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Regulation of *LANCEOLATE* by *miR319* is required for compound-leaf development in tomato

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Plant leaves show pronounced plasticity of size and form. In the classical, partially dominant mutation *Lanceolate* (*La*)[1], the large compound leaves of tomato (*Solanum lycopersicum*) are converted into small simple ones. We show that *LA* encodes a transcription factor from the TCP family[2,3] containing an *miR319*-binding site[4]. Five independent *La* isolates are gain-of-function alleles that result from point mutations within the *miR319*-binding site and confer partial resistance of the *La* transcripts to microRNA (miRNA)-directed inhibition. The reduced sensitivity to miRNA regulation leads to elevated *LA* expression in very young *La* leaf primordia and to precocious differentiation of leaf margins. In contrast, downregulation of several *LA*-like genes using ectopic expression of *miR319* resulted in larger leaflets and continuous growth of leaf margins. Our results imply that regulation of *LA* by *miR319* defines a flexible window of morphogenetic competence along the developing leaf margin that is required for leaf elaboration.

The tomato leaf provides a sensitive system to explore mechanisms underlying variations in leaf shape, size and complexity, owing to the presence of a broad range of leaf features among cultivars, mutant lines and crossable sibling species[5–7]. Indeed, a recent saturation mutant screen in tomato revealed that leaf shape is one of the most variable morphological traits[8]. The classical, partially dominant mutation *Lanceolate* (*La*) causes the dose-dependent, gradual conversion of compound tomato leaves into small, simple ones. Homozygous mutants show meristem-maintenance defects, and when viable they produce very small leaves with entire margins[1,9–12] (**Fig. 1**).

We identified four new partially dominant *La* alleles, *La-2–5*, similar in leaf and flower morphology to the original *La* allele (**Fig. 1a** and **Supplementary Fig. 1** online). To define the early events that distinguish the development of simple and compound leaves, we compared wild-type and *La-2*/+ shoots. As leaves are formed sequentially at the flanks of the shoot apical meristem, plastochron (P) number is used to designate the developmental stage of an initiating leaf, such that the latest emerging leaf is termed P1, the next oldest leaf P2, and so forth. Wild-type tomato P1 and P2 leaf primordia are still simple. Lateral leaflet initiation, as well as laminar expansion, are first evident in P3 primordia[6,10] (**Fig. 1b**). Trichomes, which constitute a marker of leaf differentiation[13], emerge in late P1 and reach maturation at P2 and onwards, but are absent from the meristematic regions of the leaf margins where leaflets will initiate. In contrast, P2 and P3 primordia in *La*/+ plants show precocious lateral expansion and trichome development along their margins, with reduced or no leaflet initiation (**Fig. 1c**). Therefore, leaf elaboration requires prolonged morphogenetic activity in a region of the leaf margin termed the blastozone[10,13,14].

The *LA* gene has been fine-mapped on chromosome 7 (ref. 15; **Supplementary Fig. 2** online). We noticed a phenotypic resemblance between homozygous *La* mutants and *Arabidopsis* seedlings expressing miRNA-resistant versions of *TCP* genes[4], implicated in the regulation of cell cycle and growth[2,16]. One tomato *TCP* homolog completely cosegregated with *La* (**Supplementary Fig. 2**). Sequence analysis of this gene (**Fig. 1d** and **Supplementary Fig. 3** online) revealed point mutations in the five partially dominant *La* alleles, all within a highly conserved *miR319*-binding site present in this gene, identifying it as *LANCEOLATE* (**Fig. 1e**). Confirming the identity of *LA*, leaf overexpression of the *La-2* mutant coding region (LA^m) in transgenic plants resulted in *La*-like simple leaves (**Fig. 2**). Although the mutations in *La* and *La-5* caused amino acid substitutions, those in *La-2–4* were silent. This indicated that *La* might be a gain-of-function phenotype resulting from reduced sensitivity of its RNA to *miR319*-directed regulation[4] rather than from altered activity of the LA protein. We detected products from 5′ RNA ligase–mediated RACE corresponding to miRNA cleavage between nucleotides 10 and 11 of the *miR319* recognition site in *LA* transcripts (**Supplementary Fig. 2**), confirming that *LA* is regulated by miRNA. Surprisingly, we also detected mRNA cleavage products in homozygous *La-2* plants (**Supplementary Fig. 2**), despite the presence of a mutation within the 'seed' recognition sequence that has been shown to be essential for miRNA-directed regulation[17,18]. This could explain the partially dominant nature of the *La* mutation. Sequencing of the cleavage products confirmed that mutant *La-2* transcripts could be cleaved,

