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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

Abbreviations used in this paper: CNA, CORONA; HD-Zip III, class III HD-Zip gene; ICU, INCURVATA; NAA, naphthylacetic acid; PHB, PHABULOSA; PHV, PHAVOLUTA; REV, REVOLUTA.

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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*)/*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-

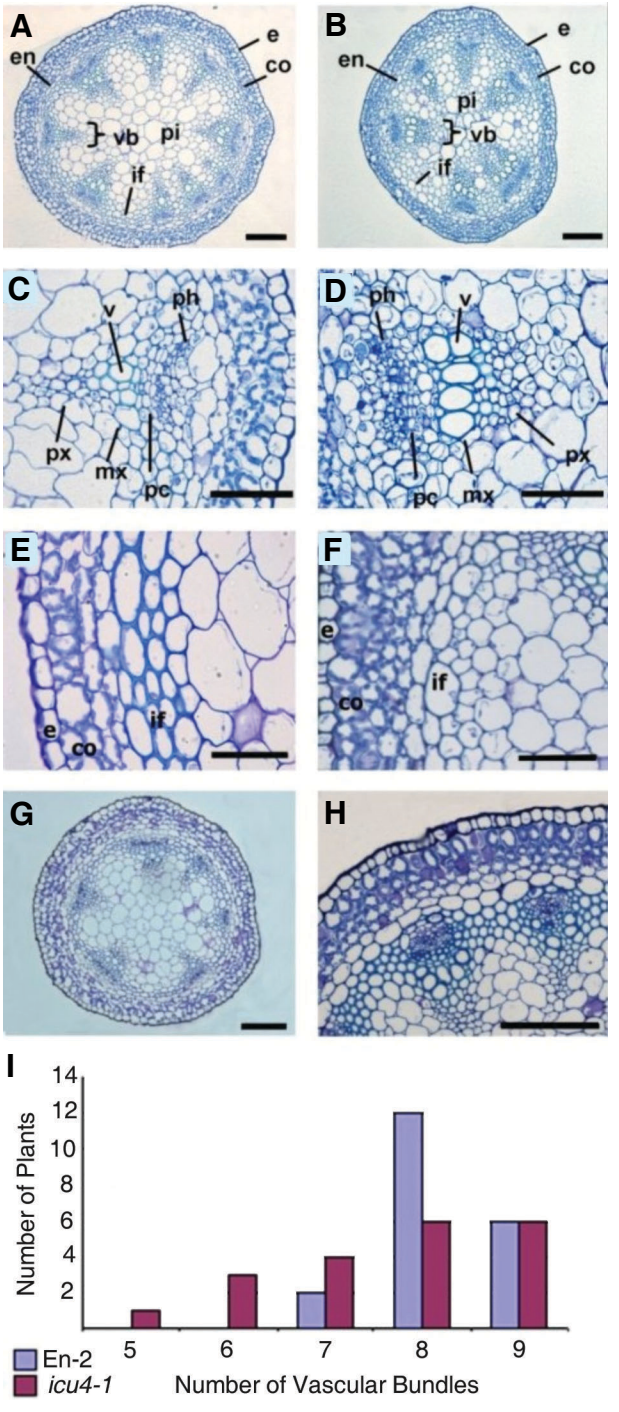

Fig. 1. Effects of the *icu4-1* mutation on vascular development in inflorescence stems. *Sections were taken from the middle portions of inflorescence stems of 60 day-old plants.* **(A)** *Transverse section of a wild-type (En-2) inflorescence stem showing nine vascular bundles.* **(B)** *Transverse section of an* icu4-1 *inflorescence stem showing six vascular bundles.* **(C)** *A close-up view of an En-2 vascular bundle.* **(D)** *A close-up view of an* icu4-1 *vascular bundle.* **(E)** *A close-up view of En-2 interfascicular fiber cells.* **(F)** *A close-up view of* icu4-1 *interfascicular fiber cells showing thin secondary walls.* **(G)** *Transverse section of the inflorescence stem from a transgenic plant harboring the 35S-*ICU4*-G189D transgene, showing a phenotype reminiscent to that of* icu4-1. **(H)** *Transverse section of the inflorescence stem from a transgenic plant harboring the 35S-*ICU4*-G189D transgene, showing a partial transformation to amphivasal polarity in the vascular bundles.* **(I)** *Number of vascular bundles were counted from 20 inflorescence stems from each genotype, wild-type (En-2) and* icu4-1. *co, cortex; e, epidermis; en, endodermis; if, interfascicular fibers; mx, metaxylem; ph, phloem; pi, pith; pc, procambium; px, protoxylem; v, vessel element; vb, vascular bundle. Scale bars indicate 100 μm in panels (A, B, G and H), and 50 μm in panels (C-F).*

