Alteration of the shoot radial pattern in *Arabidopsis thaliana* by a gain-of-function allele of the class III HD-Zip gene *INCURVATA4*

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**ABSTRACT** Class III HD-Zip (HD-Zip III) family genes play key roles in a number of fundamental developmental programs in *Arabidopsis thaliana*, such as embryo patterning, meristem initiation and homeostasis, lateral organ polarity and vascular development. Semidominant gain-of-function alleles of the HD-Zip III genes *PHABULOSA* (PHB), *PHAVOLUTA* (PHV) and *REVOLUTA* (REV) disrupt the negative regulation of these genes by a mechanism of microRNA interference. We provide evidence that the gain-of-function icu4-1 allele of *INCURVATA4*, a gene encoding the HD-Zip III transcription factor ATHB15/CORONA (CNA), stimulates the production of vascular tissues, supporting a role for ICU4 in promoting vascular development. Occasionally, homozygous mutants for this allele show a reduced number of thick shoot vascular bundles, although normal collateral polarity remains unchanged. Genetic analysis of icu4-1 and phb-1D, a gain-of-function allele of the related PHB gene, revealed antagonism in lateral organ polarity between both mutations and a synergistic interaction in shoots, with transformation of the polarized collateral bundles into a radialized amphivasal pattern. These results indicate that the precise regulation of HD-Zip III genes confers positional information which is required to establish the number and pattern of vascular bundles in the stem. In addition, we present results that suggest an interaction between ICU4 function and auxin signaling.

**KEY WORDS:** Arabidopsis, class III HD-Zip gene, shoot radial pattern, vascular development

**Introduction**

The vasculature, a distinguishing feature of vascular plants, consists of an intricate network of conducting tissues that interconnect the different parts of the plant body, allowing the transport of water and solutes such as mineral nutrients, photoassimilates and signal molecules. Vascular plants already existed in the Silurian period, 438 to 408 million years ago, the evolution and diversification of their vascular systems being among the key events for efficient land colonization by plants. The vasculature is composed of the vascular meristematic tissues procambium and vascular cambium, responsible for the development of primary and secondary vascular tissues, respectively, and two differentiated tissues, xylem and phloem, each consisting of several specific cell types. Given the basic functions carried out by the vascular system, its accurate differentiation and patterning is a crucial process in plant development (Ye, 2002; Ye *et al.*, 2002).

Plant vascular patterns are species-specific traits, which suggests that they are subject to genetic control and may represent good models for the study of genetic and molecular mechanisms involved in pattern formation. Several studies have pointed to the importance of auxin for the differentiation of vascular tissues (Jacobs, 1952; Young, 1953; Aloni, 1987), as well as the implication of other plant hormones like cytokinin (Fukuda, 1997) and brassinosteroids (Yamamoto *et al.*, 1997; Caño-Delgado *et al.*, 2004). In fact, auxin plays a determinant role not only in vascular...
cell differentiation, but also in producing the continuity of vascular strands that finally become organized in a defined pattern (Sachs, 1981; 1991; Berleth et al., 2000). Auxin is synthesized in the apical tissues and moves basipetally in the shoot, according to a mechanism that involves its polar transport from cell to cell in a process mediated by the asymmetric localization of influx and efflux auxin carriers (Muday and DeLong, 2001; Muday and Murphy, 2002).