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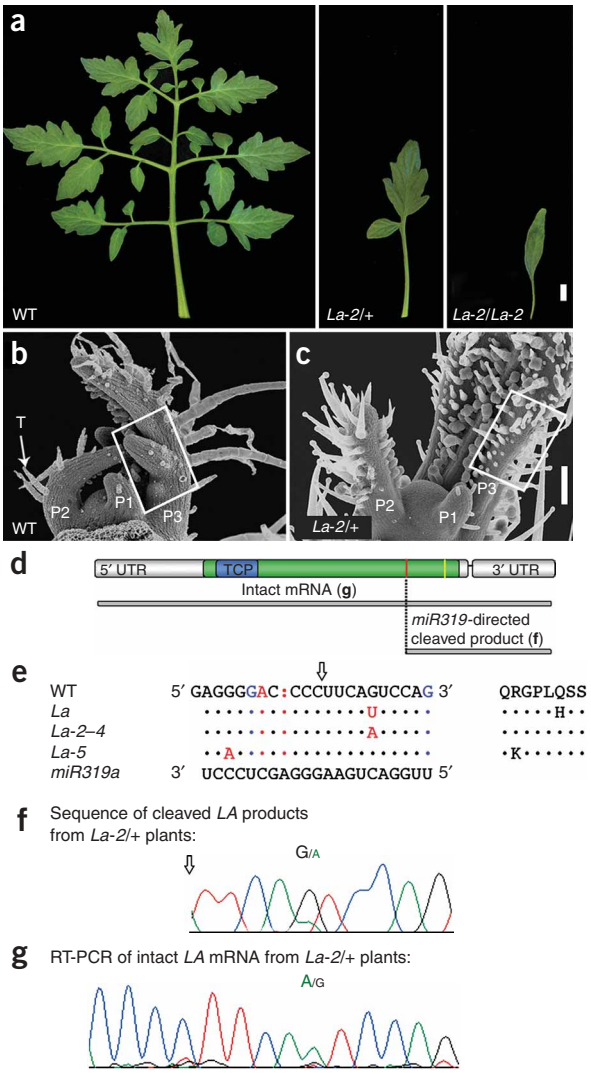

Figure 1 The molecular basis for the *La* syndrome. (**a**) Phenotypes of wild-type (WT) and *La-2* tomato leaves. (**b**,**c**) Scanning electron micrographs (SEMs) of shoot apices. In wild-type shoots (**b**), leaflet initiation (boxed) occurs long after leaf initiation, in areas maintaining marginal meristem activities. Trichomes (T) appear first on the abaxial surface. Margins of *La-2*/+ leaves (**c**) lack leaflets and develop trichomes precociously. (**d**) The *LA* gene structure showing the coding region (green), the TCP domain (blue), the miRNA-binding site (red), the *la-6* mutation site (yellow), a single intron (a thin line in the 3′ UTR), the cleavage site (open arrow) and the RNA molecules sequenced in **g** and **f** (bottom). (**e**) Sequence of the *miR319*-recognition site in the various *La* alleles. Black, blue and red represent complementary residues, G-U wobbles and mismatches, respectively. (**f**) Sequenced *LA* cleavage products from *La*/+ plants. (**g**) Sequenced intact *LA*/*La-2* mRNAs obtained from RT-PCR with primers flanking the cleavage site. Control chromatograms are shown in **Supplementary Figure 2**. Scale bars, 1 cm (**a**); 100 μm (**b**,**c**).

