TAA1, and PIN1 was obviously elevated within 2 h (Figure S5B), suggesting that these genes might be direct targets of IDDs. To examine whether IDD can directly bind to the promoter regions of YUC5, TAA1, or PIN1, we performed chromatin immunoprecipitation (ChIP) assays with both β33S::IDD16-GFP and β33S::IDD14-GFP transgenic plants. It has been reported that the maize ID1 and ID domain proteins could bind to a specific 11 bp DNA consensus motif, T-T-T-G-T-C-G/T/G-T/a-T/a-T/a-T [35]. As such, we targeted similar possible IDD-binding motifs in the promoter and/or upstream coding regions of YUC5, TAA1, PIN1, YUC2, and YUC3, and carried out the ChIP analysis (Figure 7B). As expected, we found that the three fragments containing a putative IDD-binding motif in YUC5, TAA1, or PIN1 were greatly enriched by IDD16 after GFP immunoprecipitation, whereas no binding activity was detected in the promoter regions of YUC2 or YUC3 (Figure 7B). Such enrichment was detected in a control DNA fragment of the QUA-QUINE STARCH (QQS) promoter, which was previously reported to be a target of IDD14 [38], but no enrichment was detectable in a control DNA fragment in the ACTIN2 (ACT2) promoter which lacked the putative IDD-binding motif (Figure 7B). Likewise, similar enrichments of these DNA fragments were further confirmed by ChIP assayed with IDD14 protein (Figure S5C). In addition, we indeed visualized the enhanced or attenuated PIN1 accumulation in the roots of idd14-1D and idd triple mutant carrying a βPIN1::PIN1-GFP construct, respectively (Figure S6). These results illustrate that IDD can directly target YUC5, TAA1, and PIN1, to activate their expression.

Alteration of Auxin Accumulation Suppresses Organ Morphogenesis in idd Mutants

Since idd14-1D contains a high auxin level while the idd triple mutant has low endogenous auxin content, we attempted to genetically modify auxin biosynthesis to examine whether this could suppress or restore the phenotype observed in gain- and loss-of-function idd mutants. We first generated an idd14-1D yuc2 yuc6 triple mutant through genetic crosses, and observed that loss-of-function yuc2 yuc6 completely suppressed the leaf phenotypes of idd14-1D (Figure 8A, S7), and partially attenuated the hypersensitivity of idd14-1D inflorescence stems to gravistimulation (Figure 8B). Further, when we overexpressed YUC2 in the idd triple mutant, the ectopic expression of YUC2 fully rescued the fertile silique defect (Figure 8C), but only partially restored the silique orientation in the idd triple mutant (Figure 8D). These results provide further evidence that auxin biosynthesis is genetically downstream of this IDD subfamily.

As the amyloplast movement in endodermal cells of idd15 stem has been reported to be defective under gravistimulation [39–41], we also investigated whether ectopic expression of YUC2 has an effect on amyloplast movement in endodermal cells of the idd triple mutant stems. As shown in Figure S8, the retarded amyloplast movement in endodermal cells of the idd triple mutant stems under gravistimulation was not rescued by ectopically expressed YUC2 (Figure S8), suggesting that altered auxin accumulation does not affect IDD-mediated amyloplast responsiveness to gravistimulation.

Discussion

The Distinct IDD Subfamily Is Critical for Organ Development and Plant Architecture Formation

The IDD family has been defined as a plant-specific transcription factor family [35,51], and previous characterizations of a few IDD members have indicated that IDDs are involved in the regulation of transition to flowering and starch metabolism [36–38]. IDD14, IDD15, and IDD16 belong to a subfamily that is distinct from the other IDD members in Arabidopsis, rice, and maize [34]. The mutation of IDD15 in Arabidopsis and rice reduces gravitropic response in inflorescence stems [39–41,48]. However, because the loss-of-function idd14 mutant does not have obvious organ phenotype, and IDD16 has not yet been characterized, the functions of this subfamily are still largely unknown.

In this study, we characterized both gain- and loss-of-function mutants of this IDD subfamily, and discovered that these three IDD genes function redundantly and cooperatively in regulating
organ morphogenesis and gravitropic responses. Gain-of-function of each IDD led to a small, narrow, and down-curled leaf phenotype. Although idd single mutants did not have obvious organ morphological phenotypes (except the reduced gravitropic response observed in idd15), our further characterization of the idd double and triple mutants clearly demonstrates that IDD14 and IDD16 act redundantly to regulate the morphology of aerial organs and affect fertility, while IDD15 and IDD16 cooperatively control the gravitropic responses and plant architecture.

