Dynamic growth program regulated by LANCEOLATE enables flexible leaf patterning

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SUMMARY

During their development, leaves progress through a highly controlled yet flexible developmental program. Transcription factors from the CIN-TCP family affect leaf shape by regulating the timing of leaf maturation. Characterization of mutants in the tomato (*Solanum lycopersicum*) CIN-TCP gene *LANCEOLATE* (*LA*) led us to hypothesize that a threshold LA-like activity promotes leaf differentiation. Here, we examined the relationship between LA activity, leaf maturation, and final leaf size and shape. Leaves of diverse shapes from various Solanaceae species or from different positions on the tomato plant differed in the timing of growth and maturation, and these were often associated with altered *LA* expression dynamics. Accordingly, genetic manipulations of *LA* activity in tomato altered leaf growth and maturation, leading to changes in leaf size and shape. *LA* expression sustained until late stages of tomato leaf development, and stage-specific overexpression of miR319, a negative regulator of CIN-TCP genes, confirmed that LA-like proteins affect leaf development through these late stages. Together, our results imply that dynamic spatial and temporal leaf maturation, coordinated by *LA*-like genes, enables the formation of variable leaf forms.

KEY WORDS: Solanaceae, TCP, Leaf development, Tomato

INTRODUCTION

Leaves are flat, lateral organs that are produced by the shoot apical meristem (SAM). Leaf development has been divided into three overlapping stages: initiation (I), primary morphogenesis (PM), and secondary morphogenesis (SM) or histogenesis (Dengler and Tsukaya, 2001; Donnelly et al., 1999; Kaplan, 2001). At the I stage, the leaf emerges at the flanks of the SAM and, depending on the species, either encircles the SAM flanks or appears as a rod-shaped protrusion. During PM the leaf expands laterally, and, in some species, marginal structures such as leaflets are produced from a specialized morphogenetic zone termed the marginal blastozone (Hagemann and Gleissberg, 1996). At SM, tissue differentiation occurs, which is manifested by the development of morphological markers such as trichomes, provascular strands and guard cells, and the leaf grows substantially, mainly by cell expansion. In spite of the division into three distinct stages, leaf maturation has been shown to be a dynamic process that is characterized by continuous morphological and molecular changes (Efroni et al., 2008; Freeling, 1992). Moreover, leaf maturation is not simultaneous, such that at any given time point during leaf development, regions of the leaf differ in their relative maturation state (Avery, 1933). The complex and dynamic maturation process raises the question of whether tuned manipulation of this dynamics underlies the flexibility and variability in leaf shapes that are observed in nature.

Leaf structure varies from a simple lamina with smooth margins to a compound leaf with reiterated substructures termed leaflets and lobed margins. The compound leaves of tomato (*Solanum lycopersicum*) show a high level of flexibility of form and size, which is manifested by a high variability between cultivars, a wide range of mutants affecting leaf shape, and sensitivity to growth conditions (Brand et al., 2007; Kessler et al., 2001; Menda et al., 2004; Shalit et al., 2009). In some species, including tomato, leaflets are thought to be initiated from the leaf margin through a mechanism that is partly equivalent to the formation of leaves from the SAM flanks (Barkoulas et al., 2008; Berger et al., 2009; Blein et al., 2008; Hagemann and Gleissberg, 1996; Koenig et al., 2009). This process is thought to require prolonged maturation that enables a spatially and temporally extended morphogenetic potential at the leaf margins. In particular, a sufficiently long PM stage has been shown to be crucial for leaflet formation. The duration of PM and the specific morphogenetic events that take place during this stage are thought to underlie much of the variability in leaf shape and size in nature (Blein et al., 2010; Canales et al., 2010; Hagemann and Gleissberg, 1996). We have previously shown that in tomato, downregulation of the activity of the TCP transcription factor LANCEOLATE (LA) is essential for the extended maintenance of morphogenetic potential at the leaf margins (Ori et al., 2007).

TCP transcription factors affect many aspects of plant development, including growth of axillary meristems, flower symmetry and leaf development (Broholm et al., 2008; Doebley et al., 1997; Kosugi and Ohashi, 1997; Luo et al., 1996; Martin-Trillo and Cubas, 2010; Poza-Carrion et al., 2007). There are two main classes of TCP genes, which have been suggested to affect growth antagonistically (Herve et al., 2009; Li et al., 2005). CIN-TCPs comprise a subclade of class II TCP genes that have been shown to dramatically affect the shape and size of leaves and flower organs in *Antirrhinum*, *Arabidopsis* and tomato by promoting organ maturation (Crawford et al., 2004; Efroni et al., 2008; Koyama et al., 2007; Nag et al., 2009; Nath et al., 2003; Ori et al., 2007; Palatnik et al., 2003). Some of the CIN-TCP genes are subject to negative regulation by microRNA 319 (miR319) (Palatnik et al., 2003). Manipulation of CIN-TCP activity in species with simple leaves, such as *Arabidopsis* and *Antirrhinum*, affects leaf size and smoothness (Efroni et al., 2008; Nath et al., 2003; Palatnik et al., 2003; Schommer et al., 2008). By contrast, misexpression of the CIN-TCP gene *LA* in tomato results in the conversion of the

compound leaf into a simple one, and leaves that overexpress miR319, which is likely to downregulate all miR319-sensitive CIN-TCP genes, exhibit indeterminate growth at the leaf margins (Caruso, 1968; Dengler, 1984; Mathan and Jenkins, 1962; Ori et al., 2007). These results led us to hypothesize that a threshold activity of LA promotes the transition from the PM to the SM stage of leaf development, and that tight regulation of LA activity ensures proper timing of this transition. This hypothesis predicts a dynamic spatial and temporal expression of *LA* during leaf development and a correlation between *LA* expression and the progress of leaf development.

Interestingly, transient manipulation of CIN-TCP activity at different stages of *Arabidopsis* leaf development results in very different phenotypes (Efroni et al., 2008). This implies that developmental cues are interpreted in a developmental context-dependent manner, and might suggest that dynamic spatial and temporal regulation of leaf maturation is utilized to produce a highly flexible, yet robust, array of leaf shapes.

Here, we tested these models by measuring the dynamics of leaf growth and *LA* expression during leaf development in tomato and related species with variable leaf forms. We show that differential growth dynamics, accompanied by a corresponding difference in the timing of *LA* expression, are correlated with variable leaf shapes and sizes. We further show that manipulations of LA activity lead to corresponding alterations in leaf growth dynamics and final size and shape, and that *LA* is expressed and is required throughout the late stages of tomato leaf development. These results imply that dynamic spatial and temporal regulation of leaf maturation is one of the mechanisms underlying leaf shape variability.

