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RESEARCH PAPER

# 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-transcription 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 laminae 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 centro-lateral 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; Wyrzykowska *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

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Abbreviations: Col, Columbia; DIC, differential interference contrast; EMS, ethyl methane sulphonate; Gln, glutamine; GA, gibberellic acid; Ler, Landsberg erecta; *lug*, *leunig*; *ron*, *rotunda*; SAM, shoot apical meristem; *STM*, *SHOOTMERISTEMLESS*.

*et al.*, 2001; Fleming, 2002). In addition to the above-mentioned internal factors, leaf growth is also modulated by environmental factors such as water, light, and CO<sub>2</sub> 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 (Long *et al.*, 1996; Ori *et al.*, 2000). The ectopic expression of *KNOX* 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; Semiarti *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 laminae, 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  $\mu\text{m}^{-2}$  s<sup>-1</sup> 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).

### *Cellular analyses by differential interference contrast microscopy*

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  $\times 200$  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 ( $n\geq 1000$ ). In case of a skewed distribution, data were transformed to logarithmic values (lnX). Shapes of epidermal cells were evaluated in terms of numbers of cell protrusions in leaf width and length directions according to Tsuge *et al.* (1996). For polarity determination, a grid (unit=2 cm<sup>2</sup>) was positioned on top of the drawings ( $n=5-10$ ) of the upper epidermis cells obtained after DIC microscopy, with one axis of the grid parallel to the direction of leaf length. 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.

Half leaf laminae 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  $\mu\text{m}$  sections) with a Ralphy 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 laminae 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 88 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

InDel/SNP name <sup>a</sup>	BAC accession number	Forward primer (5'→3')	Reverse primer (5'→3')	Fragment size (bp)		InDel size or SNP base
				Col	Ler	
CER448548	AL021636	TGTAGCCTACATAAT GTAGTTGGT	TTCTTTAGTCTCTAA CTTGTTG	203	193	20/–20
CER454915	ATL23H3	ACCACACCAGACATC CTCTC	GAGACGACCATCGGAA TAAC	85	71	14/–14
CER435932 <sup>b</sup>	ATL23H3	CGAGTTCTTGGCCTAC CTTGA	CGTAAGCTGCGTTTTGT ACGTT	120	120	C/T
CER431255 <sup>b</sup>	AL022537	CCACAATCTAAAGTCT TGCGAA	AGTFACTGCCACCAAGTC TGTG	137	137	C/A
CER452248	AL022537	GTGATGTATGCAAAG GATTGTG	AAAACCTCACCTGCTT CCTC	81	78	3/–3
CER451516	AL031804	CATTTGCATTTGGT CAGCA	CTGATCTCAACGA CGATA	68	55	13/–13

<sup>a</sup> InDels and SNPs were taken from the Cereon Arabidopsis Polymorphism Collection.

<sup>b</sup> SNP markers.

**Table 2.** Primers used in quantitative reverse transcriptase PCR amplifications

Gene	Oligonucleotide sequences (5'→3')	
	Forward primer	Reverse primer
<i>AS1</i>	TGTTACGTGCTTACGTTAGACA	AAGACTTGGCGTCACGGTTCA
<i>AS2</i>	GGATGTTCCGGCCGGTAAACA	GGCTCTCGGCTGCTGAAACT
<i>GA20ox1</i>	CGACGACATGAGCCGCTCAA	ACACCTTCCCAAATGGCTGAAA
<i>GA2ox1</i>	TCTAAATGATGGCTCATGGATCT	GTCATCACCTGGAGAGAGTCA
<i>GA3ox1</i>	TCAACTACTGCGATATCGTTGAA	AAGTGAATTTAGTGCTAACCAT
<i>KNAT1</i>	CCATTCAGGAAGCAATGGAGTT	ACTCTTCCCATCAGGATTGTTGA
<i>KNAT2</i>	CTCTTTCAGATGATGGTGCAGTT	GCGTAGTAGCTGGTCTTCAGATC
<i>KNAT6</i>	GGGAGTTTCTGAGGATGGTGTA	TTTGAGGTCCCGGTCTTCACA
<i>LFY</i>	CCCACCAAGGTGACGAACCA	ACAGTGAACGTAGTGTCCGATT
<i>LUG</i>	AGCTGATAAAATGTTGGATGTCTA	GGATCCGATGACACTTTTCCTT
<i>OTC</i>	TGAAGGGACAAAGGTTGTGTATGTT	CGCAGACAAAGTGAATGGA
<i>STM</i>	TGGTGCTCCAACCTTCTGACA	GTCAAGGCCAAGATCATGGCT
<i>YAB3</i>	TCACGG TCACCGACAAAAGGT	GTCCTTGCTGTGAGTGTTCCT