but showed that in *La-2*/+ plants, most of the cleavage products originated from the wild-type allele of the gene (**Fig. 1f**). Conversely, sequencing of RT-PCR products encompassing the cleavage site indicated that in *La-2*/+ plants, the steady-state pool of intact *LA* mRNAs consisted mainly of the mutant form (**Fig. 1g** and **Supplementary Fig. 2**). Thus, the partial resistance to miRNA-directed cleavage was sufficient to stimulate profound molecular and morphological phenotypes. Notably, sequencing of *LA* orthologs from Solanaceae species with variable leaf architectures revealed an invariant *miR319*-binding site (**Supplementary Fig. 3b**), implying that additional mechanisms underlie the difference between simple and compound leaves.

Our results implied a graded regulation of *LA* by *miR319*. Using quantitative RT-PCR, we could not detect any difference in the amounts of intact *LA* mRNA between wild-type and *La*/+ apices, which contain the shoot apical meristem and P1–P4 leaf primordia (**Supplementary Fig. 4** online). We hypothesized that the decrease in *miR319* sensitivity resulted in a spatial and/or temporal change in *LA* expression, rather than altering the total amount of *LA* mRNA. To test this prediction, we assayed the relative expression territories of *miR319* and *LA* in wild-type and *La*/+ shoots by RNA *in situ* hybridization. In wild-type shoots, low amounts of *LA* mRNA were detected in the inner layers of the meristem and initiating leaf primordia. Much higher amounts were evident in the distal, upper (adaxial) part of the developing leaf (**Fig. 2a**,**c**), with highest expression in leaflet primordia (**Fig. 2a**,**b**). In contrast, high amounts of *miR319* were detected in the shoot apical meristem and the proximal adaxial domain of the developing leaf (**Fig. 2e**,**f**,**h**), resulting in an expression gradient opposing and overlapping that of *LA*. Overlapping expression domains between *LA* and *miR319* included the shoot apical meristem, young leaf primordia and developing leaflets (**Fig. 2b**,**c**,**e**,**g**). *miR319* therefore modulates the timing and location of *LA* expression, rather than clearing it. Indeed, we observed a precocious increase in *LA* transcript relative to the wild-type at the *La-2*/+ meristem flanks where the next leaf is to be initiated (P0) and in the initiating leaf primordia (P1), both regions overlapping with *miR319* expression (compare **Fig. 2a** with **Fig. 2d** and corresponding insets). In contrast, no change in *miR319* expression was detected in the *La-2*/+ mutant apices (not shown). The absence of leaflet primordium, a tissue with a relatively high abundance of *LA* mRNA, from developing *La*/+ leaves accounts for the similar mRNA amounts found in wild-type and mutant apices using quantitative RT-PCR. A tuning model for the interplay between *LA* and *miR319* implied that increased activity of each component within the other expression territory would alter leaf morphology. To test this prediction, we ectopically expressed wild-type *LA* in initiating leaf primordia using the *FIL* promoter[19] (*FIL*>>*LA*) (**Fig. 2i**). The resulting leaves were simpler and smaller than wild-type ones, but not as simple as leaves of *FIL*>>*LA*^{m} plants (**Fig. 2j**). Thus, fine-tuning of *LA* amounts is achieved by superimposing regulation by *miR319* on the *LA* transcriptional pattern.

