Incurvata13, a Novel Allele of Auxin Resistant6, Reveals a Specific Role for Auxin and the SCF Complex in Arabidopsis Embryogenesis, Vascular Specification, and Leaf Flatness[^1][^2][^3]

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Auxin plays a pivotal role in plant development by modulating the activity of SCF ubiquitin ligase complexes. Here, we positioned cloned Arabidopsis (Arabidopsis thaliana) incurvata13 (icu13), a mutation that causes leaf hyponasty and reduces leaf venation pattern complexity and auxin responsiveness. We found that icu13 is a novel recessive allele of AUXIN RESISTANT6 (AXR6), which encodes CULLIN1, an invariant component of the SCF complex. Consistent with a role for auxin in vascular specification, the vascular defects in the icu13 mutant were accompanied by reduced expression of auxin transport and auxin perception markers in provascular cells. This observation is consistent with the expression pattern of AXR6, which we found to be restricted to vascular precursors and hydathodes in wild-type leaf primordia. AXR1, RELATED TO UBQUITIN1-CONJUGATING ENZYME1, CONSTITUTIVE PHOTOMORPHOGENIC SIGNALOSOME5A, and CULLIN-ASSOCIATED NEDD8-DISSOCIATED1 participate in the covalent modification of CULLIN1 by RELATED TO UBQUITIN. Hypomorphic alleles of these genes also display simple venation patterns, and their double mutant combinations with icu13 exhibited a synergistic, rootless phenotype reminiscent of that caused by loss of function of MONOPTEROS (MP), which forms an auxin-signaling module with BODENLOS (BDL). The phenotypes of double mutant combinations of icu13 with either a gain-of-function allele of BDL or a loss-of-function allele of MP were synergistic. In addition, a BDL:green fluorescent protein fusion protein accumulated in icu13, and BDL loss of function or MP overexpression suppressed the phenotype of icu13. Our results demonstrate that the MP-BDL module is required not only for root specification in embryogenesis and vascular postembryonic development but also for leaf flatness.

The plant hormone auxin is a key player in multiple morphogenetic processes (Friml et al., 2003; Scarpella et al., 2006; Weijers et al., 2006; Donner et al., 2009). Transcription of many auxin-responsive genes is regulated by auxin/indole-3-acetic acid (Aux/IAA)-AUXIN RESPONSE FACTOR (ARF) signaling modules (Mockaitis and Estelle, 2008; De Smet et al., 2010). In the absence of auxin, interaction between an Aux/IAA protein and its partner ARF results in ARF inactivation; in the presence of auxin, ubiquitination and degradation of the Aux/IAA protein by the SCF[^1],[[^2],[^3] E3 ubiquitin ligase complex and the 26S proteasome allow the ARF to directly inhibit or repress its target genes (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b; Mockaitis and Estelle, 2008).

The core of the SCF complex includes three major components: RING-BOX1 (RBX1), CULLIN1 (CUL1; also called AUXIN RESISTANT6 [AXR6]), and S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1). There are 21 SKP1 homologs in Arabidopsis (Arabidopsis thaliana; Liu et al., 2004). The fourth component of the SCF complex is a variable F-box protein, of which the Arabidopsis genome encodes 700 to 900 (Hua et al., 2011). TRANSPORT INHIBITOR RESPONSE1 (TIR1; Gray et al., 2001; Dharmasiri et al., 2005a) and AUXIN SIGNALING F-BOX1 (AFB1) to AFB3 (Dharmasiri et al., 2005b; Parry et al., 2009) are the F-box proteins that target Aux/IAA proteins for ubiquitination.

CUL1 is posttranslationally modified by RELATED TO UBQUITIN (RUB; also called NEDD8 [for neuronal precursor cell-expressed developmentally downregulated8]), a small peptide with 60% identity to ubiquitin (Rao-Naik et al., 1998; del Pozo and Estelle, 1999; Bostick et al., 2004). The covalent modification of CUL1 requires a three-step enzymatic pathway similar to the ubiquitination pathway (Hotton and Callis, 2008; Hua and Vierstra, 2011), where AXR1 and E1 C-TERMINAL RELATED1 encode the two subunits of the E1 ubiquitin-activating enzyme and RUB-CONJUGATING-ENZYME1.
(RCE1; Dharmasiri et al., 2003) functions as E2. The SCF subunit RBX1 probably acts as the E3 enzyme for rubylation (Kamura et al., 1999). Derubylation of CUL1 is carried out by the CSN5 metalloprotease subunit of the CONSTITUTIVE PHOTOMORPHOGENIC9 signalsome (Cope et al., 2002; Dohmann et al., 2005). Another layer of the regulation of the SCF complex involves CULLIN-ASSOCIATED NEDD8-DISSOCIATED1 (CAND1), a protein that can sequester the derubylated CUL1-RBX1 core of the SCF complex, thereby preventing complex assembly (Min et al., 2003; Goldenberg et al., 2004).

Several Aux/IAA-ARF auxin-signaling modules play important roles in different developmental processes. The BODENLOS-MONOPTEROS (BDL-MP or IAA12-ARF5) module, for example, is known to participate in cell specification (Weijsers et al., 2006) and organogenesis (De Smet et al., 2010). Basal structures are missing in Arabidopsis seedlings homozygous for either strong loss-of-function alleles of MP or a gain-of-function allele of BDL; these seedlings lack hypocotyl and root and exhibit reduced cotyledon vasculature (Berleth and Jürgens, 1993; Hamann et al., 1999). Basal defects of these mutants are caused by misspecification of the hypophysis, the upper part of the suspensor, during early embryogenesis (Hardtke and Berleth, 1998; Hamann et al., 1999). MP is directly repressed by BDL (Hamann et al., 2002; Hardtke et al., 2004), but the auxin-dependent MP-BDL regulatory loop is not simple: the MP protein activates the expression of MP and BDL (Lau et al., 2011). Although the precise mechanism is still unknown, MP plays a crucial role in hypophysis specification via a non-cell-autonomous signal (Weijsers et al., 2006; Schlereth et al., 2010). MP also plays a postembryonic role in vein specification (Hardtke et al., 2004; Wenzel et al., 2007; Schuetz et al., 2008; Donner et al., 2009; Krogan et al., 2012): it directly activates the expression of ARABIDOPSIS THALIANA HOMEBOX GENE8 (ATHB8; Donner et al., 2009), which encodes an HD-Zip III transcription factor involved in preprocambial cell fate specification (Baima et al., 1995). The levels of PIN-FORMED1 (PIN1), an auxin transporter that is expressed in procambium formation domains (Scarpella et al., 2006; Wenzel et al., 2007), are reduced in mp mutants (Wenzel et al., 2007). Moreover, pin1 mutants genetically interact with mp in a synergistic manner (Schuetz et al., 2008), and transgenes expressing truncated MP proteins that lack the homodimerization and heterodimerization domains cause increased leaf vascularization (Krogan et al., 2012).

A number of Arabidopsis mutants exhibit hypostatic (curled up) leaves, a phenotype that can be explained by impaired coordination of the growth of adaxial and abaxial leaf tissues (McConnell et al., 2001; García et al., 2006; Pérez-Pérez et al., 2010; Jover-Gil et al., 2012). We isolated a large set of incurvata (icu) mutants, which display hypostatic vegetative leaves and include alleles of 14 genes (Berná et al., 1999; Serrano-Cartagena et al., 1999, 2000). Ten of the initial set of ICU genes have been cloned, allowing their functional classification in three different pathways, related to chromatin-mediated cellular memory (Barrero et al., 2007), microRNA biogenesis and action (Jover-Gil et al., 2005, 2012), and auxin signaling (Pérez-Pérez et al., 2010). Two of the genes in the last group are SHORT HYPOCOTYL2 (SHY2; also called IAA3) and AXR3 (also called IAA17; Pérez-Pérez et al., 2010). For example, icu6, a semidominant allele of AXR3, causes reduced size of leaf adaxial pavement cells and abnormal expansion of palisade mesophyll cells. Hence, the differential growth of the adaxial and abaxial leaf tissues of icu6 is thought to be a consequence of the limited space available for the internal mesophyll, which is defined by the epidermal layers (Pérez-Pérez et al., 2010). We also studied mutants exhibiting aberrant leaf venation patterns (Candela et al., 1999, 2007; Alonso-Peral et al., 2006; Robles et al., 2010), three of which carried loss-of-function alleles of HEMIVENATA (HVE), the gene encoding CAND1 (Alonso-Peral et al., 2006; Candela et al., 2007), which is known to physically interact with AXR6.

