Mutations in the MicroRNA Complementarity Site of the **INCURVATA4** Gene Perturb Meristem Function and Adaxialize Lateral Organs in Arabidopsis\(^{1\text{W}}\)

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Here, we describe how the semidominant, gain-of-function icu4-1 and icu4-2 alleles of the **INCURVATA4** (**ICU4**) gene alter leaf phyllocladaxis and cell organization in the root apical meristem, reduce root length, and cause xylem overgrowth in the stem. The **ICU4** gene was positionally cloned and found to encode the ATHB15 transcription factor, a class III homeodomain/leucine zipper family member, recently named CORONA. The icu4-1 and icu4-2 alleles bear the same point mutation that affects the microRNA complementarity site of **ICU4** and is identical to those of several semidominant alleles of the class III homeodomain/leucine zipper family members **PHABULOSA** and **PHAVOLUTA**. The icu4-1 and icu4-2 mutations significantly increase leaf transcript levels of the **ICU4** gene. The null *lst-1* allele of the **HASTY** gene, which encodes a nucleocytoplasmic transporter, synergistically interacts with icu4-1, the double mutant displaying partial adaxialization of rosette leaves and carpels. Our results suggest that the **ICU4** gene has an adaxializing function and that it is down-regulated by microRNAs that require the **HASTY** protein for their biogenesis.

MicroRNAs (miRNAs) are small regulatory RNAs present in organisms as diverse as plants and humans. In plants, most of the miRNAs studied guide cleavage of their mRNA targets after miRNA-mRNA pairing. In Arabidopsis (*Arabidopsis thaliana*), miRNAs perfectly or almost perfectly match their mRNA targets, which prompted several authors to perform computational analyses to predict miRNA targets. Many putative miRNA target genes found in this way encode transcription factors that control specific aspects of plant development (for review, see Bartel and Bartel, 2003; Bartel, 2004; Chen, 2005; Du and Zamore, 2005; Jover-Gil et al., 2005; Kim, 2005).

Class III homeodomain/leu zipper (HD-Zip III) genes share a conserved miRNA complementarity site, which is cleaved after miRNA-mRNA pairing (for review, see Bowman, 2004). Three well-known HD-Zip III family members are **PHABULOSA** (**PHB**; McConnell and Barton, 1998), **PHAVOLUTA** (**PHV**; McConnell et al., 2001), and **REVOLUTA** (**REV**, also known as **IFL1** and **AVB1**; Alvarez, 1994; Zhong and Ye, 1999; Zhong et al., 1999). The expression of **PHB**, **PHV**, and **REV** is generalized in the incipient leaves, but becomes restricted to the adaxial domain after primordium emergence (Eshed et al., 2001; McCall et al., 2001; Emery et al., 2003; Heisler et al., 2005). The absence of **PHB**, **PHV**, and **REV** from the abaxial domain of early leaves allows expression of the **KANADI** (**KAN**; Eshed et al., 1999; Kerstetter et al., 2001) and **YABBY** (**YAB**; Siegfried et al., 1999; Kumaran et al., 2002; Emery et al., 2003) abaxializing proteins. These recent studies extended classical surgical experiments (Sussex, 1954) and provided evidence that certain genes are required in lateral organs to specify the identities of their adaxial and abaxial domains.

**HASTY** (**HST**) is the Arabidopsis ortholog of the genes encoding mammalian exportin 5 and MSN5 of yeast (*Saccharomyces cerevisiae*), two importin β-like receptors (Telfer and Poethig, 1998; Bollman et al., 2003). Because human exportin 5 exports miRNA precursors from the nucleus to the cytoplasm (Gwizdek et al., 2003; Yi et al., 2003; Bohmsack et al., 2004), a similar role has been proposed for **HST** (Hunter and Poethig, 2003).

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based on its localization at the nuclear periphery and
the phenotypic effects of hst alleles, which are remi-
niscent of those caused by mutations in genes of the
miRNA pathway. However, the demonstration that
miR159 precursors are cleaved within the nucleus
(Papp et al., 2003) suggested that HST might instead
export mature miRNAs to the cytoplasm. Further-
more, although loss of function of HST causes a
generalized reduction of miRNA levels, it does not cause
miRNAs to accumulate in the nucleus (Park et al., 2005).

In an attempt to identify genes required for leaf
morphogenesis, we searched for Arabidopsis leaf mu-
tants (Berná et al., 1999; Serrano-Cartagena et al., 1999),
some of which displayed involute leaves, a phenotype
that we named Incurvata (Icu). Two of these mutants, icu4-1 and icu4-2, previously isolated by G. Röbbelen
(Bürger, 1971; Kranz, 1978), were found to be allelic
and semidominant and to synergistically interact with
hst alleles (Serrano-Cartagena et al., 2000). Here, we
describe the positional cloning of the ICU4 gene, which
codes the HD-Zip III transcription factor ATHB15,
also named CORONA (CNA; Green et al., 2005; Prigge
et al., 2005). Our results suggest that the ICU4 gene is
down-regulated by miRNAs that require the HST pro-
tein for their biogenesis and that it encodes a protein
with adaxializing activity.