Five class III HD-Zip (HD-Zip III) gene family members have been identified in the Arabidopsis genome: PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1), ATHB8, and ATHB15. CORONA (CNA) and INCURVATA4 (ICU4). These genes are involved in several key developmental processes, including vascular development. The expression of ATHB8 is restricted to procambial cells (Baima et al., 1995), and its overexpression in transgenic plants promotes vascular cell differentiation, increasing the formation of xylem tissue (Baima et al., 2001). Transcripts of the adaxial identity genes PHB, PHV, and REV accumulate in the adaxial side of lateral organs and are absent from the abaxial side (McConnell et al., 2001; Otsuga et al., 2001), the adaxial region being the closest to the meristem and the abaxial the farthest. Semidominant gain-of-function alleles of PHB, PHV, and REV disrupt the regulation of these genes by microRNA (miRNA), causing the ectopic accumulation of their mRNA in the abaxial side of leaves, which produces abaxial-to-adaxial transformations (McConnell et al., 2001; Emery et al., 2003; Zhong and Ye, 2004). These alleles have also been reported to affect the pattern within leaf veins, as they cause the transformation of the normal collateral arrangement, consisting of adaxial xylem and abaxial phloem, into a radialized amphivasal pattern, with xylem surrounding phloem (McConnell and Barton, 1998; Zhong and Ye, 2004). In addition, the semidominant alleles of REV also produce this same transformation in the vascular bundles that extend longitudinally within the inflorescence stem (Emery et al., 2003; Zhong and Ye, 2004). Accordingly, vascular bundles of triple mutants for loss-of-function alleles in the abaxial KANADI family genes KAN1, KAN2, and KAN3 also display an amphivasal pattern (Emery et al., 2003).

A genetic analysis of icu4-1 and icu4-2, two semidominant gain-of-function alleles of the HD-Zip III gene CNA/ICU4, which codes for the ATHB15 transcription factor (Prigge et al., 2005), has shown that both carry the same point mutation perturbing the binding site of miR165/166, giving rise to increased levels of the mutant mRNA in every organ, and that the gene product possesses adaxial activity (Ochando et al., 2006). In this report, we show that homozygosis for the icu4-1 allele results in an increase of vascular tissues in inflorescence stems, which supports the idea that ICU4 promotes vascular development. Moreover, plants carrying gain-of-function mutations in both ICU4 and PHB display shoots with amphivasal bundles, indicating a crucial role for the precise regulation of HD-Zip III gene expression in the establish-
ment of the radial pattern of inflorescence stems. In addition, altering the auxin signaling pathway in the icu4-1 mutant also modifies the shoot radial pattern, which suggests a collaboration between ICU4 (and also other HD-Zip III genes) and auxin in its establishment. Our results represent novel findings on the complexity of the interactions between mutant alleles of HD-Zip III genes.

Results

Inflorescence stem vascular pattern of the icu4-1 mutant and transgenic plants overexpressing icu4-1 cDNA

Transverse sections in nonelongating internodes of the En-2 wild-type inflorescence stems (Fig. 1A) show a radial pattern similar to that described in other Arabidopsis accessions (Altamura et al.; 2001; Ye et al., 2002). From the outside to the inside, the inflorescence stem shows an epidermal cell layer, three layers of cortex and one layer of endodermis. Below the endodermis, eight or nine vascular bundles are arranged in a eustele, as they form a ring-like pattern around a pith of large parenchyma cells. Located between the vascular bundles, the interfascicular regions display three or four layers of thick interfascicular fiber cells adjacent to the endodermis (Fig. 1A and E). The vascular bundles have a polarized collateral pattern, with phloem close to the peripheral region of the inflorescence stem, xylem near the central region and the procambium positioned in-between (Fig. 1C).

The icu4-1 allele, a semidominant gain-of-function allele of the HD-Zip III family member ATHB13/CNA/ICU4, gives rise to the accumulation of large amounts of the mutant mRNA in the inflorescence stem (Ochando et al., 2006). The homozygous icu4-1 mutant shows an alteration in the shoot vascular pattern, consisting of a reduction in the number of vascular bundles compared with the wild type, as seen in inflorescence stems with only five or six bundles (Fig. 1B). This phenotype is shown with incomplete penetrance (Fig. 1I). Vascular bundles are generally larger in icu4-1, and many of them adopt a rectangular or trapezoidal shape instead of the common isosceles triangle shape observed in its wild-type ancestor En-2 (Fig. 1B and D). This is probably due to an increase of vascular tissues in the mutant bundles, which show extra layers of procambium, an overproliferated phloem, and a more modest increase of xylem, although the metaxylem contains very large vessels (Fig. 1D; Table 1). Transverse sections of En-2 and icu4-1 shoots display the same area (A_s; Table 1). Therefore, given that the average total area of vascular tissues per inflorescence stem is greater in the icu4-1 mutant (A_v; Table 1), the latter displays a conspicuous increase in the amount of vascular tissue related to the shoot area, as compared with En-2 (A_v/A_s; Table 1), indicating that the formation of large vascular bundles in icu4-1 is independent of shoot thickness. Considering the gain-of-function nature of the icu4-1 allele, this fact suggests that ICU4 promotes vascular tissue formation in the bundles. Another trait occasionally observed in the mutant is a poor lignification of interfascicular fiber cells (Fig. 1E and F).

