The rotunda2 mutants identify a role for the LEUNIG gene in vegetative leaf morphogenesis

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Abstract

Leaf development in Arabidopsis thaliana is considered to be a two-step process. In the first step, a leaf primordium is formed that involves a switch from indeterminate to leaf developmental fate in the shoot apical meristem cells. The second step, known as leaf morphogenesis, consists of post-initiation developmental events such as patterned cell proliferation, cell expansion, and cell differentiation. The results are presented of the molecular and genetic analyses of the rotunda2 (ron2) mutants of Arabidopsis, which were isolated based on their wide and serrated vegetative leaf lamina. The RON2 gene was positionally cloned and was identical to LEUNIG (LUG); it encodes a transcriptional co-repressor that has been described to affect flower development. Morphological and histological analyses of expanded leaves indicated that RON2 (LUG) acts at later stages of leaf development by restricting cell expansion during leaf growth. Real-time reverse-transcriptase polymerase chain reaction was used to quantify the expression of KNOX, WUSCHEL, YABBY3, LEAFY, ASYMMETRIC LEAVES, and GIBBERELLIN OXIDASE genes in expanding and fully expanded rosette leaf laminas of the wild type and ron2 and lug mutants. SHOOTMERISTEMLESS was expressed in wild-type leaves and down-regulated in the mutants. The results indicate that RON2 (LUG) has a function in later stages of leaf development.

Key words: Cell division, cell expansion, leaf development, real-time reverse-transcriptase PCR.

Introduction

The vegetative leaves of model dicotyledonous species such as Arabidopsis thaliana and Antirrhinum majus are initiated as leaf primordia from the peripheral zone of the shoot apical meristem (SAM). Their lateral growth is patterned along three newly formed axes. Patterning along the proximo-distal axis (length direction) and the centrolateral axis (width direction) generates the petiole and leaf lamina domains; patterning along the dorsi-ventral axis (thickness direction) of the leaf lamina into tissue layers restricts growth and determines the sheath-like structure of the leaf blade. Early leaf growth is mainly due to cell division processes that cease gradually from the tip to the base of the organ, from its margin to the midvein and from the ventral to the dorsal side of the lamina (Pyke et al., 1991; Van Lijsebettens and Clarke, 1998; Donnelly et al., 1999). Interfering with early growth by modulation of cell cycle regulatory genes results in changes in leaf size and shape (De Veylder et al., 2001; Fleming, 2002; Wyzykowski et al., 2002). Later growth is assumed to be due to polar and non-polar cell expansion processes (Tsuge et al., 1996). Expansion growth can be perturbed by modifying the expression of genes that code for enzymes involved in hormone biosynthesis or cell wall composition, resulting in altered leaf size and shape (Cho and Cosgrove, 2000; Pien...
et al., 2001; Fleming, 2002). In addition to the abovementioned internal factors, leaf growth is also modulated by environmental factors such as water, light, and CO₂ availability, affecting leaf size and shape. These parameters influence the number of cell cycles during leaf formation (Tardieu and Granier, 2000), as well as the polar and non-polar cell expansion processes that contribute to leaf organogenesis (Granier and Tardieu, 1998).

In Arabidopsis, mutational and transgenic evidence indicate that leaf growth is controlled at the transcriptional level by either transcription factors or regulatory complexes, such as Mediator and Elongator, associated with the RNA polymerase II transcription initiation and elongation complexes, respectively (Autran et al., 2002; Nelissen et al., 2003). A well-known group of transcription factors that affect leaf development is that of the KNOX homeobox genes that promote indeterminacy of cells in the SAM and are repressed at the leaf initiation site and in leaf primordia genes that promote indeterminacy of cells in the SAM and elongation complexes, respectively (Autran et al., 2002; Nelissen et al., 2003). A well-known group of transcription factors that affect leaf development is that of the KNOX homeobox genes that promote indeterminacy of cells in the SAM and are repressed at the leaf initiation site and in leaf primordia genes in leaf primordia of Arabidopsis is correlated with the lobing or dissection of the leaf lamina (Lincoln et al., 1994; Chuck et al., 1996). In addition, Bharathan et al. (2002) have recently shown that, in a variety of plant species, leaf lobing coincides with KNOX expression. The KNOX genes are negatively regulated by the YABBY3 (YAB3), ASYMMETRIC LEAVES1 (AS1), and ASYMMETRIC LEAVES2 (AS2) transcription factors (Siegfried et al., 1999; Byrne et al., 2000; Semiari et al., 2001; Kumaran et al., 2002). The YAB3 function in the repression of indeterminate growth is independent of its function in the specification of leaf polarity. Several other transcription factors are involved in leaf polarity (Kerstetter et al., 2001; McConnell et al., 2001). The AINTEGUMENTA transcription factor controls organ size by regulating the number and the extent of cell divisions during organogenesis (Mizukami and Fischer, 2000).