Ampli $Taq$  Gold DNA polymerase (Perkin-Elmer/Applied Biosystems), 0.4  $\mu$ M of primers, and 1  $\mu$ l 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 Ampli $Taq$  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  $2^{-\Delta\Delta C_T}$  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 house-keeping *ORNITHINE TRANSCARBAMYLASE* (*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  $2^{-\Delta\Delta C_T}$ , being  $\Delta\Delta C_T = (C_{T, \text{gene of interest}} - C_{T, \text{orc}})_{\text{mutant}} - (C_{T, \text{gene of interest}} - C_{T, \text{orc}})_{\text{wild type}}$ . Confidence intervals were obtained by evaluating the expressions  $2^{-(\Delta\Delta C_T + SD)}$  and  $2^{-(\Delta\Delta C_T - SD)}$ , with SD the standard deviation of the  $\Delta\Delta C_T$  value.

## Results

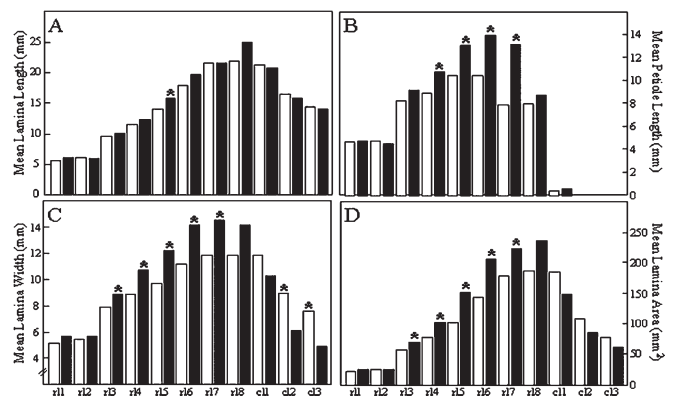
### Leaf phenotype of the *ron2* mutants

In *Arabidopsis*, the size and shape of leaves varies 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.



**Fig. 1.** Leaf morphology of *ron2* mutants. Series of fully expanded leaves from wild type (upper row) are compared with leaves of *ron2-1* and *ron2-2* homozygous plants. From left to right: two cotyledons, rosette leaves 1 to 7, and two to three cauline leaves. The mutant rosette leaves are wider and more serrated than those of the *Ler* wild type. Bar=1 cm.



**Fig. 2.** Mean of the lamina length (A), petiole length (B), lamina width (C), and lamina area (D) of eight rosette leaves (r11 to r18) and three cauline leaves (c11 to c13) ( $n=15$ ), of 5-week-old *ron2-1* mutant (black bars) and wild-type (*Ler*) plants (white bars). The asterisks indicate a statistically significant difference between mutant and wild type ( $t$ -test,  $P < 0.05$ ).