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 */CU4* (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 *ATHB15*/*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_{VB}; 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_{VB}/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 */CU4* 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-*/CU4*-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

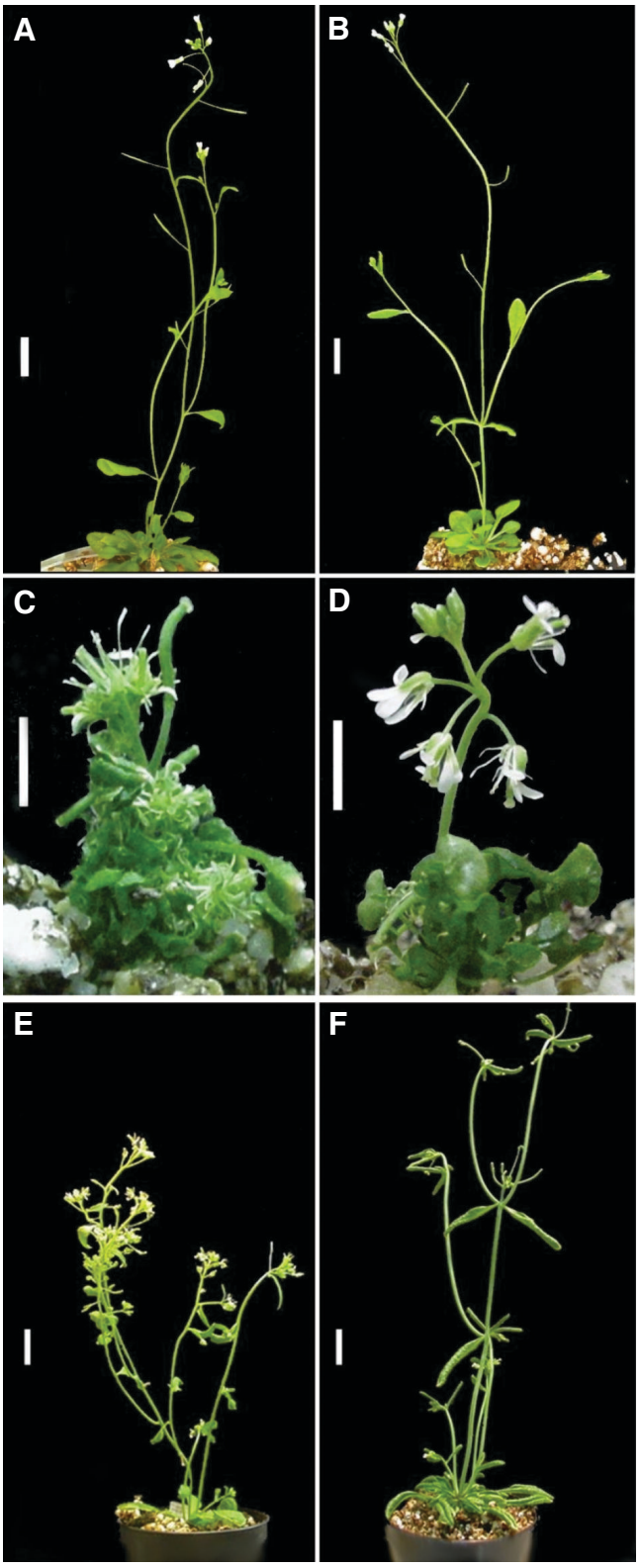

Fig. 2. Morphological phenotypic traits of double mutants involving the *icu4-1* allele. *8-week-old* **(A)** *En-2 wild-type accession,* **(B)** icu4-1/icu4-1, **(C)** phb-1D/PHB, **(D)** phb-1D/PHB;icu4-1/ICU, **(E)** fil-*3*/fil-3, *and* **(F)** fil-*3*/fil-3;icu4-1/icu4-1 *plants. Scale bars indicate 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).