The *La* gain-of-function phenotypes indicated that LA may promote leaf growth cessation and tissue differentiation. To test this, we screened through ethyl methane sulfonate mutagenesis of *La-2*/+ seeds for reduced-activity *la* alleles. In this background, an intragenic mutation with impaired LA activity would be expected to suppress the *La*/+ gain-of-function phenotype in a dominant manner[20]. Among 2,000 mutant plants examined in the M1 generation, one wild-type–looking sector[21] was identified (**Fig. 3a**). Progenies from this branch segregated for a nonsense mutation (designated the *la-6* allele) upstream of 23 highly conserved C-terminal amino acids, *in cis* to the original mutation in the miRNA-binding site (**Supplementary Fig. 5** online). Homozygous *la-6* plants had highly lobed leaf margins (**Fig. 3b** and **Supplementary Fig. 5**), but rather normal patterns of leaflet initiation.

The relatively mild *la-6* phenotype implied that other *miR319*-regulated family members may act redundantly with *LA* (**Supplementary Fig. 3**). To examine this possibility, ectopic expression of the Arabidopsis *miR319a* precursor in initiating tomato leaves was

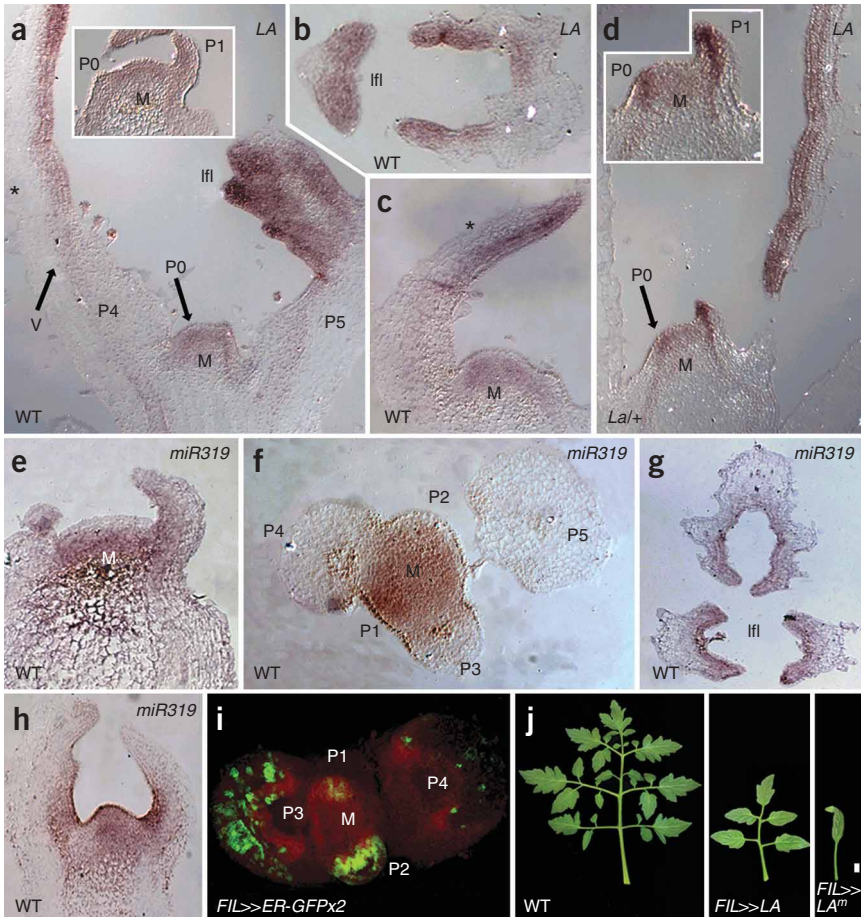

Figure 2 Spatial distribution of *LA* and *miR319*. (**a**–**h**) *In situ* hybridization of longitudinal sections of wild-type (**a**–**c** and **e**–**h**) or *La-2*/+ (**d**) plants, probed with *LA* antisense (**a**–**d**) or LNA-miR319 (**e**–**h**) probes. Asterisks, distal leaf tissues with higher *LA* expression than that in proximal tissues. In wild-type, strongest LA expression is detected in initiating leaflets (lfl, **a**,**b**). In *La-2*/+, strong expression is already evident in P0 (**d**, arrow and inset). (**i**) The *FIL* promoter driving two copies of the gene for green fluorescent protein targeted to the endoplasmic reticulum (*FIL>>ER-GFPx2*) in P1–P4 initiating tomato leaves, extending expression to earlier and later stages relative to *LA* and *miR319*, respectively. (**j**) Gradual reduction in leaf complexity results from expression of wild-type *LA* or the *La-2* form (LA^m) in leaves using the *FIL* promoter. M, meristem; V, leaf vein.

employed to simultaneously downregulate activities of all potential *miR319* targets (**Fig. 3c–j**). Intact *LA* mRNA amounts were markedly reduced in *miR319*-overexpressing plants (**Supplementary Fig. 4**). Whereas the first pair of wild-type leaves completed their morphogenesis and growth within 4 weeks, the same leaves of *miR319*-overexpressing plants continued to grow for more than 3 months (**Fig. 