RESULTS
Positional Cloning of the ICU4 Gene

We followed a map-based strategy for cloning the
ICU4 gene, which we previously mapped to chromo-
some 1 between the T27K12-Sp6 and nga128 micro-
satellite markers (Serrano-Cartagena et al., 2000). The
genotyping of 130 icu4-1 homozygotes selected from
an F2 mapping population derived from a Columbia-0
(Col-0) × icu4-1/icu4-1 cross allowed us to narrow
down the candidate region to five overlapping bacte-
rial artificial chromosome clones (Fig. 1A). One such
bacterial artificial chromosome, F5F19, contained the
At1g52150 gene, a member of the HD-Zip III family
also known as CNA and ATHB15. Because semidom-
inant alleles of other genes of this family perturb leaf
morphology, we sequenced the gene in the icu4-1 and
icu4-2 mutants. Both alleles were found to carry the
same point mutation, a G-to-A transition, in the fifth
position of exon 5, within a complementarity site for
the miR165 and miR166 miRNAs (Fig. 1, A and B).
This mutation is predicted to cause a Gly-to-Asp amino
acid change within the sterndiogenetic acute regulatory-
related lipid transfer (START; Ponting and Aravind,
1999) domain, which is highly conserved in HD-Zip
III family members of Arabidopsis and maize (Ze a m a y s;
Fig. 1, B and C; McConnell et al., 2001; Rhoades et al.,
2002; Juarez et al., 2004). The mutation found in icu4-1
and icu4-2 is identical to those of semidominant alleles
of the PHB and PHV genes (Fig. 1B; McConnell et al.,
2001) and different from those of semidominant alleles
of the REV gene of Arabidopsis and rolled leaf1 (rld1) of
maize. All these mutations impair pairing of miR165/
166 and their targets (Emery et al., 2003; Juarez et al.,
2004; Zhong and Ye, 2004; Fig. 1B). We did not find
additional mutations in the icu4-1 and icu4-2 mutants
compared to their wild-type ancestor Enkheim-2
(En-2) after sequencing all the exons and introns of the
At1g52150 gene and several hundred base pair
upstream of the initiation codon and downstream of
the stop codon. Given that there is little information on
their isolation by Röbbelen, we cannot rule out that
icu4-1 and icu4-2 represent two independent isolations
of a single mutation.

As indicated in http://www.ncbi.nlm.nih.gov/
UniGene, the At1g52150 gene is 4,968 bp long and
includes 18 exons. Its transcriptional activity is sup-
ported by six full-length Col-0 cDNA sequences de-
posited in databases. Its predicted protein product
contains the three domains characteristic of HD-Zip
III family members (Schrick et al., 2004; Fig. 1C): a
homeodomain (HOX; residues 17–77), a basic region
Leu zipper (bZIP) domain (69–115), and a START
lipid-binding domain (152–366). Comparison of the
At1g52150 sequence in several wild-type lines revealed
a high degree of polymorphism. We found that resi-
dues in positions 181, 238, 622, and 629 are Val, Gln,
Gln, and Ala, respectively, in Col-0, but Ile, Asp, Leu,
and Thr in En-2 and ecotype Landsberg erecta (Ler).
Residue 641 was Ile in Col-0 and Ler, but Thr in En-2.
Of these, only the Val residue at position 181 was con-
served among the members of the HD-Zip III family.

Functional Nature of icu4 Mutations

The most conspicuous phenotypic trait of the icu4-1
and icu4-2 mutants is rosette leaf incurvature (i.e. the
lamina curls upward; Fig. 2, A–C and E–G; Serrano-
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icu4-1 heterozygotes (Fig. 2, C and G) was weaker than
in icu4-1/icu4-1 homozygotes (Fig. 2, B and F), but still
clearly distinguishable from the wild type (Fig. 2, A and
E), particularly at early stages of leaf expansion (Fig.
2G). The expressivity of the leaf phenotype was variable
in ICU4/icu4-1 heterozygotes, ranging from slightly
affected plants with only the first two leaves incurved,
to plants having all the rosette leaves with incurved margins and an irregular lamina surface (Fig. 2C).

We performed a dosage analysis to ascertain whether
the semidominance of the icu4-1 allele results from a gain-of-function mutation or, alternatively, from a loss-
of-function mutation at a haploinsufficient locus. To
this end, we crossed the tetraploid line CS3151 to
icu4-1/icu4-1 plants and studied the phenotype of the
resulting triploid F1 progeny. Incurvature was ob-
served in the expanding leaves of ICU4/ICU4/icu4-1
triploids (Fig. 2, D and H) similar to that displayed by
ICU4/icu4-1 diploid plants (Fig. 2, C and G). The
leaves of the triploid progeny of CS3151 × En-2 con-
trol crosses were not incurved, confirming that the phe-
notype of ICU4/ICU4/icu4-1 was an effect of a gain
of function in the icu4-1 allele.

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In addition, an RNA interference (RNAi) construct for the At1g52150 gene (35S::ICU4-RNAi) was transferred to icu4-1/icu4-1 plants. Six primary transformants for the 35S::ICU4-RNAi construct displayed flattened leaves similar to those of the wild type, a phenotypic trait that cosegregated with the construct in the T2 and subsequent generations (Fig. 2I), which is consistent with the hypothesis that icu4-1 and icu4-2 are gain-of-function mutations. The ability of the transgene to suppress the mutant phenotype further supports the idea that the icu4 mutations affect the expression of the At1g52150 gene. En-2 plants transformed with the 35S::ICU4-RNAi construct did not show any mutant phenotypic trait.