The genetic analysis of a collection of 255 ethyl methane sulphonate (EMS)-induced leaf mutants (Berná et al., 1999), which were found to fall into 94 loci, suggests that the number of independent loci required for the making of a leaf is limited. Several mutants of this collection that are defective in vegetative leaf growth along either the width or the length axes, or along both, were studied. Together with their positional cloning (Peters et al., 2004), these mutants were analysed morphologically and histologically to determine whether a perturbation in cell number or cell expansion causes their vegetative leaf phenotypes. The EMS-induced rotunda2 (ron2) mutants (Berná et al., 1999), which were isolated based on their wide rosette leaf laminas, were characterized. RON2 is identical to LEUNIG (LUG), as concluded from allelism tests and sequence analysis of two ron2 alleles. Morphological analyses of expanded leaves indicated that cell expansion processes are perturbed by the ron2-1 mutation and are responsible for the increase in lamina width. Gene expression of a number of transcription factors involved in indeterminacy and polarity was analysed in the ron2 mutants to identify potential targets of RON2 (LUG) in vegetative development.

Materials and methods

Plant material and growth conditions

Seeds of the Arabidopsis thaliana (L.) Heynh. ecotypes Columbia (Col-0) and Landsberg erecta (Ler) were supplied by the Nottingham Arabidopsis Stock Centre (stock numbers N1092 and NW20, respectively). The ron2-1 and ron2-2 mutants (Berná et al., 1999) were available at the Universidad Miguel Hernández (Elche, Spain). The lug-1, lug-3, and lug-16 mutants (Liu and Meyerowitz, 1995; Conner and Liu, 2000) were kindly provided by Z Liu (University of Maryland, College Park, MD). Plants were grown in a soil/vermiculite (3:1, v:v) mixture in a 16/8 h light/dark regime at 22 °C, 100 μm m⁻² s⁻¹ light intensity, and 70% relative humidity.

Morphological analysis

Fully expanded leaves collected 35 d after sowing were analysed morphologically. Leaves were arranged on agar plates according to their time of emergence and scanned with the computer program Image Beta 3b (Scion Corporation, Frederick, MD, USA). Leaf length, width, and area were determined (n=15) and the significance of the mean differences (P<0.05) was analysed by a t-test with the Statistical Package for the Social Sciences (release 10.0.5) (SPSS Inc., Chicago, IL, USA).

Morphological analysis

Fully expanded first or second and third leaves of 28- and 35-d-old seedlings, respectively, grown in vitro on GM medium (Valvekens et al., 1988) were cleared with 100% (v/v) methanol (overnight) and 90% (v/v) lactic acid (overnight), mounted onto a microscope slide and visualized by differential interference contrast (DIC) optics. Cells of the upper epidermis and palisade parenchyma were viewed under ×200 magnification and drawn inside a 14 cm diameter circle on paper with a camera lucida. After scanning, the cell area was analysed with the Scion Image computer program. A t-test using SPSS was applied on normally distributed data sets of 10 leaves (n=1000). In case of a skewed distribution, data were transformed to logarithmic values (lnX). Shapes of epidermal cells were evaluated in terms of the tendency of cells to protrude in the directions of cell expansion. The number of protrusions crossing the length or the width direction per grid unit or per cell was counted and graphically represented. Each data point in the graph represented the mean of 15 grid units or cells. The measurements per unit length and width of the grid indicate the tendency of cells to protrude in the directions of cell expansion. The measurements per cell define the complexity of the cell, in terms of protrusions in each direction.

In vitro

Half leaf laminas of fully expanded leaves were fixed in 90 ml EtOH 70% (v/v), 5 ml acetic acid, and 5 ml formaldehyde 40% (v/v), embedded in historesin (Leica, Heidelberg, Germany), transversally and serially sectioned with a microtome (5 μm sections) with a Ralph glass knife, stained with toluidine blue (0.05% [w/v] in phosphate buffer, pH 6.8), and analysed. The number of palisade cells was counted under the microscope in consecutive transverse sections at the widest part of the lamina. A t-test on the means was performed by SPSS.
DNA extraction and insertion/deletion and single-nucleotide polymorphism analysis

DNA was extracted according to Edwards et al. (1991). Insertion/Deletion (InDel) and single-nucleotide polymorphisms (SNPs) in the region of interest were identified from the Cereon Arabidopsis Polymorphism Collection (Cereon Genomics, Cambridge, MA, USA) (http://www.arabidopsis.org/Cereon/index.html). The InDel and SNP primers designed to fine map the RON2 locus are summarized in Table 1. PCR primers flanking the InDels/SNPs were designed according to Thareau et al. (2003). The conditions used for PCR amplification were: 2 min at 94 °C; 10 cycles of 15 s at 94 °C, 30 s at 63 °C, 30 s at 72 °C with a touch-down of 1 °C per cycle; 35 cycles of 15 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C, and 2 min at 72 °C.