Several classes of 35S-ICU4-G189D transgenic plants, which overexpress the mutant icu4-1 cDNA, were previously isolated (Ochando et al., 2006). One of them included plants with a leaf phenotype similar to that of the icu4-1 mutant. These transgenic

Fig. 2. Morphological phenotypic traits of double mutants involving the icu4-1 allele. 8-week-old (A) En-2 wild-type accession, (B) icu4-1/icu4-1, (C) phb-1D/PHB, (D) phb-1D/PHB;icu4-1/ICU, (E) fil-3/fil-3, and (F) fil-3/fil-3;icu4-1/icu4-1 plants. Scale bars indicate 5 mm in (C,D) and 1 cm in (A, B, E and F).
plants also showed the same phenotype of reduction in the number of vascular bundles than icu4-1 (Fig. 1G). Other 35S-ICU4-G189D transgenic plants of stronger phenotype showed radialized and trumpet-shaped leaves. The number of vascular bundles was similar to that of the En-2 accession in these latter plants, but the arrangement of cell types within the bundles was altered. The phloem adopted a rounded shape and, in most vascular bundles, was surrounded by xylem, so that there was a partial transformation of the wild-type collateral bundles to amphivasal types (Fig. 1H). A third class of 35S-ICU4-G189D transgenic plants showed completely radialized leaves and never flowered (Ochando et al., 2006).

icu4-1 interacts with mutations in adaxial and abaxial identity genes

Plants of the homozygous icu4-1 mutant show altered phyllotaxis and frequently exhibit two cauline leaves with their associated paraclades emerging from the same node (Fig. 2A and B), as previously described (Ochando et al., 2006). A semidominant allele of the PHB gene, phb-1D, transforms the abaxial tissues of lateral organs into adaxial tissues (McConnell and Barton, 1998). The phb-1D PHB plants are bushy due to the formation of extra axillary buds next to the ectopic adaxial leaf tissues, and exhibit a variable degree of radial symmetry in all lateral organs (Fig. 2C). Given the high similarity of ICU4 and PHB, and the molecular and phenotypic similarities of their semidominant alleles, we expected a mutual enhancement of the icu4-1 and phb-1D mutations. However, the phb-1D PHB:icu4-1/ICU4 diheterozygotes do not show the expected strong phenotype. Instead, these plants display a weaker phenotype (Fig. 2D), being less bushy and with fewer radialized organs than phb-1D PHB individuals, which indicates that icu4-1 partially suppresses the adaxial transformations caused by phb-1D. To determine whether the interaction between these two alleles also affects vascular patterning of shoots, we have compared cross-sections of heterozygous phb-1D PHB and double heterozygous icu4-1/ICU4,phb-1D PHB inflorescence stems. The number and position in the eustele of vascular bundles are both unaffected in phb-1D heterozygotes, although several bundles have an increased size and, more seldom, one or two of them may adopt a partial amphivasal pattern (Fig. 3A). Inflorescence stems of the double heterozygote also show a normal number and position of vascular bundles, but surprisingly these exhibit a conspicuous amphivasal pattern (Fig. 3B and C). Thus, unlike the adaxialized phenotype observed in lateral organs, in which icu4-1 antagonizes the effect of phb-1D, this amphivasal phenotype shows that, in the vascular bundles, there is a synergistic interaction between both alleles. These results indicate that the HD-Zip III genes ICU4 and PHB functionally interact in different ways depending on the organ, antagonistically in leaves and synergistically in vascular bundles.