We searched for insertional, putatively null alleles of ICU4 in public collections (see “Materials and

Figure 1. Cloning and structural analysis of the ICU4 gene. A, Map-based cloning of the ICU4 gene, with indication (in parentheses) of the number of informative recombinants found relative to each of the markers used for linkage analysis. B, Complementarity site for the miR165 and miR166 miRNAs in the mRNA of the rld1 gene of maize and those of HD-Zip III family genes of Arabidopsis. The effects of some gain-of-function mutations disrupting the miR165/166 complementarity site is shown for both mRNA and protein sequences (McConnell et al., 2001; Emery et al., 2003; Juarez et al., 2004; this work). The amphivasal vascular bundle 1 (avb1; Zhong and Ye, 2004) mutation is identical to rev-10d. C, Alignment of HD-Zip III family proteins in a Col-0 background. The dashed, continuous, and dotted lines indicate the HOX, bZIP, and START domains, respectively. Identical and similar residues are colored in black and gray, respectively.
Methods”) and found two, icu4-3 and icu4-4, which did not cause any mutant phenotype in homozygosis, neither on their own nor in double-mutant combinations with null alleles of the ATHB8 gene (data not shown). This result is also in close agreement with the behavior of our RNAi construct, as well as with the lack of a discernible phenotype recently found in homozygotes for the null cna-2 allele (Prigge et al., 2005). Our results and those of Prigge et al. (2005) are in striking contrast with those of Kim et al. (2005), who recently reported severe phenotypic alterations in transgenic plants bearing an antisense ATHB15 construct.

To further analyze the involvement of At1g52150 on the phenotype of icu4 mutants, the cDNAs of the wild-type ICU4 and mutant icu4-1 (ICU4-G189D) alleles were fused to the constitutive cauliflower mosaic virus 35S promoter and transferred to the En-2 wild type. All transgenic plants overexpressing the wild-type ICU4 cDNA were late flowering but displayed almost normal leaf morphology (data not shown). In contrast, we identified two classes of transgenic plants overexpressing the icu4-1 mutant cDNA, one of them including five phenotypically wild-type lines that might result from the silencing of the transgene. The remaining 12 35S::ICU4-G189D transformants were late flowering or did not flower at all, and exhibited a wide spectrum of mutant phenotypes, ranging from four lines with moderately incurved leaves (Fig. 2J) to more severely affected plants, showing radialized leaves (Fig. 2L) or an intermediate phenotype with radialized and trumpet-shaped leaves (Fig. 2K), which were apparently adaxialized. The most affected transformants (Fig. 2L, three primary transformants) never flowered. Five primary transformants with an intermediate phenotype also displayed partially radialized floral organs (Fig. 2, M and N) and abnormal pistils, resulting in sterility.

The four 35S::ICU4-G189D lines with moderate phenotype were very similar to the icu4-1 mutant and the mATHB15 transgenic plants (Kim et al., 2005), which overexpress a construct carrying silent mutations in its miRNA complementarity site. The very strong phenotypes of some of our 35S::ICU4-G189D transgenics are likely to be due to the 5′- and 3′-untranslated sequences, which might cause expression of the transgene at high levels and were not completely incorporated into mATHB15 (Kim et al., 2005).

The Morphological Phenotype of icu4 Mutants

The phenotype of icu4-1 and icu4-2 mutants was pleiotropic and more severe at 25°C than at 20°C (data not shown), as described for semidominant phb-1d alleles (McConnell and Barton, 1998). Some icu4-1 and icu4-2 homozygotes, when grown at 25°C, displayed two inflorescences that bolted simultaneously.
Characterization of the Arabidopsis INCURVATA4 Gene

(data not shown), suggesting that the shoot apical meristem had split during the vegetative phase. The epidermis of $icu4-1/icu4-1$ leaves contained smaller pavement cells and more stomata than the wild type, as observed on both sides of the leaves by scanning electron microscopy (Fig. 3, A–D). Finger-like outgrowths were occasionally observed on the abaxial surface of mutant leaves (data not shown). In addition, all trichomes found on the adaxial epidermis of $icu4-1$/ $icu4-1$ vegetative leaves had supernumerary branches (from four to six; Fig. 3E). Interestingly, the abaxial epidermis of all $icu4-1$/ $icu4-1$ vegetative leaves completely lacked trichomes. Mesophyll cell size was normal, as shown by confocal microscopy (data not shown). Most cauline leaves of $icu4-1$ homozygotes were incurved, although in some cases its lamina curled down, whereas the margin and the apex curled up (Fig. 3, F and G).

The ICU4 gene has previously been shown to be expressed in the vasculature, as seen in plants carrying a pATHB-15::β-glucuronidase (GUS) transgene (Ohashi-Ito and Fukuda, 2003). To test whether the gain-of-function $icu4-1$ mutation altered the leaf venation pattern, we studied the expression of the pATHB-8-GUS reporter transgene, which is restricted to provascular cells and has previously been used to characterize the development of the vascular system in leaves and stems (Baima et al., 1995; Kang and Dengler, 2002; Kang et al., 2003). The pattern of GUS staining revealed no differences between the wild-type and $icu4-1$/ $icu4-1$ mutant plants (data not shown).