For the InDel markers, two reactions, representing the markers flanking the region of interest at either side, were run on a polyacrylamide gel. Depending on the size of the products that resulted from the PCR, the samples could be loaded simultaneously or serially. For the SNP markers, sequence reactions were run on the recombinants with the USB Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences, Little Chalfont, UK) and loaded on a polyacrylamide gel.

RNA isolation and reverse transcription

Total RNA was isolated from 50–100 mg of vegetative leaves collected from Ler and from ron2-1, ron2-2, and lug-1 homozygotes 21 d after sowing. From each plant a mixture of leaves was obtained, some of which were fully expanded (the first and the second) and the remaining ones still expanding (from the third to the fifth). Leaf laminas were excised from the petioles and immediately frozen in liquid nitrogen. RNA was isolated with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). In the elution step, RNA was resuspended in a volume of 8 μl of RNase-free water and incubated at 37 °C for 30 min with DNase I in a final volume of 100 μl. DNase I was inactivated at 70 °C for 15 min. RNA was precipitated and finally resuspended in 40 μl of RNase-free water. cDNA was obtained by reverse transcription of 3–5 μg of RNA with the SuperScript II Reverse Transcriptase according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Real-time quantitative RT-PCR

cDNA from Ler and the ron2-1, ron2-2, and lug-1 mutants was amplified on the ABI PRISM 7000 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). A primer pair was designed for each of the genes under study to obtain a PCR product of approximately 100 bp. The 5’ and 3’ halves of one of the oligonucleotides of each pair corresponded to the sequences of two exons flanking an intron, so that genomic DNA could not be amplified. The primers used are presented in Table 2.

Amplification reactions were prepared in a volume of 25 μl by adding 12.5 μl of the SYBR-Green PCR Master kit containing the

Table 1. InDel and SNP primers used to identify Col/Ler polymorphisms for the map-based cloning of the RON2 locus

<table>
<thead>
<tr>
<th>InDel/SNP namea</th>
<th>BAC accession number</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>Fragment size (bp)</th>
<th>InDel size or SNP base</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER448548</td>
<td>AL021636</td>
<td>TGTAGCCTACATAAT</td>
<td>TTCTTTAGTGTCTCTAA</td>
<td>203</td>
<td>193</td>
</tr>
<tr>
<td>CER454915</td>
<td>ATL23H3</td>
<td>GTGATGTATGCAAAG</td>
<td>GTGATGTATGCAAAG</td>
<td>198</td>
<td>182</td>
</tr>
<tr>
<td>CER435932b</td>
<td>ATL23H3</td>
<td>CAGGTCTCTGCTTCTT</td>
<td>CAGGTCTCTGCTTCTT</td>
<td>192</td>
<td>176</td>
</tr>
<tr>
<td>CER431255b</td>
<td>AL022537</td>
<td>CATCTCTTCTCTCTCA</td>
<td>CATCTCTTCTCTCTCA</td>
<td>192</td>
<td>176</td>
</tr>
<tr>
<td>CER452248</td>
<td>AL022537</td>
<td>GATGTATGCTCAGC</td>
<td>GATGTATGCTCAGC</td>
<td>192</td>
<td>176</td>
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<tr>
<td>CER451516</td>
<td>AL031804</td>
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<td>CER431255b</td>
<td>AL022537</td>
<td>GATGTATGCTCAGC</td>
<td>GATGTATGCTCAGC</td>
<td>192</td>
<td>176</td>
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<tr>
<td>CER452248</td>
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<td>GATGTATGCTCAGC</td>
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<td>176</td>
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<tr>
<td>CER451516</td>
<td>AL031804</td>
<td>CATTCTCTCTCTTCA</td>
<td>CATTCTCTCTCTTCA</td>
<td>192</td>
<td>176</td>
</tr>
</tbody>
</table>

a InDels and SNPs were taken from the Cereon Arabidopsis Polymorphism Collection.

b SNP markers.