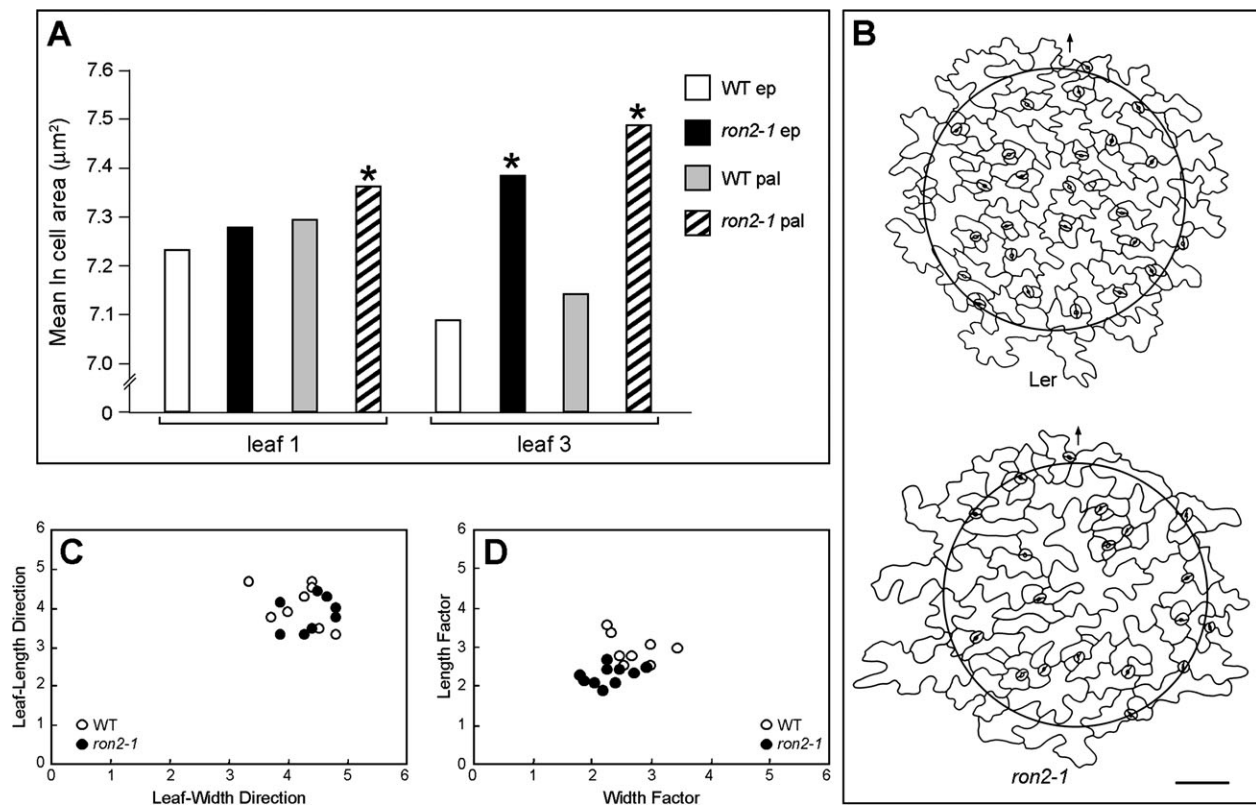
2D). The differences were statistically significant from the third (or the fourth for petiole length) to the seventh rosette leaf. The cauline leaves were significantly narrower in the mutant (from the second cauline leaf onward; Fig. 2C), which confirms earlier observations (Liu and Meyerowitz, 1995).

### 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.

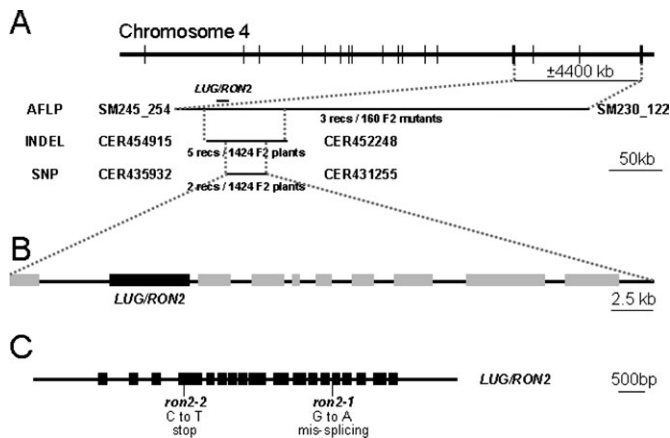


**Fig. 3.** Characterization of epidermal and palisade cells of the *ron2-1* mutant and wild-type leaves. (A) Mean area of epidermal (ep) and palisade parenchyma (pal) cells of the fully expanded first and third rosette leaves of the *ron2-1* mutant and wild type (WT). Asterisks indicate a statistically 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 F<sub>2</sub> 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 F<sub>1</sub> progeny was screened for individuals displaying deformed adult leaves (serrated margins) or siliques (horn-like protrusions at the tip of the carpels). Six F<sub>1</sub> 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 F<sub>1</sub> 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