Figure 3. Some morphological and ultrastructural phenotypic traits of $icu4-1$/ $icu4-1$ homozygous plants. A to E, Scanning electron micrographs are shown of the adaxial (A and B) and the abaxial (C and D) epidermis of En-2 (A and C) and $icu4-1$/ $icu4-1$ (B and D) first leaves, and a four-branched $icu4-1$/ $icu4-1$ trichome (E). F and G, Wild-type En-2 (F) and incurved $icu4-1$/ $icu4-1$ cauline leaves (G). H and I, Cauline leaf and axillary shoot formation in En-2 (H) and $icu4-1$/ $icu4-1$ (I) plants. J, Different degrees of late flowering in $icu4-1$/ $icu4-1$ individuals. K to M, En-2 and $icu4-1$/ $icu4-1$ roots grown in vertically oriented plates (K), which are magnified (L and M), respectively. N to P, Confocal micrographs of propidium iodide-stained En-2 (left) and $icu4-1$/ $icu4-1$ (right) root tips (N), with a drawing of the founder and cortex initial cells. Transverse sections of wild-type (O) and $icu4-1$/ $icu4-1$ (P) stems, if, Interfascicular fibers; mx, metaxylem; pc, procambium; ph, phloem; px, protoxylem; vb, vascular bundle. Scale bars indicate 10 μm (A–D), 100 μm (E, O, and P), 1 cm (J), 2 mm (F, G, and K), 1 mm (H and I), 500 μm (L and M), and 20 μm (N). Pictures were taken 21 (A–E), 30 (F–I), 50 (J), 8 (K–M), and 5 (N) d after sowing.
The phyllotaxis was found to be altered in icu4-1/icu4-1 rosettes. In the wild type, the divergence angle between the first and second rosette leaves is 180° and approaches 137.5° for the remaining leaves (Kang et al., 2003). In contrast, we observed a 180° angle until the fifth or sixth leaf in icu4-1/icu4-1 rosettes (Fig. 2, B and C), extending the number of nodes with subdecussate phyllotaxis, which is characteristic of juvenile leaves. The frequent presence of two cauline leaves, with their associated axillary shoots, arising from a single node suggests that the allocation of cells to make cauline leaves is also altered in icu4-1 (Fig. 3, H and I). Because phyllotactic defects are often associated with abnormal size or shape of the meristem, we compared icu4-1/icu4-1 and wild-type meristems, finding no differences between them (Supplemental Fig. 1).

The icu4-1 and icu4-2 homozygotes were late flowering. Bolting was found to occur 24.9 ± 3.9 d after sowing in En-2 (n = 25 plants), but 39.3 ± 2.6 d after sowing in icu4-1/icu4-1 plants (n = 35). Some of the latter bolted as late as 50 d after sowing (Fig. 3J). As expected, the delayed flowering was correlated with an increase in the number of rosette leaves, which was 14.9 ± 2.0 for En-2 (as determined 40 d after sowing) and 39.0 ± 7.7 for icu4-1/icu4-1 (50 d after sowing).

The root system of icu4-1/icu4-1 plants had longer root hairs and more secondary roots than the wild type (Fig. 3, K–M). In addition, icu4-1/icu4-1 primary roots were 56.25% shorter than in the wild type, as determined 14 d after sowing. In agreement with this, we found a disorganized root apical meristem with extra cells within or next to the quiescent center of some icu4-1/icu4-1 primary roots (Fig. 3N). The shoots of icu4-1/icu4-1 plants usually had fewer, thicker, vascular bundles than the En-2 wild type. Transverse sections of icu4-1/icu4-1 shoot vascular bundles showed enlarged metaxylem tracheids and extra layers of procambial cells located between overproliferated phloem and xylem cells, as well as a poor lignification of the interfascicular fibers (Fig. 3, O and P). Transverse sections of leaves, however, did not reveal obvious structural differences between the veins of icu4-1/icu4-1 and wild-type plants (data not shown).

The icu4 hst Double Mutants

We previously described a synergistic interaction between the icu4 and hst mutations based on the rosette phenotype of the icu4-1 hst-5 double mutants (Serrano-Cartagena et al., 2000). We obtained an additional double mutant using hst-1, a null allele that had been thoroughly characterized by previous authors (Fig. 4A; Telfer and Poethig, 1998; Bollman et al., 2003). The icu4-1/icu4-1; hst-1/hst-1 double-mutant plants showed variable expressivity because most of their vegetative leaves were helically rotated (Fig. 4B), and some of them were radialized and completely covered by trichomes (Fig. 4C), which suggests that they were partially adaxialized.

In addition, we characterized the phenotype of stage 12 and stage 14 gynoecia (Smyth et al., 1990; Ferrándiz et al., 1999) using optical and scanning electron microscopy. At stage 12, wild-type En-2 (Fig. 5A) and mutant icu4-1/icu4-1 (Fig. 5B) pistils were indistinguishable, although after pollination icu4-1/icu4-1 siliques were slightly smaller, probably due to a modest decrease of male fertility. On the other hand, most stage 12 hst-1/hst-1 pistils were almost normal, although they were medi ally flattened and their stigmata (Fig. 5C) were larger than those of Ler. Flowers appearing late in hst-1/hst-1 inflorescences frequently had pistils that were unfused in the apical region, as previously described (Bollman et al., 2003). Transverse sections of En-2 and icu4-1/icu4-1 fruits did not show any difference at stage 14 (Fig. 5D), whereas those of hst-1/hst-1 showed an incompletely fused septum (Fig. 5E). Double-mutant gynoecia were strikingly abnormal despite the weak phenotypes of both icu4-1/icu4-1 and hst-1/hst-1 single-mutant plants. Major deformities were observed in most icu4-1/icu4-1; hst-1/hst-1 gynoecia, which displayed reduced carpel fusion, incomplete septum formation, and production of styal and stigmatic tissues at abnormal positions (Fig. 5F), as well as external placenta (which is an adaxial tissue in the wild type) bearing ovules, instead of the typical cells of the abaxial replum (Fig. 5G and H). We observed a failure of the outer integument to completely cover the inner integument and the nucellus (Fig. 5H), indicating an alteration in the polarity of the outer integument. Lack of fusion in the septum was enhanced in double-mutant ovaries in which loss of transmitting tract can be observed (Fig. 5K), unlike those of the hst-1/hst-1 single mutant (Fig. 5E).