Here, we report the characterization of icu13, a novel recessive allele of AXR6 that causes leaf hypostasy as a consequence of a strong reduction of AXR6 protein levels. Our genetic and molecular results support the hypothesis that AXR6 acts through the MP-BDL module, which is crucial for embryo basal patterning and vascular specification in the early stages of leaf development. Hence, altering the amount of active CUL1, via misregulation of rubylation and derubylation, phenocopies the strong defects of mp and bdl mutants. As the leaf incurvature of icu13 can be rescued by either overexpression of MP or loss of function of BDL, we suggest that the MP-BDL module is also responsible for leaf flatness.

RESULTS

Positional Cloning of icu13 and Detection of Its mRNA and Protein Products

For positional cloning of the recessive icu13 mutation, a mapping population of 481 F2 phenotypically mutant plants derived from an icu13 × Landsberg erecta cross was used for linkage analysis as described previously (Ponce et al., 1999, 2006). The oligonucleotides used for simple sequence length polymorphism marker scoring are listed in Supplemental Table S1. The icu13 mutation mapped to a 139-kb interval on top of chromosome 4, encompassing 38 annotated genes, one of which was AXR6 (Fig. 1, A and B). We sequenced the AXR6 transcription unit in the icu13 mutant and its corresponding wild type, Enkheim-2 (En-2), finding in exon 15 a C→T transition that creates a new splicing donor site, as confirmed by comparison of the sequences of PCR amplification products obtained from En-2 and icu13 complementary DNAs (cDNAs; Supplemental Fig. S1). Two different AXR6 splice variants are present in icu13 seedlings: one variant...
 differs from the wild-type En-2 mRNA only in a silent point mutation (GGC → GGT, both codons encoding Gly), but the other variant lacks the last five nucleotides of the 15th exon as a consequence of the new splicing donor site (GC→GT) introduced by the mutation. Translation of the latter mRNA is predicted to produce a truncated protein product; whereas the AXR6 wild-type protein includes 738 amino acids, the mutant protein is predicted to contain only 492, 19 of which, at its C terminus, are different from those of the wild type (Supplemental Fig. S2).

To quantify the relative levels of the two splice variants produced by the icu13 allele, we performed quantitative reverse transcription (qRT)-PCR amplifications using two primer pairs: one primer pair (1F +1R) amplifies a cDNA region upstream of the icu13 mutation that is shared by both splice variants, and the other primer pair (1F+2R) amplifies only the long splice variant (Fig. 1C). Our results indicated that AXR6 mRNA levels in En-2 are more than twice those in the icu13 mutant. Also, only 43.2% of the AXR6 mRNA in the icu13 mutant corresponded to the longer splice variant, which can be assumed to be functionally wild type (Fig. 1D). Taken together, these results indicate that the amount of mRNA than can be translated into wild-type AXR6 proteins in icu13 is 18% of that seen in the wild type En-2.

As we could not exclude translation of the short, aberrant splice variant of icu13, we tried to detect its predicted truncated protein product (Supplemental Fig. S2) by western blotting using the available anti-CUL1 antibody (Gray et al., 1999). Although we could not detect any band with the size predicted for the truncated protein (57 kD), we confirmed that both wild-type inactive (CUL1) and wild-type rubylated (CUL1-RUB) protein species were strongly decreased in icu13 seedling extracts compared with the wild type (Fig. 1E).

Phenotypic Characterization of the icu13 and eta1 Mutants

The enhancer of tir1-1 auxin resistance (eta1) recessive allele of AXR6 carries a missense mutation (Quint et al., 2005; Fig. 1B). Both eta1 and icu13 caused mild leaf hyponasty (Fig. 2, A and B). Transverse sections of first-node vegetative leaves indicated that icu13 leaves contained smaller adaxial pavement cells, more densely packed palisade mesophyll cells, and fewer air spaces in the spongy mesophyll than wild-type En-2 (Supplemental Fig. S3). More vegetative leaves were produced by icu13 (13.60 ± 1.34 at 21 d after stratification [das]) than En-2 (11.85 ± 0.91; P < 0.01; Fig. 2B), despite bolting occurring earlier in icu13 (20.98 ± 1.71 das) than in En-2 (23.64 ± 1.34 das; P < 0.01). Flowering stems were much shorter in icu13 and eta1 mutants than in their respective wild types (Fig. 2C) and also displayed reduced apical dominance (increased inflorescence branching): at 45 das, icu13 plants exhibited more flowering stems (44.22 ± 6.48; n = 9) than En-2 (23.67 ± 3.74; n = 9; P < 0.01). Similar results were obtained for eta1 (52.00 ± 5.7; n = 5) compared with Columbia-0 (Col-0; 33.40 ± 5.70; n = 5; P < 0.01).

We found that the complexity of the vascular network was reduced in icu13 and eta1 first- and third-node leaves compared with their wild types. Third-, fourth-, and higher order veins were missing from

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**Figure 1.** Positional cloning of icu13 and its effects on AXR6 expression. A, Map-based cloning strategy used to identify the icu13 mutation, showing the markers used for linkage analysis, their physical map positions, and the number of informative recombination events identified (in parentheses). B, Structure of the AXR6 gene, indicating the nature and positions of known mutations. Boxes and lines between boxes indicate exons and introns, respectively. White boxes correspond to the 5’ and 3’ untranslated regions. Vertical arrows and triangles indicate point mutations and T-DNA insertional mutations, respectively. C, Magnification of the 14th, 15th, and 16th exons of AXR6 showing the oligonucleotides (not drawn to scale) used as qRT-PCR primers. A vertical arrow marks the site of the icu13 mutation. D, qRT-PCR analysis of AXR6 expression in the icu13 mutant. Bar graphs indicate the relative expression levels (comparative threshold cycle method) obtained using the oligonucleotide pairs shown in the abscissa. E, Western blot probed with a primary antibody against CUL1 (anti-CUL1). The large subunit of Rubisco (anti-RbcL) was used as a loading control.
icu13 and eta1 venation patterns in these leaves (Fig. 2D). The venation density and number of branching points per millimeter squared, which are indicators of vascular complexity (Candela et al., 1999), were reduced in both icu13 and eta1, and the number of free-ending veins per mm of venation length was significantly increased only in icu13 (Table I).

We also tested the auxin responses of icu13 and eta1. Seedlings of these mutants displayed reduced inhibition of primary root growth when treated with exogenous IAA (Fig. 2E; \( P < 0.01 \)) and displayed a small increase in the number of lateral roots after the transfer of mutant seedlings to IAA-supplemented plates (Fig. 2F). We confirmed this reduction in auxin responses by examining the expression of the DR5rev:GFP reporter (Friml et al., 2003) in primary root meristems. DR5rev:GFP expression in icu13 and eta1 roots, in contrast to expression in the wild type, was restricted to the distal region of the meristem (quiescent center and columnella) and excluded from the vasculature (Supplemental Fig. S4). These results are consistent with a reduction in auxin responses in the icu13 and eta1 mutants, a reduction that can be explained by impaired SCFTIR1/AFB1-3 function.

### Phenotypic Characterization of Heterozygotes for AXR6 Alleles

Twelve mutations of AXR6 have already been described, including loss- and gain-of-function alleles