MATERIALS AND METHODS
Plant material
Tomato (*Solanum lycopersicum* cv M82, *sp*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*) and potato (*Solanum tuberosum*) seedlings were grown initially in a growth room at 16 hours day:8 hours night conditions at 24-25°C under fluorescent light. Four-week-old seedlings were transferred to greenhouse conditions with natural day length and 20°C at night and 25°C during the day. All transgenic genotypes were generated using the LhG4 transactivation system (Moore et al., 1998), in which driver lines expressing the synthetic transcription factor LhG4 under the control of a specific promoter are crossed to responder lines containing a gene of interest under the control of an *E. coli* operator that is recognized by the LhG4 transcription factor but not by any endogenous plant transcription factor. The cross results in the expression of the gene of interest under the control of the selected specific promoter. The expression domains of the *BLS* and *650* promoters have been described previously in detail (Shani et al., 2009). The expression domains of the *LA* and *NGA* promoters are described in this study (Fig. 2B-F; see Fig. S7 in the supplementary material).

Growth analysis and tissue collection
As leaves are produced successively on the plant, at a given time point each leaf is at a different developmental stage. Therefore, each collected or measured leaf is characterized by its position on the plant (for example, L1 is the first leaf produced and L5 is the fifth), and by its developmental stage. Thus, L5 P1 is the fifth leaf when it has just initiated from the SAM, and it becomes L5 P2 after the next primordium initiates. For each developmental stage, photographs of fifth leaves (L5) or the first leaf (L1) from at least five different plants were taken and the leaf images measured. For stages P1-P6, the length was measured at least three times for each image using ImageJ 1.25q (NIH, USA). For advanced stages, measurements were taken using a ruler.

For analysis of gene expression, tissue was collected at successive stages of leaf development such that the first or fifth leaf of the plant (L1 or L5, respectively) was at the corresponding developmental stage in all samples.

For very young leaf primordia at the P1-P3 stages, the leaf was collected with younger leaf primordia and the SAM. The P4 and P5 stages were collected both with and without the SAM and younger primordia.

Molecular analysis
For quantitative (q) RT-PCR, RNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions, except that samples were incubated for 30 minutes at room temperature after addition of the lysis buffer. cDNA synthesis was performed using the Verso cDNA Kit (Thermo Scientific) with 1 µg of RNA. qRT-PCR analysis was carried out using a Corbett Rotor-Gene 6000 real-time PCR machine, with TaqMan probes (PrimerDesign) and Premix Ex Taq (TaKaRa) for *LA* or SYBR Premix Ex Taq II (TaKaRa) for all other genes. Levels of *LA* mRNA were calculated relative to *EXPRESSED* (*EXP*) as an internal control as follows: in each biological repeat, the levels of *LA* and *EXP* were separately calculated relative to a standard curve obtained by a dilution series of a reference sample. The *LA* expression level in each biological repeat was calculated by dividing the *LA* expression value by that of *EXP*. Average expression values were then calculated and presented as 'relative *LA* expression'. *EXP* has been shown to be expressed at a similar level throughout tomato development (Exposito-Rodriguez et al., 2008). Similar results were obtained when *TUBULIN* (*TUB*) was used as a reference. Levels of *LA* orthologs were calculated relative to *TUB* orthologs as an internal control. Primers used for the qRT-PCR analysis are detailed in Table S1 in the supplementary material.

Isolation of the *LA* promoter
The *LA* promoter was isolated from the BAC clone SLmboI0123m13 (Tomato Functional Genomics Database). BAC DNA was digested with *Bam*HI and the fragments subcloned into the pBlueScript II KS(+) vector. Clones that contain *LA* upstream sequences were selected by hybridization with a probe from the 5′UTR of the *LA* gene. Positive clones were sequenced, and a 4125 bp fragment located upstream of the ATG was then amplified from the BAC clone and cloned upstream of the LhG4 sequence. Primers used for *LA* promoter cloning are listed in Table S1 in the supplementary material.

Isolation of *LA* orthologs
Fragments of the *LA* and *TUB* orthologs from eggplant, pepper and potato were amplified from cDNA or genomic DNA using tomato primers and primers derived from partial sequences of each species. The different fragments were assembled into contigs, in which only the sequences corresponding to primers from the 5′ and 3′ UTRs are tomato sequences. In parallel, we used the BLASTN and TBLASTX search programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Sol Genomics Network (SGN; http://solgenomics.net/) to identify partial sequences of *LA* and *TUB* orthologs, which were assembled with the amplified sequences.

Multiple sequence alignment and phylogenetic tree construction were conducted using CLC Main Workbench 5.6.1 (CLC Bio).

Imaging, microscopy and GUS staining
Tissue sectioning and microscopy were performed as described (Goldshmidt et al., 2008; Shani et al., 2009). Images of P5 and older leaf primordia were captured using a SMZ1500 fluorescence stereomicroscope (Nikon) equipped with a Nuance camera (CRi) as follows: NLS-mRFP and chlorophyll were imaged using a 540/40-nm filter for excitation and a long-pass 600-nm filter for emission. The multispectral acquisition range was 580-720 nm, captured in 10-nm steps. Following image acquisition, the spectral processing feature of the CRi software was used to mark the areas of the chlorophyll and NLS-mRFP spectral emission signatures in green and red pseudo color, respectively, into our spectral library. The saved spectral signatures were then unmixed and mapped across the leaf images, again with green for chlorophyll and red for NLS-mRFP.

Scanning electron microscopy (SEM) was performed using a JEOL 5410 LV microscope as described previously (Brand et al., 2007). GUS staining was performed as described previously (Ori et al., 2000).

RESULTS

Dynamic expression of *LA* mRNA during leaf development

Leaf phenotypes of gain- and loss-of-function *la* alleles led to the hypothesis that LA promotes the transition from the PM to the SM phase of leaf development, and that the timing of this transition underlies much of the variation in leaf size and shape (Dengler, 1984; Ori et al., 2007). We examined the dynamics of *LA* mRNA expression during wild-type tomato leaf development, to test whether it correlates with this transition. The developmental stage of young leaf primordia is followed by plastochrons, the intervals between successive leaf primordia. Thus, P1 is the youngest leaf primordium, it becomes P2 when the next primordium initiates, and so on. *LA* expression was followed during the development of the fifth leaf produced by the plant. Owing to their small size, leaves at the P2-P4 stages were collected with the SAM and younger leaf primordia. Relatively low expression of *LA* mRNA was detected in shoot apices and in young leaf primordia at the P1-P4 stages. The transition from the P4 to the P5 stage of development was accompanied by a steep increase in *LA* expression (Fig. 1A). To verify that the observed increase in *LA* levels at the transition from P4 to P5 was not due to the presence of the SAM and younger leaf primordia, we compared *LA* levels between P4 and P5 primordia with and without the SAM and younger leaf primordia. A similar increase was observed in these comparisons (Fig. 1B).