In addition, the replum of the double mutant was wider than those of the single mutants (Fig. 5K). In addition, other floral organs showed abnormal development. Stamens of wild type and icu4-1/icu4-1 were indistinguishable and had pollen sacs in the adaxial surface (Fig. 5I), whereas a displacement to more lateral positions was occasionally observed in those of icu4-1/icu4-1; hst-1/hst-1 double mutants (Fig. 5J). Taken together, the strong adaxial transformations developed in the double mutant suggest that the ICU4 product has an adaxializing function, which is in agreement with the adaxial transformations shown by plants containing the 35S::ICU4-G189D construct.
Other Genetic Interactions

Other mutations have been described that determine the presence of ectopic ovules in the abaxial replum (Eshed et al., 1999, 2001). This is the case with double-mutant combinations involving alleles of KAN1 (see introduction), PICKLE/GYMNOs (PKL/GYM; Ogas et al., 1997, 1999), which encodes a putative CHD3 chromatin remodeling factor, and the YABBY family member CRABS CLAW (CRC; Bowman and Smyth, 1999). To identify genetic interactions, we made double mutants with icu4-1 and the kan1-2, pkl-1, or crc-1 loss-of-function alleles. The icu4-1/icu4-1;
kan1-2/kan1-2 and icu4-1/icu4-1:pk1-1/pk1-1 double mutants showed phenotypes that might be considered merely additive (data not shown).

Siliques from icu4-1/icu4-1;crc-1/crc-1 double mutants showed a phenotype that was stronger than those of their ICU4/ICU4;crc-1/crc-1 siblings. Plants homogenous for the strong crc-1 allele displayed short and thick siliques that were opened at the apex (Fig. 5L) and had unfused septa (Fig. 5P). Ectopic ovules are displayed on the abaxial replum of crc-1/crc-1 fruits at low frequency (Alvarez and Smyth, 1999). Double-mutant siliques showed a larger fissure in which ovules were easily seen (Fig. 5, M and N) and a higher frequency of ectopic ovules (Fig. 5, M and O). Eight of 30 fruits from three different double mutants displayed ectopic ovules, whereas none of the 30 crc-1/crc-1 fruits derived from the same F2 revealed this phenotype. In addition, icu4-1/icu4-1;crc-1/crc-1 siliques were very wide and flat (Fig. 5Q) and their septa showed a stronger disconnection than those of crc-1/crc-1 siliques (Fig. 5P). Thus, the mutant phenotype caused by homozygosis of crc-1 is enhanced by the gain-of-function allele icu4-1.

Expression Analysis

We detected ICU4 transcripts in roots, vegetative leaves, shoots, flower buds, and open flowers of Col-0 by semiquantitative reverse transcription (RT)-PCR (Fig. 6A). Quantitative, real-time RT-PCR (qRT-PCR) amplifications were also performed using RNA from flowers, leaves, and aerial tissues (whole plants with no roots) of En-2 and icu4-1/icu4-1 plants (Fig. 6B). Transcript levels of ICU4 were lower in the leaves than in the aerial tissues of En-2. However, transcript levels were 8-fold higher in icu4-1/icu4-1 leaves than in En-2 leaves, as is to be expected if icu4-1 escapes cleavage by the miRNA machinery, but only 2-fold in the aerial tissues of the mutant compared with those of the wild type. Further qRT-PCR expression analyses were made separately for leaves of the first and second, third to fifth, and sixth to last vegetative nodes, as well as for roots, shoots, and shoot apices of En-2 and icu4-1 homozygotes (Fig. 6, C and D). Overexpression of the gene was higher in the first two leaves of the icu4-1 mutant (Fig. 6D), consistent with the more severe phenotype displayed by these leaves (Fig. 2, B and F). ICU4 was also overexpressed in mutant shoots, roots, and shoot apical meristems (Fig. 6C).

Meristematic activity due to ectopic expression of class I KNOX genes (Chuck et al., 1996; Gallois et al., 2002) might explain the outgrowths seen in icu4-1/icu4-1 leaves. Consequently, we studied by qRT-PCR the expression of KNAT1, KNAT2, and KNAT6 (Lincoln et al., 1994; Semiarti et al., 2001). In addition, an indirect effect of the icu4-1 allele on the expression of class I KNOX genes is possible by the negative regulation of abaxializing genes. The YABBY genes, for instance, repress the expression of KNOX genes (Kumaran et al., 2002). Therefore, we also quantitated the expression of the abaxializing KAN1 (Eshed et al., 2001) and YAB3 (Kumaran et al., 2002) genes. Only KNAT2 was found to be misexpressed, with a 3-fold up-regulation in mutant leaves (Fig. 6E).

Overexpression of PINHEAD (PNH) has been described as causing leaf incurvature, most likely due to increased cell division in the abaxial domain (Newman et al., 2002). PNH encodes a member of the Argonaute protein family that is expressed in the shoot apical meristem, provascular tissues, and the adaxial domain of wild-type leaf primordia (Lynn et al., 1999; Newman et al., 2002). We studied the expression of PNH by qRT-PCR and found a 2.4-fold up-regulation in icu4-1/icu4-1 leaves (Fig. 6E).
DISCUSSION

icu4-1 and icu4-2 Are Gain-of-Function Alleles of the ICU4 Gene

We used the identical icu4-1 and icu4-2 semidominant mutations (Serrano-Cartagena et al., 2000) to positionally clone the ICU4 gene, which encodes the ATHB15 transcription factor, an HD-Zip III family member. A dosage analysis performed in triploids indicated that icu4-1 is a gain-of-function allele and constitutive overexpression of icu4-1 in transgenic plants with a wild-type background caused an extreme phenotype. The latter might be due to the generalized transcription driven by the 35S promoter, whereas expression of the endogenous ICU4 gene is restricted to specific organs and tissues. In addition, the mutant phenotype of icu4-1/icu4-1 individuals was suppressed by an RNAi construct designed to target transcripts of the ICU4 gene. Taken together, these results show that icu4-1 and icu4-2 are gain-of-function alleles of the ICU4 gene. Given that expression of this gene is known to be spatially restricted in wild-type plants (Frigge et al., 2005; Williams et al., 2005), the gain of function of semidominant icu4 alleles can be explained by ectopic derepression or by an increase in transcript levels within the wild-type realm of action of the gene.

