Lateral suppressor and Goblet act in hierarchical order to regulate ectopic meristem formation at the base of tomato leaflets

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SUMMARY

In seed plants, new axes of growth are established by the formation of meristems, groups of pluripotent cells that maintain themselves and initiate the formation of lateral organs. After embryonic development, secondary shoot meristems form in the boundary zones between the shoot apical meristem and leaf primordia, the leaf axils. In addition, many plant species develop ectopic meristems at different positions of the plant body. In the compound tomato leaf, ectopic meristems can initiate at the base of leaflets, which are delimited by two distinct boundary zones, referred to as the proximal (PLB) and distal (DLB) leaflet boundaries. We demonstrate that the two leaflet boundaries differ from each other and that ectopic meristem formation is strictly limited to the DLB. Our data suggest that the DLB harbours a group of pluripotent cells that seems to be the launching pad for meristem formation. Initiation of these meristems is dependent on the activities of the transcriptional regulators *Goblet* (*Gob*) and *Lateral suppressor* (*Ls*), specifically expressed in the DLB. Gob and Ls act in hierarchical order, because *Ls* transcript accumulation is dependent on Gob activity, but not vice versa. Ectopic meristem formation at the DLB is also observed in other seed plants, like *Cardamine pratensis*, indicating that it is part of a widespread developmental program. Ectopic meristem formation leads to an increase in the number of buds, enhances the capacity for survival and opens the route to vegetative propagation.

Keywords: tomato, *Cardamine pratensis*, meristem, boundary zone, compound leaf, *Lateral suppressor*, *Goblet*.

INTRODUCTION

Elaboration of aerial plant architecture can be traced back to the activity of meristems, groups of pluripotent cells, positioned at the tips of growing shoots. In seed plants, the primary shoot meristem (SAM) is established during embryonic development at the boundary of cotyledon primordia (Aida *et al.*, 1999). After germination, secondary meristems initiate in leaf axils at the junction between leaves and the stem. Subsequently, new meristems start to form leaf primordia and develop into buds. Such buds can grow out immediately or after a resting period, establishing new axes of growth. Different from the classical shoot model (Steeves and Sussex, 1989), many plant species develop ectopic meristems at different positions of the plant body. A well known example is the formation of shoots on leaves (epiphylly), which has been reported for several angiosperm species (Dickinson, 1978).

Shoot meristem formation is preferentially associated with specific regions of the plant body, the so-called boundary zones, which separate different parts of a plant. A typical boundary zone is established in the region between the newly formed leaf and the meristem, the leaf axil. Histological studies have demonstrated that leaf axils in tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana* are characterized by a low rate of cell divisions, resulting in a repression of growth (Hussey, 1971; Breuil-Broyer *et al.*, 2004). This growth retardation leads to mechanical stress and, as a result, cells in the axillary boundary develop stiff cell walls with microtubules in parallel orientation (Heisler *et al.*, 2010). Furthermore, the auxin efflux facilitator PIN1 is preferentially localised at the anticlinal cell walls of the boundary, promoting a depletion of the plant hormone auxin from the leaf axil (Heisler *et al.*,

2010). Recent experiments have demonstrated that this low auxin environment is a prerequisite for the formation of axillary meristems later in development (Wang *et al.*, 2014a,b). The precise localisation of a narrow zone of growth retardation in the leaf axil is also a consequence of brassinosteroid (BR) signaling. BRs were shown to activate expression of *BZR1*, a transcriptional regulator that down-regulates expression of the boundary-specific *CUPSHAPED COTYLEDON* (*CUC*) genes (see below) in cells adjacent to the boundary, thereby enabling an accurate spatio-temporal control of their mRNA accumulation (Gendron *et al.*, 2012). In addition, the transcription factor LATERAL ORGAN BOUNDARIES (LOB) negatively regulates accumulation of BRs in organ boundaries through the BR-inactivating enzyme BAS1 (Bell *et al.*, 2012), which is encoded by a direct target gene of LOB.

As mentioned above, the boundary zone of the leaf axil is characterized by specific gene expression profiles. Key factors involved are evolutionarily highly conserved NAC-domain transcription factors, encoded by the *CUC1–CUC3* genes in Arabidopsis (Aida *et al.*, 1997; Vroemen *et al.*, 2003) and the *Goblet* gene in tomato (Berger *et al.*, 2009). These proteins repress growth in the meristem-to-leaf boundary (Breuil-Broyer *et al.*, 2004) and contribute to meristem initiation through their activation of *KNOXI* gene expression (Aida *et al.*, 1999). In both species, axillary meristem formation is strongly compromised in mutants harbouring loss-of-function alleles of *CUC* (Hibara *et al.*, 2006; Raman *et al.*, 2008) and *Gob* genes (Busch *et al.*, 2011), respectively. microRNA164 modulates transcript accumulation of *CUC1* and *CUC2* (Laufs *et al.*, 2004) fine-tuning the number of secondary meristems per leaf axil (Raman *et al.*, 2008). In recent years, a complicated regulatory network required for boundary zone establishment and maintenance has been described (reviewed in Aida and Tasaka, 2006; Žádníková and Simon, 2014).