Such redundant and cooperative roles of the three IDD genes in organ morphogenesis and gravitropism are consistent with their differential and overlapping expression patterns in particular organs. IDD14 and IDD16, but not IDD15, were expressed in juvenile leaves, whereas IDD15 and IDD16, but not IDD14, were highly expressed in inflorescence stems. The three IDD genes were all expressed in floral organs, and the abnormal flower phenotype in idd14-1 IDD16-RNAi was enhanced in the idd triple mutant.

Interestingly, although the IDD genes were also expressed in hypocotyls and roots, we could not observe obvious changes in their morphology or gravitropic response in either gain- or loss-of-function idd mutants, except the slightly waved roots in idd triple mutant seedlings. This may be attributable to decreased expression of WAG1, a gene that is an indirect target of IDD and has been identified as a suppressor of root waving (Figure 7A) [52].

### IDD Modulates Auxin Biosynthesis and Transport

Previous studies have shown that IDD15 and IDD14 are involved in the regulation of starch metabolism [30,40]. Here,
with a combination of phenotypic, genetic, and molecular approaches, we demonstrated that IDD14, IDD15, and IDD16 modulate auxin accumulation by affecting auxin biosynthesis and transport, thereby modifying organ morphogenesis and architecture formation. First, the organ phenotype in both gain- and loss-of-function \textit{idd} mutants appears to be related to altered auxin homeostasis and distribution. For example, the narrow, epinastic leaves in the plants overexpressing \textit{IDD} are similar to those in auxin overproduction mutants or transgenic plants, such as \textit{yucca} (\textit{yuc1D}), \textit{yucca6-1D}, and \textit{p35S::YUC} transgenic plants [6,7,9], whereas the rotund and curly rosette leaves and abnormal floral phenotype in the loss-of-function \textit{idd} mutants are similar to those observed in the mutants defective in auxin biosynthesis or transport [9,53]. Disruption of \textit{IDD} genes also influenced the silique and branch angles, root waving, and gravitropism of inflorescence stems, which have been well documented to be related to polar auxin transport [2,54–57]. Second, expression analyses using \textit{DR5:GUS} and \textit{DR5:GFP} reporter clearly indicated that gain- or loss-of-function of \textit{IDD} enhanced or reduced auxin gradients, which was further confirmed by the increased or decreased endogenous auxin content and transport ability. Third, the expression of several genes involved in auxin biosynthesis and transport were altered in both gain- and loss-of-function \textit{idd} mutants, and the IDD proteins could directly bind to the promoter regions of \textit{YUC5}, \textit{TAA1}, and \textit{PIN1} to activate their expression. In addition, genetic manipulation of auxin biosynthesis could fully or partially restore the pleiotropic phenotypes in \textit{idd14-1D} or the \textit{idd} triple mutant. These results demonstrate that IDD indeed modulates auxin gradients by promoting auxin biosynthesis and transport.

**IDD-Mediated Auxin Gradients and Starch Metabolism May Coordinate Gravitropic Response**

The gravitropic response in plants requires a coordination of three sequential processes: gravity perception, signal transduction, and asymmetric growth response [58]. It is widely believed that the starch-filled amyloplasts (statoliths) within specific gravi-sensing cells (statocytes) perceive gravity stimulation [59,60]. Some other molecules, such as InsP$_3$ and Ca$^{2+}$ have been found to be involved in gravity signaling [60]. A large body of evidence indicates that auxin plays a key role in gravitropic signaling and asymmetric organ growth, and that it may possibly be involved in gravi-sensing [2,60]. For example, many mutants related to auxin biosynthesis and especially transport such as \textit{taa1}, \textit{aux1}, \textit{pin1}, and \textit{pin2}, exhibit a defect in gravitropic responses [2,61]. Other mutants with altered silique or branch architecture, such as \textit{plethora} (\textit{plt}) in \textit{Arabidopsis} and \textit{laz1} ($laz1$) in rice, also show altered auxin accumulation or transport within their organs [54,56,62].