Table 2. Primers used in quantitative reverse transcriptase PCR amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequences (5’→3’-3')</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS1</td>
<td>TGTTCATGTCCTACCTAA</td>
<td>GAGACTTTGGCGCTAC</td>
<td>AAGACCTTTGGCGCTAC</td>
</tr>
<tr>
<td>AS2</td>
<td>CCAACTCCAGGCGCTCA</td>
<td>GCTACCCTCGTCGTAACG</td>
<td>GCTACCCTCGTCGTAACG</td>
</tr>
<tr>
<td>GA20ox1</td>
<td>CAGGACAGCTAGGCTAGCGCTCA</td>
<td>GAGTGGCTTGCGCTGTAACG</td>
<td>GAGTGGCTTGCGCTGTAACG</td>
</tr>
<tr>
<td>GA20ox1</td>
<td>CTCAAACTGATGGCTAGGCTAGGCTAGG</td>
<td>GATGATGGCGCGCTGTAACG</td>
<td>GATGATGGCGCGCTGTAACG</td>
</tr>
<tr>
<td>GA3ox1</td>
<td>TGTAGCGCAGGGTCTAAGGCTAA</td>
<td>GGGTGGCGCGCTGTAACG</td>
<td>GGGTGGCGCGCTGTAACG</td>
</tr>
<tr>
<td>KNAT1</td>
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<td>AACCTTGGGAAAGGAAAGGTG</td>
<td>AACCTTGGGAAAGGAAAGGTG</td>
</tr>
<tr>
<td>KNAT2</td>
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<td>GCTTGAGGTGGGAGGTGGG</td>
<td>GCTTGAGGTGGGAGGTGGG</td>
</tr>
<tr>
<td>KNAT6</td>
<td>CCAAGAGGGTCAGGACTACGAGGTA</td>
<td>TTCTGGTGGGAGGTGGG</td>
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</tr>
<tr>
<td>LUG</td>
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<td>GAGTTCGCTGTCGTAACG</td>
<td>GAGTTCGCTGTCGTAACG</td>
</tr>
<tr>
<td>OTC</td>
<td>TGAGGAGGAAAGGAAAGGTG</td>
<td>GAGTCGAGGTGGGAGGAAAGGT</td>
<td>GAGTCGAGGTGGGAGGAAAGGT</td>
</tr>
<tr>
<td>STM</td>
<td>TGGTGCTTCAACCTGCTACG</td>
<td>GTCAAAGGCAAAGGCAAAGGT</td>
<td>GTCAAAGGCAAAGGCAAAGGT</td>
</tr>
<tr>
<td>YAB3</td>
<td>TCACGGTCACCGACAA</td>
<td>GTCTTGTCGTCGTAACG</td>
<td>GTCTTGTCGTCGTAACG</td>
</tr>
</tbody>
</table>
AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems), 0.4 μM of primers, and 1 μl of cDNA. Each reaction was made in triplicate. PCR amplifications were done in 96-well optical reaction plates heated first to 50°C for 2 min and then to 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of denaturation at 95°C for 15 s and annealing-extension at 60°C for 1 min. Once finished, a dissociation kinetics analysis was performed to determine whether only the expected product was amplified.

Relative quantification of gene expression data was carried out with the 2^(-ΔΔC_T) or comparative C_T method (Livak and Schmittgen, 2001). The threshold cycle (C_T) indicates the cycle number at which the amount of amplified transcript reaches a fixed threshold. Expression levels were normalized with the C_T values obtained for the housekeeping ORNITHINE TRANSCARbamILASE (OTC) gene (Quesada et al., 1999), which was used as an internal reference gene. Once normalized, the amount of transcript was determined for the genes under study in the mutants relative to their expression in the wild type, by evaluating the expression 2^(-ΔΔC_T), being ΔΔC_T=(C_T, gene of interest−C_T, OTC)mutant−(C_T, gene of interest−C_T, OTC)wild type. Confidence intervals were obtained by evaluating the expressions 2^(-ΔΔC_T+SD) and 2^(-ΔΔC_T−SD), with SD the standard deviation of the ΔΔC_T value.

Results

Leaf phenotype of the ron2 mutants

In Arabidopsis, the size and shape of leaves vary during the life cycle (Röbbelen, 1957), a phenomenon called heteroblasty. Rosette leaves are classified as early juvenile (leaves 1 and 2), late juvenile (leaves 3 and 4), and adult (leaves 5 to 7). Cauline leaves are formed after bolting at the inflorescence. Fully expanded leaves were measured at specific positions in the rosette. They can be considered as standardized material in which cell size and cell number are representative of total cell expansion and cell division contributing to the final leaf size. In Arabidopsis, leaves 1 and 2 are initiated simultaneously at opposite positions. Subsequently, leaf 3 is formed perpendicularly to the axis defined by the first two leaves, and leaf 4 and the following ones are initiated according to a spiral phyllotaxis at angles of approximately 137°.

A series of fully expanded leaves is shown from ron2-1 and ron2-2 homozygous plants at the start of flowering (i.e. 5 weeks after sowing; Fig. 1). The leaves of the mutants were wider than those of the wild type and serrated. Serration was observed from leaf 3 onward and became more pronounced in older rosette leaves. Fertility was severely reduced in both mutant alleles (data not shown). The leaf serration phenotype and reduced fertility were more pronounced in ron2-2 than in ron2-1. To quantify the differences, leaf series of ron2-1 mutant plants and the wild-type Ler were scanned and image analysed. Lamina length, petiole length, lamina width, and lamina area were measured (Fig. 2A-D). Wild-type and ron2-1 mutant leaves had similar lamina lengths (Fig. 2A). However, both the length of the petiole and the width of the lamina were larger in the mutant than in the wild type (Fig. 2B, C), resulting in longer total leaf length with larger laminas in the ron2-1 plants, as confirmed by lamina area measurements (Fig. 2D). The differences were statistically significant from the third (or the fourth for petiole length) to the seventh rosette leaf. The cauline leaves were significantly narrower in the mutant (from the second cauline leaf onward; Fig. 2C), which confirms earlier observations (Liu and Meyerowitz, 1995).