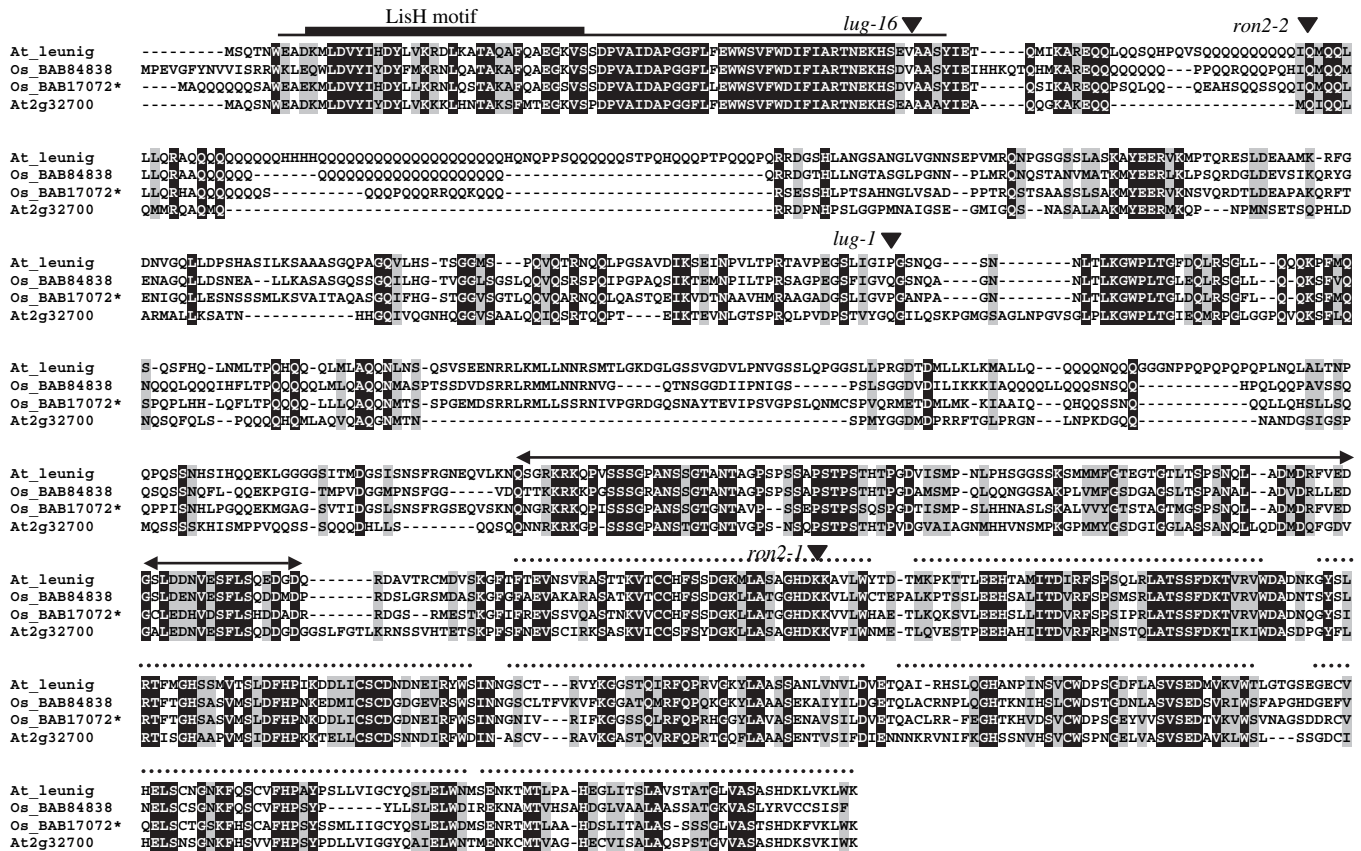
**Fig. 4.** Identification of the *RON2* gene. (A) Map-based cloning strategy. A standard set of eight AFLP primer combinations was applied to 21 F<sub>2</sub> mutant individuals and identified a 4.4-Mb area at the bottom of chromosome 4. Additional mapping with AFLP markers on 160 F<sub>2</sub> mutant individuals delineated the *RON2* locus to 390 kb. Subsequently, 1264 F<sub>2</sub> 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.

