The ULTRACURVATA2 Gene of Arabidopsis Encodes an FK506-Binding Protein Involved in Auxin and Brassinosteroid Signaling

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The dwarf ucu (ultracurvata) mutants of Arabidopsis display vegetative leaves that are spirally rolled downwards and show reduced expansion along the longitudinal axis. We have previously determined that the UCU1 gene encodes a SHAGGY/GSK3-like kinase that participates in the signaling pathways of auxins and brassinosteroids. Here, we describe four recessive alleles of the UCU2 gene, whose homozygotes display helical rotation of several organs in addition to other phenotypic traits shared with ucu1 mutants. Following a map-based strategy, we identified the UCU2 gene, which was found to encode a peptidyl-prolyl cis/trans-isomerase of the FK506-binding protein family, whose homologs in metazoans are involved in cell signaling and protein trafficking. Physiological and double mutant analyses suggest that UCU2 is required for growth and development and participates in auxin and brassinosteroid signaling.

Plant growth and development are regulated through genetic networks that finally act on the division, expansion, and differentiation of cells to generate specific developmental patterns (Tsiantis and Langdale, 1998). Brassinosteroids (BRs) are steroid hormones belonging to the group of endogenous signals required for plant growth and organogenesis, controlling processes such as cell expansion, vascular differentiation, etiolation, reproductive development, and stress responses (Bishop and Yokota, 2001; Bishop and Koncz, 2002).

The biosynthetic pathway of brassinolide, the most active BR, has been dissected thanks to the genetic and molecular analysis of Arabidopsis dwarf mutants (Altmann, 1999; Li and Chory, 1999; Bishop and Yokota, 2001). Little is known, however, about the genes involved in BR signal transduction (Altmann, 2001; Friedrichsen and Chory, 2001; Bishop and Koncz, 2002; Clouse, 2002). Among the BR-insensitive dwarf mutants isolated until now, all those carrying recessive mutations were shown to be affected in the BRI1 (BRASSINOSTEROID INSENSITIVE1) gene, which codes for a cell-surface Leu-rich repeat receptor Ser/Thr kinase (Li and Chory, 1997; He et al., 2000; Wang et al., 2001). BRI1 transduces the BR signal into the nucleus through a mostly unknown phosphorylation cascade (Friedrichsen et al., 2000), leading to the differential expression of target genes, some of which are assumed to control cell expansion (Müssig et al., 2002). On the contrary, BR-insensitive semidominant dwarf mutants carry mutations in the UCU1 (ULTRACURVATA1; Berná et al., 1999) gene, also named BIN2 (BRASSINOSTEROID INSENSITIVE2; Li et al., 2001a), which encodes a member of the SHAGGY/GSK3 family of intracellular protein kinases (Li and Nam, 2002; Perez-Perez et al., 2002), whose metazoan homologs are involved in the negative regulation of several developmental pathways, such as those of insulin or Wingless/Wnt signaling (Kim and Kimmel, 2000; Cohen and Frame, 2001). More recently, screenings based on the use of a specific BR-biosynthesis inhibitor have led to the identification of dominant mutations in the BZR1 (BRASSINAZOLE RESISTANT1; Wang et al., 2002) gene and its homolog BES1 (BRI1-EMS-SUPPRESSOR1), also named BZR2 (Yin et al., 2002), the protein products of which are positive regulators of the BR signaling pathway and cooperatively regulate the expression of BR target genes. BIN2 (UCU1) has been shown to be a negative regulator of the BR signaling pathway that destabilizes BZR1 and BES1 by phosphorylation, in an analogous way to that of SHAGGY/GSK3 over β-catenin in the Wingless/Wnt pathway (He et al., 2002; Yin et al., 2002).

In this work, we present the genetic and molecular analyses of four loss-of-function, recessive alleles of the UCU2 gene, the phenotype of which is extremely similar to that of plants homozygous for the semidominant alleles of the UCU1 gene, sharing most phenotypic traits such as dwarfism and circinate leaves.
In addition, ucu2/ucu2 plants display helical rotation of some of their organs. We identified the UCU2 gene by a map-based approach and found that it encodes an FK506 binding-like protein (FKBP), named AtFKBP42. FKBPs are a class of peptidyl-prolyl cis-trans-isomerases (PPIases) that play critical roles in regulating cellular processes through protein activation (Harrar et al., 2001; Schiene-Fischer and Yu, 2001). The twd1 (twisted dwarf1) mutant, which is phenotypically indistinguishable of the ucu2 mutants, is also defective in AtFKBP42 (B. Schulz, unpublished data; quoted in Kamphausen et al., 2002), which indicates that TWD and UCU2 are the same gene. The responses of ucu2 mutants to exogenous plant hormones and the genetic analysis of ucu2 alleles suggest that UCU2 participates in auxin and BR signaling.

RESULTS

Isolation of ultracurvata2 Mutants

In a large-scale screening for Arabidopsis mutants displaying abnormally shaped or sized leaves (Berná et al., 1999; Serrano-Cartagena et al., 1999; Robles and Micol, 2001), one of the strongest leaf phenotypes found was that of ucu (ultracurvata) mutants, whose vegetative leaves are rolled spirally downwards, in a circinate manner (Fig. 1, B–D). Five ucu lines were identified among our mutants, which were found to fall into two complementation groups. On the one hand, the UCU1 gene was represented by one recessive (Fig. 1B) and two semidominant ethyl methanesulfonate-induced alleles (Fig. 1C; Pérez-Pérez et al., 2002). On the other hand, the UCU2 complementation group included two recessive alleles, ucu2-1 (Fig. 1D) and ucu2-3, which were respectively induced by fast neutron bombardment (P. Robles and J.L. Micol, unpublished data) and T-DNA insertional mutagenesis (this work). Although it was isolated from a collection of lines carrying T-DNA insertions, we found the ucu2-3 mutation to be untagged (data not shown). Another line displaying the Ultracurvata phenotype, CS3397, initially named inl (invalida) by Rédei, was obtained from the ABRC and found to be a double mutant carrying the ucu2-2 and gi-2 (gigantea-2; Rédei, 1962) recessive mutations, the latter conferring an extremely late flowering phenotype, both of them putatively induced by x-ray mutagenesis. After backcrossing the CS3397 line to Columbia-0 (Col-0), F2 plants that displayed the Ultracurvata phenotype and normal flowering time were isolated, and the absence of the gi-2 mutation was confirmed in their F3 progeny.

Figure 1. The Ultracurvata phenotype. A to D, Rosettes from a Landsberg erecta (Ler) wild-type individual (A) and several ucu mutants: B, ucu1-3/ucu1-3; C, ucu1-2/ucu1-2; and D, ucu2-1/ucu2-1. Vegetative (E, third node) and cauline (F, eighth node) leaves from an ucu2-1/ucu2-1 individual. G and H, Inflorescence of ucu2-1/ucu2-1 (G) and ucu2-3/ucu2-3 (H) plants. I and J, Helical rotation in some organs of ucu2-1/ucu2-1 plants: elongated pistil (I) and mature siliques (J). K and L, Root epidermis and root tip (M), visualized by light microscopy. N, Root vascular tissues, visualized by confocal microscopy. Pictures were taken 23 (A–D and K–Ni), 30 (E and F), and 45 (G–J) d after sowing. Scale bars = 4 mm (A–D and K–Ni), 2 mm (E, F, and J), 200 μm (K, M, and N), and 100 μm (L).
Pleiotropy of the Phenotype of ucu2 Mutants

All the ucu2 mutant alleles studied in this work displayed complete penetrance and only minor variations in expressivity. The rosette phenotypes of ucu2-1, ucu2-2, or ucu2-3 homozygotes are indistinguishable (Fig. 1D) and very similar to the homozygotes for the ucu1-1 and ucu1-2 semidominant alleles of the UCU1 gene (Fig. 1C).