Among the three IDD members we characterized, IDD15 and its rice ortholog, LPA1, have been previously reported to affect the gravitropic response by altering amyloplast sedimentation in the endodermis [39–41,48]. Recently, IDD14 was also found to mediate starch degradation by directly activating the expression of \textit{QQS} [38], suggesting that IDD14 also participates in the regulation of starch metabolism. Our detailed characterization of gain- and loss-of-function of three \textit{IDD} provides substantial evidence that IDD-mediated auxin biosynthesis and transport contribute to the organ morphogenesis and also, to some extent, gravitropic responses, because the genetic manipulation of auxin biosynthesis does not alter the responsiveness of amyloplast to gravistimulation but partially restores the gravity sensitivity or defect in gain- or

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**Figure 8. Genetic interaction of auxin biosynthesis and IDD-mediated organ morphogenesis and gravitropism.** (A) Aerial organ morphology of 25-day-old \textit{idd14-1D}, \textit{yuc2 yuc6}, and \textit{idd14-1D yuc2 yuc6} plants. The scale bar represents 2 cm. (B) Gravitropic responses of WT, \textit{idd14-1D}, \textit{yuc2 yuc6}, and \textit{idd14-1D yuc2 yuc6} inflorescence stems. (C) 45-day-old plants of the \textit{idd} triple mutant, \textit{35S-YUC2}, and \textit{idd} triple mutant carrying a \textit{p35S::YUC2} construct. The scale bar represents 2 cm. (D) The silique orientation of WT, \textit{idd} triple mutant, \textit{35S-YUC2}, and \textit{idd} triple mutant carrying a \textit{p35S::YUC2} construct (one-way ANOVA test, $P<0.05$).

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loss-of-function idl mutants. Therefore, it is likely that IDD-mediated auxin accumulation and starch metabolism coordinately control the gravitropic responses. The IDD-regulated starch metabolism might be primarily involved in gravi-sensing while the IDD-regulated auxin gradient may be primarily involved in signaling and responses.

Materials and Methods

Plant Materials and Growth Conditions

The Arabidopsis thaliana accession Col-0 was used in this study. idd14-1D was isolated from a population generated by T-DNA activation-tagging mutagenesis. idd14-1 (CS367164) and idd15-5 (SALK_087765) were obtained from ABRC. All seeds were sterilized and germinated on 1/2 MS medium after vernalization for 2 days at 4°C, and the plants were grown in a culture room or growth chamber at 22 ± 1°C with an illumination intensity of 80–90 μmol m⁻² s⁻¹ and a 16-h light/8-h dark photoperiod, as described previously [63].

Leaf Curvature and Gravitropism Assays

Leaf curvature was determined as described previously [64,65]. Briefly, the transverse curvature (TC) index was defined as TC = 1 - cw/pw, where cw and pw are the curve width and the pressed width of leaves, respectively. The longitudinal curvature (LC) index was defined as LC = 1 - cl/pl, where cl and pl are the curve length and the pressed length of leaves, respectively. To quantify the pressed width or length of leaves, the sixth leaf of 25-day-old plants was dissected transversely or longitudinally on a desk, and then the extreme distances between the margins of the leaves were measured before and after pressing.

To quantify the gravitropic responses of roots and hypocotyls, seedlings were grown vertically for 4 days and then turned horizontally, and curvature angles were measured [58]. For quantification of the gravitropic responses of inflorescence stems, 32-day-old plants with inflorescence stems of approximately 4–8 cm were gravistimulated by rotating them 90° in darkness, and stem curvatures were measured as the angles between the growing direction of apex and horizontal base line [39,58].

Genotyping of T-DNA Mutants

The T-DNA flanking sequence of idd14-1D was amplified by TAIL-PCR [66]. Three primers, P1, P2, and pSK-LB2 were used for co-segregation analysis. P1 and P2 were located in the Arabidopsis genome flanking the T-DNA insertion site, and pSK-LB2 was a primer corresponding to the left border of the T-DNA sequence. Similarly, idd14-1 and idd15-5 were genotyped with corresponding primers (Table S2), and yuc2 and yuc6 were genotyped according to the methods from a previous study [9].

Plasmid Construction and Plant Transformation

To generate p35S::IDDs and p35S::anti-IDD14 transgenic plants, the IDD coding sequences were amplified by RT-PCR and ligated into the pGEM-T-Easy vector (Promega, USA), and then verified by sequencing. The resulting plasmids were digested with EcoRI and cloned into pVIP96 [67]. The IDD14 cDNA was also cloned into pER8 to generate a chemically inducible IDD14 construct. To investigate the tissue-specific expression of IDD14, IDD15, and IDD16, approximately 2-kb promoter fragments of IDD genes were amplified from genomic DNA and then fused with the β-glucuronidase (GUS) gene into pBII101. To generate the p35S::IDD16-GFP and p35S::IDD14-GFP constructs, an IDD16 or IDD14 coding sequence lacking a stop codon was amplified and then cloned in frame into pMDC83 (Invitrogen, USA). To generate the p35S::IDD16-RNAi construct, a specific IDD16 cDNA fragment was amplified and ligated inversely into pBluescript SK-GUSF [60], and then an XbaI-BglII digested fragment was subcloned into pVIP96. A TUC2 cDNA fragment was amplified by RT-PCR and cloned into pVIP96myc to generate the p35S::TUC2 construct. All the primers used are listed in Table S2.