Specific expression in the leaf axil is also a hallmark of a group of transcriptional regulators that is required for axillary meristem formation. Key among those are the orthologous LATERAL SUPPRESSOR/MONOCULM1 proteins in Arabidopsis (LAS; Greb *et al.*, 2003), tomato (Ls; Schumacher *et al.*, 1999) and rice (*Oryza sativa*, MOC1; Li *et al.*, 2003). In Arabidopsis and tomato, LAS/Ls proteins promote axillary meristem formation specifically during vegetative development. Furthermore, several *MYB* (Schmitz *et al.*, 2002; Keller *et al.*, 2006; Müller *et al.*, 2006) and *bHLH* (Komatsu *et al.*, 2003; Gallavotti *et al.*, 2004; Yang *et al.*, 2012) transcriptional regulators are expressed in subdomains of the leaf axil and modulate axillary meristem initiation at different stages of development in diverse plant species.

Beside the leaf axil, boundary zones play an important role in separating other parts of the plant body established at different stages of development, for example during flower (Huang *et al.*, 2012; Lampugnani *et al.*, 2012; Hendelman *et al.*, 2013) or compound leaf (Blein *et al.*, 2008; Berger *et al.*, 2009) development. In higher plants, two types of leaves can be distinguished: simple and compound leaves. Simple leaves have an undivided leaf blade, whereas compound leaves consist of independent units, called leaflets, which are attached to a median connecting structure, called rachis. All leaves originate as simple primordia, however, in compound leaved species (e.g. tomato) the leaf margin comprises a zone of transient organogenetic activity, the marginal blastozone (Hagemann and Gleissberg, 1996), which initiates the formation of leaflets. Several studies have shown that partially overlapping mechanisms regulate leaf initiation at the SAM and leaflet initiation at the marginal blastozone (Barkoulas *et al.*, 2008; Koenig and Sinha, 2010; Ben-Gera *et al.*, 2012). The development of compound leaves strictly requires the establishment of boundaries between individual leaflets. A recent study has demonstrated that the compound tomato leaf is transformed into a simple leaf, if loss-of-function mutations in the genes *Gob* and *Potato leaf* (*C*) are combined (Busch *et al.*, 2011).

So far very little information is known about the different roles of boundary zones at different positions of the plant body, especially with respect to their ability to form new meristems, as they do in leaf axils. In this study, we demonstrate that the tomato distal leaflet boundary (DLB), but not the proximal leaflet boundary (PLB), is competent to initiate new meristems. This ability is strictly dependent on the activities of the Gob and Ls transcriptional regulators. Gob and Ls act in a hierarchical order, as *Ls* mRNA accumulation is dependent on Gob activity, but not vice versa. The competence of DLBs to initiate meristems is widespread among higher plants, because *Cardamine pratensis* and other species show the same phenomenon. Ectopic meristem formation on leaves increases the size of the bud bank of a plant (Klimešová and Klimeš, 2007) strengthening its capacity for survival, and is the initial step towards vegetative propagation.