Although the ucu mutants were isolated on the basis of the abnormal shape and size of their vegetative leaves, they display a pleiotropic phenotype. The ucu2/ucu2 plants are dwarf, with shorter roots, hypocotyls, stems, and fruits than the wild type (Table I), compact dark-green rosettes (Fig. 1D), and reduced inflorescence length with partial loss of apical dominance (Fig. 1, G and H), traits also displayed by the homozygotes for the semidominant ucu1 alleles (Fig. 1C; Table I; Pérez-Pérez et al., 2002). In addition, the three ucu2 mutant alleles cause helical rotation of several radial organs, such as roots, hypocotyls, stems, and pistils, a trait that is clearly visible at whole-organ (Fig. 1, I and J) and epidermal cell level (Figs. 1, K and L). Confocal microscopy of hypocotyls and roots allowed us to visualize in addition the whole-organ (Fig. 1, I and J) and epidermal cell level (Fig. 1N). Such spiral habits of growth in ucu2/ucu2 plants caused the distortion of roots and stems, which are wavy and disorganized (Fig. 1H). Their flowers are small, and their pedicels, stamens, pistils, and siliques are twisted (Fig. 1, I and J). Some homeotic transformations of sepals and stomata into pistil tissues were also found. Fertility is reduced in length, wide, and very wrinkled (Fig. 1F) but do not display the circinate morphology of those of ucu1/ucu1 plants.

The gravitropic response of the ucu2 roots is similar to that displayed by the wild-type ones, as already shown in the ucu1 mutants (Pérez-Pérez et al., 2002).

Leaf Architecture in the ucu2 Mutants

The vegetative leaves of ucu2/ucu2 plants show reduced expansion along the proximodistal axis (Fig. 1, D and E; Table I), which affects both the petiole and the lamina, and are indistinguishable from those of ucu1-1 or ucu1-2 homozygous plants (Fig. 1C; Table I). Their cauleine leaves are reduced in length, wide, and very wrinkled (Fig. 1F) but do not display the circinate morphology of those of ucu1/ucu1 plants.

When ucu2/ucu2 leaves are stretched on a slide for observation under a microscope, their adaxial surface wrinkles, as occurred in the semidominant ucu1/ucu1 mutants (Pérez-Pérez et al., 2002). The abaxial pavement cells of the ucu2/ucu2 mutants (Fig. 2, B, F, and G), however, are only slightly smaller than those of the wild type (Fig. 2, A and E), unlike that observed in the ucu1/ucu1 plants (Pérez-Pérez et al., 2002). No obvious differences with the wild type were found in the inner tissues of ucu2/ucu2 leaves (Fig. 2, C–G), in contrast to ucu1/ucu1 mutants, which showed a reduced number of air spaces (Pérez-Pérez et al., 2002). In addition, ucu2/ucu2 leaves displayed many more

<table>
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<th>Table 1. Some body parameters of representative ucu mutants</th>
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<tr>
<td>Values are means of at least 20 measurements ± SD. Lengths and weights are indicated in millimeters.</td>
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<tr>
<td>Body parameters</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Root length</td>
</tr>
<tr>
<td>Hypocotyl length (grown in the dark)</td>
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<tr>
<td>Number of vegetative leaves</td>
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<tr>
<td>Rosette diameter</td>
</tr>
<tr>
<td>Fresh weight</td>
</tr>
<tr>
<td>Dry weight</td>
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<tr>
<td>Petiole length</td>
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<tr>
<td>Lamina length</td>
</tr>
<tr>
<td>Lamina width</td>
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<tr>
<td>Pedicel length</td>
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<td>Siliques length</td>
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<td>Seeds per siliques</td>
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<tr>
<td>Primary stem length</td>
</tr>
<tr>
<td>No. of secondary stems</td>
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a Measurements were taken from plant material collected 11 d after sowing. b Measurements were taken from plant material collected 21 d after sowing. c Measurements were not determined. d ND, Not determined.
clustered stomata than the wild-type leaves on both the adaxial and abaxial epidermis (Fig. 2, A and B).

Bending of the *ucu2/ucu2* vegetative leaves is more pronounced along the primary vein, whose length is clearly reduced compared with the wild type (Fig. 2, H and J), whereas the higher order veins and the complexity of the venation pattern seem to be much less affected, similar to that already observed in plants homozygous for the *ucu1* semidominant alleles (Fig. 2I).

**Physiological Analyses**

The *ucu1* mutants display a de-etiolated phenotype similar to that of BR synthesis or response mutants (Pérez-Pérez et al., 2002). On the contrary, *ucu2/ucu2* plants grown in the dark displayed a wild-type photomorphogenic response because no leaves were developed (Table I; Fig. 3B). Although *ucu2/ucu2* hypocotyls were shorter than those of the wild type, their epidermal cells are not noticeably reduced in length along the proximodistal axis of the hypocotyls or in their width (Fig. 3A; data not shown).

We have shown previously that *ucu1* mutants are hypersensitive to auxin (Pérez-Pérez et al., 2002). The reduction in root length displayed by *ucu2/ucu2* plants grown in auxin-supplemented medium (20 or 50 nM 2,4-dichlorophenoxyacetic acid [2,4-D]) was similar to that found for the wild type (data not shown). Some phenotypic traits of the *ucu2/ucu2* plants, as the helical rotation displayed by some organs, are shared by mutants with disrupted auxin transport, such as lop1 (lopped1; Carland and McHale, 1996). Hence, we analyzed the responses of *ucu2/ucu2* mutants to auxin transport inhibition by specifically blocking auxin influx with 1-naphthoxyacetic acid (1-NOA; Parry et al., 2001) or auxin efflux with NPA and TIBA (Fujita and Syono, 1996), in this way disrupting net polar auxin transport. Root growth was more inhibited by 1 μM NPA in the *ucu2/ucu2* mutants than in their wild-type backgrounds (Fig. 4A). Similar results were obtained when using 1 μM TIBA (data not shown). When grown in the presence of these auxin efflux inhibitors, *ucu2/ucu2* mutants also displayed an increase in root width and in the number and length of root hairs compared with the wild type. In addition, root hairs developed closer to the root apex in the *ucu2/ucu2* mutants than in their wild-type backgrounds (Fig. 3D). We found that these traits can be phenocopied in wild-type seedlings exposed to 50 nM 2,4-D. The aerial parts of *ucu2/ucu2* seedlings also displayed hypersensitivity to auxin efflux inhibitors (Fig. 3, E and F).

The *aux1/aux1* mutant plants, which are affected in the AUX1 permease-like protein, display agravitropic roots and disrupted auxin uptake (Bennett et al., 1996; Marchant et al., 1999). Phenocopies of *aux1/aux1* mutants can be obtained by treating wild-type plants with the auxin influx inhibitor 1-NOA, which does not affect root elongation (Parry et al., 2001). Although small concentrations of 1-NOA up to 10 μM abolish gravitropism in wild-type seedlings, thus phenocopying *aux1/aux1* mutants, this trait is unaffected in *ucu2/ucu2* seedlings. In addition, we found that the *ucu2/ucu2* mutants display a moderately reduced sensitivity to 24-epibrassinolide in root elongation assays (Fig. 4B), as already shown for the
weak ucu1-3 recessive allele of the UCU1 gene (Pérez-Pérez et al., 2002).