Strikingly, *LA* expression remained relatively high until late stages of leaf development, when the leaf had already expanded, and started to very gradually decline from the P9 stage (Fig. 1A). This could reflect a situation in which different parts of the leaf are at different developmental stages. To test this, we examined *LA* expression in different parts of the wild-type fifth leaf at the P7 and P8 stages. Tomato leaf differentiation is basipetal, such that the distal parts of the leaf differentiate earlier than the proximal parts. In addition, tomato leaves are thought to retain morphogenetic activity at their margins. Marginal regions had higher *LA* expression than internal regions (Fig. 1C). The expression of *LA* in the relatively younger tissue at the leaf margin might appear contradictory to its role in promoting differentiation. We propose that LA is upregulated during late PM, when it promotes the gradual transition to SM, and that its tightly controlled expression tunes the morphogenetic activity of the leaf margin. Too much or premature LA-like activity results in termination of the morphogenetic activity, whereas too little or delayed activity results in indeterminate growth (see also below). In agreement, low-level *LA* expression is also observed in the SAM (Ori et al., 2007) (see Fig. S1 in the supplementary material). It thus seems that morphogenetic regions can handle a certain level of LA activity, and that the higher activity in leaf margins relative to the SAM reflects its more advanced maturation state.
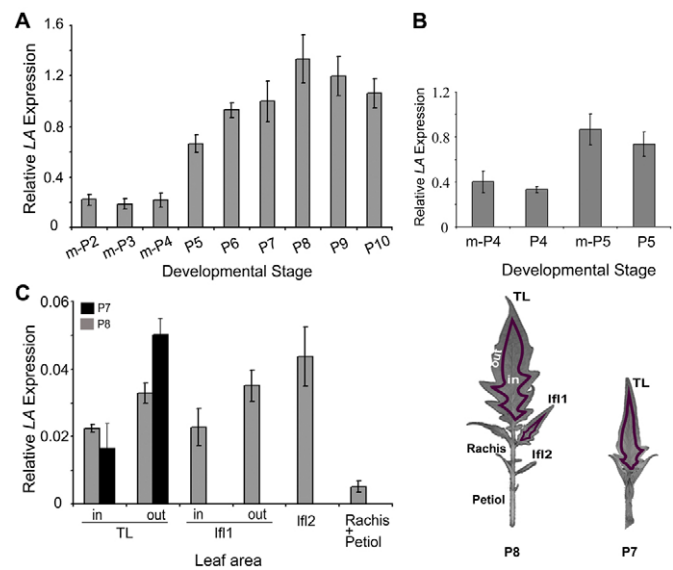

Fig. 1. Temporal and spatial expression of tomato *LA* mRNA. Levels of *LA* were assayed by real-time quantitative (q) RT-PCR relative to the reference gene *EXP*, and are shown as an average of 3-6 biological repeats (± s.e.) for the indicated developmental stages. (**A**) Dynamics of *LA* expression during wild-type fifth leaf development. (**B**) Relative contribution of the shoot apical meristem (SAM) to the *LA* expression level was assayed by comparing the corresponding mRNA levels in samples with (m-P4, m-P5) or without (P4, P5) the SAM and younger leaf primordia. (**C**) Comparison of *LA* expression in different parts of the fifth leaf at the P7 and P8 stages. The different leaf parts are illustrated to the right. TL, terminal leaflet; lfl1, first leaflet; lfl2, second leaflet. The lines illustrate the site of dissection between the inner (in) and the outer (out) part of the leaflets.

To verify the expression of *LA* in morphogenetically active leaf margins, we compared its expression level in wild-type apices containing leaf primordia at the I, PM and SM stages to that in *FIL>>Tkn2* apices, which are enriched for primordia at the I stage, and to that in *FIL>>Tkn2-SRDX* apices, which are enriched for precociously differentiated primordia (Shani et al., 2009). Of these, wild-type apices uniquely exhibit morphogenetically active leaf marginal tissues. In agreement with the expression of *LA* in this tissue, wild-type apices showed increased *LA* levels relative to *FIL>>Tkn2* and *FIL>>Tkn2-SRDX* apices (see Fig. S2 in the supplementary material). The expression of *LA* in the leaf margin is also supported by in situ hybridization analysis (Ori et al., 2007) (see Fig. S1 in the supplementary material) and by expression from the *LA* promoter (see below).

To examine the contribution of transcriptional regulation to the dynamic expression pattern of *LA*, we examined the expression pattern directed by a putative promoter contained within a ~4 kb region upstream of the *LA* translation start site (Fig. 2A). The *LA* promoter drove expression of the NLS-mRFP reporter throughout the SAM and early leaf primordia at the P1 stage, and at the P2-P5 stages expression became gradually restricted to the leaf margins (Fig. 2B-D). Later, expression gradually disappeared from distal and more developmentally advanced tissues, and dynamically correlated with younger and more marginal tissues (Fig. 2E,F). The relatively high level of expression driven by the *LA* promoter in the SAM and young leaf primordia contrasted with that of the *LA* mRNA (Fig. 1A). This is likely to reflect additional control of *LA* expression, including miR319-directed negative
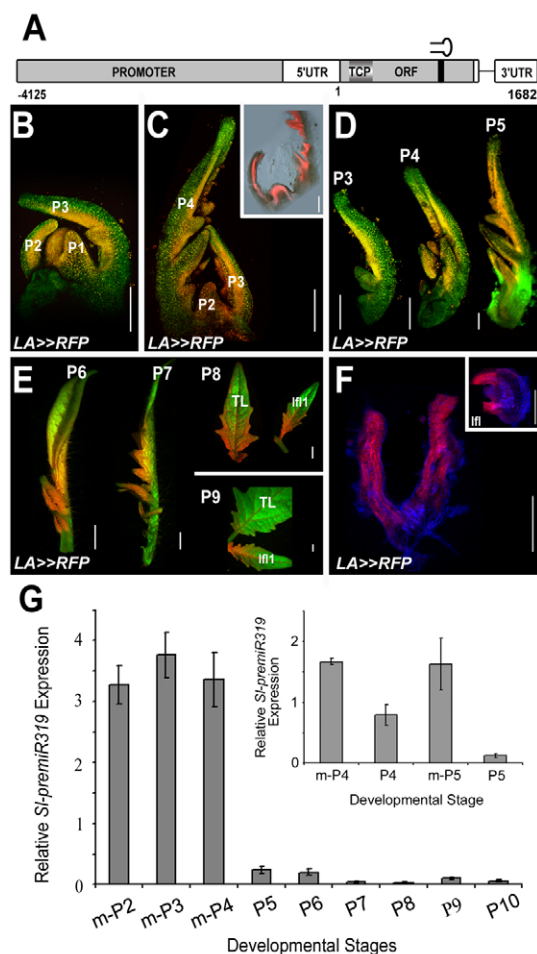
Fig. 2. Transcriptional and post-transcriptional regulation of *LA* expression. (**A**) The tomato *LA* gene and promoter. The miR319 binding site is indicated by the stem-loop. Numbers indicate position relative to the *LA* translation start site. TCP, TCP domain. (**B-D**) Expression of the *LA* promoter during early leaf development, viewed by mRFP fluorescence (red) in *LA>>RFP* plants. (B,C) SAM and young leaf primordia. Inset in C shows a longitudinal section of a SAM and young leaf primordia. (D) P3-P5 leaf primordia. (**E**) P6-P9 stages of leaf development. (**F**) Transverse section of an *LA>>RFP* leaf and leaflet (inset). (**G**) Expression of Sl-premiR319 during fifth leaf development in wild type, assayed by qRT-PCR relative to the reference gene *EXP* and shown as an average of 3-6 biological repeats (± s.e.) for the indicated developmental stages. Inset shows a comparison between expression in P4 and P5 leaves with or without the SAM and younger leaf primordia. TL, terminal leaflet; lfl1, first lateral leaflet. Scale bars: 200 µm in B-D,F; 1 mm in E.