3c–e**), maintaining morphogenetic characteristics of P3 leaves in marginal regions (**Fig. 3c**,**d** insets). Furthermore, cotyledons of *miR319*-overexpressing plants emerged as in wild-type, but then resumed marginal growth (**Fig. 3d**). Mature leaves were buckled and disorganized, but maintained the basic partitioning into rachis and leaflets. Leaflets were greatly enlarged and had highly lobed margins (**Fig. 3e**). Although P1 and P2 initiating leaves of *miR319*-overexpressing plants resembled wild-type ones, subsequent differentiation at their margins was delayed, as manifested by later trichome development, prolonged leaflet initiation (**Fig. 3g–j**) and elevated expression of the shoot apical meristem and leaf primordia marker *Tkn2*, also known as *LeT6* (refs. 22,23; **Supplementary Fig. 4**). Similarly, loss-of-function mutants of the *Antirrhinum majus LA* homolog *CINCINNATA* (*CIN*) also showed excess growth at the leaf margins. CIN has been proposed to confer sensitivity to a cell-division arrest signal[3], a model compatible with the extended morphogenetic activity in margins of *miR319*-overexpressing plants. Thus, delayed growth arrest at the leaf marginal blastozone is a prerequisite for the formation of compound leaves. *La-2*/+ substantially suppressed the *miR319*-overexpression phenotype (**Fig. 3f**), indicating that downregulation of *LA* in initiating leaves is a prerequisite for maintenance of this morphogenetic competence.

Class I knotted-like homeobox (KNOXI) proteins are major regulators of compound leaf patterning[28,29]. Overexpression of the maize *knotted1* (*kn1*) gene in tomato through the ubiquitous promoter 35S (*35S:kn1*) results in supercompound leaves, with extra reiterations of leaflet initiation[5], and deferred differentiation of leaf primordia (**Fig. 3k**). This supercompound leaf phenotype is completely suppressed by *La* mutations[5,7] (**Fig. 3l**). Furthermore, differentiation of *35S:kn1 La-2*/+ P2 and P3 leaf primordia is similar to that in *La-2*/+ plants, as manifested by the erect P2 and the early trichome initiation on the P3 margin (**Fig. 3l**). Several lines of evidence suggest that despite this epistatic relationship, LA and KNOXI proteins affect leaf compoundness through separate pathways. These include the additive effects of *kn1* and *miR319* overexpression (**Fig. 3m**), the *35S:kn1*-induced leaf up-curling and deferred trichome development in *35S:kn1 La* P1 primordia (**Fig. 3l**), and the unchanged amounts of *Tkn2* (also known as *LeT6*) mRNA in *La*/+ shoots (**Supplementary Fig. 4**).