**Double Mutant Analysis**

We obtained ucu1/ucu1;ucu2/ucu2 double mutants (Fig. 5, A and B; Tables II and III) whose overall phenotype was in all cases the same irrespectively of the strength of the ucu1 allele involved. Their rosette phenotype was strongly different from that of each ucu/ucu single mutant and quite similar to that of bri1/bri1 plants (Fig. 5G). The BRI1 BR-receptor (Li and Chory, 1997) is upstream of the UCU1 (BIN2) protein (Li and Nam, 2002; Pérez-Pérez et al., 2002) in the BR signal transduction pathway. The extreme rosette phenotype of the ucu1-1/ucu1-1;ucu2/ucu2 double mutant (Fig. 5A) makes it difficult to determine whether or not it indicates additivity or synergy. However, the extreme rosette phenotype of the ucu1-3/ucu1-3;ucu2/ucu2 double mutants (Fig. 5B) is clearly synergistic, given that it cannot be expected from the weak leaf phenotype of ucu1-3/ucu1-3 single mutant plants (Fig. 1B).

Several dwarf mutants have been described as affected in BR biosynthesis (Altmann, 1999). These include dim1 (Takahashi et al., 1995) and det2 (Li et al.,...
1996), which are specifically blocked in the early steps of the BR biosynthetic pathway. The ucu2/ucu2; dim1/dim1 (Fig. 5E) and ucu2/ucu2; det2/det2 (Fig. 5F) double mutants that we obtained displayed additive phenotypes similar to those of bri1/bri1 mutants (Fig. 5G). The helical rotation characteristic of ucu2/ucu2 organs was also displayed by all the double mutants obtained (Fig. 5D).

BR and Auxin-Induced Gene Expression

We tested whether exogenous 2,4-D or 24-epibrassinolide modulate the expression of several genes in the ucu mutants (Fig. 6). To this end, several genes were chosen, all of them already described as regulated by plant hormones. This group of genes included the following (Table IV): IAA7 (also named AUXIN RESISTANT2; AXR2) and IAA2, which are overexpressed in BR-deficient mutants and encode proteins of the Aux/indole-3-acetic acid (IAA) family (Nagpal et al., 2000; Liscum and Reed, 2002; Müssig et al., 2002) induced by IAA (Abel et al., 1995). CPD (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM; Szekeres et al., 1996) and ROT3 (ROTUNDIFOLIA; Kim et al., 1998), which encode two cytochrome P450 enzymes involved in BR biosynthesis, both of them negatively regulated by BR (Mathur et al., 1998; Bancos et al., 2002); CycD3 (CyclinD3), the product of which is involved in the progression of the cell cycle; and BEE1 (BRASSINOSTEROID ENHANCED EXPRESSION1), which encodes a transcription factor. CycD3 and BEE1 are known to be induced by BR (Friedrichsen et al., 2002; Müssig et al., 2002). We studied also the expression of a gene that has no known relationship with BR or auxin signaling, NCED3, which codes for the 9-cis-epoxycarotenoid dioxygenase, one of the enzymes involved in abscisic acid (ABA) biosynthesis, whose expression is positively regulated by ABA (Xiong et al., 2002). Transcription of the UCU1 gene was also studied.

Table II. Phenotypic segregations in the F2 progeny of crosses involving ucu mutants

<table>
<thead>
<tr>
<th>Cross (♀ × ♂)</th>
<th>F1</th>
<th>F2</th>
<th>Putative double mutants</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>ucu2-1/ucu2-1 × ucu1-1/ucu1-1</td>
<td>Ucu</td>
<td>38 Ucul</td>
<td>47 111 54 51</td>
<td>13</td>
</tr>
<tr>
<td>ucu2-1/ucu2-1 × ucu1-2/ucu1-2</td>
<td>Ucul</td>
<td>9 Ucul</td>
<td>54 104 60 56</td>
<td>14</td>
</tr>
<tr>
<td>ucu2-1/ucu2-1 × ucu1-3/ucu1-3</td>
<td>WT</td>
<td>30 WT</td>
<td>147 – 57 58</td>
<td>11</td>
</tr>
</tbody>
</table>

* The χ² values represent the fit of the data (only families segregating for the putative double mutant are shown) to an expected 3:6:3:3:1 phenotypic segregation.  

a The χ² values represent the fit of the data (only families segregating for the putative double mutant are shown) to an expected 9:3:3:1 phenotypic segregation. Corresponds to a modified 3:6:3:1:2:1 segregation, given that the ucu1-1/UCU1; ucu2-1/ucu2-1 and the ucu1-1/ucu1-1; ucu2-1/ucu2-1 individuals were phenotypically indistinguishable.
containing 50 nm 2,4-D or 100 nm 24-epibrassinolide and quantified by means of the comparative threshold cycle (C_T) method (Livak and Schmittgen, 2001), using the expression of the housekeeping OTC (OR-NITINÉ TRÁNSCARBAMILASE) gene as an internal reference. We found no significant differences in the expression of the NCED3, CycD3, BEE1, and UCU1 genes in the wild-type plants after auxin and BR treatment (data not shown). Hence, activity of these genes was not analyzed in the ucu mutants.

The ucu1 and ucu2 mutations do not affect induction by auxin of the AXR2 and IAA2 genes, as indicated by the similar strong effects of exogenous 2,4-D on the wild-type and mutant plants studied (Fig. 6, A and B). No remarkable effect of 24-epibrassinolide on AXR2 and IAA2 gene expression was observed. As expected, ROT3 and CPD were found downregulated by brassinolide in the wild types studied, an effect that is abolished or extremely reduced by the ucu2 mutations (Fig. 6, C and D). On the other hand, the expression of ROT3 and CPD in the ucu1 mutant studied was higher than in the wild type in noninductive conditions and did not change significantly by auxin or brassinolide treatment.

Isolation and Molecular Characterization of the UCU2 Gene

Using an F_2 mapping population obtained from three ucu2-1/ucu2-1 × Col-0 crosses, we determined the map position of the UCU2 gene, which was found to be closely linked to the AtDMC1 (Klimyuk and Jones, 1997) cleaved-amplified polymorphic sequence marker (1.92 ± 0.57 cm, n = 574) in a region of 12.11 cm (1.9 Mb), flanked by the nga162 (8.35 ± 1.76 cm, n = 278) and AthGAPFab (11.72 ± 2.12 cm, n = 252) markers (Fig. 7A). Allelism between UCU2 and the DIM (Takahashi et al., 1995) and AXR2 (Wilson et al., 1990) genes, the latter two mapping within the candidate interval, was ruled out after the corresponding complementation tests.

Based on the genome sequence available at Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de/proj/thal/db/index.html), we developed new simple sequence length polymorphism (SSLP) markers within the candidate region (Fig. 7B; Table V), which were used to screen for informative recombinants. The linkage analysis of 1,292 chromosomes performed using these new markers delimited the UCU2 gene to an interval of 160 kb, encompassing three BAC clones (Fig. 7B). One of the candidate genes within this region, At3g21650, included in the MIL23 BAC clone, encodes a putative B’-regulatory subunit of a protein phosphatase 2A (PP2A B’-ζ; Terol et al., 2002), which displays 54% identity and 71% similarity to the human B56 PP2A protein delta isoform (NP_006236). The latter is known to inhibit Wnt signaling in a similar way to that in which it reduces the signaling of SHAGGY/GSK3 metazoan kinases, by reducing the transcription of Wnt-responsive genes by means of β-catenin degradation through ubiquitinylation (Seeling et al., 1999; Li et al., 2001b). Given the similarities between the phenotypes of ucu1 and ucu2 mutants and that UCU1 encodes a SHAGGY/GSK3-like kinase (Pérez-Pérez et al., 2002), we considered At3g21650 a strong candidate. Hence, several primer pairs were designed to PCR amplify the genomic region containing the At3g21650 gene, but no amplification products were obtained from ucu2-1/ucu2-1 or ucu2-2/ucu2-2 genomic DNA. Because these results suggested a deletion, several primer pairs were designed for the adjacent gene, At3g21640, which codes for a putative FK506-binding protein (FKBP); once again, no PCR products were obtained. On the contrary, PCR products from both the At3g21640 and At3g21650 genes were obtained from the DNA of ucu2-3/ucu2-3 plants, and no differences were found with the wild type in the coding region of the At3g21650 transcription unit or in its 5’- and 3’-flanking regions. However, a rearrangement of 40 bp, including two small deletions in the 3′ region of the At3g21640 gene, was shown to cause a frameshift and to induce a premature stop codon in the ucu2-3/ucu2-3 mutant. In addition, we found by RT-PCR that the At3g21640 gene is expressed in ucu2-3/ucu2-3 plants, although its transcript is smaller than that of the wild type (Fig. 7D).