All constructs were introduced into Arabidopsis by Agrobacterium tumefaciens-mediated transformation via the floral dip as described previously [69]. At least 20 independent lines harboring a single T-DNA insertion from each construct were generated, and 4–5 independent lines of T3 homozygous plants were used for detailed characterization.

Gene Expression Analysis

Total RNA was isolated using a guanidine thiocyanate extraction buffer [70]. For semi-quantitative RT-PCR or qRT-PCR analysis, cDNA was synthesized from 1 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, USA). qRT-PCR was performed with a Rotor-Gene 3000 thermocycler (Corbett Research, Australia) with a SYBR Premix Ex Taq II kit (Takara, Japan). The relative expression level for each gene was normalized to the ACTIN2 and the data were collected from three biological replicates, as described previously [49].

The histochemical GUS assay was carried out according to previously described protocol [71]. The GUS activities were quantified by measuring cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucoronic acid (MUG), as described [71].

For RNA in situ hybridization, a specific cDNA region of IDD14, IDD15, or IDD16 was transcribed in vitro to generate sense and antisense probes using the Digoxigenin RNA labeling kit (Roche, Switzerland). The WT inflorescence stems were fixed and embedded in paraffin (Sigma-Aldrich, USA), and then sectioned to a 10 μm thickness. RNA in situ hybridization was performed according to a previously described method [54].

Free IAA Measurement

Aerial organs from 15-day-old plants of WT, idd14-1D, and idd triple mutant plants were used for measurement of free IAA content. The extraction, purification, and analysis of free IAA by gas chromatography-mass spectrometry was performed according to the methods described by Edlund et al. [72], except that an Agilent GC and a LECO Pegasus TOF mass spectrometer was used, with separation using a DB-5ht column (Agilent, USA) [63].

Auxin Transport Assays

Auxin transport in inflorescence stems was measured according to the methods of a previously published protocol [73]. 25-mm inflorescence segments were cut from 3-week-old plants, and the segments were submerged inversely into an auxin transport buffer (100 nM 3H-IAA, 0.05% MES, pH 5.5–5.7) in a 0.5-ml microcentrifuge tube. Control experiments were performed by submerging the base of inflorescence stems to measure acropetal IAA movement. After 12 h, 3-mm stem segments were dissected from the non-submerged ends and used to quantify the radiolabeled auxin using a scintillation counter.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed as described previously [74]. Briefly, 2 g of p35S::IDD16-GFP or p35S::IDD14-GFP transgenic plants grown on 1/2 MS plates for 16 days was harvested, and then submerged in 1% formaldehyde to crosslink the DNA with DNA-binding proteins. The chromatin pellets were extracted and sheared by sonication. 5 μl anti-GFP antibodies (Abcam, UK)
were used to immunoprecipitate the DNA-IDD16 or DNA-IDD14 complexes. DNA was released with proteinase K and then purified. The enrichment of DNA fragments was determined by quantitative PCR with the primers listed in Table S2.

**Amyloplast Staining**

Plants were grown in soil until the primary inflorescence stems bolted to a height of 4–9 cm, and then gravistimulated by rotating them upside down [40]. 1-cm-long inflorescence stems below the apex were fixed, embedded in paraffin, and sectioned to a 10-μm thickness. A periodic acid-Schiff kit (Sigma-Aldrich, USA) was used for amyloplast staining, according to the manufacturer’s instructions.

**Supporting Information**

**Figure S1** IDD14, IDD15, and IDD16 belong to a distinct IDD subfamily. (A) Phylogeny of the Arabidopsis IDD family. A neighbor-joining tree of 16 IDD members was generated using CLUSTAL W based on the amino acid sequences of IDD proteins. Number of generations = 1000. (B) Transcriptional analysis of IDD genes in transgenic plants overexpressing IDD14, IDD15, or IDD16, respectively. Two independent lines of each construct were assayed by semi-quantitative RT-PCR and the expression of At1g68140 was determined in 35S:IDD14 plants. The GAPC subunit (GAPC) gene was used as an internal control.