The role of *LA* regulation in patterning compound leaves is modeled in **Fig. 4**. *LA* gain- and loss-of-function and *miR319*-overexpressing phenotypes imply that threshold activity levels of LA and LA-like proteins promote the differentiation of leaf margins (**Fig. 4a**). Activity of LA and LA-like proteins are mitigated by *miR319* to define a spatial and temporal morphogenetic window, characterized by low LA amounts, in which leaflet-initiation programs can operate (**Fig. 4b**,**c**). This provides a molecular basis for the morphologically characterized marginal blastozone[13]. In *La* gain-of-function mutants, precocious threshold LA-like activity leads to a narrowing of this window in time and space (**Fig. 4b**,**c**), whereas ectopic expression of *miR319* throughout early leaf development substantially extends it. In agreement, the simple leaf of *La-2* plants is epistatic to a wide array of unrelated leaf shape mutants, whereas aspects of the blade morphology interact in an additive manner (**Supplementary Fig. 6** online). Thus, the interplay between *miR319* and its *LA*-like targets can lead to a range of phenotypic outputs of leaf size and form. A similar delicate balancing mechanism may be used by additional miRNA-target pairs with partially overlapping expression domains[24–27] to achieve output flexibility in other morphogenetic processes.

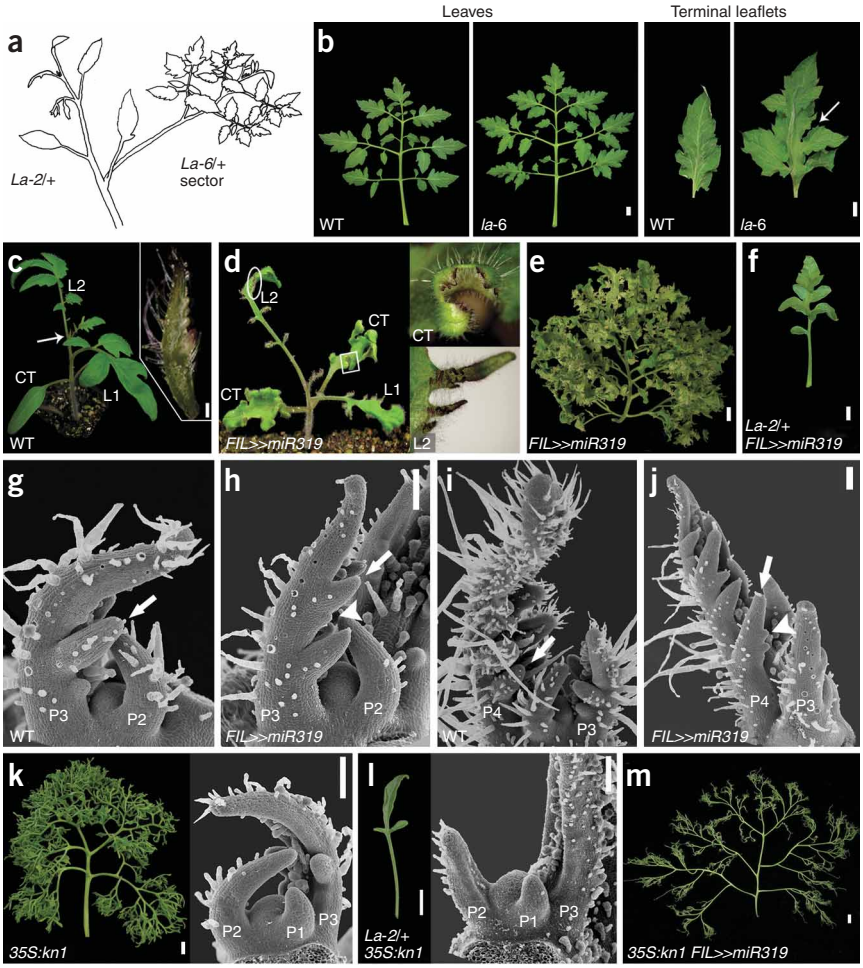

Figure 3 Loss of LA-like activities results in delayed leaf differentiation. (**a**) Illustration of an M1 *La-2/+* plant with a normal-looking *la-6/+* sector. (**b**) Young leaves and mature terminal leaflets from wild-type and homozygous *la-6* plants. Arrow points to a deep lobe in the *la-6* leaflet. (**c**,**d**) Ectopic