To determine the nature of the alterations carried by the ucu2-1/ucu2-1 and ucu2-2/ucu2-2 mutants, we performed a Southern blot using three probes spaced along the region of the MIL23 BAC clone including At3g21640 and At3g21650 (probe 3) and their two flanking genes, At3g21630 (probe 1) and At3g21660

Table III. Phenotypic segregations in the F_3 progeny of crosses involving ucu mutants

<table>
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<th>Cross (♀ × ♂)</th>
<th>F_2</th>
<th>F_3</th>
<th>Putative double mutants</th>
<th>χ^2 (3:1)</th>
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<tr>
<td>ucu2-1/ucu2-1 × ucu1-1/ucu1-1</td>
<td>UcuX</td>
<td>42</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>ucu2-1/ucu2-1 × ucu1-2/ucu1-2</td>
<td>UcuX</td>
<td>45</td>
<td>–</td>
<td>19</td>
</tr>
<tr>
<td>ucu1-3/ucu1-3</td>
<td>UcuX</td>
<td>50</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>ucu1-2/ucu1-2</td>
<td>UcuX</td>
<td>26</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td>ucu1-3/ucu1-3</td>
<td>UcuW</td>
<td>–</td>
<td>98</td>
<td>36</td>
</tr>
<tr>
<td>ucu2-1/ucu2-1</td>
<td>UcuW</td>
<td>–</td>
<td>100</td>
<td>40</td>
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No differences with the wild type were found for the genomic DNA of \( \text{ucu2-3/ucu2-3} \) plants (Fig. 7E). In regards to \( \text{ucu2-1} \), no hybridization bands were detected with probes 2 and 3, whereas the bands obtained with probe 1 were different from those of the wild type, suggesting the deletion of about 6 kb, which removes the \( \text{At3g21640} \), \( \text{At3g21650} \), and \( \text{At3g21660} \) genes. In addition, RT-PCR analyses confirmed the absence of the \( \text{At3g21640} \) transcription product (data not shown) in \( \text{ucu2-1/ucu2-1} \) and \( \text{ucu2-2/ucu2-2} \) plants (data not shown).

Complementation of the Mutant Phenotype of \( \text{ucu2-3/ucu2-3} \) Plants

With the aim of confirming that the lack of function of the \( \text{At3g21640} \) gene is responsible for the phenotype of \( \text{ucu2} \) mutants, a segment of 2,441 bp of the genomic region harboring this gene, including its whole coding region, which contains 1,235 bp of the 1,360-bp full-length cDNA, was cloned into the binary vector pART27 (see “Materials and Methods”) under the control of a \( \text{35S} \) promoter. Homozygous \( \text{ucu2-3} \) plants were transformed with the \( \text{35S:At3g21640:ocs} \) construct, and the \( T_2 \) progeny obtained was sown on kanamycin-supplemented medium. Six transformants were isolated, which unambiguously displayed wild-type vegetative and cauline leaf phenotypes, normal length of the stem and siliques, and no helical rotation of organs (Fig. 8A). The phenotypic segregations observed in their \( T_3 \) progeny (Table VI) suggested that the \( T_2 \) plants were as expected, hemizygous for the transgene. Furthermore, the presence of the \( \text{35S:At3g21640:ocs} \) transgene in the \( T_2 \) plants was confirmed by amplification of the segment of the \( \text{At3g21640} \) gene that is deleted in the \( \text{ucu2-3} \) allele (Fig. 8B).

In addition, we screened T-DNA collections for additional alleles of the \( \text{UCU2} \) gene. The Salk_012836 insertion line from the Salk Institute Genomic Analysis Laboratory (SIGnAL) collection carries a T-DNA insertion in the fifth exon of \( \text{At3g21640} \). Its mutant phenotype is indistinguishable from that of \( \text{ucu2} \) mutants and was inherited as a recessive trait (Fig. 8C). We named this allele \( \text{ucu2-4} \).

The \( \text{UCU2} \) Gene Encodes the FKBP42 of Arabidopsis

The \( \text{At3g21640} \) gene codes for a protein with homology to the FKBP type of PPIases (or rotamases; E.C. 5.2.1.8). Its mRNA is 1,360 nucleotides long (AJ224640), with an open reading frame of 1,095 nucleotides that gives rise to a protein of 365 amino acids and 42 kD, nine amino acids different from the \( \text{At3g21640} \) gene, as annotated in the MIPS genome project Web page (http://mips.gsf.de/proj/thal/db/index.html), probably as the result of the incorrect deduction of the splicing site. The \( \text{UCU2} \) gene consists of eight exons, and the \( \text{UCU2} \) protein shows significant similarity with the FKBP-type immunophilins (Galat, 2000), which are characterized by a conserved FKBP-like domain carrying the PPIase activity (Schiene-Fischer and Yu, 2001). When we compared the \( \text{UCU2} \) amino acid sequence with those of already known FKBP, we found a single PPIase
domain that includes amino acids 58 to 156. The C-terminal region of UCU2 contains three putative domains of the tetratricopeptide repeat (TPR) type, a 34-amino acid domain involved in protein-protein interactions, which mediates association into multi-protein complexes (Blatch and Lassle, 1999). When the amino acid sequence of UCU2 was compared with those of proteins found in databases, the highest degree of similarity was observed with other FKBP's: 50% similarity and 34% identity with the FKBP1 Rotamase (ROF1; Vucich and Gasser, 1996; S72485) and 46% similarity and 28% identity with PASTIC-

Table IV. Quantitative RT-PCR primer pairs used in this work

<table>
<thead>
<tr>
<th>Gene</th>
<th>Bacterial Artificial Chromosome (BAC)</th>
<th>Oligonucleotide Sequences (5' → 3')</th>
<th>PCR Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD (At5g05690)</td>
<td>M3J3</td>
<td>CTAACCTCAGAGGAAGCATGA</td>
<td>93</td>
</tr>
<tr>
<td>CycD3 (At1g43680)</td>
<td>F28A23</td>
<td>TCTGTAATCTCCCGATCATGTT</td>
<td>79</td>
</tr>
<tr>
<td>ROT3 (At1g53380)</td>
<td>F24P17</td>
<td>AAGCCTTATATACGGGAGGATT</td>
<td>87</td>
</tr>
<tr>
<td>BEE1 (At1g18400)</td>
<td>F15H18</td>
<td>CACTGAGAAAGTTCTTGGAGAA</td>
<td>104</td>
</tr>
<tr>
<td>IAA2 (At3g23030)</td>
<td>MXC7</td>
<td>TACACCTCCTACCCAATACTCAA</td>
<td>86</td>
</tr>
<tr>
<td>UCU1 (At4g18710)</td>
<td>F28A21</td>
<td>GAGATCTAAGAGGCTCCTAATTCT</td>
<td>99</td>
</tr>
<tr>
<td>AXR2 (At3g23050)</td>
<td>MXC7</td>
<td>GTCCTTTACTAAGGGAAACTAT</td>
<td>91</td>
</tr>
<tr>
<td>NCE3 (At3g14440)</td>
<td>MOA2</td>
<td>CCGTGGTTTACGGACAAAGAACA</td>
<td>103</td>
</tr>
<tr>
<td>OTC (At4g75330)</td>
<td>F1B16</td>
<td>TGAAGGGACAAACCGTTGTGATGT</td>
<td>95</td>
</tr>
</tbody>
</table>