RESULTS

Distal leaflet boundary zones of tomato leaves form ectopic meristems

Wild-type tomato plants develop compound leaves with a leaf blade subdivided into distinct units, called leaflets. Leaflets are initiated early during primordium development and are attached to the rachis via petiolules (Figure 1a). At the junction between rachis and petiolule ectopic shoots are occasionally formed (Figure 1b,c). All cultivars studied (i.e. Antimold B, M82, Lukullus, Ailsa Craig, Rheinlands Ruhm) produced ectopic shoots, although their numbers per plant varied (Figure S1). Under appropriate conditions ectopic shoot formation can be induced by removal of the
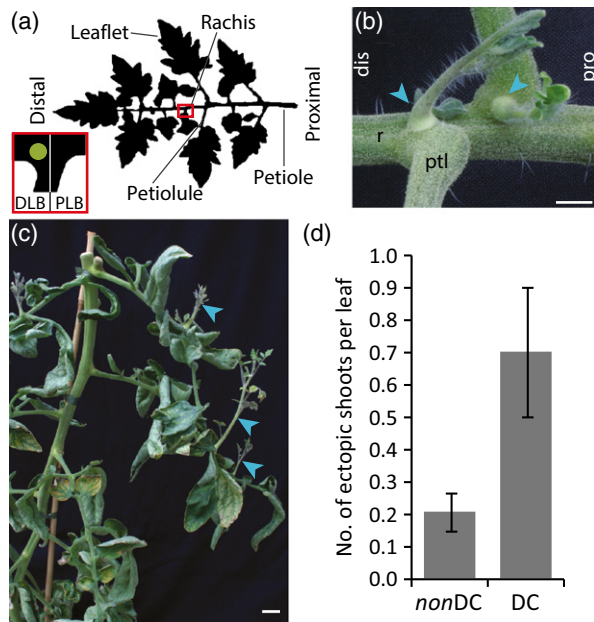
Figure 1. Ectopic shoot formation on tomato leaves.
(a) Wild-type tomato plants form ectopic shoots at the adaxial (dorsal) edge of distal (dis) boundary zones of leaflets (DLB), between rachis (r) and petiolule (ptl) (green circle indicates exact position), but not in proximal (pro) leaflet boundaries (PLB).
(b, c) Ectopic shoots (blue arrowheads) on wild-type leaves (cv. AmB).
(d) Comparison of ectopic shoot formation on wild-type tomato plants (cv. Lu) after intensive pruning, with decapitation after the 15th leaf (DC) or without (*non*DC). The number of ectopic shoots per leaf on 3-month-old plants was determined. Means ± SEM are given, *n* ≥ 6, Scale bars = 5 mm (b), 2 cm (c).

shoot apical meristem (SAM), which, in combination with intensive pruning, led to an increase in the number of ectopic shoots per leaf (Figure 1d). However, ectopic shoots never initiated on the lower (older) leaves (Figure S2), independent of the decapitation position (Figure S3), indicating that these leaves might lack this capacity.

At the rachis-petiolule junction two boundary zones can be distinguished, referred to as the PLB and DLB (Figure 1a). Visual inspection revealed that ectopic meristems exclusively form at the adaxial (dorsal) edge of the DLB (Figure 2a) in a region characterized by less trichomes and a lighter green colour (Figure 2b,c), which is absent in PLBs (Figure 2c,d). Microscopic analysis of sections through DLBs indicated that this colour difference between the DLB and its surrounding tissue is caused by a subepidermal cell layer that in the DLB comprises cells with reduced levels of chlorophyll (Figure S4). In this zone, meristems developed in a highly organized fashion (Figures 2a and S5) without callus formation, as observed at decapitation sites (Figure S6).

The histology of the adaxial sides of both leaflet boundary zones before and during ectopic meristem formation was investigated using scanning electron microscopy.

Epidermis cells within the DLB were significantly smaller compared to the cells flanking it and to those located in the PLB (Figure 2e–g). Stomata are highly specialized structures and guard cell development is considered to be a hallmark for cell differentiation within a tissue (Bergmann and Sack, 2007). Stomata development was found to be prevalent in the PLBs (Figure 2g), whereas no stomata were found in the DLBs (Figure 2f). Taken together, these analyses indicate that a specific area in DLB zones of tomato leaves comprises a coherent group of less differentiated cells that is competent to form new meristems.

Lateral suppressor activity is required for ectopic meristem formation

Lateral suppressor (*Ls*) encodes a key transcriptional regulator of axillary meristem (AM) formation that is expressed in the boundary zone between the shoot apical meristem (SAM) and leaf primordia prior to AM initiation (Schumacher *et al.*, 1999; Busch *et al.*, 2011). The expression of *Ls* precedes the formation of these boundaries and it has been hypothesised that Ls is needed to define these areas of inhibited growth (Busch *et al.*, 2011). In line with this view, *Ls* and the cell division marker *Histone H4* showed complementary expression patterns in tomato leaf axils (Figure S7). *Ls* mRNA also accumulates in DLBs (Busch *et al.*, 2011), but so far no deviation in leaf phenotype has been described for the *ls* mutant. Interestingly, also in DLBs *Ls* and *Histone H4* were found to be expressed in a complementary fashion (Figure S7). As the *Ls* expression domain comprises the region from which ectopic meristems develop, we tested the capacity of *ls-1* to form ectopic shoots. In five independent experiments, 50 *ls-1* plants, representing two different genetic backgrounds (Antimold B and Craigella), were analysed. All *ls-1* mutant plants had lost the capacity to form ectopic shoots (Figures 3a and S8). Furthermore, the distinct light green area found in wild type was absent in *ls-1* DLBs (Figure 3b,c). This indicates that a specific population of cells required for ectopic shoot formation is not present in the *ls-1* mutant. Moreover, epidermis cells in *ls-1* DLBs were significantly larger than those in wild-type DLBs, but indistinguishable from cells in either *ls-1* PLBs or wild-type PLBs (Figures 2e and 3d). In addition, a significant increase in stomata density was found in *ls-1* DLBs compared with wild type (Figure 3e–g). Combined, these data suggest an important role of Ls in ectopic meristem formation, probably dampening cell divisions and/or differentiation within a small group of DLB cells.