RON2 is required for correct cell expansion in leaves

The larger rosette leaves observed in ron2-1 mutants could be the consequence of an increase in cell number, cell expansion or a combination of both. To distinguish between these three options, the absolute cell number in the width direction of the lamina was counted in the first and the third fully expanded rosette leaves of ron2-1 mutant and wild-type seedlings of 28- and 35-d-old plants, respectively. The number of palisade cells at the largest width of half a leaf lamina in the wild type and the mutant was
55.5±3.4 and 51.3±4.3 cells in the first leaf and 82.0±12.1 and 84.1±7.2 cells in the third leaf, respectively. These results show that the number of palisade cells in the mutant does not differ significantly from that of the wild type (t-test, P>0.05) and that an aberrant cell division does not account for its larger lamina width. The serial transverse sections revealed that both the organization and polarity was not altered in the tissues of ron2-1 plants (data not shown), suggesting that ron2-1 is not a polarity mutant. Cell expansion was studied in fully expanded first and third leaves of wild type and ron2-1 mutants. The mean of the cell area in the upper epidermis and palisade cell layer of leaf 1 was larger than that of leaf 3 in the wild type, a phenomenon already observed in sunflower leaves (Granier and Tardieu, 1998). The cell area of both the upper epidermis and the palisade parenchyma was significantly larger in leaf 3 of the ron2-1 plants than that of the wild type. In first leaves, only the palisade cells were significantly larger (Fig. 3A). These data confirm previous observations of more severe phenotypic effects (more serration and increased lamina width) caused by the ron2-1 mutation in older rosette leaves. Thus, the increase in lateral growth is due to an increase in cell expansion.

The number of protrusions of the jigsaw-shaped epidermal cells were studied according to Tsuge et al. (1996) to determine whether cell polarity was affected by the ron2-1 mutation. The polarity of the upper epidermis cells of the first and third leaves of ron2-1 plants was measured by applying a standard grid to the drawings obtained from the DIC microscopy (Fig. 3B). The number of cell protrusions crossing a grid unit in the length or width direction is indicative of the polarity in each direction. In addition, the number of protrusions in each direction was measured per cell, which can be considered as a measure for cell complexity. The polarity between mutant and wild type was comparable when first (data not shown) and third leaves (Fig. 3C) were used to measure the number of protrusions per cell, suggesting a similar complexity of the mutant and wild-type epidermal cells. In the third leaf (but not in the first leaf) of ron2-1 plants, fewer protrusions were measured in length and width per grid unit than in wild-type leaves (Fig. 3D) because of significantly larger mutant cells (Fig. 3B), with fewer cell-peripheral lines crossing length and width grid units. Our results do not indicate a defect in cell polarity in the mutant ron2-1 leaves but only an increase in non-polar cell expansion.

**Fig. 3.** Characterization of epidermal and palisade cells of the ron2-1 mutant and wild-type leaves. (A) Mean area of epidermal (ep) and palisade parenchyma (pal) cells of the fully expanded first and third rosette leaves of the ron2-1 mutant and wild type (WT). Asterisks indicate a statistically significant difference between mutant and wild type (t-test, P<0.05). (B) Camera lucida drawings showing third-leaf upper epidermal cells of wild-type (Ler) and mutant (ron2-1) plants. The arrows mark the leaf length direction. Bar=50 μm. The number of cell-bordering lines crossing the grid per cell (C) and per unit length (D) were counted for samples of eight wild-type (open circles) and ten mutant (closed circles) fully expanded third leaves.
The RON2 gene is identical to LEUNIG

Homozygous ron2-1 plants (Ler ecotype) were crossed to the ecotype Col-0 and their F1 progeny was used as mapping population. The ron2-1 mutation was mapped to a 390 kb interval with an amplified fragment length polymorphism (AFLP)-based genome-wide mapping approach (Peters et al., 2004). InDel and SNP markers were used to reduce the interval around the RON2 locus further (see Materials and methods). In an interval containing nine complete genes (Fig. 4), the LEUNIG (LUG) gene was the most likely candidate to correspond to RON2, because the two available ron2 alleles, ron2-1 and ron2-2 (Berná et al., 1999) display flower and silique traits resembling those already described for the lug flower developmental mutants (Liu and Meyerowitz, 1995).