Figure 7. Positional cloning of the UCU2 gene. A, Map position of the UCU2 gene as determined by low-resolution mapping. B, Physical map of the BAC contig assumed to contain the UCU2 gene. A total of 23 recombination events were identified relative to four molecular markers (those shown in italics) within this region. C, Candidate region with indication of the mutations found in the ucu2 mutants. Boxes, Exons; lines between boxes, introns. A, S, and E, Positions of restriction sites for AccI, SacI, and EcoRI, respectively, for the enzymes used for the Southern blot shown in E. D, Amplification products corresponding to the At3g21640 gene, obtained using the primers indicated at the top and genomic DNA (gDNA) or cDNA as a template, from wild-type ( Ler) and ucu2-3 homozygous ( ucu2-3) plants. The positions of the primers used within the region containing the At3g21640 gene are indicated as 4R, 4F, 2R, and 10F in C. E, Gel blots of genomic DNA from the ucu2 mutants hybridized with probes 1 and 2. The results shown were obtained by reprobing a single membrane. The absence of some bands is indicated by asterisks, and the differential bands present in the DNA of the mutants are indicated by arrows.


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cincinate vegetative leaves and helical rotation of some organs. The cincinate leaf phenotype of the \textit{ucu1} and \textit{ucu2} mutants is presumably the consequence of a perturbation in the coordination of the growth of the adaxial and abaxial tissues along the proximodistal leaf axis. Histological analyses of \textit{ucu2}/\textit{ucu2} leaves indicated that they might be defective in the differentiation of the primary vein. In fact, leaf morphology is severely altered in venation pattern mutants (Koizumi et al., 2000; Semiarti et al., 2001) and in wild-type plants treated with auxin transport inhibitors (Mattsson et al., 1999; Sieburth, 1999). A reduction in primary vein length was also observed in the \textit{ucu1} mutants (this work), which is consistent with the abaxial origin of these tissues. Although we have shown previously that the \textit{ucu1} mutants display differences in cell size between the abaxial and adaxial epidermis (Pérez-Pérez et al., 2002), no such differences were found in the mutant leaves of \textit{ucu2}/\textit{ucu2} plants.

The small size of most organs is a phenotypic trait shared by the \textit{ucu} mutants and BR-deficient or -insensitive mutants. The leaf morphology of \textit{ucu} mutants, however, is rather different from that of all other dwarf mutants affected in BR synthesis or perception, whose small rounded leaves suffer a significant reduction in both cell size and cell number in all the tissues studied (Nakaya et al., 2002). Both the vegetative and cauline leaves of \textit{ucu} mutants are reduced in length but retain a normal width, which indicates that the expansion of the organ in these mutants is reduced along the proximodistal axis but...
not along the mediolateral axis. In addition, the ucu2 mutants displayed an etiolated phenotype when grown in the dark, contrary to that shown by BR-deficient and -insensitive dwarf mutants, which indicates that ucu2 mutants are not affected in the repression by darkness through the BR signaling pathway of the light-induced developmental program.

Only recently has the genetic analysis of Arabidopsis mutants shed light on the molecular components of handedness in plants, by the identification of genes involved in establishing left-right asymmetry patterns (Hashimoto, 2002). A number of mutants with a helical habit of growth have been identified, including lefty1 and lefty2, which are affected in the genes that encode the TUA6 and TUA4 α-tubulins, both of them involved in cortical microtubule orientation (Thitamadee et al., 2002). These results, together with the observation that proper orientation of microtubule arrays is disrupted in the spiral mutants (Furutani et al., 2000), indicate the importance of microtubule organization in establishing helical handedness in Arabidopsis. Plant hormones such as auxins and BR, among others, can perturb plant morphology through microtubule reorientation (Catterou et al., 2001). It must be noted, however, that the helical rotation found in the ucu2 mutants only affects organs that present radial symmetry in the wild type, whereas all organs are rotated in other helical mutants. No helical rotation has been shown in any BR-deficient mutants described to date (Catterou et al., 2001).

The helical rotation displayed by ucu2 mutants might be related to that caused by mutant alleles of the LOPI (LOPPED1) gene (Carland and Hale, 1996), also named TRN1 (TORNADO1; Cnops et al., 2000). Such lop1 and trn1 mutants display a pleiotropic phenotype, which is characterized by a perturbation in leaf vasculature and abnormal patterns of cell expansion that cause helical rotation of the root and deformed leaves, including twisting of the petiole (Carland and McHale, 1996). The phenotype of the lop1 mutants seems to be caused by deficiencies in the basipetal transport of IAA (Carland and McHale, 1996). The maize rs2 (rough sheath2) mutant, which displays aberrant leaf vasculature development and dwarfism, was also found to be defective in the basipetal transport of auxin in the shoot (Tsiantis et al., 1999).

The UCU2 Gene Encodes an FKBP

To gain insight into the molecular nature of the UCU2 function, we positionally cloned the UCU2 gene, which was found to encode an Arabidopsis FKBP. FKBP5s belong to the PPIase family of folding proteins, also named rotamases, whose homologs are found in prokaryotes, such as Escherichia coli, and eukaryotes such as yeast, invertebrates, mammals, and plants (Galat, 2000). Low-M₇ FKBP5s participate in signal transduction pathways through association with transmembrane receptors and usually include a single PPIase domain, similar to that found in the UCU2 protein. An example of this is mammalian FKBP12, which has been found to be associated with the TGF-β type II receptor and calcium channels (Schiene-Fischer and Yu, 2001). Multidomain PPIases are characterized by their C-terminal domain, which binds calmodulin and interacts with multiple partners (Schiene-Fischer and Yu, 2001). The multidomain PPIase FKBP52 (52-kD mammalian FKBP) is associated with Hsp90 through its TPR domain and participates in the activation of the cytoplasmic glucocorticoid receptor complex and its migration into the nucleus (Galigniana et al., 2001; for review, see Pratt et al., 2001). It has been demonstrated recently that plants hold the entire heterocomplex assembly machinery that links the glucocorticoid receptor complex to dynein, the motor protein that guides the complex to the nucleus (Harrell et al., 2002). Furthermore, wheat plants overexpressing wFKBP77 showed dwarfism, sterility, and altered leaf shape (Harrar et al., 2001). The UCU2 protein is a high-M₇ FKBP that includes three TPR domains but only one PPIase domain, hence sharing structural properties with both previously known classes of FKBP5s.