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**Table 1. Morphometry of first-node leaf venation patterns in single and double mutants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leaf Area ( \text{mm}^2 )</th>
<th>Venation Density ( \text{mm mm}^{-2} )</th>
<th>No. of Branching Points per ( \text{mm}^2 )</th>
<th>No. of Free-Ending Veins per Venation Length (mm)</th>
</tr>
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<tbody>
<tr>
<td>En-2</td>
<td>33.96 ± 7.54</td>
<td>2.80 ± 0.30</td>
<td>3.13 ± 0.69</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>icu13</td>
<td>13.58 ± 2.50</td>
<td>2.45 ± 0.25</td>
<td>2.27 ± 0.50</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>Col-0</td>
<td>29.83 ± 11.69</td>
<td>2.89 ± 0.47</td>
<td>3.23 ± 0.95</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>eta1</td>
<td>11.67 ± 2.21</td>
<td>1.89 ± 0.26</td>
<td>1.07 ± 0.42</td>
<td>0.27 ± 0.10</td>
</tr>
<tr>
<td>rce1-10</td>
<td>25.16 ± 8.56</td>
<td>2.35 ± 0.34</td>
<td>1.74 ± 0.49</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>csm5a-2</td>
<td>9.15 ± 3.17</td>
<td>3.12 ± 0.30</td>
<td>3.21 ± 0.75</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>axr2-1</td>
<td>14.85 ± 3.21</td>
<td>3.02 ± 0.18</td>
<td>3.12 ± 0.48</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>axr2-1 icu13</td>
<td>6.90 ± 2.87</td>
<td>2.55 ± 0.32</td>
<td>2.08 ± 0.69</td>
<td>0.38 ± 0.24</td>
</tr>
<tr>
<td>axr2-1 eta1</td>
<td>5.81 ± 1.64</td>
<td>2.65 ± 0.67</td>
<td>2.28 ± 1.57</td>
<td>0.40 ± 0.21</td>
</tr>
<tr>
<td>shy2-10</td>
<td>23.87 ± 7.37</td>
<td>3.05 ± 0.43</td>
<td>3.54 ± 0.88</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>shy2-10 icu13</td>
<td>7.42 ± 1.40</td>
<td>2.68 ± 0.16</td>
<td>2.46 ± 0.39</td>
<td>0.44 ± 0.16</td>
</tr>
<tr>
<td>shy2-10 eta1</td>
<td>9.62 ± 0.63</td>
<td>2.66 ± 0.15</td>
<td>2.50 ± 0.34</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td>icu13 35S\text{_pro}:MP</td>
<td>20.73 ± 3.97</td>
<td>2.63 ± 0.23</td>
<td>2.34 ± 0.47</td>
<td>0.28 ± 0.12</td>
</tr>
<tr>
<td>icu13 iaa12-1</td>
<td>15.41 ± 2.14</td>
<td>3.03 ± 0.34</td>
<td>3.02 ± 0.68</td>
<td>0.36 ± 0.05</td>
</tr>
</tbody>
</table>
To study their phenotypes in combination with icu13, we selected four mutants: a semidominant, gain-of-function allele, axr6-2 (Hobbie et al., 2000), and three recessive alleles, two of which are null (cul1-1 and cul1-2; Shen et al., 2002) and one that is likely hypomorphic (eta1; Quint et al., 2005). We studied the embryo and seedling phenotypes of plants heterozygous for all possible combinations of these five alleles of AXR6 (Fig. 3). The icu13/eta1 heterozygotes (Fig. 3I) were indistinguishable from their icu13/icu13 parent, except for their increased size, which is due to heterosis caused by their hybrid En-2/Col-0 background (data not shown). Seedlings heterozygous for icu13 and axr6-2, cul1-1, or cul1-2 lacked their basal region, including most of the hypocotyl and primary root, and died at postembryonic stages (Fig. 3, C and G; data not shown). These three heterozygotes strongly resembled homozygous axr6-1, axr6-2 (Fig. 3B), mp, or bdl mutants. However, combinations of eta1 with axr6-2, cul1-1, or cul1-2 developed roots and were fertile when grown in petri dishes (Fig. 3, D and H; data not shown). Taken together, our results indicate that the recessive icu13 mutation causes a stronger loss of function in AXR6 than the eta1 mutation.

To study the phenotypes of axr6-2/cul1-1 and axr6-2/ cul1-2 heterozygotes, siliques from several axr6-2/AXR6 × AXR6/cul1-1 and axr6-2/AXR6 × AXR6/cul1-2 crosses were collected before dehiscence and manually dissected. In 17 out of 19 siliques, one-quarter of the seeds were abortive (Supplemental Table S2). Differential interference contrast (DIC) microscopy revealed that the axr6-2/cul1-1 (Fig. 3K) and axr6-2/cul1-2 (Fig. 3L) embryos were in most cases arrested at the late globular stage (89.7%; n = 68), exhibiting several layers of cells within the suspensor.

Expression Patterns of Auxin and Vascular Markers in the icu13 Mutant

Previous analysis of AXR6 indicated that it played a role in auxin signaling. To assess the role of AXR6 and auxin signaling in vascular fate specification and in the mutant phenotype of icu13, we examined the expression of markers for auxin transport (PIN1pro:PIN1:GFP; Xu et al., 2006), auxin perception (DR5rev:GFP; Friml et al., 2003), and vascular differentiation (ATHB8pro:GUS; Baima et al., 1995; see “Materials and Methods”). PIN1 encodes the only PIN family auxin-efflux carrier that is expressed early in leaf preprocambial cells and whose expression is auxin dependent (Scarpella et al., 2006). We used the PIN1pro:PIN1:GFP marker (Xu et al., 2006) to visualize auxin transport during early development of the leaf venation pattern. PIN1:GFP

Figure 3. Vegetative and embryonic phenotypes of combinations of alleles of AXR6. A and B, Homozygotes for the axr6-2 semidominant, gain-of-function allele of AXR6 were seedling lethal. C, Heterozygotes for icu13 and axr6-2 were rootless and seedling lethal. D, Heterozygotes for eta1 and axr6-2 developed roots and were viable. E and F, The cul1-1 and cul1-2 recessive, null alleles were both embryonic and gametophyte lethal. A white arrowhead indicates an aborted ovule in a dissected silique. G and H, cul1-1/icu13 but not cul1-1/eta1 heterozygotes were rootless and seedling lethal. A black arrowhead indicates the root. I, Rosettes of icu13/eta1 heterozygotes were indistinguishable from those of their homozygous parents. J to L, At the globular and heart stages, wild-type embryos differed from embryos heterozygous for axr6-2 (l) and either cul1-1 (K) or cul1-2 (L), which exhibited more than one row of suspensor cells (white arrowheads). Photographs of rosettes and dead seedlings were taken 16 das. Bars = 2 mm (A–E and G–I), 0.2 mm (F), and 25 μm (J–L).


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expression was lower in *icu13* than in wild-type En-2 primordia at 3 das (Fig. 4, A and F). PIN1:GFP expression in leaf primordia was detected at 4 das in the preprocambial cells of more than three vascular loops in *icu13* (F–I, P–T, and Z–AD) and En-2 (A–E, K–O, and U–Y) backgrounds. Photographs were taken of plants grown on medium supplemented (E, J, O, T, Y, and AD) or not (A–D, F–I, K–N, P–S, U–X, and Z–AC) with 1 μM NPA. Values in parentheses indicate the number of primordia behaving as the ones shown/total primordia studied. Bars = 25 μm (A, B, F, G, K, L, P, Q, U, V, Z, and AA) and 50 μm (C–E, H–J, M–O, R–T, W–Y, and AB–AD).
with an enhanced expression of PIN1:GFP (Fig. 4, E and J). However, the venation patterns of icu13 leaf primordia treated with NPA were always less complex than those of the wild type.

In contrast to PIN1pro:PIN1:GFP, the expression of DR5rev:GFP was initially indistinguishable in icu13 and En-2 and was restricted to the apex of leaf primordia at 3 das (Fig. 4, K and P). However, from 4 to 7 das, DR5rev:GFP expression broadened in the wild type but expanded much less in icu13 (Fig. 4, L–N and Q–S). Consistent with decreased auxin signaling in icu13 leaves, DR5rev:GFP expression was substantially reduced in the quiescent center and the columella region of icu13 root meristems at 7 das (Supplemental Fig. S4). NPA treatment increased vein densities and DR5rev:GFP expression in En-2 and icu13, but the icu13 venation pattern was much simpler (Fig. 4, O and T).

ATHB8 encodes an MP-inducible HD-Zip III transcription factor that restricts preprocambial cell fate specification downstream of auxin (Baima et al., 1995; Donner et al., 2009). ATHB8pro:GUS expression was similar in the central region of leaf primordia of En-2 and icu13 at 3 das (Fig. 4, U and Z) and more complex in En-2 than in icu13 from 4 das onward (Fig. 4, V–Y and AA–AD).

In eta1 leaf primordia, the expression patterns of DR5rev:GFP, PIN1pro:PIN1:GFP, and ATHB8pro:GUS were similar to those in icu13 (data not shown). Taken together, our results indicate that a loss-of-function mutation in AXR6, such as the icu13 and eta1 mutations, alters vascular development, beginning at the earliest stages of auxin perception and transport and extending to the later expression of specific vascular differentiation genes.