Strong loss-of-function alleles of REV cause a reduction in the initiation of lateral shoot and flower meristems (Talbert et al., 1995; Otsuga et al., 2001). In addition, these alleles produce a mutant phenotype of the interfascicular fiber cells that normally develop adjacent to the endodermis, whose differentiation is blocked in rev homozygotes (Fig. 3F) (Zhong et al., 1997; Zhong and Ye, 1999). This phenotype, which is suppressed by the double homozygosis of null alleles in ICU4 and ATHB8 (Prigge et al., 2005), is also rescued in a double mutant carrying the

**TABLE 1**

|                      | Phloem Area$^a$ (µm²) | Procambium Area$^a$ (µm²) | Xylem Area$^a$ (µm²) | Stem Area (A) 3$^b$ (µm²) | Vascular Bundle Area (A) 2$^b$ (µm²) | AVB / A 2
|----------------------|----------------------|--------------------------|----------------------|--------------------------|-------------------------------|------------------
| wild-type            | 1035.53              | 456.09                   | 4356.5               | 45436.69                 | 68444.99                      | 0.152            
| (En-2)               | 165.96 ± 93.46       | 978.71 ± 165.96          | 16252.37 ± 27.25     | 42536.15 ± 0.018          | 18708.39 ± 0.018             | 0.101            
| icu4-1               | 293.64 ± 277.05      | 1315.65 ± 93.46          | 131194.8 ± 18078.39  | 454366.9 ± 0.190          | 84316.94 ± 0.190             | 0.190            

$^a$ Each value represents the average of ten icu4-1 and ten icu4-1 vascular bundles from three different inflorescence stems ± standard deviation.

$^b$ Each value represents the average of six ICU4 and six icu4-1 inflorescence stems ± standard deviation.

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**Fig. 3.** Histological and morphological phenotypic traits of double mutants involving the icu4-1 allele. (A) Cross-section through a phb-1D/PHB inflorescence stem. Arrow denotes a partially amphivasal bundle. (B) Cross-section through a phb-1D/PHB:icu4-1/ICU4 inflorescence stem. (C) A close-up view of a phb-1D/PHB:icu4-1/ICU4 amphivasal vascular bundle. (D) Cross-section through a fil-3/fil-3 inflorescence stem. (E) Cross-section through a fil-3/fil-3;icu4-1/ICU4 inflorescence stem. (F) Cross-section of a rev-6/rev-6 inflorescence stem showing the absence of normal interfascicular fiber cells. (G) Cross-section of a rev-6/rev-6;icu4-1/icu4-1 inflorescence stem showing the presence of thin interfascicular fiber cells. Scale bars indicate 50 µm in panel (C), 100 µm in panels (E-G) and 250 µm in panels (A, B and D).
icu4-1 allele and rev-6, a null allele of REV. Thus, icu4-1 rev-6 plants displayed interfascicular fiber cells in their normal position, although they showed the reduced thickness previously observed in icu4-1 (Fig. 3G). A similar result was obtained in a double mutant with the rev-7 allele (Talbert et al., 1995), which also differentiated icu4-1-like interfascicular fiber cells next to the endodermis (data not shown). These results suggest that overexpression by the icu4-1 allele can compensate for the loss of REV function, at least in the formation of interfascicular fiber cells.

The FIL gene, a YABBY family member, regulates the development of flower and inflorescence meristems, and the abaxial identity of lateral organs (Chen et al., 1999; Sawa et al., 1999). The hypomorphic fil-3 allele produces weak defects in flower formation and partial adaxialization of flower organs (Fig. 2E). This phenotype increases with the contribution of the icu4-1 allele. The double mutant shows low number of flowers, long pedicels and internodes, flowers subtended by cauline leaves, and stronger adaxialization of flower organs (Fig. 2F). Cross-sections of fil-3 inflorescence stems usually reveals six or seven vascular bundles (Fig. 3D). Transverse sections of icu4-1 fil-3 inflorescence stems also show a stronger phenotype. There are only three or four vascular bundles, numbers never observed in either of the two single mutants (Fig. 3E). The icu4-1 fil-3 double mutant was easily distinguished from F2 wild-type and single mutant plants, indicating that the increase in the mutant phenotype is not due to mixing accessions and/or loss of the Er allele. The double mutant with the icu4-1 allele reduces the number of bundles in the eustele, and demonstrates the participation of abaxial identity genes, like FIL, in the proper establishment of this pattern.