We found the UCU2 protein to be the same as AtFKBP42, some of whose functional characteristics have been reported recently (Kamphausen et al., 2002), including its localization in the plasma mem-

### Table VI. Segregation of kanamycin resistance in the progeny of ucu2-3/ucu2-3 plants subjected to transformation

<table>
<thead>
<tr>
<th>Transgene Used</th>
<th>T₂ Non-supplemented medium</th>
<th>T₂ Supplemented medium</th>
<th>T₃</th>
<th>Kan² Kan³</th>
<th>T₃</th>
<th>Kan² Kan³</th>
<th>χ² (3:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S:At3g21640:ocs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>77</td>
<td>34</td>
<td>1.589</td>
<td>142</td>
<td>63</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>76</td>
<td>38</td>
<td>3.789</td>
<td>100</td>
<td>58</td>
<td>2.364</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>70</td>
<td>32</td>
<td>1.882</td>
<td>98</td>
<td>48</td>
<td>4.898</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>140</td>
<td>50</td>
<td>0.112</td>
<td>84</td>
<td>47</td>
<td>1.528</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>59</td>
<td>24</td>
<td>0.486</td>
<td>63</td>
<td>23</td>
<td>3.172</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>21</td>
<td>8</td>
<td>0.011</td>
<td>48</td>
<td>19</td>
<td>0.065</td>
<td></td>
</tr>
</tbody>
</table>

See Table 2 legend. Kan² and Kan³, Kanamycin-resistant and -sensitive individuals, respectively.
brane and in the tonoplast and the interaction of its TPR domain with the COOH-terminal region of the heat shock protein, Hsp90. Such an interaction with Hsp90 is similar to that already shown for other FKBPs (Owens-Grillo et al., 1996; Young et al., 1998) as the mammalian PPlases, FKBP51 and FKBP52, which is necessary for the maturation of nonactivated steroid hormone receptor complexes (Richter and Buchner, 2001). AtFKBP42 also may be part of an analogous steroid hormone receptor complex in Arabidopsis, probably through their interaction with the BRI1 BR receptor. No PPlase activity has been detected for AtFKBP42 (Kamphausen et al., 2002), in contrast to that found for mammalian FKBP51 and FKBP52 (Schiene-Fischer and Yu, 2001). At least another FKBP, FKBP71, is known to be involved in the development of Arabidopsis. Mutations in the PAS1 (PASTICCINO1) gene, which encodes the FKBP71 (Vittorioso et al., 1998), display altered cell division and elongation, probably as a consequence of the perturbation of cytoquinin signaling (Faure et al., 1998).

All the ucu2 alleles studied here are likely to be null, given that the ucu2-1 and ucu2-2 mutants carry a deletion of the whole At3g21640 gene and that their mutant phenotypes are indistinguishable from that of ucu2-3 and ucu2-4. The deletion of the At3g21630 and At3g21650 genes in the ucu2-1 mutant and that of At3g21650 and At3g21660 in the ucu2-2 mutant do not cause a visible phenotype, possibly due to gene redundancy. The At3g21650 gene, for instance, codes for the PP2A B′-c, which has at least seven paralogs in the genome of Arabidopsis (Terol et al., 2002).

**Auxin and BR Responses Are Perturbed in the ucu Mutants**

A large body of evidence supports the hypothesis of a connection between the auxin and BR signaling pathways. Some auxin-regulated genes, such as Aux/IAA and ARF (AUXIN RESPONSE FACTOR) genes, have been found overexpressed in BR-deficient mutants (those of ARF7, AXR3 [IAA17], SHY2 [IAA3], IAA2, AXR2 [IAA7], and IAA22 genes), or expressed in response to BR (such as IAA3 and IAA19; Müssig et al., 2002). In addition, altered responses to auxins have been described in some BR-deficient or -insensitive dwarf mutants, such as in the curl-3 tomato (Lycopersicon pinninellifolium) mutant, which is hypersensitive to 2,4-D and is affected in a homolog of the BRI1 receptor (Koka et al., 2000; Bishop and Koncz, 2002), in the sax1 (hypersensitive to ABA and auxin1) mutant, whose hypersensitivity to auxin is restored after BR treatment (Ephritikhine et al., 1999a; 1999b) or in the ucu mutants (Pérez-Pérez et al., 2002; this work). A reduction of IAA levels was also observed in the BR-deficient lkb mutants of pea (Pisum sativum; Nomura et al., 1997).

Our results on the morphological, physiological, and genetic analysis of the ucu mutants of Arabidopsis indicate the implication of the UCU genes in the signaling pathways of auxin and BRs. Further support to this hypothesis is given by the quantitative differences found between the ucu1 and ucu2 mutants and the wild types studied here with regard to the effects of 24-epibrassinolide on the expression of the CPD and ROT3 genes, which are involved in BR biosynthesis. Down-regulation by exogenous BR of CPD and ROT3 is abolished by the ucu1 and ucu2 mutations, which indicates that UCU1 and UCU2 are required for BR-regulated gene expression.

Our auxin transport inhibition analyses suggest that UCU2 might be involved in the regulation of the transport of auxin. Contrary to that expected from the hypersensitivity displayed by the ucu1 mutants to auxin (Pérez-Pérez et al., 2002) and by the ucu2 mutants to auxin transport inhibitors (this work), auxin-induced expression of AXR2 and IAA2 genes was found to be similar in the wild-type and in the ucu mutants. Further analyses will be required to ascertain whether or not the growth responses of the ucu mutants to exogenous auxin or auxin transport inhibitors are mediated by the AXR2 and IAA2 genes.

It has been proposed that ATP-binding cassette (ABC) plant proteins, which are related to those of the multidrug resistance (MDR) family of animal proteins (often referred to as P-glycoproteins), could be involved in auxin transport in plants (Gaedeke et al., 2001; Noh et al., 2001; Luschnig, 2002). Support for this hypothesis is provided by mutant alleles of the AtMDR1 gene, which are defective in basipetal auxin transport and cause epinastic cotyledons and wrinkled leaves (Noh et al., 2001). Furthermore, the phenotype of a double mutant lacking both AtMDR1 and its closest homolog AtPGP1 (Noh et al., 2001) is similar to that of the ucu2 mutants described in this work, with a dwarf phenotype, leaves curled down, and a distorted habit of growth.

AtMDR1 and AtPGP1 are likely to be required for auxin efflux, preventing excessive auxin accumulation (Luschnig, 2002). In a similar way, the ucu2 epinastic leaf phenotype can be explained by the accumulation of intracellular auxin as a result of a defect in its polar transport, either directly if UCU2 participates in the auxin transport complex or indirectly through the posttranslational modification of the AtMDR1 and AtPGP1 transporters. The analysis of ucu2/ucu2;atpgp1/atpgp1;atmdr1/atmdr1 triple mutants would help to test such a hypothesis. It is known that FK506 immunosuppressant drugs are able to inhibit P-glycoprotein-mediated MDR in mammals, which suggests that FKBPs might positively regulate ABC transporter function (Hemenway and Heitman, 1996). Increased auxin levels have been found in the atmdr5 mutants of Arabidopsis. The wild-type ABC transporter AtMRP5 is also able to transport organic anions when expressed in yeast, such as estradiol-17-(β-d-glucuronide), a product of the catabolism of steroids in animals (Gaedeke et al., 2004).


**UCU1 and UCU2 Function and Interaction**

The similarity between the leaf phenotypes of *ucu1* and *ucu2* mutants, together with their insensitivity to BR, prompted us to consider the possibility that the *UCU1* and *UCU2* genes participate in some developmental process related with BR signal transduction. We found that the phenotype of *ucu1 ucu2* double mutants is indistinguishable from that of *bri1* single mutants, which suggests that UCU1 and UCU2 act in convergent pathways, both probably downstream of the BRI1 receptor, to cooperatively trigger BR responses. The UCU2 protein can be a positive regulator of BR signaling, acting by controlling hormone transport through ABC transporters. Another possibility is that UCU1 and UCU2 participate in the same pathway and that the semidominant, gain-of-function *ucu1* alleles interfere with the function of UCU2.