**Figure S2** IDD14-GFP protein is localized in nucleus. (A) Phenotype of 25-day-old p35S:IDD14-GFP transgenic plant. The scale bar represents 1 cm. (B) Nuclear localization of the IDD14-GFP protein in transgenic plants. GFP fluorescence, DAPI staining, overlaid and bright fields (BF) are shown from left to right. The scale bar represents 10 μm.

**Figure S3** Gravitropic response and root morphology of gain- and loss-of-function idd mutants. (A) Gravitropic responses of inflorescence stem in idd and idd14-1D plants. 32-day-old plants were gravistimulated by rotation by 90° in the dark for 0, 3, or 24 h. The scale bar represents 2 cm. (B–C) Kinetics of the gravitropic response of the hypocotyls (B) and primary roots (C) in WT, idd14-1D, and idd triple mutant plants. At least 20 seedlings from each genotype were used. The data are shown as mean values ± one SE. (D) Morphology of primary roots of WT, idd14-1D, and idd triple mutant plants. Seedlings grown vertically for 6 days were photographed. Note that the primary roots in the idd triple mutant showed a slightly waving phenotype. The scale bar represents 0.5 cm.

**Figure S4** Auxin responses of idd mutants and IDD genes. (A) Expression of DR5::GUS in WT, idd14-1D, and idd triple mutant seedlings treated with/without auxin. The 3-day-old seedlings were treated with various concentrations of IAA for 6 h and subjected to GUS staining assays. The scale bar represents 2 mm. (B) Transcripts of IAA5 and IAA29 in WT, idd14-1D, and idd triple mutant before and after auxin treatment. (C) Transcript levels of IDD14, IDD15, and IDD16 in WT plants before and after auxin treatment. Semi-quantitative RT-PCR was performed with the RNAs isolated from 10-day-old seedlings treated with 1 μM IAA for the time durations indicated. GAPC was used as an internal control.

**Figure S5** Identification of IDD-regulated genes involved in auxin biosynthesis and transport. (A) Relative expression levels of the genes involved in auxin biosynthesis and transport in WT, 35S:IDD15, 35S:IDD16, and idd14-1D IDD16-RNAi mutant plants. RNAs isolated from aerial organs of 3-week-old plants were subjected to qRT-PCR analysis, and data are from three biological replicates and shown as mean values ± one SD (Student’s t-test, *P<0.05 and **P<0.01). (B) Expression analyses of IDD-regulated genes in transgenic plants carrying an inducible IDD14 construct. 15-day-old transgenic plants were transferred into a liquid medium containing DMSO or 10 μM β-estradiol for the indicated time durations, and subjected to RNA isolation and qRT-PCR analysis. Data are from three biological replicates and shown as mean values ± one SD. Note that YUC5, TAA1, and PIN1 are rapidly induced by the activation of IDD14. (C) ChiP assay performed with p35S::IDD14-GFP transgenic plants by anti-GFP antibody. The DNA fragments with a possible IDD-binding motif in the promoter and upstream regions of YUC5, TAA1, PIN1, YUC2, and YUC3 (a–c) were assayed by ChiP, and the enrichment of their qPCR products are shown as mean values ± one SD from three biological replicates. An IDD-targeted fragment in the QOS promoter and a fragment in the ACT2 promoter were used as the positive and negative controls, respectively.

**Figure S6** PIN1 accumulation in gain- and loss-of-function idd mutants. GFP fluorescent signals in primary roots of WT, idd14-1D, and idd triple mutant plants containing a pPIN1::PIN1-GFP construct. GFP fluorescence, bright fields (BF), and overlaid images are shown from left to right. The scale bar represents 50 μm.

**Figure S7** Suppression of idd14-1D phenotypes by yuc2 yuc6. (A–C) The blade areas (A), transverse curvature (TC) index (B), and leaf index (C) of WT, idd14-1D, yuc2 yuc6, and idd14-1D yuc2 yuc6 leaves. At least 10 sixth leaves from each genotype were used for determination of the leaf area, TC index, and leaf index, respectively. Data are shown as mean values ± one SD (one-way ANOVA test, P<0.05).

**Figure S8** Alteration of auxin biosynthesis does not affect amyloplast sedimentation in idd mutants. Plants were gravistimulated by turning upside down for 0 or 20 min, and longitudinal sections of inflorescence stems were prepared and then stained with a periodic acid-Schiff kit. Arrowheads indicate the retarded movement of amyloplasts in the endodermal cells of the triple idd mutant and triple idd mutant carrying a p35S::YUC2 construct. The scale bar represents 10 μm.

**Table S1** Phenotypic characterization of caf1-D plants.

**Table S2** Primers used in this study.

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