expression of *miR319* with the *FIL* promoter (*FIL>>miR319*). Marginal growth lasts a few days in wild-type plants (arrow and inset in **c**) but months in cotyledons (CT) and leaves (L) of *FIL>>miR319* plants. Square and oval: leaf margin regions magnified in insets CT and L2, respectively. (**e**,**f**) The buckled large leaflets of mature *FIL>>miR319* leaves (**e**) are suppressed in the *La-2/+* background (**f**). (**g**–**j**) SEM images of shoot apices with P2–P3 leaves (**g**,**h**) and P3–P4 leaves with initiating leaflets (**i**,**j**). Initiating first- and second-order leaflets are indicated by arrows and arrowheads, respectively. (**k**) A leaf and an SEM of a shoot from *35S:kn1* plants. Development of leaf primordia is slightly deferred, as evident by the absence of trichomes in P1 leaves. (**l**) A leaf and an SEM of a shoot from *35S:kn1 La-2/+* plant. Whereas the simple leaf phenotype of *La-2/+* is epistatic to the *35S:kn1* supercompound leaf, and P2–P3 primordia differentiate precociously, similarly to their behavior in *La-2/+* leaves, the deferred maturation of P1, characteristic of *35S:kn1*, is maintained. (**m**) Additive phenotypes of *FIL>>319* and *35S:kn1*. Scale bars, 1 cm, **b**–**f** and leaves in **k**–**m**; 100 μm, insets in **c**,**d**,**g**–**j** and SEM in **k**.

We propose that a fundamental difference between tomato simple and compound leaves is governed by the timing and location of growth arrest and tissue differentiation at the marginal blastozone. A 'long enough' morphogenetic phase, achieved by the spatial and temporal regulation of LA-like activities, is a prerequisite for leaf elaboration, and may therefore underlie a portion of the widespread variability in leaf size and form.