Spatial Expression Pattern of the AXR6 Gene

To examine AXR6 expression, an AXR6pro:GUS transcriptional fusion was obtained and transferred into plants. Two independent T2 transgenic families in the Col-0 background and five in the En-2 background exhibited similar expression patterns. AXR6 was broadly expressed during embryogenesis from the 16-cell dermatogen (Supplemental Fig. S5A) to the early torpedo (Supplemental Fig. S5, B–D) stages and remained ubiquitous in both the embryo and the suspensor. From the torpedo stage onward, strong GUS staining was associated with provascular tissues and the shoot apical meristem (Supplemental Fig. S5E). During postembryonic development, AXR6 expression was high in the shoot (Fig. 5A) and root apical meristems (Fig. 5C), vascular associated cells, and cotyledon and leaf hydathodes (Fig. 5, A and B). In the flowers, AXR6pro:GUS was mainly expressed in the vasculature, mature stigmas, ovules, and mature pollen grains (Fig. 5, D–F). We also examined AXR6pro:GUS expression during the early development of the
first pair of leaves, from 3 to 10 days, in phenotypically mutant and wild-type siblings from several F3 families segregating for the icu13 mutation. The initially broad AXR6pro::GUS expression in leaf primordia (Supplemental Fig. S5, F and K) was rapidly restricted to vascular associated cells and hydathodes (Supplemental Fig. S5, G–J and L–O). NPA treatment of either wild-type or icu13 seedlings increased vein density, which was correlated with an increase in the expression of the AXR6pro::GUS marker in their leaves (Supplemental Fig. S5, I and N).

Distribution of the AXR6 Protein

To study AXR6 protein distribution and subcellular localization, we used an AXR6::GFP fusion expressed under the control of the AXR6 promoter. We obtained two T2 families in the Col-0 background and four in the En-2 background, all of them expressing the AXR6pro::AXR6::GFP construct. AXR6::GFP was detected early in embryogenesis, from the dermatojen stage onward (Fig. 5, G–K). The pattern of early embryonic AXR6::GFP expression was ubiquitous, similar to that seen for AXR6pro::GUS. However, increased AXR6::GFP signal was detected in provascular tissues from the heart stage onward (Fig. 5, I–K). Postembryonic expression of AXR6::GFP was strong in the division zone of the root meristem (Fig. 5L), with both nuclear and cytoplasmic subcellular localization (Fig. 5, M and N) as described previously for the AXR6 protein (Farrás et al., 2001; Shen et al., 2002). In the root differentiation zone, AXR6::GFP was detected only in pericycle cells (data not shown). AXR6::GFP was broadly expressed in young leaf primordia (Fig. 5O), but in mature cotyledons and leaves, it was restricted to vascular associated cells (Fig. 5P) and some cells within the hydathodes (Fig. 5Q). We also performed a time-series analysis of AXR6::GFP localization during leaf development (Supplemental Fig. S5, P–T). In contrast to other auxin signaling (DR5rev) or vascular (PIN1, ATHB8) markers (see above), specific procambial expression of AXR6::GFP was not seen in leaf primordia earlier than 5 days (Supplemental Fig. S5Q). As leaf development proceeded, AXR6::GFP was restricted to vascular tissues (Supplemental Fig. S5, R–T). In addition, we obtained similar NPA-dependent patterns of AXR6::GFP to those previously found for AXR6pro::GUS (Supplemental Fig. S5S).

Genetic Interactions of icu13 and eta1 with Loss-of-Function Alleles of Genes Required for SCF Function

To investigate whether the precise regulation of SCF function is essential for leaf development and vein patterning, we examined the interaction of icu13 with mutations affecting genes that regulate the covalent modification of CUL1 by RUB. We obtained double mutant combinations of either icu13 or eta1 with axr1-12 (del Pozo et al., 2002), hve-2 (Alonso-Perala et al., 2006), rce1-10 (this work), and csn5a-2 (Dohmann et al., 2005; Fig. 6A), loss-of-function mutations that affect RUB modification of CUL1 (see introduction).

The axr1-12 and hve-2 mutants display reduced venation pattern complexity and leaf size compared with their wild-type counterparts (Dharmasiri et al., 2003; Alonso-Perala et al., 2006; Robles et al., 2010). We found a transfer DNA (T-DNA) insertion in the first intron of RCE1 that reduced its expression to 6% of the wild-type level (Supplemental Fig. S6), and we named this allele rce1-10. Leaves of homozygous rce1-10 plants were slightly hypsonastic (Fig. 6A) and normally sized, but they exhibited reduced venation density and number of branching points veins per millimeter squared (Fig. 6B; Table I). Leaves of the csn5a-2 mutant were of reduced size but with a venation density and number of branching points per millimeter squared similar to those of Col-0. The number of free-ending veins per venation length, however, was significantly decreased in csn5a-2 mutant leaves compared with those of Col-0 (Fig. 6B; Table I).

All the double mutant combinations of axr1-12, hve-2, rce1-10, or csn5a-2 with icu13 or eta1 (Supplemental Tables S3 and S4) shared a seedling-lethal phenotype that we interpreted as synergistic (Fig. 6, C–F). These double mutants strongly resembled homozygous axr6-1 and axr6-2 (Hobbie et al., 2000), mp (Berleth and Jürgens, 1993), or bdl (Hamann et al., 1999) single mutants, with a lack of roots and hypocotyls, strong defects in vein differentiation, and only incipient leaf development. Since this more-than-additive phenotype showed variable expressivity, we classified the embryo-lethal double mutants into five phenotypic classes, from strong (nongerminated seeds) to weak (seedlings developing more than two leaves; Fig. 6, C–F; Supplemental Table S3). However, no clear pattern emerged from our phenotypic classification that would allow us to make conclusions on differences in the strength of the genetic interactions found. We further dissected siliques from F2 plants that were homozygous for icu13 and heterozygous for axr1-12, rce1-10, csn5a-2, or hve-2, and vice versa. All double mutant embryos for all four genetic combinations displayed failures in the division of the hypophysis and hypophysis-derived cells, giving rise to embryonic phenotypes (Fig. 6, G, H, J, and K) similar to those previously observed in homozygous for icu13 and heterozygous for axr1-12, rce1-10, csn5a-2, or hve-2, and vice versa. All double mutant embryos for all four genetic combinations displayed failures in the division of the hypophysis and hypophysis-derived cells, giving rise to embryonic phenotypes (Fig. 6, G, H, J, and K) similar to those previously observed in homozygous for axr6 with loss-of-function mp (Berleth and Jürgens, 1993) or gain-of-function bdl (Hamann et al., 1999) and axr6 (Hobbie et al., 2000) mutations. The most extreme double mutant phenotypes that we found were in some cases more severe than those usually observed in mp, bdl, axr6-1, and axr6-2 homozygotes (Fig. 6, I and L). In summary, we found genetic interactions between loss-of-function alleles of AXR6 and four genes required to regulate SCF activity by the rubylation-derubylation pathway, suggesting that both activation and inhibition of CUL1 by RUB attachment and release, respectively, are essential for proper SCF function.
Genetic Interactions of *icu13* and *eta1* with Gain-of-Function Alleles of *Aux/IAA* Genes

The main role of the SCF^{TIR1/AFB1-3} complex is the ubiquitin-mediated destabilization of *Aux/IAA* repressors, which are negative regulators of auxin signaling (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b). We next examined the interaction of *icu13* and *eta1* with hypermorphic alleles of *Aux/IAA* genes, such as the dominant *shy2-10* (this work; see “Materials and Methods”) and *axr2-1* (Timpte et al., 1994; Supplemental Table S5) or the semidominant *axr3-3* (Leyser et al., 1996) and *icu6* (Berná et al., 1999; Pérez-Pérez et al., 2010) alleles (Supplemental Table S6). All plants of the eight double mutant genotypes analyzed were dwarfed and exhibited compact rosettes, with leaves of reduced size and increased hyponasty compared with their single mutant siblings (Fig. 7A), phenotypes that we interpreted as additive. Leaves of the *shy2-10* and *axr2-1* mutants were reduced in size, but their vascular phenotypes were similar to the wild type, the only exception being an increased number of free-ending veins per mm of venation (Table I). The *shy2-10 icu13*, *shy2-10 eta1*, *axr2-1 icu13*, and *axr2-1 eta1* double mutants displayed additive venation phenotypes, as we found a significant reduction in their leaf area and intermediate values in vascular complexity traits compared with those seen in both single mutants alone (Fig. 7B; Table I). All the double mutant combinations, including a loss-of-function allele of *AXR6* and a gain-of-function allele of *SHY2*, *AXR2*, or *AXR3*, were viable and fertile, in contrast to the interactions that we observed between loss-of-function alleles of *AXR6* and *AXR1*, *HVE/CAND1*, *RCE1*, or *CSN5A* (Fig. 6).