ron2-1 heterozygous plants were used as pollen donors in crosses to either lug-1 or lug-16 homozygotes. Their F1 progeny was screened for individuals displaying deformed adult leaves (serrated margins) or siliques (horn-like protrusions at the tip of the carpels). Six F1 progenies from these crosses segregated for mutant and wild types in an overall 1:1 ratio (17:14 mutants:wild types). One cross between a lug-1 homozygote and a ron2-1 homozygote resulted in three mutant F1 plants. These genetic results indicated that the ron2-1 mutation is an allele of the LUG gene. Sequence analysis of the LUG gene in ron2-1 and ron2-2 showed a single nucleotide change in the LUG gene in each ron2 allele (Fig. 4). The ron2-1 mutation consists of a G to A change at the splice acceptor site of the 14th exon of the LUG gene, which causes incorrect splicing. PCR amplifications with primers located in exons 12 and 15 of the RON2 (LUG) gene revealed a single product of the expected size in the case of wild-type cDNA, and several weak products from ron2-1 cDNA, indicating that the splicing was incorrect in ron2-1 plants (data not shown). The C to T change found in the ron2-2 mutant introduced a stop codon at amino acid position 110, in a Gln-rich region of the LUG protein (Fig. 5), which causes a stronger mutant phenotype in ron2-2 than in ron2-1 individuals. This observation correlated with a lower LUG transcript level in ron2-2 than in ron2-1 (Fig. 6). DNA sequencing proved that the ron2 alleles are two new alleles of the LUG gene.

The RON2 (LUG) (At4g32551) gene encodes a WD40 protein. BLAST searches revealed the existence of several plant homologues: an additional gene of Arabidopsis (At2g32700 with 55% identity and 73% similarity), two rice (Oryza sativa) genomic clones (OsBABI7072 and OsBABA84838), and several expressed sequence tags from lettuce, tomato, and Medicago. Sequence comparison between RON2 (LUG) and its homologues confirmed the presence of four conserved domains (Fig. 5) previously described by Conner and Liu (2000). The N-terminal domain (from residues 6 to 74 in RON2) is slightly longer than the LUF5 domain predicted by Conner and Liu (2000) (Fig. 5), and is very conserved among plants. This domain is similar to the N-terminal region of the transcriptional activators FLO1 and FLO8 (Saccharomyces cerevisiae) and the members of the family of single-stranded DNA-binding proteins described in human, chicken, mouse, frog, zebrafish, and fruit fly (Castro et al., 2002). The Lissencephaly type-1-like (LisH) motif, which is present in RON2 (LUG) (Fig. 5) is an α-helical motif with a putative function in the regulation of microtubuli dynamics (NCBI CDD SMART 00667.6, LISH). A second domain of RON2 (LUG) is Gln-rich and is the least conserved among the genomic clones of Arabidopsis and rice (Fig. 5). As mentioned above, the ron2-2 allele introduces a stop codon at the beginning of this Gln-rich region (Fig. 5). The third domain of RON2 (LUG) of approximately 100 amino acids was significantly similar to proteins of some other plant species, such as rice. The C-terminal region of RON2 (LUG) consists of seven WD40 repeats and is affected by the ron2-1 mutation. The Gln-rich domain and the seven WD40 repeats are shared with a family of transcriptional co-repressors that includes Tup1 in yeast and Groucho in Drosophila (Conner and Liu, 2000).

Fig. 4. Identification of the RON2 gene. (A) Map-based cloning strategy. A standard set of eight AFLP primer combinations was applied to 21 F2 mutant individuals and identified a 4.4-Mb area at the bottom of chromosome 4. Additional mapping with AFLP markers on 160 F2 mutant individuals delineated the RON2 locus to 390 kb. Subsequently, 1204 F2 individuals were screened with flanking InDel markers and 18 recombinants were selected (data not shown). Recombinants from both screens were used for fine-mapping by means of InDels and SNPs and delineated the locus to 85 and 40 kb, respectively, flanked by the SNP markers CER435932 and CER431255. (B) The 40 kb region contained 10 genes of which LUG was the candidate gene, and was spanned by the overlapping bacterial artificial chromosome clones L23H3 and F4D11. (C) Gene structure of RON2, with exons represented as boxes. The site of the mutation and nucleotide change in the two ron2 alleles are indicated.

Gene expression analyses in the ron2 and lug mutants

Leaf lobing/serration in a number of species, including Arabidopsis, is induced by, or is correlated with, ectopic KNOX gene activity in leaf primordia (Lincoln et al., 1994; Chuck et al., 1996; Bharathan et al., 2002). Leaf
compoundsness in pea, which is considered as a partial indeterminate growth state, is due to LEAFY (LFY) expression in the leaf primordia (Weigel et al., 1992; Hofer et al., 1997). A genetic interaction between RON2 (LUG) and LFY during flower formation has been demonstrated in Arabidopsis (Liu and Meyerowitz, 1995).