Effects of auxin on eustele organization

Many of the mutations in HD-Zip III genes affect processes regulated by auxin, suggesting a connection between the function of these genes and auxin signaling. One of the processes in which both pathways are involved is vascular development. Therefore, to verify a possible interaction between ICU4 and auxin signaling in the radial patterning of shoots, we investigated the effect of treatments with naphthylacetic acid (NAA), a synthetic auxin, on the phenotype of En-2 and icu4-1 shoots. To verify the efficacy of the treatment, we monitored the induction of the auxin-response genes IAA1 and IAA19 (Fig. 4). At 8 hours of NAA treatment, we observed induction of both genes in the En-2 accession. However, when the treatment was extended to 56 hours, there were no difference between NAA- and mock-treated plants, as seen by

![Fig. 4. Semiquantitative RT-PCR analysis of IAA1 and IAA19. Induction of the expression of IAA1 and IAA19 in leaves after 8 hours of naphthylacetic acid (NAA) treatment as compared to mock-treated (DMSO-treated) plants. Similar levels of IAA19 expression were detected in NAA-treated and control (DMSO-treated) plants after 56 hours of treatment. ACTIN2 (ACT2) expression was analyzed as an internal control.](image)

the similar levels of IAA19 mRNA, suggesting that the effect of NAA on the shoot radial pattern occurs early at bolting, coinciding with the beginning of the treatment.

Control plants with mock treatment showed the same phenotypes as previously described for En-2 and icu4-1 plants. En-2 plants treated with NAA phenocopied the inflorescence stem phenotype that characterizes the icu4-1 mutant. Five out of seven plants showed stems with only six bundles (Fig. 5A), while the remaining two plants showed stems with seven bundles. In these stems, some vascular bundles had the same aspect as those of icu4-1, exhibiting a rectangular or trapezoidal shape, and overproliferated procambium, phloem and xylem, as well as large metaxylem vessels (Fig. 5B). Treatment of icu4-1 plants with NAA enhanced the mutant phenotype. Most shoots had three (three out of seven shoots) or four (two out of seven shoots) large vascular bundles (Fig. 5C), numbers never seen in untreated icu4-1 plants. The remaining two plants showed five and seven vascular bundles. Treatment of heterozygous phb-1D plants with NAA resulted in a similar phenotype of reduction in the number of vascular bundles (Fig. 5D). As a whole, these results indicate that the shoot phenotypes produced by the wild-type, icu4-1 and phb-1D alleles are influenced by auxin, and suggest that HD-Zip III genes and the auxin signaling pathway might be collaborating in the establishment of the radial pattern of shoots.

As auxin has an essential role in lateral root formation and development (Casimiro et al., 2003), and HD-Zip III genes participate in the development of lateral roots (Hawker and Bowman, 2004), we studied the root system in homozygous icu4-1 and heterozygous phb-1D plants at 14 days after germination. The length of the primary root of the icu4-1 mutant showed an average of 6 cm, indicating a shortening as compared to the average of 8.4 cm observed in the En-2 accession (P<0.0001), as previously.
reported (Ochando et al., 2006), while the lengths of primary roots in the heterozygous phb-1D mutant and the Ler accession were not significantly different, with averages of 4.5 and 4.6, respectively (P>0.05). Both mutants showed a conspicuous reduction in the number of lateral roots, 1.9 lateral roots/cm in icu4-1 and 1.5 lateral roots/cm in phb-1D, as compared to 3.1 lateral roots/cm in En-2 (P<0.0001) and 3.2 lateral roots/cm in Ler (P<0.0001). Therefore, the icu4-1 and phb-1D alleles, in addition to their involvement in modifying vascular patterning, also alter the development of lateral roots.