TABLE 1

HISTOLOGICAL ANALYSIS OF INFLORESCENCE STEMS

	Phloem Area[a] (μm²)	Procambium Area[a] (μm²)	Xylem Area[a] (μm²)	Stem Area (A_S)[b] (μm²)	Vascular Bundle Area (A_{VB})[b] (μm²)	A_{VB} / A_S
wild-type (En-2)	1035.53 ± 165.96	456.09 ± 93.46	4356.5 ± 978.71	454366.9 ± 162523.7	68444.99 ± 22536.15	0.152 ± 0.018
icu4-1	1597.96 ± 293.64	1123.05 ± 277.05	5920.36 ± 1315.65	451585.9 ± 131194.8	84316.94 ± 18708.39	0.190 ± 0.018

[a] Each value represents the average of ten *ICU4* 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.

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.

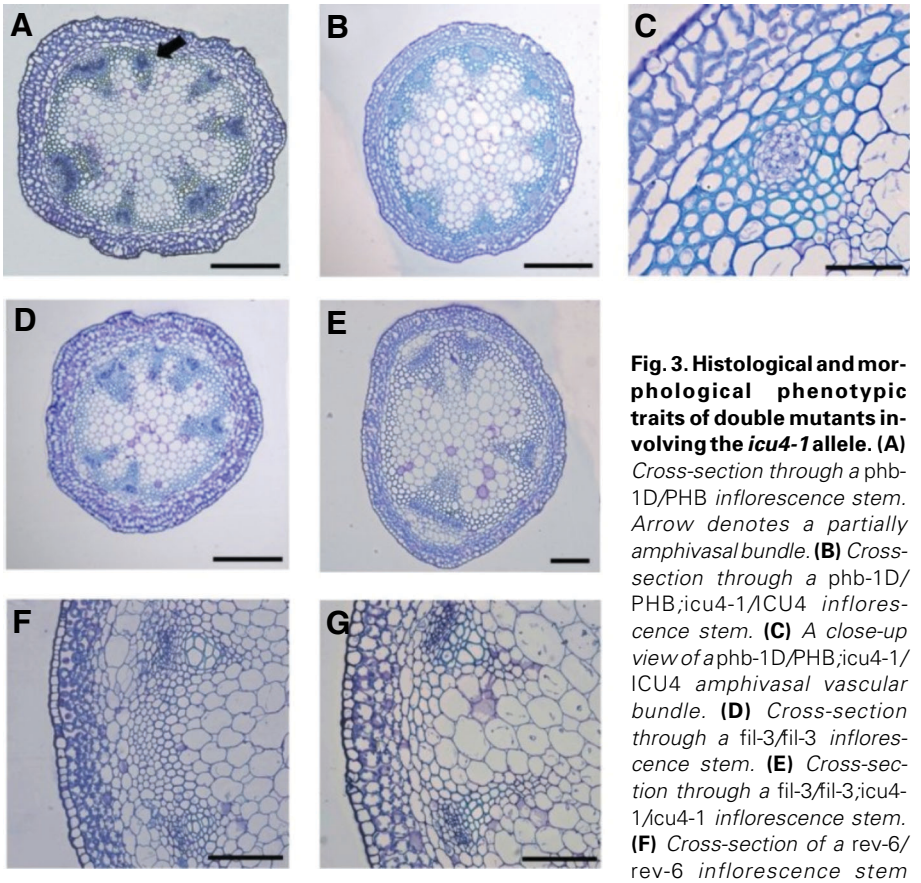

Fig. 3. Histological and morphological phenotypic traits of double mutants involving the *icu4-1* allele. (A) *Cross-section through a* phb-1D/PHB *inflorescence stem. Arrow denotes a partially amphivasal bundle.* **(B)** *Cross-section through a* phb-1D/PHB;icu4-1/ICU4 *inflorescence stem.* **(C)** *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/icu4-1 *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).*