With the aim of ascertaining whether overexpression of KNOX or LFY genes in ron2 and lug mutant leaves could
account for their increased serration, total RNA was isolated from Ler vegetative leaves, which was reverse transcribed and amplified by conventional PCR, with primers corresponding to LFY and the KNOX genes STM, KNAT1, KNAT2, and KNAT6 (Table 2, primers were chosen over an intron). A mixture of fully expanded (first and second) and expanding (from the third onward) leaf laminae collected without petioles to exclude shoot apical and auxillary meristematic cells was used to extract RNA. All the tested transcripts yielded visible bands in ethidium bromide-stained agarose gels. The expression of the above-mentioned genes was quantified by real-time PCR in expanding leaves collected from *ron2-1*, *ron2-2*, and *lug-1* homozygous plants (Fig. 6) as described by Pérez-Pérez et al. (2004). The KNOX genes were expressed in the mixture of expanding and fully expanded laminae. The expression of KNAT1, KNAT2, KNAT6, and LFY was normal or slightly reduced in the mutants. However, the STM expression level in each mutant tested was reduced 10–20-fold compared with that of the wild type (Fig. 6). These results show that leaf serration in the studied mutants is not due to ectopic overexpression of STM or LFY in expanding leaves and that mutations in the RON2 (LUG) gene cause down-regulation of STM. WUSCHEL (WUS) transcript was not detectable (data not shown) in the leaf samples, indicating that the samples were not contaminated with shoot apical or auxillary meristems (the WUS transcript was detectable in rosette samples containing SAM, which were used as control).

STM overexpression represses GA20 OXIDASE1 (GA20ox1), the key enzyme for gibberrellin (GA) biosynthesis, and GA20ox and GA3ox genes are down-regulated by exogenous GA, whereas the GA2ox1 gene is up-regulated by GA treatment (reviewed in Olszewski et al., 2002). GA20ox1, GA3ox1, and GA20ox1 transcript levels were tested in the mutants, but no differences were found with respect to the wild type (Table 2; Fig. 6). Expression of AS1 and AS2 at the leaf primordium initiation site in the SAM represses STM. AS1 and AS2 transcript levels were normal in the mutants and did not account for the reduction in STM transcript level (Table 2; Fig. 6).

The floral organ identity gene AGAMOUS (AG) is regulated by LUG in the two outer whorls of the flower (Liu and Meyerowitz, 1995; Conner and Liu, 2000; Franks et al., 2002). AG expression was measured on the leaf samples described above and no significant differences with the wild type were found (data not shown).

The expression of the LUG gene was also measured. It was expressed in the leaves in the wild type and a reduced expression was observed in the tested mutant alleles (Fig. 6).

**Discussion**

The RON2 gene has been cloned based on mutant alleles that perturb leaf growth (Berna´ et al., 1999; Peters et al., 2004) and it was found to be identical to LUG, previously identified by flower mutants. LUG acts together with SEUSS (SEU) and APETAL2 (AP2) to repress AGAMOUS (AG) in the outer two whorls of the flower (Conner and Liu, 2000; Franks et al., 2002). By means of quantitative, real-time RT-PCR, loss of function alleles of RON2 (LUG) did not affect AG expression in leaf tissues. Hence, AG does not seem to be a target gene of RON2 (LUG) in expanding or fully expanded leaves. This observation is consistent with earlier ones in which the ectopic expression of AG in the leaves of the *curly leaf and incurvata2* mutants (Goodrich et al., 1997; Kim et al., 1998; Serrano-Cartagena et al., 2000) results in phenotypic alterations that are completely different from those displayed by *ron2* and *lug* plants.

The RON2 (LUG) gene codes for a WD40 repeat protein, with structural homology to the general transcriptional co-repressor Tup1 of yeast, *Groucho of Drosophila*, and Transducin-like Enhancer of split (TLE) of mammals that affect growth and pattern formation (Hartley et al., 1988; Williams and Trumbly, 1990; Parkhurst, 1998; Conner and Liu, 2000). Tup1 is part of the Ssn6-Tup1 repressor complex that controls different pathways in yeast through different sequence-specific DNA-binding proteins, each of which binds a specific set of target genes (Smith and Johnson, 2000). In Arabidopsis, RON2 (LUG) may act through a similar mechanism, with SEUSS being a candidate gene for the Ssn6 function (Franks et al., 2002). The LUG gene may regulate a number of growth-related processes in the vegetative phase of plant development, in which its expression was demonstrated by quantitative RT-PCR, in addition to more specific developmental processes such as those in flower formation (Conner and Liu, 2000; Franks et al., 2002).