Discussion

The ICU4 gene contributes to pattern establishment in the eustele

Our results provide evidence that the CNAI ICU4 gene, which codes for the HD-Zip III transcription factor ATHB15 (Prigge et al., 2005), promotes vascular development through the stimulation of procambial cell proliferation. This is consistent with a previous work on ICU4/ATHB15 and its Zinnia elegans ortholog ZehB-13, indicating that both genes are expressed in procambial cells, and suggesting that they might function in the differentiation or maintenance of these cells (Ohashi-Ito and Fukuda, 2003). Thus, homozygous plants for the semidominant gain-of-function icu4-1 allele, which disrupts the negative control of ICU4 by miR165/166, overexpress the gene (Ochando et al., 2006), and show more than twice the procambium, and a consequent increase of phloem and xylem, compared with the wild-type, supporting the notion that ICU4 promotes vascular development, as proposed for ATHB8, its closest paralog (Baima et al., 2001). Nevertheless, accurate quantification of a pattern element, like the amount of vasculature in shoots, should refer to the area of the developmental field in which such an element develops. Thus, the complexity of the leaf venation pattern has been quantified by the length and the number of vein branch points relative to the lamina area (Candela et al., 1999). We measured the total area of vascular bundles per inflorescence stem relative to the shoot area, finding that this value is approximately 25% greater in the homozygous icu4-1 mutant, which corroborates that this mutant produces more vascular tissue than the wild-type.

Most inflorescence stems of the En-2 accession show a eustele with eight vascular bundles, which are reduced to six or five in icu4-1 shoots, although with a penetrance that approaches 20%. This phenotype represents a true reduction in the number of components of this shoot pattern element, since: 1) we have never observed fewer than seven vascular bundles in inflorescence stems of En-2, 2) the reduced number of bundles is always observed and even decreased in some double mutant combinations, like icu4-1 fil-3, and 3) a recent work has shown that mATHB15 transgenic plants, which overexpress an ICU4 cDNA with silent mutations in the region complementary to miR165/166, also exhibit low number of vascular bundles and poor lignification of interfascicular fiber cells (Kim et al., 2005).

Auxin is involved in the establishment of vessel diameter and density along the plant axis (Aloni, 2004). Interestingly, the icu4-1 mutant overexpresses PINOID (PID) (Ochando et al., 2006), a gene that positively regulates the polar auxin flow. Thus, taking into account that vascular strands are induced along the preferred pathways of auxin flow, a higher rate of transport in the thick developing bundles of the icu4-1 mutant might deplete the auxin in the surrounding tissues that would be unable to differentiate new vascular elements, giving rise to the occasional reduction of bundle numbers. Alternatively, if there was a process of lateral inhibition by which vascular strands impeded the induction of additional bundles nearby through the action of an inhibitor, as suggested by the phenotype of the cov1 mutant (Parker et al., 2003), the thick vascular bundles of icu4-1 would produce high levels of the inhibitor, resulting in the reduced number of vascular bundles. Treatments of icu4-1 plants with the synthetic auxin NAA produced an enhancement of the mutant phenotype. We think that this finding supports the inhibitor hypothesis, as plants treated with NAA would have an excess of auxin that would preclude its depletion in the proximity of developing bundles.

The ICU4 gene contributes to the establishment of polarity in shoots

Unlike other mutants carrying semidominant alleles of HD-Zip III genes, icu4-1 never shows the transformation of the normal collateral organization of tissues in vascular bundles to an amphivasal pattern. Nevertheless, double heterozygous plants carrying the icu4-1 and phb-1D alleles exhibit a synergistic phenotype in their bundles, which show a remarkable amphivasal pattern, and some transgenic plants overexpressing the mutant icu4-1 cDNA (35S-ICU4-G189D transformants) display strongly adaxialized lateral organs (Ochando et al., 2006) and partially amphivasal vascular bundles (this work). These results indicate that the ICU4 product has both an adaxial activity and a function in the specification of the central-peripheral axis in shoot vascular bundles, as proposed for PHB and REV proteins (Emmery et al., 2003; Dinneny and Yanofsky, 2004; Zhong and Ye, 2004). That ICU4 and other HD-Zip III genes collaborate in the patterning of shoot vascular bundles is also suggested by the aberrant amphivasal bundles observed in inflorescence stems of triple mutants for null alleles of ICU4, PHB and PHV (Prigge et al., 2005).