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

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-1* 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 F_2 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⁻ phenotype of *fil-3* plants. The low number of vascular bundles seen in *icu4-1 fil-3* stems shows again that misregulation of *ICU4* by 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

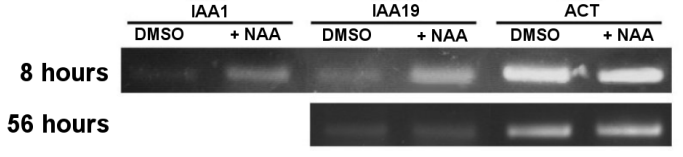

Fig. 4. Semiquantitative RT-PCR analysis of *IAA1* and *IAA19*. *Induction of the expression of* IAA1 *and* IAA19 *in leaves after 8 hours of naphthylacetic acid (NAA) treatment as compared to mock-treated (DMSO-treated) plants. Similar levels of* IAA19 *expression were detected in NAA-treated and control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

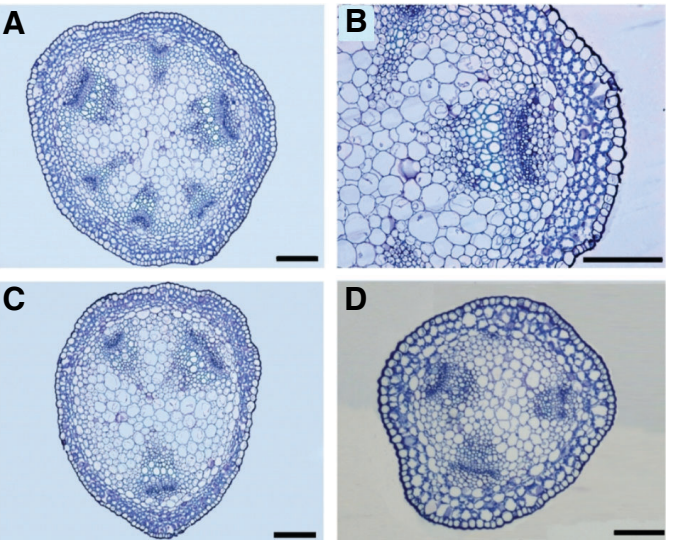

Fig. 5. Effects of naphthylacetic acid (NAA) treatments. (A) *Transverse section of an En-2 inflorescence stem treated with 1 μM NAA, showing a reduced number of large collateral bundles.* **(B)** *A close-up view of a vascular bundle from (A).* **(C)** *Transverse section of an* icu4-1 *inflorescence stem treated with 1 μM NAA, showing an enhancement of the mutant phenotype.* **(D)** *Transverse section of a* phb-1D *inflorescence stem treated with 1 μM NAA, showing only three vascular bundles. Scale bars indicate 100 μm.*

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 L*er* 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 L*er* (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 *CNA*/*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).

Synergistic 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 *CNA*/*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 P_{REV}:*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 amphivasal 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 L*er* accession, and *rev-1* (N3826) (Talbert *et al.*, 1995), in the No-0 accession. The *fil-3* mutant, in a L*er* 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*-G189D 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 gown 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.

Semiquantitative RT-PCR (SQRT-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'-ggattacccggagcacaag and 5'-ggagctccgtccatactcac (Yamazoe *et al.*, 2005); *IAA19*, 5'-tggtgacaactgcgaatacg-3' and 5'-tcactcgtctactcctctag-3' (Perri *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 F_2 segregants by a differential phenotype, and confirmed by self-pollination of putative double mutants and production of F_3 non-segregating progenies. In every F_2 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 F_1 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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