Genetic Interactions of *AXR6* with *BDL* and *MP*

As we mentioned in the introduction, seedlings homozygous for *bdl*, a semidominant, gain-of-function allele of *BDL*, do not develop basal structures due to...
defective early hypophysis specification (Hamann et al., 2002; Fig. 8, A and G), a phenotype that is similar to that caused by strong loss-of-function alleles of MP (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Fig. 8I). We found this phenotype to be reminiscent of that caused by strong loss of SCF function either directly (strong loss- and gain-of-function alleles of AXR6; Fig. 3) or indirectly (mutations affecting the regulators of SCF activity; Fig. 6). In our standard growth conditions, bdl/BDL heterozygotes (Fig. 8B) displayed a leaf incurvature similar to that seen in icu13 and eta1 homozygotes. In light of our finding that icu13 and eta1 do not interact with gain-of-function alleles of some Aux/IAA genes (Fig. 7), the dose-dependent phenotype of the bdl allele suggested to us that the leaf and vascular phenotypes of icu13 and eta1 primarily arise from the stabilization of BDL, whose levels are known to be precisely titrated during embryogenesis for proper specification of embryonic basal structures (Hamann et al., 2002). In several F2 families derived from selfed AXR6/icu13;bdl/BDL and AXR6/eta1;bdl/BDL plants, we found more rootless, early-lethal seedlings than expected, a significant proportion of which were heterozygous for the bdl mutation and homozygous for either icu13 (21%; n = 24; Fig. 8C) or eta1 (12.5%; n = 24; Fig. 8D). About one-quarter of the F2 seeds studied did not germinate, and as no double mutant was found among the germinated seedlings, we hypothesize that icu13 bdl and eta1 bdl double homozygotes might have an embryo-lethal phenotype. We then dissected siliques from AXR6/icu13;bdl/BDL and AXR6/AXR6;bdl/BDL selfed plants to visualize the embryonic phenotypes of the offspring by DIC microscopy. In all cases, wild-type embryos were found in siliques from homozygous icu13 and eta1 plants (Fig. 8, E and F), as expected since these mutations do not alter the root initiation program during embryogenesis. Moreover, whereas bdl/bdl embryos only display weak phenotypes derived from hypophysis misSpecification (Fig. 8, G and H), putative icu13 bdl double homozygote embryos show more severe defects, such as apparently twin embryos (Fig. 8, K and L) or embryos displaying strong division defects and overproliferation in the suspensor (Fig. 8, L–N). Similar embryo-lethal phenotypes were found in the progeny of AXR6/eta1;bdl/BDL selfed plants (data not shown).

Figure 7. Genetic interactions of icu13 and eta1 with gain-of-function alleles of SHY2, AXR2, and AXR3. Rosettes (A) and first-node leaf venation patterns (B) from double mutant combinations of either icu13 or eta1 and dominant alleles of SHY2 and AXR2 (shy2-10 and axr2-1) or semidominant alleles of AXR3 (axr3-3 and icu6) are shown. Photographs were taken 16 (A) and 21 (B) das. Bars = 2 mm.
BDL is known to interact with and inhibit the MP transcription factor, which is required for basal patterning of embryos and vascular development (Hamann et al., 2002; Hardtke et al., 2004; Weijers et al., 2006). To confirm that the genetic interaction found between AXR6 and BDL is mediated by MP, we crossed the weak mp-S319 allele (Cole et al., 2009) to icu13 or eta1. As described previously, only 7% to 14%

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**Figure 8.** Genetic interactions of icu3 and eta1 with bdl and mp. A to D, The bdl semidominant mutation causes seedling lethality when homozygous (A) as well as when heterozygous (B) with its wild-type allele in icu13 (C) and eta1 (D) homozygous backgrounds. E to Q, The embryonic phenotypes of homozygous bdl and mp-S319 are made more extreme by icu13. icu13 (E and F), bdl (G and H), and mp-S319 (I and J) single mutants and the icu13 bdl (K–N) and icu13 mp-S319 (O–Q) double homozygotes are shown at globular (E, G, and I) and heart (F, H, and J) stages. White arrows indicate abnormal divisions at the basal pole cells derived from the hypophysis. R to U, Confocal micrographs of roots stained with propidium iodide and expressing the BDL::BDL:GFP transgene in the En-2 (R and S) and icu13 (T and U) backgrounds, treated (S and U) or not (R and T) with the MG132 proteasome inhibitor. V, Average values of BDL::GFP fluorescence intensity in seedlings treated or not with MG132. Double and triple asterisks indicate $P < 0.05$ and $P < 0.01$, respectively. Photographs were taken 16 (A–D) and 4 (R–U) das. Bars = 2 mm (A–D) and 10 μm (E–U).
accumulates in the results are consistent with the hypothesis that BDL:GFP expression (Supplemental Fig. S7D). These re-
pavement cells of cleared icu13 GFP was found mainly in the nucleus of abaxial roots treated or not with MG132 (Fig. 8, U and V). BDL:
final of BDL:GFP was caused by overexpression of the shown). We discarded the idea that the stronger sig-
larms that BDL:GFP accumulation in double mutant phenotypes, we quantified the number of abortive seeds in the siliques of icu13/mp-S319; MP/mp-S319 (n = 30 from 109 seeds) and eta1/eta1;MP/mp-S319 (data not shown) plants. We then determined the embryonic phenotypes by DIC microscopy. We found abnormal cell divisions in the hypophysis and hypophysis-derived cells in homozygous mp-S319 embryos (Fig. 8, I and J) as described previously (Schlereth et al., 2010). All the abortive seeds contained dead embryos with mutant phenotypes of different degrees of severity, some of which are shown in Figure 8, O to Q.

We reasoned that the icu13 phenotype might be caused by the inactivation of MP caused by the stabili-
zation of its Aux/IAA repressor, BDL, as a conse-
quence of partial inactivation of the SCFTIR1/AFB1-3 complex (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b). To test our hypothesis, we obtained 10 icu13 and nine En-2 transgenic lines expressing the BDLpro-
BDL:GFP construct, which were used to study BDL stabilization. We found faint expression of the BDL:GFP protein in the central region of primary roots in young En-2 seedlings (Fig. 8R), which is indicative of rapid protein turnover via 26S proteasome degradation (Gray et al., 2001). Consistent with a reduced function of SCFTIR1/AFB1-3 in the icu13 mutants (see above), we found an accumulation of BDL:GFP in the vascular domain of the root meristem in icu13 plants (Fig. 8T). To confirm that BDL:GFP accumulation in icu13 is earlier and independent of proteasome function, we incubated young seedlings in the presence of MG132, a protea-
some inhibitor (Jensen et al., 1995). We found that a short MG132 treatment increased the accumulation of BDL:GFP in wild-type roots (Fig. 8, S and V), whereas no significant differences were found between icu13 roots treated or not with MG132 (Fig. 8, U and V). BDL:GFP was found mainly in the nucleus of abaxial pavement cells of cleared icu13 leaf primordia (Supplemental Fig. S7, A–C), whereas no BDL:GFP signal was found in wild-type leaves (data not shown). We discarded the idea that the stronger signal of BDL:GFP was caused by overexpression of the BDL gene in the icu13 background, by quantifying BDL expression (Supplemental Fig. S7D). These results are consistent with the hypothesis that BDL:GFP accumulates in the icu13 mutant due to a defective SCFTIR1/AFB1-3 function, which is upstream of proteasomal degradation.

Taken together, the genetic interactions found be-
tween AXR6, BDL, and MP suggest that the regulatory pathway involving these three genes is mainly re-
sponsible for the specification of embryonic basal structures and for the specification of postembryonic vascular tissues and that tight regulation of BDL activity through auxin-mediated SCF regulation is required.

Loss of Function in BDL and Gain of Function in MP Suppress the Phenotype of icu13

To investigate if the phenotype of icu13 depends on the MP-BDL module, we assessed to what extent it can be modified by MP overexpression and obtained independent T1 lines carrying the 35Spro:MP transgene (Hardtke et al., 2004) in the icu13 (n = 24) and En-2 (n = 4) backgrounds. In 10 35Spro:MP icu13 T2 families, we observed segregation for a mild leaf hyponasty phe-
notype, clearly distinguishable from that of the icu13 homozygotes (Fig. 9A). In all cases (n = 13), plants with this phenotype were icu13/icu13 and carried the 35Spro:MP construct, likely as homozygotes. We confirmed further this partial rescue in T3 35Spro:MP families that exhibited a mild leaf incurvature and found that all were transgenics for the 35Spro:MP construct and homozygous for the icu13 mutation (Fig. 9B).