Plants overexpressing a construct with silent mutations in the miRNA target (mATHB15 transgenic plants) showed reduced numbers of collateral bundles (Kim et al., 2005). This phenotype is similar to that of 35S-ICU4-G189D transgenic plants exhibiting weak adaxial transformations in their leaves. Other class of 35S-ICU4-G189D plants showing stronger leaf aberrations, which suggests that they might be expressing the transgene at higher levels, presented a partial transformation to amphivasal polarity in their bundles. Thus, moderate expression of an miRNA-resistant form of ICU4 might cause reduced numbers of collateral bundles (mATHB15 plants, 35S-ICU4-G189D plants with weak adaxial transformations and some icu4-1 stems), while a higher expression would produce a change in the polarity of the bundles (35S-ICU4-G189D plants with stronger adaxial transformations). This suggests the participation of a mechanism of positional information for establishing the number of vascular bundles and their characteristic collateral pattern, which involves the transcriptional activity of ICU4 and other HD-Zip III genes, and their post-transcriptional regulation by miRNAs, which affects both the spatial expression of the genes and the abundance of transcripts. As suggested by the phenotypes of plants treated with NAA, this process would be influenced by auxin, which has been previously shown to interact with KANADI and HD-Zip III genes for pattern...
formation along the central-peripheral axis of the embryo (Izhaki and Bowman, 2007) and to induce the expression of several HD-Zip III genes, including ICU4 and PHB (Zhou et al., 2007).

Synergetic and antagonistic interactions between mutant alleles of HD-Zip III genes

A recent report has described the complexity of the interactions among HD-Zip III genes (Prigge et al., 2005). Our results make new contributions to this line of work. We have shown that icu4-1 partially suppresses the abaxial-to-adaxial transformations caused by phb-1D in lateral organs, which suggests that the ICU4 product, though itself showing adaxial activity (Ochando et al., 2006), antagonizes the function of the PHB transcription factor in establishing adaxial identity in lateral organs. However, we observed a synergistic interaction between both alleles in shoot vascular bundle patterning, which suggests that ICU4 and PHB have overlapping functions in the bundles. Although there is a common mechanism at work to specify the adaxial-abaxial polarity in lateral organs and the pattern of shoot vascular bundles (Emery et al., 2003; Zhong and Ye, 2004), these results point out that there must be some differences between both processes. Moreover, we have seen that the icu4-1 allele is epistatic over loss-of-function alleles of REV, playing the same role as the double combination of null alleles of CNAI/ICU4 and ATHB8 in eliminating the Rev- phenotype (Prigge et al., 2005). Therefore, the overproduction of ICU4 caused by icu4-1 compensates for a loss of REV function, in agreement with the modest suppression of the Rev- phenotype by the FREV:CNA construct, which expresses the ICU4 wild type cDNA under the control of the REV promoter (Prigge et al., 2005).

These results pose the question of why loss-of-function and gain-of-function mutations in HD-Zip III genes produce similar phenotypes, such as the amphivalval vascular bundles seen in the double heterozygous icu4-1 phb-1D and the cna phb phv triple null mutant, or the rescue of the Rev- phenotype by cna/icu4 alleles with different genetic behaviors. Based on the ability proposed for HD-Zip III transcription factors to dimerize (Sessa et al., 1998; Ohashi-Ito et al., 2002), we suggest that the production of homo and heterodimers, with different activities depending on the protein(s) involved in the dimers, might account for the complex interactions shown by HD-Zip III genes. In this context, the phenotype of specific mutants should result from the types of dimers formed in the different tissues, which would depend ultimately on the relative level of each HD-Zip III product. This hypothesis would imply that HD-Zip III transcription factors are not fully equivalent in function, as has already been suggested (Prigge et al., 2005). However, although the formation of functionally different dimers might shed light on the behavior of HD-Zip III genes, given that the mechanism of action of these genes appears to be more resistant to loss-of-function mutations than to gain-of-function mutations, further studies will be needed to ascertain how HD-Zip III genes interact to establish developmental processes in specific organs.