Recessive mutations in the *AXR1* gene confer resistance to auxin and cause morphological alterations, such as wrinkled leaves. *AXR1* encodes a protein related to the ubiquitin-activating enzyme E1, which is a key component in a complex that controls ubiquitin-mediated protein stability (Leyser et al., 1993). In addition, it is known that alterations in the ubiquitin pathway, caused by overexpressing a modified ubiquitin that inhibits protein degradation, could originate curled down leaves in tobacco (*Nicotiana tabacum*; Bachmair et al., 1990). Furthermore, the auxin efflux carrier EIR1 (also named AGR or AtPIN2), which is involved in the gravitropic response of roots, is posttranscriptionally modified and requires the function of *AXR1*, suggesting that auxin transport is regulated through protein stability (Sieberer et al., 2000). UCU1 (BIN2) is likely to be a negative regulator of the BR transduction pathway, acting through the destabilization of the BES1 and BZR1 transcription factors (He et al., 2002; Yin et al., 2002). Such negative regulation may be dependent on protein degradation mediated by ubiquitin, which is analogous to protein degradation mediated by of SGG/GSK3 in the Wingless/Wnt pathway of metazoans (Barker et al., 2000; Clouse, 2002). On the other hand, metazoan SGG/GSK3 kinases regulate signaling pathways other than that of Wingless/Wnt, such as those of insulin or cell cycle control (for review, see Cohen and Frame, 2001). It has been described recently that BRI1 interacts with BAK1, another Leu-rich receptor-like protein kinase, which suggests a conserved downstream activation pathway between BR signal transduction in plants and the TGF-β signaling cascade in metazoans (Li et al., 2002). In fact, the interplay between the TGF-β and Wingless/Wnt signaling pathways is important for the specification of cell fates during animal development (Capdevila and Izpisúa-Belmonte, 2001). If a similar cross talk between pathways occurs in plants, the phenotype of *ucu1 ucu2* double mutants might suggest the implication of both UCU1 and UCU2 in an interaction between the BRI1 pathway and the activation of the ABC transporters. This would explain the similarities of *ucu1* and *ucu2* leaf phenotypes and their phenotypic differences with BR-deficient or -insensitive mutants.

A role for the *UCU2* gene can be proposed in the cross talk between the auxin and BR signal transduction pathways, which would be achieved primarily by regulation of auxin homeostasis through activation of one or both of the AtMDR1 and AtPgp1 ABC transporters or by the regulation of the BRI1 and BAK1 heterocomplex. Further molecular analyses will be required to gain insight into such a mechanism of cross talk between plant hormones.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Seeds from Arabidopsis *Lor* and Col-0 accessions were supplied by the Nottingham Arabidopsis Stock Center (Loughborough, UK). The CS397 line and the *DIM1/dim1-1* (CS100), *det2-1/det2-1* (CS6159), *BR1/bri1-1* (CS3723), *aux1-7/aux1-7* (CS3074), and *aux2-1/aux2-1* (CS3077) mutants were supplied by the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The *ucu2-1* and *ucu2-3* alleles, respectively, were isolated after fast neutron mutagenesis in a Lcr background (P. Robles and J.L. Miclo, unpublished data) and T-DNA mutagenesis in a Col-0 background (this work). The NS12836 insertion line (*ucu2-4/ucu2-4*) was supplied by the Nottingham Arabidopsis Stock Center and is described at the SIGnAL Web site (http://signal.salk.edu). The nomenclature rules of Meinke and Koornneef (1997) for phenotypes, genes, alleles, mutations, and proteins are followed in this paper.

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 luxes. Crosses were performed as described by Bernà et al. (1999).

**Photomicrography and Morphometric Analysis**

Morphometric analyses of *ucu2* mutants were carried out as described previously (Candela et al., 1999; Pérez-Pérez et al., 2002). Photographs were taken with a Cybershot FV-505 digital camera (Sony, Tokyo) using a resolution of 1,024 × 768 pixels. Photomicrography and confocal microscopy of whole leaves and leaf sections were performed as described by Serrano-Cartagena et al. (2000) and Pérez-Pérez et al. (2002). Images were digitally processed with the Adobe Photoshop 6.0 program (Adobe Systems Incorporated, San Jose, CA).

**Physiological Analyses and Hormone Treatment**

Photomorphogenic and gravitropic responses were studied as described by Pérez-Pérez et al. (2002). Photomorphogenic development was monitored 13 and 21 d after sowing.

For the root elongation assays of hormone-treated plants, at least 30 seedlings of each genotype were grown in vertically oriented agar plates supplemented with 0, 10, 50, or 100 nM epibrassinolide (no. E1641, Sigma, St. Louis), a mixture of synthetic BR; 0, 10, 20, or 50 nM 2,4-D (no. 11215-019, Life Technologies/Gibco-BRL, Cleveland), a synthetic auxin; 0, 1, 5, or 10 μM 1-NOA (no. 25,541-6, Aldrich, Milwauke, WI), an auxin influx transport inhibitor. To determine root lengths, primary roots grown in the above-mentioned supplemented media were stretched by forceps and photographed 13 d after
sowing. Statistical analysis of the data was performed with the SPSS version 7.5 software package (Statistical Products & Service Solutions, Chicago), and plots were obtained with the SigmaPlot 2000 version 6.0 program (Statistical Products & Service Solutions).

**Hormone-Induced Gene Expression Analyses**

Plants grown on agar plates as described above were collected 20 d after sowing and transferred to flasks containing 50 mL of one-half-strength Murashige and Skoog (no. M 0221, Duchaef Biochemie B.V., Haarlem, The Netherlands) liquid medium, which were incubated for 24 h at 225 rpm, 20°C ± 1°C, and 60% to 70% relative humidity under continuous illumination of 7,000 luxes. Ethanol solutions of 2,4-D or 24-epibrassinolide were then added to the liquid cultures to reach a final concentration of 50 or 100 nm, respectively. After incubation for 12 h in the same conditions, plants were immediately frozen in liquid nitrogen and stored at −80°C. RNA was extracted from frozen samples of 50 to 100 mg, using the Qiagen RNeasy Plant Mini Kit (no. 74904, Qiagen USA, Valencia, CA), following the instructions of the manufacturer. The eluate was incubated for 30 min at 37°C with 40 units of DNaseI. After inactivation of DNaseI at 70°C for 15 min, RNA was ethanol precipitated and resuspended in 40 μL of RNase-free water. About 4 μg of the RNA extracted from each sample was reverse transcribed using the SuperScript II RNase H RT following the instructions of the manufacturer (no. 18064-022, Invitrogen, Carlsbad, CA). The cDNA solution obtained in this way was diluted by adding 60 μL of distilled water.