As BDL accumulated to higher levels in icu13 (Fig. 8, R–V), we hypothesized that a stronger phenotypic suppression might be achieved with a lack-of-function allele of BDL. We crossed icu13 and eta1 to the iaa12-1 mutant (Overvoorde et al., 2005; this work), which bears a T-DNA insertion in the second exon of BDL, abolishing its expression (Supplemental Fig. S8). In the F2 progeny, the icu13/icu13;iaa12-1/iaa12-1 double
DISCUSSION

Leaf vascular patterning is assumed to be established by a polar flux of auxin. Consistent with the canalization hypothesis (Sachs, 1981), cells that primarily respond to auxin express the auxin efflux facilitator PIN1 in a polar fashion (Scarpella et al., 2006, 2010; Wenzel et al., 2007). PIN1 positioning within a row of cells allows a more efficient auxin transport through them than through their neighbors, which results in narrower PIN1 expression domains. Cells suited for auxin flow will later differentiate into functional veins (Scarpella et al., 2006, 2010; Wenzel et al., 2007). Hence, PIN1 expression represents the first stage of vascular differentiation, and the irreversible stage of preprocambial cell identity is marked by the later expression of the transcription factor ATHB8 (Baizabal et al., 1995; Scarpella et al., 2006). The direct link between these two stages is led by auxin signaling, as MP directly activates ATHB8 expression (Donner et al., 2009).

icu13 leaves display a simple venation pattern with a lack of higher order veins and an increased number of free-ending veins. A reduction of PIN1:GFP expression in the young primordia was the earliest defect observed during icu13 leaf development. Low PIN1 expression in icu13 primordia might cause a reduction in auxin transport through PIN1-expressing cells. Although we did not measure auxin levels directly, the lower responses of the DR5revGFP auxin reporter found in icu13 leaf primordia is consistent with the hypothesis that auxin levels in provascular cells are lower in icu13 than in En-2. Alternatively, defective function of the SCFTIR1/AFB1-3 complex in icu13 leaf primordia may directly cause reduced auxin responses. The expression of late vascular markers, such as ATHB8proGUS, correlated to those of PIN1:GFP in both wild-type and icu13 seedlings. Since PIN1 expression depends on MP (Weijers et al., 2006; Wenzel et al., 2007; Schuetz et al., 2008) and MP binds to the ATHB8 promoter to activate its expression (Donner et al., 2009), the simpler expression patterns of PIN1:GFP and ATHB8proGUS in icu13 leaf primordia will mirror active MP levels. However, the earliest differences observed between icu13 and En-2 leaves involve PIN1:GFP but not ATHB8proGUS, suggesting that ARFs other than MP might activate PIN1 expression during the initial stages of leaf development. Consistent with these data, quadruple mutants of the YUCCA (YUC) genes, which encode flavin monooxygenases required for auxin biosynthesis, fail to develop veins (Cheng et al., 2007).

The expression pattern of the AXR6 gene and AXR6 protein fully overlapped, as expected if the SCF complex acts cell autonomously. For SCFTIR1/AFB1-3 the auxin molecule acts as the diffusible factor that determines its function (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b). During embryogenesis, the AXR6:GFP fusion protein was broadly expressed in the embryo and the suspensor up to the heart stage and was later restricted to the central domain of the embryo, mirroring the MP and BDL expression domains within the vascular precursor cells (Hardtke and Berleth, 1998; Hamann et al., 2002; Hardtke et al., 2004; Cole et al., 2009; Rademacher et al., 2011). Postembryonically, the AXR6 gene and protein patterns remain comparable to MP expression in the developing veins and the hydathodes within the leaf primordia (Wenzel et al., 2007). Interestingly, the earliest expression patterns for AXR6 in leaf primordia did not fully overlap with auxin signaling, measured by DR5revGFP reporter expression, which suggests the involvement of other SCF complexes in the leaf initiation process.

Loss-of-function mutations in genes required to regulate SCF assembly through the rubylation-deubylation pathway, such as AXR1, HVE, RCE1, and CSN5A (del Pozo et al., 2002; Dohmann et al., 2005; Alonso-Peral et al., 2006; Hotton and Callis, 2008; Hua and Vierstra, 2011; see introduction), displayed reduced venation density in their leaves (Dharmasiri et al., 2003; Alonso-Peral et al., 2006; Robles et al., 2010; this work). Similar to the results described for axr1-12 etal1 (Quint et al., 2005), axr1-30 cul7-1 (Gilkerson et al., 2009), and etal2-1 etal1 (Zhang et al., 2008), the double
mutants obtained in this work involving icu13 or eta1 and mutations in the SCF rubylation-derubylation pathway displayed seedling lethality phenotypes resembling homozygous mp, bdl, axr6-1, or axr6-2. We hypothesize that, when AXR6 levels are reduced (as in the icu13 or eta1 mutants), the turnover between active and inactive SCF complexes is limiting for optimal SCF function. Our results are in agreement with the current model, where the cyclic rubylation-derubylation of the AXR6 subunit determines SCF activity (Gray et al., 2001, 2002; del Pozo et al., 2002; Hotton and Callis, 2008).

The pleiotropic phenotype of icu13 mutants is likely due to the importance of the AXR6 protein as a scaffold for SCF function, which is a key integrator of intracellular hormone-regulated responses and environmental stimuli (Vierstra, 2009). Our results indicate, however, that most of the phenotype of icu13 might be related to auxin signaling defects alone. Auxin signaling through SCF acts on the stability of Aux/IAA repressors, of which there are 29 members in Arabidopsis (Overvoorde et al., 2005; Guilfoyle and Hagen, 2007; Mockaitis and Estelle, 2008). Gain-of-function mutants in several Aux/IAA repressors with partially overlapping phenotypes have been described; this suggests functional redundancy between Aux/IAA homologs (Overvoorde et al., 2005; Mockaitis and Estelle, 2008; Pérez-Pérez et al., 2010). Although shy2-10, axr2-1, axr3-3, and icu6 displayed subtle alterations in their venation patterns (this work), we did not observe any enhancement of their vascular defects either by icu13 or eta1. On the contrary, the semidominant phenotype of bdl was clearly enhanced by icu13 or eta1 mutations. We also found that BDL:GFP was stabilized in the icu13 mutants, and the loss of function of BDL partially restored the wild-type phenotype of icu13 leaves. Taken together, our results indicate that the primary cause of the icu13 phenotype is BDL stabilization caused by defective SCF function, which limits the 26S proteasome-mediated degradation of BDL. Since the main function of BDL is to inhibit MP activity (Hamann et al., 2002; Hardtke et al., 2004), the phenotype of icu13 might be due to inactivation of MP function. In support of this hypothesis, icu13 and eta1 also enhanced the phenotype of a weak hypomorphic MP allele, mp-S319. Also, leaf upward curling and vascular phenotypes in icu13 were partially rescued by the gain of function of MP. However, we did not obtain a full rescue of leaf flatness and vascular development in icu13 by manipulation of the MP-BDL module, which might be due to the functional redundancy between BDL and its closest homolog, IAA13 (Weijers et al., 2005), or to the repression of the MP gain of function by the endogenous BDL (Krogan et al., 2012). Nevertheless, it should not be forgotten that icu13 might display an altered ubiquitination profile and the accumulation of many other SCF targets (Maor et al., 2007; Manzano et al., 2008). As proposed previously for the leaf incurvature of the icu6 mutant (Pérez-Pérez et al., 2010), our observations suggest that the adaxial tissues in icu13 leaves are mostly defective in cell expansion and that the upward curling of icu13 leaves might be explained by the differential growth of adaxial and abaxial tissues and by the limited space available for the internal mesophyll.