regulation. The expression of Sl-premiR319, one of at least seven precursors of the *LA* negative regulator miR319 in tomato, was followed to assess its contribution to regulation of the *LA* transcript. Misexpression of this precursor in developing tomato leaves led to phenotypes similar to those caused by At-premiR319 misexpression (see Fig. S3 in the supplementary material), confirming its relevance. High levels of Sl-premiR319 expression were observed in samples containing the SAM and the P1-P3 primordia. Sl-premiR319 expression was reduced by 85% at the transition between the P4 and P5 primordia, and a further 80% reduction occurred at the P7 stage (Fig. 2G). Sl-premiR319 and *LA* thus show opposite expression gradients, but are co-expressed

in young leaf primordia (Figs 1 and 2). These results confirm the relevance of this microRNA precursor to the regulation of *LA* mRNA levels, and suggest that the low expression of *LA* in the SAM and young leaf primordia results from negative post-transcriptional regulation by miR319, whereas the *LA* expression level in older leaf primordia is consistent with that directed by its promoter (Figs 1 and 2).

In summary, *LA* mRNA expression shows dynamic spatial and temporal expression during leaf maturation, which is controlled at multiple levels that are likely to include transcriptional regulation via the *LA* promoter and post-transcriptional regulation by miR319. Elevated *LA* expression appears to precede the accelerated growth stage and remains high in growing parts of the leaf.

Differential dynamics of *LA* expression and leaf growth in Solanaceae species with variable leaf shapes

In tomato, elevated *LA* expression preceded the transition to the SM stage of leaf development, and precocious leaf maturation in gain-of-function *La* mutants led to smaller and simpler leaves (Fig. 1) (Ori et al., 2007). The miR319 binding sequence is intact in orthologs from Solanaceae species with both simple and compound leaves (see Fig. S4 in the supplementary material). This raised the question of whether differential dynamics of *LA* expression and a differential maturation schedule are correlated with some of the differences between simple and compound leaves. To start to address this question, we compared early leaf development and the expression dynamics of *LA* in tomato with those of three additional Solanaceae species: eggplant and pepper, with simple leaves, and potato, with compound leaves. *LA* orthologs from eggplant, pepper and potato were isolated and termed *Solanum melongena LA* (*Sm-LA*), *Capsicum annuum LA* (*Ca-LA*) and *Solanum tuberosum LA* (*St-LA*). Phylogenetic analysis confirmed that these are the likely tomato *LA* orthologs (see Fig. S4 in the supplementary material).

Eggplant leaves seem to progress to the SM phase at a much earlier developmental stage than tomato leaves, as manifested by early straightening, lateral expansion and trichome development throughout the primordia, although time-wise their development was slower (Fig. 3A). *Sm-LA* expression was relatively low in the SAM and very young leaf primordia, similar to *LA* expression in tomato, but the steep increase in *Sm-LA* expression occurred between P3 and P4, earlier than in tomato. The decrease in expression also began earlier, at the P7 stage, and was sharper (Fig. 3B). The early development of dense, long trichomes on eggplant primordia complicates the identification of the stage at which the marginal blastozone terminates. However, close examination of P2 and P3 primordia suggested that the marginal blastozone is still visible at these stages (Fig. 3A; see Fig. S5 in the supplementary material). Thus, eggplant leaves mature at an earlier developmental stage than in tomato and experience precocious elevation in *LA* expression.