A Point Mutation at a miRNA Complementarity Site Causes Overexpression of the ICU4 Gene

All the gain-of-function mutations described so far in the HD-Zip III family members PHB, PHV, and REV lie within the region that encodes the START domain of their protein products. However, the presence within this region of a sequence complementary to two miRNAs that differ in a single nucleotide, miR165 and miR166 (Rhoades et al., 2002), made disruption of miRNA-mRNA pairing the most likely explanation for the phenotype of semidominant phb, phv, and rev alleles (for review, see Bowman, 2004). The transcripts of HD-Zip III genes are cleaved at the miRNA complementarity site in a variety of plant species, indicating that this posttranscriptional regulatory mechanism dates back more than 400 million years (Floyd and Bowman, 2004).

The icu4-1 and icu4-2 alleles bear the same nucleotide substitution, a G-to-A transition affecting the miR165/166 complementarity site of ICU4, identical to those already described for several semidominant alleles of PHB and PHV. The miR165/166 complementarity site is mutated also in gain-of-function alleles of other HD-Zip III family members, such as REV and ril1 (Emery et al., 2003; Juarez et al., 2004; Zhong and Ye, 2004). Consequently, defective cleavage of mutant transcripts due to impaired miRNA-mRNA pairing provides a molecular explanation for the gain of function of icu4-1 and icu4-2. Cleavage of ATHB15 transcripts mediated by miR166 has been recently demonstrated in vivo (Kim et al., 2005).

Impaired miRNA-mRNA pairing causes accumulation of ICU4 transcripts, as indicated by its up-regulation in all the tissues studied in the icu4-1 mutant, which was higher in organs with a more conspicuous phenotype, such as leaves and shoots. The severity of leaf morphological aberrations correlated with the level of ICU4 overexpression, which was higher in juvenile leaves. Weaker incurvature and less overexpression were seen in leaves from the third to the adult nodes. A different result was obtained by Green et al. (2005) with the cna-1 mutant, in which CNA mRNA levels were similar to those of the wild type. The putative dominant negative cna-1 allele carries a point mutation in a conserved domain of CNA different from that containing the miRNA complementarity site (Green et al., 2005).

The ICU4 Gene Is Required for Shoot and Root Apical Meristem Patterning and Stem Vascular Differentiation

Based on the phenotypic characterization of the icu4-1 hst-5 double mutants, we previously proposed that ICU4 might play a role in regulating shoot apical meristem function (Serrano-Cartagena et al., 2000). The shoot apical meristem was also impaired in our icu4-1 and icu4-2 single mutants, as inferred from their abnormal phyllotaxis, paired cauline leaves, and axillary shoots, and occasionally seen twin rosettes. This result, along with the enlarged shoot meristems seen in cto cna double mutants and the triple null phb phv cna (Green et al., 2005; Frigge et al., 2005), indicates a role for the ICU4 gene in shoot meristem function.

Several HD-Zip III genes are known to be required for lateral root development (Hawker and Bowman, 2004) and shoot radial patterning (Emery et al., 2003; Zhong and Ye, 2004). Consistent with this, ICU4 also has a role in patterning shoot vascular bundles, as indicated by the overproliferation of metaxylem tracheids, the development of extra procambium layers, and the frequent reduction in the number of vascular bundles observed in the shoots of icu4-1/icu4-1 plants. Our results go along with those of Ohashi-Ito and Fukuda (2003), who characterized the expression profile of the ATHB15 gene of Arabidopsis and that of its Zinnia ortholog ZeHB-13, which were found to be involved in the differentiation of procambial and xylem cells, where they are expressed. Thus, both genes might act as transcriptional regulators in early vasculature development.

Although the ATHB15 promoter drives the expression of a reporter gene in the vascular cell files that start next to the quiescent center of primary root meristems (Ohashi-Ito and Fukuda, 2003), no root phenotypes have been described to be a consequence of perturbations in ATHB15 function. We have found that roots of icu4-1 homozygotes are shorter, initiate more secondary roots, and contain longer root hairs than the wild type and display an aberrant cell pattern in the root apical meristem.
The ATHB15 Transcription Factor Has Adaxializing Activity

Dorsoventral polarity, a property of plant lateral organs such as leaves and floral organs, is thought to depend on an adaxializing signal emanating from the shoot apical meristem (Sussex, 1954). The adaxial and abaxial domains of developing lateral organs are patterned by the activities of HD-Zip III adaxializing factors, and KANADI and YABBY abaxializing factors (Eshed et al., 1999; Siegfried et al., 1999; Kerstetter et al., 2001; Kumanar et al., 2002; Emery et al., 2003). As proposed in the model of Waites and Hudson (1995), the flatness of the leaf lamina would be explained by localized growth at locations where the adaxial and abaxial identities meet.