The vegetative leaves of *ron2* and *lug* homozygous plants display a wider lamina, an increased lamina area, and a longer petiole than those of the wild type. L1 and L2 layers contribute largely to the size and shape of leaves (Dolan and Poethig, 1998). The cell area in the leaf laminae of the L1-derived adaxial epidermis and the L2-derived palisade parenchyma is significantly enlarged in a non-polar manner by the *ron2-1* mutation. The absolute cell number in the width direction of the leaf lamina is unaffected in ron2-1 plants. Thus, the increase in cell area in these mutants is not due to a compensatory mechanism that balances for a decrease in cell number in leaf organ formation in several mutants and transgenic lines (Mizukami and Fischer, 2000; De Veylder et al., 2001). The increased leaf cell size observed in the *ron2-1* mutant suggests a role for the RON2 (LUG) gene in cell expansion-related processes during leaf growth in the restriction of non-polar cell expansion.

In *ron2* rosettes, total leaf length is significantly increased because of the extension in petiole length, whereas lamina length is unaltered. The growth of the petiole and the lamina is known to be differentially affected by light
and GA (Tsukaya et al., 2002). These authors described differential cell expansion behaviour in the petiole and the lamina in Arabidopsis lines carrying mutations in phytochrome- or GA-related genes, suggesting that the RON2 (LUG) gene may have a function in photomorphogenetic processes.

Another phenotypic trait of the ron2 and lug mutations is the serration of the rosette leaf laminae. Margin lobing in Arabidopsis leaves is induced by ectopic de-repression of KNOX genes (Lincoln et al., 1994; Chuck et al., 1996). A quantitative analysis of gene expression by the sensitive real-time RT-PCR method of the KNOX genes STM, KNAT1, KNAT2, and KNAT6 at different leaf growth stages of ron2 and lug mutant plants showed that all were expressed during the post-initiation leaf developmental stages, which had not been reported before (Semiarti et al., 2001). These KNOX genes are predominantly expressed in different domains of the SAM and are suppressed during leaf initiation, as demonstrated by mRNA in situ hybridization (Lincoln et al., 1994; Long et al., 1996; Ori et al., 2000). The KNOX genes were active again at later stages of leaf development, as shown by quantitative RT-PCR. The KNOX genes were not up-regulated in ron2 and lug mutants, indicating that leaf serration is not due to ectopic meristematic activity caused by KNOX gene overexpression. This observation is consistent with the phenotypic analyses that showed enlarged cells, but normal cell numbers, in mutant rosette leaves. However, the STM expression level was significantly reduced in the ron2 and lug mutants. Because the leaf margin in plants homozygous for the weak allele stm-2 is entire (Hay et al., 2002), the reduction of STM levels in the ron2 mutants is probably not related to leaf serration.

Overexpression of STM suppresses the activity of GA20ox1, the gene encoding the key enzyme for GA biosynthesis (Hay et al., 2002; Sakamoto et al., 2001). Down-regulation of STM might de-repress GA20ox1, which, in turn, would induce ectopic accumulation of GA, a plant hormone known to promote cell differentiation and expansion. A number of phenotypic traits of the ron2 and lug mutants can be explained as GA effects, i.e. the enlarged cells in the leaf lamina, the enlarged petioles, and leaf serration. Addition of GA to wild-type leaves has been shown to phenocopy the phenotype of the serrate mutant (Serrano-Cartagena et al., 1999). However, GAox gene expression levels were normal in the ron2 and lug mutants. Thus, ectopic GA biosynthesis is probably not the cause of the mutant phenotype.

In Arabidopsis, floral meristem identity is controlled by the LFY gene, which is highly expressed in floral meristems but only weakly in cauline leaf primordia (Weigel et al., 1992). Analysis of a lug lfy double mutant indicated a genetic interaction between the LUG and LFY genes in flower organogenesis (Liu and Meyerowitz, 1995). UNIFOLIATA, the pea orthologue of LFY, is expressed in leaf primordia, rendering it indeterminate and resulting in the compoundness of the leaf (Hofer et al., 1997). The quantitative RT-PCR data showed a 2-fold reduction in the expression level of LFY between the mutants and the wild type. The serration of the ron2 and lug rosette leaves is probably not the consequence of ectopic expression of the LFY gene because its expression is not up-regulated but slightly reduced. This observation adds to the experimental evidence that leaf serration in ron2 and lug has nothing to do with reversion to indeterminate growth.

In conclusion, these data demonstrate that the RON2 (LUG) gene is involved in the control of leaf size and shape through non-polar cell expansion processes. The RON2 (LUG) gene might exert its function by transcriptional regulation of general growth-related genes during leaf development, in analogy to its function in growth in yeast. These gene expression analyses could not identify conclusively a putative target of LUG during the vegetative phase, but indicated a role for LUG at the photomorphogenic stage during leaf development.

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