**Real-Time Quantitative PCR**

Reaction mixes of 25 μL were prepared including 12.5 μL of the SYBR Green PCR Master Kit (no. 4309155, Applied Biosystems, Foster City, CA) containing the AmpliTaq Gold polymerase, 0.4 pmol of a primer pair, and 1 μL of cDNA. PCR amplifications were carried out in 96-well optical reaction plates on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Three independent amplifications were performed from each cDNA sample. The thermal cycling program started with a step of 2 min at 50°C and 10 min at 95°C to activate the polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Dissociation kinetics analyses of the amplification products were performed to check their specificity. Only the expected products were found to be amplified. The primer pairs used and the sizes of the expected amplification products are shown in Table IV. One of the primers of each pair contained the sequences of the ends of two contiguous exons to avoid amplification of genomic DNA. Relative quantitation of gene expression was carried out using the 2^−ΔΔCt method (Livak and Schmittgen, 2001). Expression levels of the target genes were normalized using the Ct values obtained for the OTC (ORNITINE TRANSCARBAMILASE) housekeeping gene (Quesada et al., 1999), which was used as an endogenous reference. The relative amount of target gene transcripts was calculated as the difference in Ct for the target gene products and the reference gene products, given by 2^−ΔΔCt, where ΔΔCt was calculated as: (Ct target gene − Ct reference gene)houseplant − (Ct target gene − Ct reference gene)wild type in noninductive conditions; or (Ct target gene − Ct reference gene)induced − (Ct target gene − Ct reference gene)noninduced in supplemented media. Confidence intervals were obtained by evaluating the expressions 2^−2ΔΔCt = m and 2^ΔΔCt = s, where s is the s.d. of the ΔΔCt value. Data were represented in Figure 6 as the relative gene expression normalized to an internal reference (OTC) and relative to gene expression in noninductive conditions in the wild type.

**Positional Cloning**

Low-resolution gene mapping was performed by using SSLP (Bell and Ecker, 1994) and cleaved-amplified polymorphic sequence (Konieczny and Ausubel, 1993) markers. Homozygous ucu2-1 plants were crossed to Col0, and ucu2-2 and ucu2-3 homozygotes were crossed to Ler. Phenotypically mutant F2 individuals were collected and frozen to extract their DNA as described by Ponce et al. (1999). Multiplex PCR amplification conditions and a fragmenting association of amplified microsatellites were as described by Ponce et al. (1999). Map distances were determined using Kosambi’s map function (Kosambi, 1944). Novel SSLP markers developed in this work are described in Table V.

Sequencing reactions were carried out on PCR amplification products, previously purified by ethanol precipitation, using ABI PRISM BigDye Terminator Cycle Sequencing version 2.0 Ready Reaction kits (Applied Biosystems) according to the instructions provided by the manufacturer, in a reaction volume of 5 μL, and GeneAmp PCR System 2400 (Applied Biosystems) thermal cycler. Sequencing electrophoresis and detection were performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences obtained were assembled with the Multiple Alignment Construction & Analysis Workbench program (version 2.0.5, Greg Schulter, National Center for Biotechnology Information, 1999). Sequences with homology to that of the UCU2 gene were identified in the GenBank database (Benson et al., 2002; http://www.ncbi.nlm.nih.gov/) using the BLAST program (Altschul et al., 1997). Analyses of amino acid sequences were undertaken by using the tools available at the PROSITE database (Falquet et al., 2002; http://www.expasy.org/prosite/).

**Southern-Blot Analysis**

Genomic DNA was isolated from whole rosette leaves as previously reported (Dellaporta et al., 1983). Five micrograms of DNA was digested with Scal or AccI and electrophoresed (15 h at 20 V in a 0.8% [w/v] agarose gel in 0.5× Tris-borate/EDTA buffer) and then treated with depurination (no. N1907, Sigma), denaturation (no. N1531, Sigma), and neutralization (no. N1532, Sigma) solutions following the protocol provided by the manufacturer. Transfection to a Hybrid N membrane (Amersham, Buckinghamshire, UK) was performed overnight in 2× SSC buffer (no. S6639, Sigma). Before prehybridization, the membrane was oven dried (10 min at 80°C) and cross linked (15 s under 70,000 μC cm−2). Prehybridization (1 h at 46°C) and hybridization (1 h at 46°C) were carried out in 20 and 30 mL, respectively, of a water solution of DIG Easy Hyb Granules (no. 1,796,895, Boehringer Mannheim/Roche, Basel), prepared as indicated by the manufacturer, in a hybridization oven. The membrane was hybridized with 30 ng μL−1 denatured (10 min at 95°C) digoxigenin-11-dUTP (no. 1,570,552, Boehringer Mannheim/Roche) probe, which had been synthesized as previously described (Ponce et al., 1998). The primers used to PCR synthesize the probes were the following: SeqMIL22.13F (5′-TCTTGAGACAGCAGTCTGTTC-3′) and SeqMIL22.13R (5′-CAGGTGACATGATGACAGC-3′) for probe I, and SeqMIL23.11F (5′-GTCAGCTTAGAAGAATCTGGAC-3′) and SeqMIL23.11R (5′-ACCCTTCTGATGGTGTTCGTC-3′) for probe 2. Washes and detection were performed using the DIG Wash and Block buffer set (no. 1,585,762, Boehringer Mannheim/Roche) as indicated by the manufacturer. Detection was performed with anti-DIG-AP Fab fragments (no. 1,093,274, Boehringer Mannheim/Roche) and the chemiluminescent phosphatase CDP-Star substrate (no. 1,685,627, Boehringer Mannheim/Roche). Autoradiograms were obtained on Hyperfilm ECL (Amersham-Pharmacia Biotech, Uppsala) exposed for about 2 h. Membranes were re-probed after being washed with water to remove the substrate and incubated twice in 0.3 μM NaOH and 0.1% (w/v) SDS for 20 min at 37°C and rinsed in 2× SSC buffer to remove the probe.

**Functional Complementation**

To clone candidate genes in the pART7 vector (Gleave, 1992), the following oligonucleotides were designed. All had a 5′ tail (shown in lowercase), including restriction sites for KpnI or XbaI (in bold): ClonMIL23.10F, 5′-gctgtacctTCTCGTAGATGCACTGGAC-3′; and ClonMIL23.4R, The ClonMIL23.10F and ClonMIL23.4R primers flanked a 2,441-bp fragment of the At3g21640 gene that contains its whole open reading frame. The PCR product obtained was digested with KpnI and XbaI and cloned into the pART7 vector (Gleave, 1992). Competent Escherichia coli DH5α cells were transformed, and the transformants were isolated on Luria-Bertani broth plates supplemented with 100 μg mL−1 ampicillin and later tested by PCR amplification for the presence of the transgene. Plasmid DNA was isolated and digested with NotI, and the restriction fragments were cloned into the pART7 vector in DH5α cells. Positive clones were selected on Luria-Bertani broth agar plates supplemented with 25 μg mL−1 streptomycin, 5% (w/v) X-Gal, and 50 mg mL−1 isopropylthio-β-galactoside, and tested by PCR for the presence of the DNA construct via plasmid DNA and used to transform chemical competent Agrobacterium tumefaciens C58Cs1 cells. Transformant A. tumefaciens cells were selected as described above and used to obtain liquid cultures that were used for in planta transformations.
of Arabidopsis wild-type and mutant plants (Bechtold and Pelletier, 1998). The T₂ seeds obtained were sown in agar plates supplemented with 50 μg ml⁻¹ kanamycin. PCR amplifications using primers flanking the AtISG21640 transcription unit were performed to confirm the presence of the transgene in the kanamycin-resistant T₂ plants.

Gene Expression Analysis
To determine whether or not the expression of candidate genes was affected in the ucu2 mutants, RT-PCR amplifications of RNA extracted from leaves and flower buds were performed as described by Ponc et al. (2000). The primer pairs used corresponded to the 5’ region (SeqMIL23.10F, 5’-CTCT-GTGAGTGACTTCGACG-3’; and SeqMIL23.2R, 5’-CTCTTAGAGCTCTCATTAGC-3’) or the 3’ region (SeqMIL23.4F, 5’-GAGCAATTTGCTACCT-GCAA-3’; and SeqMIL23.4R, 5’-GGTAGATCTTTCACGTTGG-3’) of the AtISG21640 gene. Only the SeqMIL23.4F and SeqMIL23.4R primer pair was found useful for distinguishing between wild-type and ucu2-3 sequences.

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