Interestingly, the early development of pepper leaves, which are also simple, is very different than that of eggplant. Pepper leaf primordia appear to go through an extended I stage, up to stage P6, as manifested by the bending towards the SAM and lack of trichomes throughout the primordium margin (Fig. 3A). In general, pepper primordia develop trichomes of a different shape and at lower density than those of eggplant (Fig. 3A; see Fig. S5 in the supplementary material). No steep increase in *Ca-LA* expression was observed up to the P5 stage in pepper leaves (Fig. 3C). Thus,
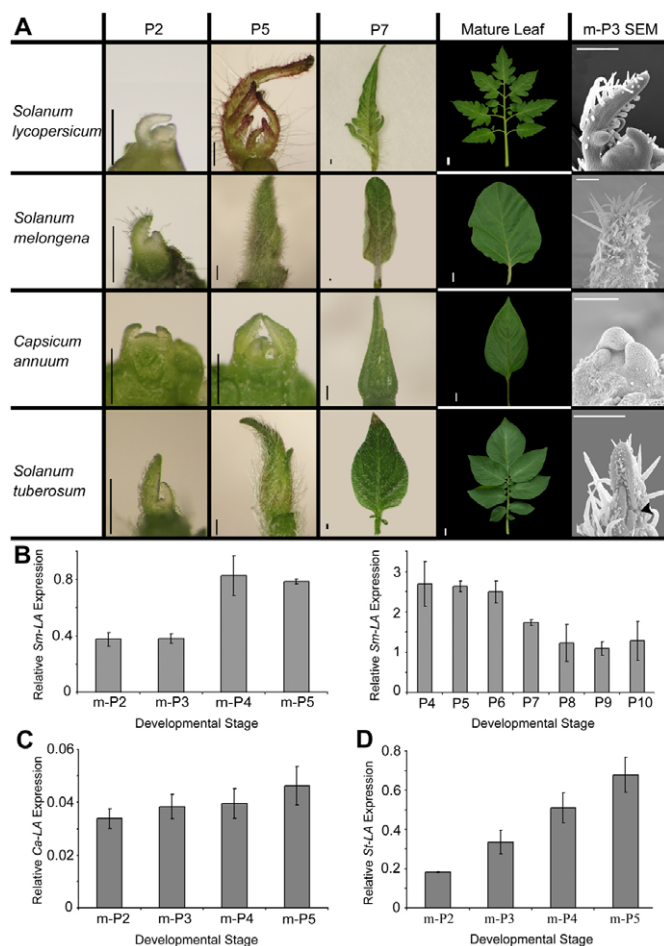
Fig. 3. Leaf development and *LA* expression in Solanaceae species. (**A**) Fifth leaves from tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*) and potato (*Solanum tuberosum*) at the indicated developmental stages. Arrowhead points to the dent between the terminal leaflet and the first lateral leaflet in potato. (**B-D**) Levels of *Sm-LA* (B), *Ca-LA* (C) and *St-LA* (D) were assayed by real-time qRT-PCR and are shown relative to the reference genes *Sm-TUB*, *Ca-TUB* and *St-TUB*, respectively, as an average of 3-6 biological repeats (± s.e.) for the indicated stages. Scale bars: 0.5 mm for P2-P7; 1 cm for mature leaf; 200 μm for SEM image.

eggplant and pepper leaves both have a relatively short PM, but in pepper the I stage, accompanied by relatively low *LA* expression, is long, whereas in eggplant both the I and PM stages are short, correlating with an early rise in *LA* expression.

Potato leaves are compound with a relatively large terminal leaflet and several pairs of lateral leaflets. Young leaf primordia straighten relatively early in their development, similar to eggplant leaves, but retain a region of trichome-less tissue at the leaf margin, similar to tomato (Fig. 3A). Lateral leaflets are formed relatively late in leaf development and are separated from the terminal leaflet by a dent (Fig. 3A, arrowhead). Subsequently, additional leaflets are formed and grow in a very moderate basipetal gradient. Accordingly, *St-LA* mRNA levels showed a gradual increase during early stages of leaf development (Fig. 3D).

Thus, the Solanaceae species examined show very different dynamics of leaf maturation and correspondingly different *LA*-like expression dynamics.

Variability in size and shape of successive tomato leaves is correlated with changes in *LA* expression and growth dynamics

Successive leaves formed on the tomato plant display a gradient of increasing size and complexity, such that the first few leaves are smaller and simpler than later leaves (see Fig. S6 in the supplementary material) (Poethig, 1997). The correlation between the maturation schedule and final leaf shape in leaves of different Solanaceae species suggested that differential maturation timing could also underlie the difference between successive leaves. To test this, we compared the dynamics of leaf growth and maturation between the first and fifth leaves produced by the tomato plant. The first leaf showed accelerated maturation relative to the fifth leaf, as manifested by the timing of leaf straightening and expansion, trichome development and leaflet initiation. However, morphogenetic activity of the first leaf ceased earlier than that of the fifth leaf, resulting in a smaller and simpler leaf despite the earlier initiation of leaflets (Fig. 4A).

The SM phase in leaf development is characterized by accelerated growth (Anastasiou et al., 2007). We thus followed the length of the first and fifth leaves as a quantitative marker of leaf maturation. During the I and PM stages, the leaf primordia grew relatively slowly and the basic leaf subcomponents, including primary and secondary leaflets, were formed (Fig. 4A,B). This was followed by a phase of accelerated growth, before growth finally slowed again (Fig. 4B,C; Fig. 5D). The first leaf grew slightly faster than the fifth leaf since its incipience, entered the accelerated growth phase much earlier, and the transition to slower growth was earlier and sharper than that of the fifth leaf, such that it virtually ceased growing (Fig. 4A-C). This earlier and faster development led to the smaller and simpler final leaf shape. The correlation between elevated *LA* expression and leaf maturation prompted us to test whether the difference in the timing of growth between the different leaves of a plant is correlated with increased *LA* levels during early leaf development. *LA* mRNA levels in the first leaf were higher than in the fifth leaf at all tested stages of development (Fig. 4D).

In summary, the earlier elevation of *LA* levels and growth in the first relative to the fifth tomato leaf is correlated with faster maturation and a smaller and simpler final leaf shape. The timing of elevation in *LA* levels correlates with that of the accelerated growth phase of the leaf and might be part of the mechanism that underlies flexibility in leaf shape within and among species.

Manipulation of *LA* expression dynamics alters the maturation schedule and final leaf shape

The correlation between *LA* expression, growth dynamics, maturation schedule and final shape among successive tomato leaves and among leaves from different species (Figs 1, 3 and 4) suggested that *LA* expression marks and promotes the transition from the PM to the SM phase of leaf development. To test this, we compared the dynamics of *LA* and Sl-premiR319 expression, the dynamics of growth and maturation and final leaf shape of wild-type leaves with those of mutants and transgenic plants with altered *LA* expression.