The expression of REV, PHV, PHB, and ATHB15 is restricted to the adaxial domains of lateral organ primordia (McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005; Williams et al., 2005). On the contrary, members of the KANADI and YABBY families are expressed abaxially in lateral organs (Sawa et al., 1999a, 1999b; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). Consistent with the model of Waites and Hudson (1995), gain-of-function phi, pbh, and rev mutants have adaxialized leaves (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Zhong and Ye, 2004). Furthermore, loss-of-function kan alleles cause abaxial tissue reduction together with an expansion of the realm of expression of REV, PHB, and PHV (Eshed et al., 1999, 2001).

The icu4-1 and icu4-2 gain-of-function mutations delay juvenile-to-adult phase change and flowering and increase the number of vegetative leaves, which lack abaxial trichomes. These phenotypic traits are the opposite of those associated with loss-of-function hst mutations, which accelerate phase change and cause early flowering and the presence of abaxial trichomes on juvenile leaves (Telfer and Poethig, 1998; Serrano-Cartagena et al., 2000; Bollman et al., 2003). These observations suggest that the ICU4 and HST genes are involved in related processes, an idea reinforced by the synergistic phenotype of the icu4-1 hst-5 (Serrano-Cartagena et al., 2000) and icu4-1 hst-1 (this work) double mutants. Given that loss-of-function hst mutations reduce miRNA levels (Park et al., 2005), their mutant phenotype is expected to be pleiotropic as a consequence of the simultaneous overexpression of different miRNA target genes, which might include ICU4.

Leaves of the icu4-1 hst-1 double mutant were helically rotated and occasionally radialized and presented many trichomes, suggesting that they are partially adaxialized. Also, the recessed outer integuments of the mature ovules of these double mutants were reminiscent of those caused by mutations in the INNER NO OUTER (INO) gene, a member of the YABBY family that is essential for the formation and asymmetric growth of the ovule outer integument (Villanueva et al., 1999). These observations, together with the synergistic phenotype of the ago1-51 icu4-1 double mutant, which displays adaxialized leaves (S. Jover-Gil, H. Candela, J.M. Barrero, P. Robles, J.L. Micol, and M.R. Ponce, unpublished data), and the adaxial transformations observed in 35S::ICU4-G189D transformants support the notion that ICU4 has an adaxializing activity. Although it has been previously suggested that the CNA (ICU4) gene might be involved in specifying polarity, this role did not become obvious from the study of cna loss-of-function alleles (Prigge et al., 2005).

Fruits of the hst-1 mutant, which are medially flattened and occasionally opened at the apical end, show some resemblance to those of homozygotes for loss-of-function alleles of the CRC gene (Alvarez and Smyth, 1999), a YABBY family member that is required for abaxial cell fate specification in developing carpels (Bowman and Smyth, 1999). Whereas crc single mutants do not show a clear loss-of-abaxial fate phenotype, their double-mutant combinations with kan1 alleles condition the presence of an external placenta with ovules, indicating that both genes cooperate to promote abaxial identity in carpels (Eshed et al., 1999). External ovules in the medial domain of the carpels are also observed in kan1 hst double mutants (Eshed et al., 2001), which show a synergistic interaction, suggesting that HST works in parallel with KAN1 in the specification of lateral organ polarity (Bollman et al., 2003). Interestingly, gynoecia of icu4-1 hst-1 double mutants show a similar strong interaction and often display the replacement of the abaxial replum by a placenta with ovules. In addition, siliques of icu4-1 crc-1 double mutants show an enhancement of the mutant phenotype conferred by the crc-1 allele. These phenotypes further indicate that ICU4 has an adaxializing activity.

As expected from the predicted impairment of miRNA-mediated regulation caused by the icu4-1 mutation, ICU4 itself was found to be up-regulated in the icu4-1 mutant. We found that leaf incurvature is at least partially caused by ectopic PNH expression in the icu4-1 mutant, which in turn indicates a positive regulatory effect of ICU4 on PNH. The KNAT2 meristematic gene was also found up-regulated in icu4-1/ icu4-1 leaves, suggesting that ICU4 also promotes meristematic activity, which might account for the abaxial protuberances observed in the mutant leaves.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Crosses

Several Arabidopsis (Arabidopsis thaliana L. Heynh.) lines studied in this work were supplied by the Nottingham Arabidopsis Stock Centre. These include the En-2 wild type, the N400 and N401 mutants (respectively carrying the icu4-1 and icu4-2 alleles, both in an En-2 genetic background), the N517186 and N531314 T-DNA insertion lines (respectively carrying the icu4-3 and icu4-4 alleles), and the N523733, N563586, and N579212 T-DNA insertion lines (which we named athb8-3, athb8-4, and athb8-5, given that the only ATHB8 alleles already published are athb8-1 and athb8-2), which are described at the SIGnAL Web site (Alonso et al., 2003; http://signal.salk.edu), and the crc-1, pkl-1, and hst-1 mutants. The CS3151 tetraploid line was supplied by the Arabidopsis Biological Resource Centre. The kan-2 mutant was provided by J.L. Bowman, and the pATHB8-GUS transgenic line by S. Baima.
Cultures were performed as described by Ponce et al. (1998), at 20°C ± 1°C and 60 to 70% relative humidity under continuous illumination of 7,000 lux. Crosses were performed as described in Berná et al. (1999). For dosage analysis, tetraploid CS151 plants were fertilized with icu4-1 pollen.