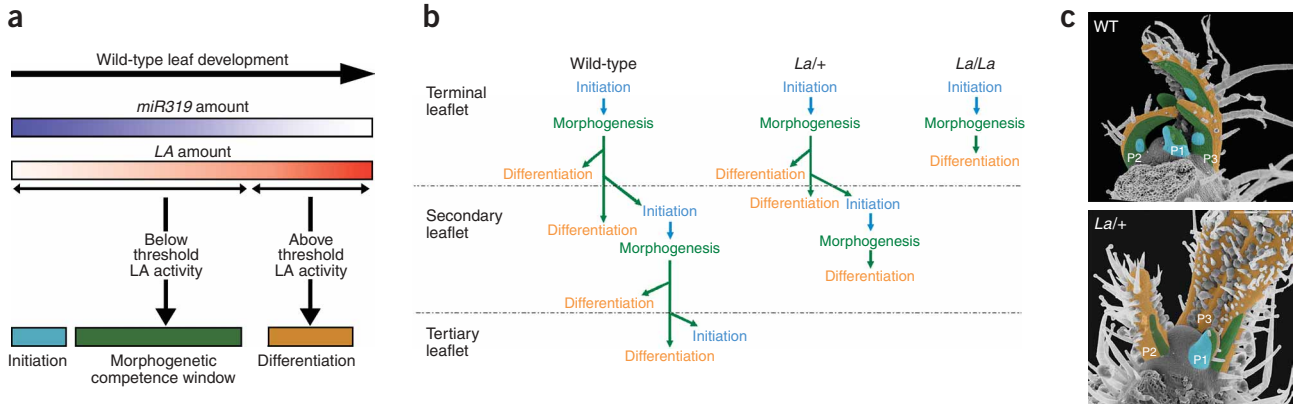

Figure 4 Patterning tomato compound leaves. (**a**) *LA* amounts (red gradient) increase with leaf maturation due to a combination of transcriptional regulation and post-transcriptional inhibition by *miR319*, which shows an opposite gradient (purple). Below-threshold LA activity enables leaf and leaflet initiation (blue) and facilitates a prolonged morphogenetic competence window (green). LA activity above the threshold promotes differentiation (orange). Initiating primordia (blue) do not reiterate primordia initiation until they reach the morphogenetic competence stage (green). (**b**) The difference between the compound wild-type and the small simple *La* leaves stems from shortening of the morphogenetic competence window by precocious above-threshold LA activity. Within this window, leaf margins can progress to tissue differentiation, prolonged morphogenetic competence or a new leaflet initiation, the last only at a minimum distance from flanking leaflets or differentiated regions. The choice among these fates is governed by the relative temporal and spatial length of the morphogenetic competence window. (**c**) A schematic color code of the different regions in leaf primordia of wild-type (WT) or *La* shoots, superimposed on the SEMs from **Figure 1**, according to the three different developmental phases detailed in **a**.

METHODS

Plant materials. For seed sources, see Acknowledgments. All mutants and transgenic plants were in the background of M82, or were backcrossed to M82 two to five times. Plants were grown in a greenhouse with 17–30 °C night-day fluctuations. For ectopic expression, OP:gene fusions were generated by PCR cloning and handled as previously described[24]. In each case, seven to ten kanamycin-resistant responder lines were recovered and crossed to the *FIL:LhG4* driver line.

Isolation of *la-6*. The tomato recessive *goblet* (*gob*) mutation[8] is lethal at the seedling stage, and maps 4 cM from *La*. To isolate a *la* loss-of-function allele, we mutagenized progenies of *La-2* +/+ *gob* plants with ethyl methane sulfonate as previously described[8] and screened M1 plants for wild-type sectors.

Molecular analysis. Database searches uncovered ESTs for four putative tomato *miR319*-regulated TCP homologs (**Supplementary Fig. 3**). A phylogenetic tree of the La-like proteins was based on ClustalX alignment (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) of 78 amino acids encompassing the TCP domain and was manually refined and analyzed by TreePuzzle (http://www.tree-puzzle.de). The numbers represent bootstrap percentage from 1,000 trials.

Analysis of miRNA. Analysis was as previously described[24].

***In situ* hybridization and microscopy.** We fixed tissue samples in formalin–acetic acid–alcohol, processed them in paraffin and performed *in situ* hybridization as described previously[26]. Locked nucleic acid (LNA) *in situ* hybridization was performed as previously described[30]. LNA oligonucleotides were synthesized by substituting every third nucleotide with a LNA monomer (Exiqon) and labeled with the DIG Oligonucleotide 3′-End Labeling Kit (Roche). Probes were not further purified after labeling. We diluted the resulting 20 µl probe solution with 20 µl formamide, and used 1 µl (2.5 pmol) per slide in a total of 200 µl hybridization solution. Hybridizations were performed at 55 °C, and slides were washed in ×1 SSC, 50% formamide at 55 °C. Scanning electron microscope (SEM) analysis and 5′ RNA ligase–mediated RACE were as previously described[24].

Primers. Primers used for cloning, sequencing, RT-PCR, cleavage analysis and LNA-modified *in situ* hybridization probes are listed in **Supplementary Table 1** online.

Accession codes. GenBank *LA* cDNA, EF091571; *LA* genomic sequence, EF091573; *SlTCP3* cDNA, EF091574; *SlmiR319a/b* precursor, EF091572.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

All authors contributed to the experiments and their interpretation. N.O., D.Z. and Y.E. directed the experiments, and N.O. and Y.E. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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