Leaves of the gain-of-function allele *La-2*, in which *LA* is mutated at the miR319 binding site, differentiate precociously (Fig. 5A) (Ori et al., 2007). Accordingly, relatively high *LA* mRNA expression was observed in developing *La-2/+* leaves since their incipience, in contrast to the low expression seen during the early development of wild-type leaves (Fig. 5B). The decrease in Sl-premiR319 expression occurred earlier in *La-2/+* than in wild-type
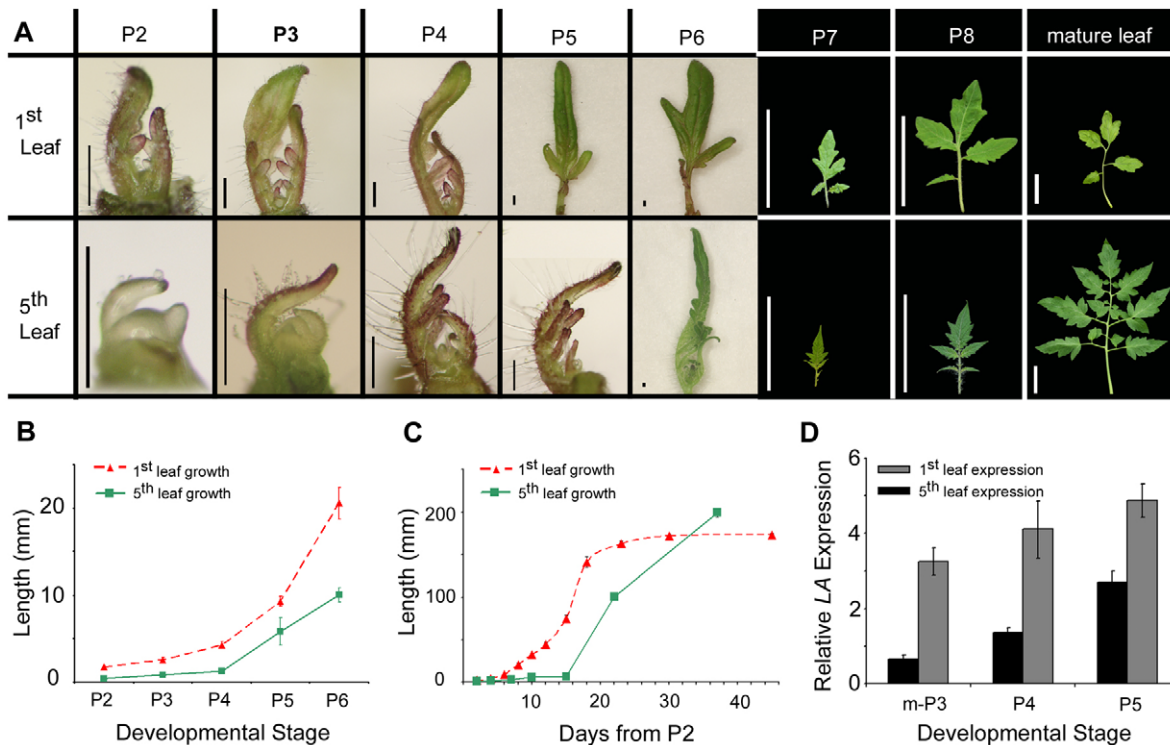
Fig. 4. The differential size and shape of successive tomato leaves correlates with altered dynamics of growth and *LA* expression.
(**A**) First and fifth leaves of wild-type tomato at different developmental stages. (**B**) Leaf growth at early developmental stages of the first and fifth tomato leaves. Shown are averages ± s.e. (*n*=7-10 biological repeats). (**C**) Leaf growth represented as length at different time points relative to the P2 stage. Shown are averages ± s.e. (*n*=7-10 biological repeats). (**D**) *LA* expression, as assayed by qRT-PCR and shown relative to the *EXP* reference gene, as an average ± s.e. (*n*=3 biological repeats). Scale bars: 0.5 mm for P2-P6; 5 cm for P7 to mature leaf.

leaves (Fig. 2D; Fig. 5C), and almost no expression could be detected in P5 and in older leaf primordia, which is likely to be secondary to the earlier maturation of these leaves (Fig. 5C). The elevated expression of *LA* in young leaf primordia led to a shift in the accelerated growth phase to an earlier developmental stage in *La-2/+* leaves (Fig. 5D). However, the transition to slower growth was also earlier in *La-2/+* leaves, leading to a smaller final leaf than that of the wild type (Fig. 5A,D).

In tomato, *LA* and three additional CIN-TCP genes possess a miR319 recognition site (see Fig. S4 in the supplementary material). *FIL>>*miR319 leaves, which overexpress the precursor of miR319 from *Arabidopsis* in leaves, display a substantial delay in leaf growth and a dramatically wider final leaf form with indeterminate marginal growth (Fig. 5A,D) (Ori et al., 2007).

In summary, manipulations of the timing of elevated *LA* expression resulted in corresponding alterations in the dynamics of leaf growth and maturation, in turn leading to substantial changes in leaf size and shape. Increased LA activity thus appears to precede and promote the transition from the PM stage, which is characterized by morphogenetic capacity and slow growth, to the SM stage, which is characterized by fast growth. The extended PM stage in the wild type enables the leaf to reach a larger final size than that of *La-2/+*, in addition to the shape elaboration.

Tomato leaves retain morphogenetic potential throughout their development

Examination of the temporal dynamics of *LA* expression suggested that LA remains active at late stages of leaf development, even after the leaf has expanded (Figs 1 and 2). To understand the role of LA

and additional miR319-regulated LA-like proteins at different spatial and temporal domains in the developing tomato leaf, they were transiently downregulated in specific domains by expressing At-premiR319 via a series of specific promoters. *LA>>*miR319 leaves showed prolonged indeterminate growth, especially at their margins, more orders of leaflets and delayed growth, leading to a large, more complex and less organized final leaf shape, similar to *FIL>>*miR319 leaves (Fig. 5A; Fig. 6B) (Ori et al., 2007).

The *BLS* promoter was used to express miR319 slightly after primary leaflet initiation beginning in P4 primordia in morphogenetically active regions of the leaf and leaflets and in the distal domains of the leaf (Shalit et al., 2009; Shani et al., 2009). *BLS* expression thus slightly overlaps with, but extends that of, Sl-premiR319. Previous analyses showed that within its expression domains, *BLS* drives expression at comparative levels to the *FIL* promoter (Shani et al., 2009). As expected from the expression domain, primary leaflets of *BLS>>*miR319 initiated normally, but later showed indeterminate marginal growth and highly lobed leaf margins (Fig. 6C). Interestingly, ectopic miR319 affected leaf shape considerably even when expressed at very late stages of leaf development and in relatively mature tissue (Fig. 6D,E), confirming that LA-like proteins still play a significant role in leaf patterning in these domains.