Positional Cloning, Sequencing, and Sequence Analysis

Mapping was carried out as described by Ponce et al. (1999). Based on the genome sequence available at the Cereon database (http://www.arabidopsis.org/Cereon), we developed 12 new single-nucleotide polymorphism and insertion/deletion markers within the candidate region (Supplemental Table II), which were used to screen for informative recombinants. PCR products spanning the At1g52150 gene from En-2, icu4-1, and icu4-2 homoygotes were sequenced in 5–μL reactions using internal primers and the ABI PRISM BigDye terminator cycle sequencing kit. Sequencing electrophoreses were performed on an ABI PRISM 3100 genetic analyzer.

Amino acid sequences of HD-Zip III family members were aligned using ClustalX, version 1.5b, and shaded with Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Identity and similarity percentages were obtained by aligning protein sequences; those for the miR165/166 complementarity site were obtained by aligning nucleotide sequences, whose accession numbers were as follows: ICU4 (ATHB-15; NP_157627.1), ATHB-8 (NP_195014.1), ATHB-14 (PHB; NP_181083.1), REV (IFI; NP_200877.1), ATHB-9 (PHV; NP_174337.1), ZeHB-13 (BADI0502.1), ZeHB-2 (CAC48276.1), ICU4 (NM_104966.2), ZeHB-3 (AJ312504.1), ZeHB-13 (AB109682.1), ATHB-8 (NM_119441.3), ATHB-9 (PHV; NM_102785.3), ATHB-14 (PHB; NM_120925.2), REV (NM_125462.2), MIR159 (AY501430.1), ZehB-1 (AJ312535.1), ZehB-3 (AJ120855.1), ZehB-10 (AB084380.1), ZehB-11 (AB084381.1), ZehB-12 (AB084382.1), Hox9 (AY425761.1), Hox10 (AY425991.1), PpHB10 (AB021281.2), and Hb-1 (AY497721.1).

Microscopy

Whole-rose and single-leaf pictures were taken in a MZ6 stereomicroscope (Leica) or in a SMZR800 stereomicroscope (Nikon). For light microscopy, plant material was fixed with formaldehyde acetic acid/Triton (1.85% formaldehyde, 45% ethanol, 1% glacial acetic acid, and 1% Triton X-100) and embedded in JB4 resin (Electron Microscopy Sciences) as described in Serrano-Cartagena et al. (2000). Transverse sections of 5-μm leaves or 3- to 4-μm silicles were made on a microtome (Microm International HM305), stained with 0.1% toluidine blue, and observed under brightfield illumination using a Zeiss Axioscope microscope.

Generation of Transgenic Plants

The 35S::ICU4 construct was made by placing the ICU4 cDNA under the control of the tandemly repeated 35S promoter of the pBIN-JT vector (Ferrándiz et al., 2000). The full-length wild-type ICU4 cDNA was obtained from the RIKEN Genomic Sciences Center (clone RFL09-35-K18; Seki et al., 1998, 2002). The 35S::ICU4-G189D construct, which consisted in the icu4-1 mutant allele cloned into pBIN-JT, was generated after PCR amplification of the RFL09-35-K18 clone with two primer pairs. Two of these primers were designed to create a G-to-A transition in the fifth position of the fifth exon of ICU4 (5′-CTGAAATGAGCTCTGATCCGATTCTCAGTCCG-3′ and its exact complement). The 35S::ICU4-RNAi construct was designed for RNAi and included a genomic fragment of the coding region of the wild-type ICU4 allele amplified using the primers 5′-CTTCGGTTCGACAAACAC3′ and 5′-TGGAGTTGAGGAAACCCCTG-3′. The PCR amplification product was ligated in sense and antisense orientations into pCF6 (C. Ferrándiz, unpublished data), both fragments being separated by the sixth intron of the FRUITFULL gene (Cujo et al., 1998), and then transferred to pBIN-JT for Agrobacterium-mediated transformation.

All constructs obtained in this work were fully sequenced to confirm their structural integrity prior to being transferred into plants by the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on 50 μg/mL kanamycin-supplemented medium (Weigel and Glazebrook, 2002).

RNA Isolation and RT-PCR Analyses

Total RNA was extracted from plant material, which was collected, immediately frozen in liquid N2, and stored at −80°C. RNA was extracted with TRIzol (Invitrogen) and further purified with an RNAeasy plant mini kit (Qiagen), according to the instructions of the manufacturers. RNA concentration was determined in a spectrophotometer and its quality checked by visualization in an agarose gel.

For semiquantitative RT-PCR, RNA was extracted from 80 to 100 mg of roots, vegetative leaves, shoots, mature flowers, and flower buds of Col-0, collected 21 (roots and vegetative leaves) and 30 (shoots, mature flowers, and flower buds) days after sowing. First-strand cDNA was synthesized with random hexamers using the SuperScript first-strand synthesis kit, according to the manufacturer’s instructions (Invitrogen). PCR amplifications were performed as described in Pérez-Pérez et al. (2004). The housekeeping ORNITHINE TRANSCARRIBOMILASE gene (Quesada et al., 1999) was used as a positive control.

For qRT-PCR, RNA was isolated from 50 to 100 mg of plant material collected 21 (vegetative leaves, shoot apices, and roots), 30 (flowers and aerial tissues), and 38 (shoots) days after sowing. qRT-PCR was performed using first-strand cDNA as a template on an ABI PRISM 7000 sequence detection system (Perkin-Elmer/ Applied Biosystems). The primers used are shown in Supplemental Table II. Amplification reactions and relative quantification of gene expression data were carried out as described in Livak and Schmittgen (2001), Chomczynski (1999), and Lazurec-Perez et al. (2004).

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