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.

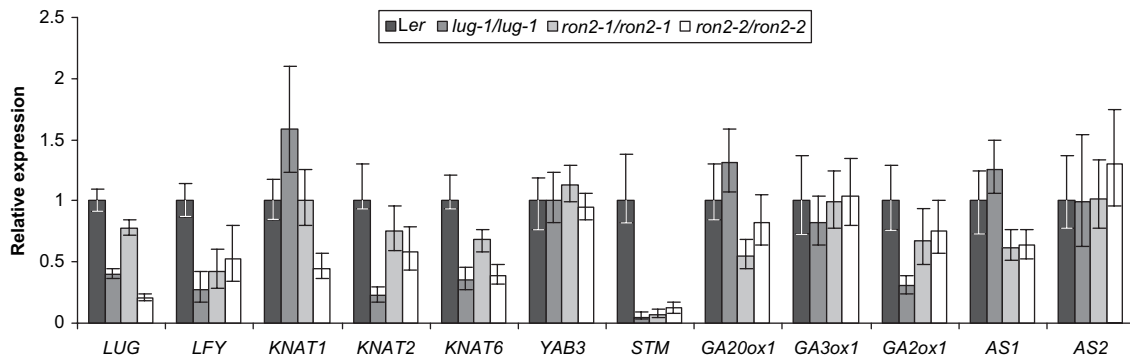
The *RON2* (*LUG*) (At4g32551) gene encodes a WD40 protein. BLAST searches revealed the existence of several plant homologues: an additional gene of *Arabidopsis* (At2g32700 with 55% identity and 73% similarity), two rice (*Oryza sativa*) genomic clones (OsBAB17072 and OsBABA84838), and several expressed 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  $\alpha$ -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).

*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



**Fig. 5.** Alignment of the deduced amino acid sequences of the *RON2* (*LUG*) gene, its *Arabidopsis* homologue At2g32700 and two rice homologues Os\_BAB84838 and Os\_BAB17072\* based on full-length cDNAs and manual re-annotation of Os\_BAB17072\*. The protein sequences were aligned with the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Identical and similar residues are presented in black and in grey, respectively. The LUF5 domain is indicated by a black line above the sequence and the LisH motif by a bold line. The arrowed lines bound a contiguous region of homology between plant sequences. The seven WD40 repeats are indicated by a dotted line above the sequence. Arrowheads give the position of the mutations in the different *ron2* and *lug* alleles used in this paper.



**Fig. 6.** Real-time, quantitative RT-PCR analysis of the expression of several genes in *ron2* and *lug* vegetative leaves. Bars indicate relative levels of expression, determined by the expression  $2^{-\Delta\Delta C_t}$ , for each of the studied genes in the wild-type *Ler* and the *ron2* and *lug* mutants after normalization with those of the *ORNITHINE TRANSCARBAMILASE* and compared with those of *Ler* (see Materials and methods). Error bars indicate the range of variation of the  $2^{-\Delta\Delta C_t}$  values of the gene under study among triplicate reactions.

compoundness 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 axillary 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 *KNOX* 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 axillary 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 gibberellin (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 *GA2ox1* 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 (Berná *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 *APETALA2 (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 lamina 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 over-expression. 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 morphogenetic stage during leaf development.

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