The *NGA* promoter drives expression in leaf margins of expanded leaves (Alvarez et al., 2009) (see Fig. S7 in the supplementary material). Expression of miR319 by the *NGA* promoter led to indeterminate growth at the leaf margin (Fig. 6D). The *650* promoter drives expression in tomato leaf tissues at the SM stage, in abaxial and distal domains of leaf primordia (Shalit
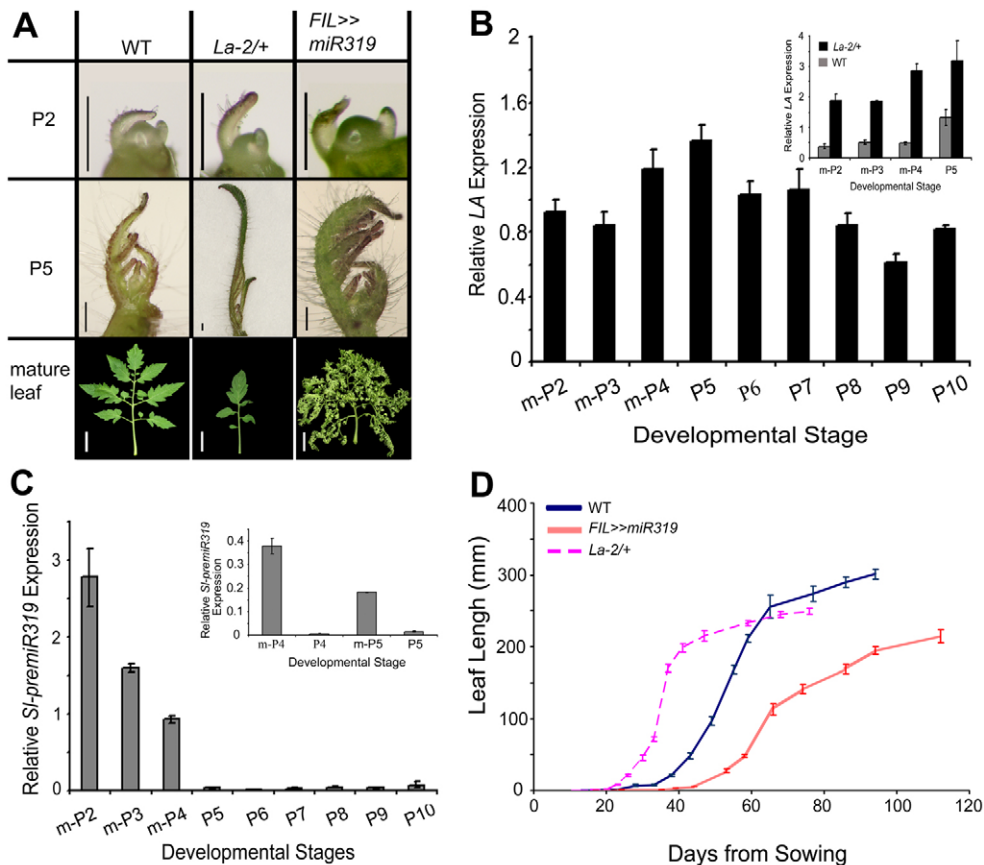
Fig. 5. Manipulation of LA activity alters growth and maturation dynamics and final leaf shape. (**A**) Fifth leaves of the indicated genotypes at the indicated developmental stages. Scale bars: 0.5 mm for P2 and P5; 5 cm for mature leaf. (**B,C**) Levels of *LA* (B) and Sl-premiR319 (C) during *La-2/+* fifth leaf development. RNA levels were assayed by real-time qRT-PCR relative to the reference gene *EXP*, and are shown as an average of 3-6 biological repeats (± s.e.) for the indicated developmental stages. Inset in B shows a comparison between wild-type and *La-2/+* tomato plants at early developmental stages in the same RT-PCR run. Inset in C shows a comparison of Sl-premiR319 expression between P4 and P5 with and without the SAM and younger leaf primordia. (**D**) Growth curves showing the length of the fifth leaf during development. Each point represents the mean length value (± s.e.) calculated from measurements of 5-9 leaves.

et al., 2009; Shani et al., 2009). Expression of miR319 using the *650* promoter resulted in leaves with a similar basic form to wild type but with highly lobed leaf margins, similar to leaves of *la-6* loss-of-function mutants (Fig. 6E,F) (Ori et al., 2007). Thus, *LA* might be the main miR319-regulated gene that is active during these stages. Interestingly, the prolonged morphogenetic activity stemming from miR319 overexpression was also manifested by the extended activity of these developmental stage-specific promoters when driving the expression of both miR319 and *RFP* (Fig. 6G-J). The extended activity of these promoters is likely to be secondary to the enhanced growth at the leaf margin, rather than a result of these promoters being direct LA targets.

In summary, the tomato leaf retains morphogenetic potential throughout its development, and the activity of LA-like proteins keeps this potential under control and tunes it.

DISCUSSION
Leaf morphogenesis is a controlled and predictable process, yet leaves in nature show an enormous variability in size and form. Our results imply that fine-tuning of the timing and location of leaf maturation underlies part of this variability. Dynamic spatial and temporal activities of LA and LA-like proteins are shown to constitute one of the mechanisms that set the pace of leaf growth and maturation and enable the flexibility of leaf shape and size within tomato and among related species.