Materials and Methods

Plant materials and growth conditions

Several Arabidopsis thaliana (L.) Heyhn. lines studied in this work were supplied by the Nottingham Arabidopsis Stock Centre (NASC). These included the Enkheim-2 (En-2) wild-type, the icu4-1 mutant (N400) in an En-2 genetic background (Serrano-Cartagena et al., 2000), the mutant phb-1D (N3761) (McConnell and Barton, 1998) in the Ler accession, and rev-1 (N3826) (Talbert et al., 1995), in the No-0 accession. The ff-3 mutant, in a Ler genetic background, was provided by G. Drews (Chen et al., 1999), and the rev-6 mutant in a Col background by S. E. Clark (Otsuga et al., 2001; Prigge et al., 2005). The kanamycin-resistant transgenic line 35S::ICU4::GUS189D overexpresses the mutant icu4-1 cDNA (Ochando et al., 2006).

Seeds were first sterilized by washing for 8 minutes in 40% commercial bleach with 0.1% Triton X-100, followed by four washes with sterile water. The seeds were then spread in Petri dishes containing GM (Germination Medium: 0.5 X Murashige-Skoog with 1% sucrose) supplemented with 50 µg/ml kanamycin when required. To assist uniform germination, seeds were kept at 4°C for 24 hours in the dark. Plants were grown in a Sanyo MLR-350H growth chamber under constant cool-white fluorescent light (7000 lux). All strains were grown at 21°C, with the exception of those containing the phb-1D allele that were grown at 18°C. Three weeks after germination, plants were transferred to pots containing a 2:2:1 mixture of perlite, vermiculite and sphagnum moss, and watered twice a week with a mineral nutrient solution (Lincoln et al., 1990).

For auxin experiments, plants were grown on GM, and 15 days after sowing they were transferred to pots and watered with the mineral nutrient solution supplemented with 0.1, 0.5 or 1 µM of naphthylacetic acid (NAA, Duchefa Biochemie). All the solutions were adjusted to contain the same volume of dimethyl sulfoxide (DMSO), used as a solvent for NAA. Results are presented for the highest concentration, 1 µM NAA. Similar, though weaker, phenotypes were obtained when lower concentrations were used. Control plants were watered with the mineral nutrient solution supplemented with DMSO.

Semi quantitative RT-PCR (SORT-PCR)

Plants were grown as indicated above and total RNA was isolated from leaves using the Nucleospin RNA plant kit (Macherey-Nagel) according to the manufacturer’s instructions. Reverse transcription was performed as previously described (Ripoll et al., 2006). cDNA fragments were amplified using the following primers: IAA1, 5’-ggattacgccccgaccaag and 5’-ggagctcgcctcatcac (Yamazoe et al., 2005); IAA19, 5’-llgggcacatgcctgcaag-3’ and 5’-tcagcgtgtacctctag-3’ (Perrin et al., 2005). After 25 PCR cycles, the amplification products (208 bp for IAA1 and 150 bp for IAA19) were run in 2% agarose gels and visualized by ethidium bromide staining. ACTIN2 (ACT2) expression was monitored as an internal control (An et al., 1996).

Genetic interactions

With the exception of phb-1D PHB plants, homozygous parental lines were cross-fertilized. Double mutants were identified among the F2 segregants by a differential phenotype, and confirmed by self-pollination of putative double mutants and production of F3 non-segregating progenies. In every F2 segregation involving parental lines with different accession backgrounds we chose five double mutants and five single homozygous mutants for each allele involved for further analysis, in order to discard a possible effect of mixing backgrounds on the observed phenotypes. To obtain the icu4-1::ICU4::phb-1D PHB plants, the icu4-1 mutant was fertilized with pollen from the phb-1D PHB heterozygote. These double heterozygous plants were compared with mutant F1 plants from a cross between phb-1D PHB and En-2.

Microscopy

For plastic sections, tissue was vacuum infiltrated with FAE (45% ethanol, 5% glacial acetic acid, 1.85% formaldehyde, 1% Triton X-100) and fixed for 2 hours at room temperature. Samples were dehydrated through an ethanol series (70%, 80%, 90% and 95%) and embedded in JB4 resin (Electron Microscopy Sciences). Sections 3-4 µm were cut with a Microm HM350S microtome, stained with 0.1% toluidine blue and observed using an ECLIPSE E800 microscope (Nikon) equipped with a
COLORVIEW-III digital camera (Nikon). Images were analyzed with the analySIS software (Soft Imaging System GmbH).

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