The icu13 bdl, eta1 bdl, icu13 mp-S319, and eta1 mp-S319 double mutants die during embryogenesis, with morphological alterations ranging from arrest at the globular stage with additional divisions of suspensor cells, as also seen in axr6-2/cul1-1 and axr6-2/cul1-2 heterozygotes, to cell overproliferation of the embryo and the suspensor, producing root-like structures. The latter phenotype was also found in double homozygotes of icu13 and axr1-12, rce1-10, csn5a-2, or hve-2. Such strong defects were never seen in bdl and mp mutants alone, but similar phenotypes have been found in embryos affected in auxin biosynthesis (yuc1 yuc4 yuc10 yuc11 quadruple mutants; Cheng et al., 2007), auxin transport (pin1 pin3 pin4 pin7 quadruple mutants; Friml et al., 2003), or auxin signaling (arf1 arf2 arf6 triple mutants; Rademacher et al., 2011). These similarities suggest that the strongest embryonic phenotypes that we have found are caused by the specific disruption of auxin target regulation through the SCFTIR1/AFB1-3 complex. It may be possible that these root-like structures arise from early proliferation of a specific cell population within the proembryo. Further studies using specific cell identity and cell-patterning markers (Lukowitz et al., 2004) will be required to determine the nature of the aberrant structures formed in these double mutants. It was proposed that MP acts in specifying the hypophysis via a non-cell-autonomous signal (Weijers et al., 2006; Schlereth et al., 2010), because the mp and bdl mutants are defective in the specification of the hypophysis but MP and BDL are expressed in the lower tier of subepidermal cells of the early globular embryos and not in the hypophysis (Hardtke and Berleth, 1998; Hamann et al., 2002; Weijers et al., 2006; Schlereth et al., 2010). DR5 maximum activity is observed in the hypophysis at this stage, indicating that there is auxin perception in response to auxin accumulation in this cell via PIN1 (Friml et al., 2003). Auxin signaling might be dependent on other ARFs to inhibit cell divisions in the hypophysis and its daughter cells, which will later give rise to the quiescent center, stem cells, and the columella cells of the root apical meristem (Dolan et al., 1993). Recently, detailed cellular expression of most ARFs during embryogenesis has been described (Rademacher et al., 2011), and there are some ARFs specifically expressed in the hypophysis and suspensor cells. ARF2 and ARF13 are expressed along the suspensor including the hypophysal cell (Rademacher et al., 2011), and both act as repressor ARFs in protoplast transfection assays (Ulmasov et al., 1999; Tiwari et al., 2003), which makes them candidates to inhibit the root initiation program during embryogenesis. Alternatively, the strong embryonic phenotypes found in the double mutants studied here, such as icu13 axr1-12, icu13 rce1-10, icu13
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cns5a-2, icu13 hae-2, icu13 bdl, etal bdl, icu13 mp-s319, and etal mp-s319, could be caused be unrestricted periclinal cell divisions of suspensor cells, as has been proposed for axr6-1 (Hobbie et al., 2000).

In conclusion, the similar synergistic phenotypes obtained in double homozygotes for loss-of-function alleles of AXR6 and mp-s319, bdl, or any loss-of-function alleles of genes associated with the rubylation-derubylation pathway (AXR1, RCE1, CSN5A, and HVE) indicate that tight regulation of SCF activity is essential to control Aux/IAA-ARF modules in response to the auxin signal. Validated among the Aux/IAA-ARF modules, we validated that the MP-BDL module is required for leaf vein specification, leaf flatness, and basal patterning during embryogenesis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (Arabidopsis thaliana) plants studied in this work are homozygotes for the mutations indicated unless otherwise stated. The Nottingham Arabidopsis Stock Centre (NASC) provided seeds of the icu13 mutant (NASC stock code N349), which was donated to the Arabidopsis Information Service Form Mutants collection by A.R. Kranz (Kranz and Kirchheim, 1987; Serrano-Cartagena et al., 1999). The etal (Quint et al., 2009), axr6-2/AXR6 (Hobbie et al., 2000), AXR6-E1-1 and AXR6-E1-2 (Shen et al., 2002), bdl/BDL (Hamann et al., 1999), and MP/mp-S319 (Cole et al., 2009) seeds were kindly provided upon request by the scientists mentioned in “Acknowledgments.” Seeds of the hae-2 (SALK_094979; N594979; Alonso-Peréz et al., 2006), axr1-12 (N3076; del Pozo et al., 2002), axr2-1 (N3007; Timpte et al., 1994), and axr3-3 (N57505; Leyser et al., 1996) mutants and of the wild-type accessesions En-2, Col-0, and Landsberg (N57505; Leyser et al., 1996; Berná et al., 1999; Pérez-Pérez et al., 2010). The AXR6pro::GUS (Curtis and Grossniklaus, 2003) in the En-2 background (previously named icu1 [N379]; Serrano-Cartagena et al., 1999, 2000) contains a mutation identical to icu1-3 (Tian and Reed, 1999) in the En-2 background (this work). We previously described a few aspects of the icu1 allele of AXR3 (Berná et al., 1999; Pérez-Pérez et al., 2010). The icu1::GUS (G394804; N437746), csn5a-2 (SALK_027705; N527705; Dohmann et al., 2005), and icu1-1 (SALK_138884; N638884 [it contains the same insertion as N252133]; Overvoorde et al., 2005) T-DNA insertion lines were obtained from the NASC and arabiWeb site (http://signal.salk.edu/). We confirmed the presence and positions of all T-DNA insertions by PCR amplification using the gene-specific primer pairs listed in Supplemental Table S1 and the left border primers of the T-DNA insertion as indicated. Lines carrying the DR5::GFP, PIN1::PIN1-GFP, and ATHB8::GUS transgenes have been described elsewhere (Bämna et al., 1995; Friml et al., 2003; Xu et al., 2006).

Plants were grown under sterile conditions on 150-mm petri dishes containing 100 mL of one-half-strength Murashige and Skoog (MS) agar medium with 1% Suc at 20°C ± 1°C, 60% to 70% relative humidity, and continuous illumination at approximately 80 μmol m−2 s−1, as described by Ponce et al. (1998). To study the effects of exogenous auxin on root growth, seedlings were grown for 4 d on vertically oriented plates containing MS medium and then transferred to plates containing MS medium supplemented with 0, 50, 100, or 200 mM L-IAA (Sigma-Aldrich) that were kept vertically oriented for 6 d before their root phenotypes were scored. To study the effects of NPA, seedlings were grown on standard plates containing 0 or 1 μM NPA (Sigma-Aldrich).

Construction of Transgenes

A single colon is used to indicate promoter-coding sequence fusions and fusions of coding sequences. For the sake of clarity, a single colon indicates that a gene is driven by its native promoter. To construct the AXR6::GUS transgene, a 2.3-kb fragment from the upstream region of the start codon of the AXR6 gene was PCR amplified from Col-0 genomic DNA with Phusion High-Fidelity DNA Polymerase (Finnzymes, Thermo Fisher Scientific). The PCR product obtained was purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences) and then cloned into the pGEM-T Easy221 vector using the BP Clonase II kit (Life Technologies) as indicated by the manufacturer. Chemically competent Escherichia coli DH5α cells were transformed by the heat-shock method, and the orientation and structural integrity of the inserts carried by the transformants isolated were verified by sequencing. The target sequences were transferred via an LR Clonase II (Life Technologies) reaction to the pMDC165 Gateway-compatible destination vector containing the gusA gene (Curtis and Grossniklaus, 2003). For the AXR6::AXR6::GFP and BDL::BDL::GFP transgenes, 6.4- and 3.7-kb segments of the AXR6 and BDL genes, respectively, were amplified from Col-0 genomic DNA, cloned into pGEM-T Easy221, and subcloned into pMDC107, which harbors an in-frame version of the GFP6 gene (Curtis and Grossniklaus, 2003), using the Gateway technology as described above. All these constructs were mobilized into Agrobacterium tumefaciens GV3101 (CS811 Rif′) cells, which were used to infect En-2, icu13, Col-0, and etal plants by the floral dip method (Clough and Bent, 1998). T1 transgenic plants were selected on plates supplemented with 15 μg mL−1 hygromycin B (Invitrogen). For the 35S::MP construct (Hardtke et al., 2004), A. tumefaciens GV3101 cells were transformed with plasmid DNA, and the transformants were used to infect En-2 and icu13 plants. T1 seeds were sown on river sand watered with a 15 mL L−1 solution of BASTA (Finale; Bayer CropScience) as described by Hadi et al. (2002). Primers used in the PCR amplifications performed to obtain the AXR6::GUS, AXR6::AXR6::GFP, and BDL::BDL::GFP constructs are described in Supplemental Table S1, together with those used to genotype antibiotic- and herbicide-resistant T1 plants.