Balancing morphogenesis and differentiation
LA-like proteins are shown here to play an essential role in promoting the transition from the PM to the SM stage of leaf development in tomato. However, the examination of tomato mutants and transgenic plants suggests that LA-like proteins constitute only one component of the control of this transition. The ratio between SINGLE FLOWER TRUSS (SFT) and SELF PRUNING (SP) activities was recently shown to promote maturation in multiple developmental contexts, including flowering time and leaf development. An increase in the SFT/SP ratio results in simpler leaves due to precocious termination of marginal blastozone activity (Shalit et al., 2009). The relationship between SFT/SP and LA remains to be determined, but the fact that loss of *SP* enhances the *La-2* phenotype suggests that these factors might act in parallel or at successive stages of leaf development to terminate indeterminate growth. Whereas LA and SFT/SP promote maturation, KNOXI proteins have been shown to promote an extended PM stage in tomato and other species (Barth et al., 2009; Canales et al., 2010; Floyd and Bowman, 2010; Hay and Tsiantis, 2006; Janssen et al., 1998; Shani et al., 2009; Uchida et al., 2010). Alternatively, KNOXI proteins have been interpreted to act within the morphogenetic window to reiterate the basic leaf shape, rather than extend this window (Efroni et al., 2010; Hareven et al., 1996; Ori et al., 2007). That KNOXI protein activity is context dependent (Shani et al., 2009) suggests that they might possess both roles. Interestingly, mutations that simplify the tomato leaf are in most cases epistatic to those that increase complexity (Efroni et al., 2010; Hareven et al., 1996; Kessler et al., 2001; Ori et al., 2007) (O.B.H. and N.O., unpublished observations). Similarly, in *Medicago truncatula* the simple-leaf phenotype of the *sgl* mutant is epistatic to that of the more compound *palm* mutant, and overexpression of *PALM* suppresses the lobed leaf phenotype caused by KNOXI gene overexpression (Chen et al., 2010). This suggests that the morphogenetic window in which leaflets are generated requires both the delayed activity of differentiation factors and a sufficient activity of PM-promoting factors.
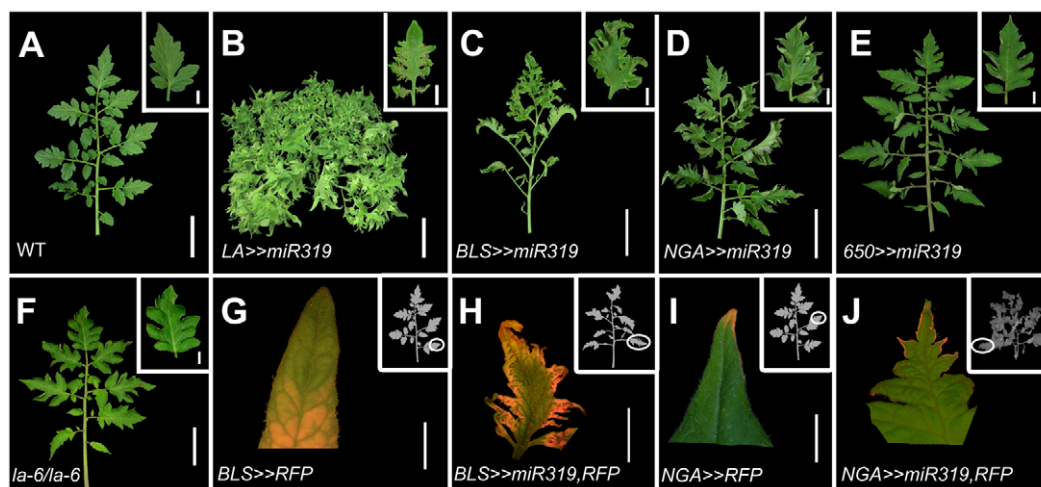
Fig. 6. Downregulation of LA-like activity affects leaf shape throughout its development. (**A-F**) Mature fifth leaves and the terminal leaflets (insets) of wild type (A), transgenic tomato plants expressing miR319 under the control of the indicated promoters (B-E) and a *la-6* mutant tomato plant (F). (**G-J**) Leaflets of the fifth leaves of the indicated genotypes, showing fluorescence of RFP when driven by the indicated promoters, with or without co-expression of miR319. Insets indicate the location of the leaflet in the context of the whole leaf. Scale bars: 5 cm in A-F; 1 cm in G-J.

CUC-like transcription factors and auxin have been shown to be involved in patterning morphogenetic events at the leaf margin in both simple and compound leaves (Barkoulas et al., 2008; Berger et al., 2009; Blein et al., 2010; Floyd and Bowman, 2010; Koenig et al., 2009; Nikovics et al., 2006). It thus appears that the timing of initiation of marginal structure with respect to the maturation schedule and the developmental stage of the leaf will determine the nature of these marginal structures.

Variations in leaf growth and maturation among species

The very different early development of potato, tomato, eggplant and pepper leaves and the corresponding differential dynamics of *LA* expression might suggest that differential timing and location of tissue maturation is utilized for flexible leaf patterning. In agreement, different genetic manipulations that affect *Arabidopsis* leaf size were recently shown to affect distinct aspects of leaf growth and maturation (Gonzalez et al., 2010), and mutant analysis in *Medicago truncatula* has identified novel factors that affect leaf growth and shape (Chen et al., 2010). Although in the eggplant leaf the transition to SM occurs at a relatively early stage of development, the leaf eventually reaches a larger final size than in tomato. This implies that whereas a prolonged PM is necessary for the elaboration of marginal appendages such as leaflets and lobes, leaf size is determined by events that occur in both the PM and SM phases.

Species- and stage-specific sensitivity to factors that affect leaf shape

Tomato and *Arabidopsis* show distinct stage-specific sensitivity to LA-like activity. Whereas expression of miR319 via the *FIL* promoter dramatically affects overall leaf structure, the effect of its expression from the *BLS* promoter was much milder, implying high sensitivity to LA-like activity during early stages of leaf development, in which the *FIL*, but not the *BLS*, promoter is expressed. By contrast, in *Arabidopsis* expression of miR319 via the *BLS* or the constitutive *35S* promoter results in comparable phenotypes (Efroni et al., 2008). Similar differences were shown in the sensitivity of *Arabidopsis* and tomato leaves to KNOXI

activity (Shani et al., 2009). Thus, part of the difference between the simple *Arabidopsis* and the compound tomato leaf appears to stem from events that take place at the very first stages of leaf development. Tomato also differs from *Arabidopsis* in its sensitivity to miR319 activity during late stages of leaf development (Efroni et al., 2008), in agreement with the late expression of *LA* and the maintenance of morphogenetic capacity until very late stages of tomato leaf development (Shani et al., 2010) (this study). Our results suggest a dual role for LA-like proteins in developing tomato leaves: promoting the transition from PM to SM and keeping the morphogenetic activity of the leaf margin in check throughout development. Interestingly, TCPs have recently been implicated in promoting leaf senescence in *Arabidopsis* (Schommer et al., 2008). Thus, although the simple *Arabidopsis* leaves lose morphogenetic capacity earlier than those of tomato, TCPs appear to be involved in promoting maturation throughout the existence of the leaf in this species too.

In contrast to repression of LA-like activity, repressing the activity of tomato KNOXI genes only affects leaf development at very early stages (Shani et al., 2009). Thus, the tomato leaf shows differential temporal and spatial sensitivity toward different patterning factors. Overall, patterning of the tomato leaf is a dynamic and complex process that spans the entire duration of leaf development, from very early until very late stages. These findings may explain the genetic and developmental flexibility of tomato leaf shape (Brand et al., 2007; Kessler et al., 2001; Menda et al., 2004).

Freeling (Freeling, 1992) proposed that different domains of the maize leaf progress through parallel, defined maturation schedules, which when appropriately coordinated give rise to a normal leaf, and further hypothesized that only at specific points during the maturation schedule is the leaf competent to respond to a developmental cue. The recent finding of a molecular maturation schedule in *Arabidopsis* supports this hypothesis in the context of simple leaves (Efroni et al., 2008). Reiteration of leaflet initiation in the tomato compound leaf appears to 'restart' the proposed maturation schedule in initiating leaflets. Efroni et al. (Efroni et al., 2010) recently suggested that leaves and leaflets are essentially different owing to the distinct developmental context of their

initiation and differences in their ontogeny. The cumulative evidence suggests that these organs utilize overlapping but distinct genetic components, and current research implies that the degree of overlap differs among species.

In summary, our results suggest that regulation of the timing and location of leaf maturation is a central mechanism that enables flexibility in plant organ shape, and that LA-like proteins are important tools in this regulation.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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