Morphological and Histological Analyses

For root growth studies, root images were obtained with a flattened scanner. Root length was digitally measured (n ≥ 17 per genotype and hormone concentration tested) with the NIS Elements AR 2.30 image-analysis package (Nikon). Flowering time was scored by counting the number of icu13 and En-2 plants (n = 165 per genotype) that had a visible flowering stem at 17 to 27 dats. Rosette leaf number was scored in the same plants at 21 dats. To assay the degree of apical dominance in mutant and wild-type plants, we recorded the flowering stem number at 45 dats. For embryo visualization, siliques from 6-week-old plants were manually excised and dissected, and their seeds were mounted in an 8:1:3 mix of chloral hydrate:glycerol:water and observed under DIC optics as described previously (Weijers et al., 2001). GUS staining of embryos was performed as described by Weijers et al. (2003).

Rosette and leaf images were taken 16 and 21 dats, respectively, with an MZe stereomicroscope (Leica Microsystems) equipped with a DMI1200 digital camera (Nikon Instruments). Leaf section micrographs were taken as described by Serrano-Cartagena et al. (2000). Venation morphometry was performed on diagrams that were obtained from micrographs of 10 cleared first-node leaves per genotype, as described previously (Candela et al., 1999; Alonso-Peréz et al., 2006; Robles et al., 2010).

All data were analyzed using the Statistical Package for the Social Sciences (SPSS 16.0.2) software, and their normality was assessed by Kolmogorov-Smirnov tests with Lilliefors correction. When the data fitted a normal distribution, Student’s t tests were used to compare mean values; otherwise, the nonparametric Mann-Whitney U test was used instead.

Confocal laser scanning microscopy (CLSM) images were obtained digitally and processed using the software package EZ-C1 for a Nikon C1 confocal microscope (Nikon Instruments). Leaf section micrographs were taken as described by Serrano-Cartagena et al. (2000). Venuation morphometry was performed on diagrams that were obtained from micrographs of 10 cleared first-node leaves per genotype, as described previously (Candela et al., 1999; Alonso-Peréz et al., 2006; Robles et al., 2010).

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Confocal laser scanning microscopy (CLSM) images were obtained digitally and processed using the software package EZ-C1 for a Nikon C1 confocal microscope (Nikon Instruments). GFP was excited at 488 nm with an argon ion laser, and its emission was analyzed between 515 and 530 nm. To visualize GFP from embryos and leaf primordia, chlorophyll was solubilized by incubating seedlings in 1 mL methanol:acetic acid for 30 min at −20°C and then transferring the samples to phosphate buffer, pH 6.8, prior to confocal microscopy. To avoid nonspecific fluorescence in the green channel, in some cases we also excited at 543 nm with a helium-neon laser and analyzed the emission between 605 and 675 nm, recording the nonspecific fluorescence in red. For CLSM micrographs of roots, we stained seedlings with a 10 μg mL−1 solution of propidium iodide (Sigma-Aldrich), which visualizes cell walls in the red channel. To confirm the nuclear localization of AXR6::GFP and BDL::GFP, we stained seedlings with a 10 μg mL−1 solution of 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) for 5 min, washed them extensively with water, and further excited them at 408 nm with a modulated diode laser. All images from a given GFP marker at a defined time point were taken using the same CLSM settings. For comparison of GFP fluorescence from icu13 and wild-type seedlings, we used F2 or F3 siblings unless otherwise stated.
Quantification of the BDL-GFP Fusion Protein

To quantify BDL-GFP levels, 4-d-old seedlings from three En-2 and three icu13 T2 families carrying the BDL<sub>prom</sub>-BDL::GFP transgene were transferred to liquid MS medium supplemented with 50 μM MG132 (PeptaNova), a proteasome inhibitor, or 0.5% dimethyl sulfoxide (Sigma-Aldrich). After 6 h of incubation under standard experimental conditions, seedlings were washed three times for 5 min with liquid MS medium to remove residual dimethyl sulfoxide or MG132. CLSM images were then taken from propidium iodide-stained roots (see above) and processed using ImageJ as follows. First, the green channel of each CLSM image was converted to eight-bit format images using a defined threshold. Then, we selected an equivalent region of interest for each image, as a line drawn in the center of the root that extends from the quiescent center up to the elongation zone. A plot profile of the gray values along this line was recorded for each image, which positively correlates with the GFP intensity as described by Scacchi et al. (2010). Arbitrary units were converted to percentages of GFP intensity, defining the 100% value as the mean of icu13 values. GFP intensity data were analyzed using SPSS by applying Kolmogorov-Smirnov tests by applying Kolmogorov-Smirnov tests with Lilliefors correction, and Student’s t tests to compare mean values.

Western Blot

Protein samples were obtained from 30 to 40 seedlings collected at 6 das and extracted with buffer containing 100 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. SDS-PAGE was performed by adding 20 μL of each protein extract using the Mini-Protein Cell kit (Bio-Rad Laboratories). Electrophoresis was performed on nondenaturing membrane (Amersham, GE Healthcare) as described in the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) method. We used anti-CUL1 (Gray et al., 1999) at a 1:3,000 dilution as a primary antibody and anti-RbCL (Agrisera) at a 1:10,000 dilution as a loading control. A goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Agrisera) was used as a secondary antibody, which was detected with the Immun-Star HRP Chemiluminescent Detection Kit (Bio-Rad) as described by the manufacturer and Lumi-Film Chemiluminescent Detection Films (Roche Diagnostics).

qRT-PCR

Total RNA was extracted from 60 to 100 mg of rosette tissue collected 14 das using the citric acid method (Ohtake-Sánchez and Vicente-Carbajosa, 2008). cDNA was reverse transcribed using Maxima Reverse Transcriptase (Fermentas, Thermos Fisher Scientific). Either Power SYBR Green PCR Master Mix (Life Technologies) or Maxima SYBR Green qPCR Master Mix (Fermentas) kits were used as indicated in the manufacturers’ instructions. At least one oligonucleotide in each primer pair was designed to span the ends of two adjacent exons so that genomic DNA would not be amplified. Relative quantification of gene expression data was performed using the comparative threshold cycle method (Livak and Schmittgen, 2001). Each reaction was made using three biological replicates, each with three technical replicates; the expression levels were normalized to the cycle threshold values obtained for the housekeeping gene OTC (Queneda et al., 1999).

Double Mutant Analyses

Double mutant phenotypes caused by the combination of recessive mutations in two genes were independently confirmed in several F3 families derived from selfed F2 plants exhibiting the phenotype of one of the single mutant parents. In some cases, the putatively double mutant F3 seedlings were genotyped to confirm that they were double homozygotes, using the oligonucleotides shown in Supplemental Table S1. Genetic interactions of icu13 with dominant or semidominant mutations in Aux/IAA genes were analyzed in F2 plants, three of 16 of which exhibited the double mutant phenotype. To exclude phenotypes dependent on the genetic background, all the genetic interactions of icu13 were also assayed in parallel with eta1. The χ<sup>2</sup> statistic was used to calculate two-tailed P values to test whether the observed phenotypic ratios fit expected segregations.

Supplemental Data

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

Supplemental Figure S1. Missplicing caused by the icu13 mutation.

Supplemental Figure S2. Alignment of human CUL1 and the two CUL1 proteins encoded by icu13.

Supplemental Figure S3. Transverse sections from first-node leaves of En-2 and icu13.

Supplemental Figure S4. Visualization of DR5::GUS expression in icu13 and eta1 roots.

Supplemental Figure S5. Visualization of AXR6::GUS activity and AXR6::GFP localization in embryos and leaf primordia.

Supplemental Figure S6. Molecular nature of rce1-10, a new hypomorphic allele of BCE1.

Supplemental Figure S7. Expression of BDL-GFP in icu13 leaves and qRT-PCR analysis of BDL in transgenic BDL<sub>prom</sub>-BDEL::GFP plants.

Supplemental Figure S8. Molecular nature of iaa12-1 and its effects on BDL expression.

Supplemental Figure S9. Partial suppression of auxin resistance in icu13 by gain of function of MP or loss of function of BDL.

Supplemental Table S1. Primer sets used in this work.

Supplemental Table S2. Seed phenotypes in the F1 progeny of axr6-2/AXR6 × AXR6/cul1-1 crosses.

Supplemental Table S3. Lethality in single and double mutants.

Supplemental Table S4. Phenotypic segregation in the F3 of crosses between mutants carrying loss-of-function alleles of AXR6 and genes of the rubylation-denrubylation pathway.

Supplemental Table S5. Phenotypic segregation in the F2 progeny of crosses between etal or icu13 and axr2-1 or shy2-10.

Supplemental Table S6. Genotypic segregation in the F2 progeny of crosses between etal or icu13 and axr3-3 or icu6.

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Vein and Hypophysis Specification by AXR6


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