Goblet promotes ectopic shoot formation on leaves

The tomato mutant *goblet* (*gob*) was previously reported to be compromised in both axillary meristem formation (Busch *et al.*, 2011) and complex leaf development (Brand *et al.*, 2007; Berger *et al.*, 2009). *Gob* encodes a *NAM/CUC*
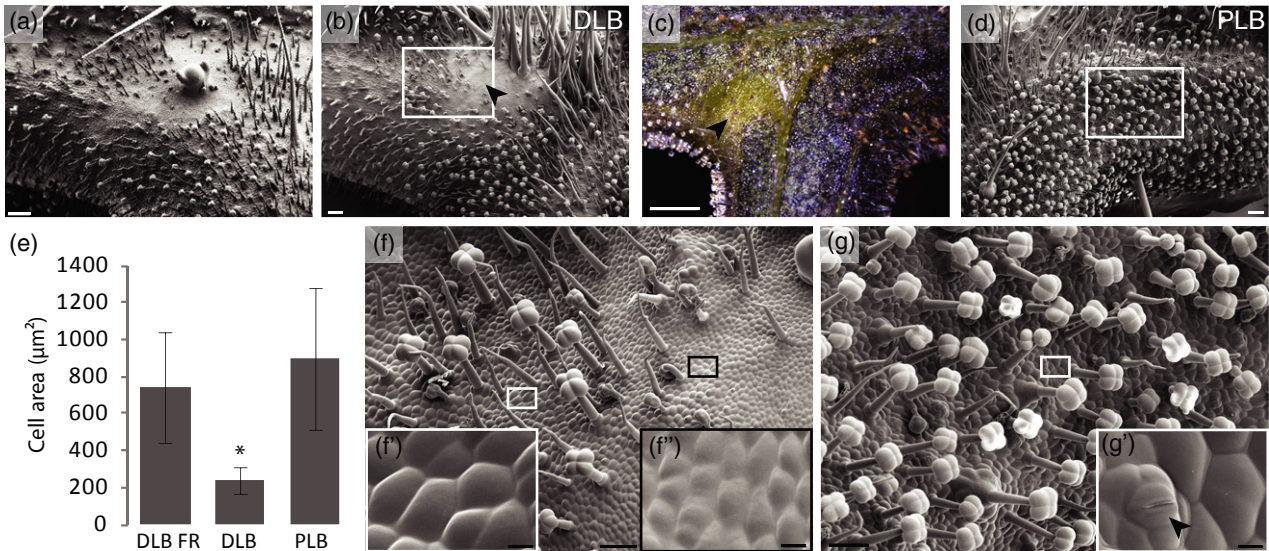
Figure 2. Tomato leaves form ectopic shoot meristems in distal leaflet boundaries.
(a) Scanning electron micrograph (SEM) of a young ectopic bud in a distal leaflet boundary (DLB) (cv. Lu).
(b) SEM picture of a DLB, showing the region competent for meristem initiation (black arrowhead) (cv. AmB). A close-up of the boxed area is shown in (f).
(c) Stereomicroscope image of a DLB and a proximal leaflet boundary (PLB). Black arrowhead marks the lighter region in wild-type DLBs (cv. AmB).
(d) SEM picture of a PLB forming trichomes (cv. AmB). A close-up of the boxed area is shown in (g).
(e) The surface area of epidermis cells in DLBs, PLBs and in the regions flanking DLBs (DLB FR) of wild-type plants (cv. AmB). Means $\pm$ SD are given, $n = 120$. *Significant difference ($P < 0.05$, Student's t-test).
(f) Small cells in the meristematic zone of a DLB [close-up view in (f″)] in comparison to the flanking region [close-up view in (f′)] with trichomes.
(g) Trichome and stomata [arrowhead in (g′)] development in PLBs. Scale bars = 200 μM (a, b, d), 2 mm (c), 100 μm (f, g), 10 μm (f′, f″, g′).

homolog that is specifically expressed in the leaf axil and in the DLB, resembling the *Ls* expression domains. The *gob-3* loss-of-function mutant lacks an embryonic SAM and, therefore, terminates shoot development at the seedling stage. However, after removal of cotyledons, plants can be regenerated from wound-induced callus tissue, formed at the tip of the hypocotyl stump (Brand *et al.*, 2007) (Figure S6c). For accurate comparison, control plants of the corresponding wild type (cv. M82) were also regenerated using the same procedure. In these experimental conditions, wild-type plants did not form ectopic shoots. However, these plants produced bulges of tissue on all DLBs (primary, secondary and intercalary) (Figure 4a,b). In these protrusions, which consisted mainly of small densely cytoplasmic cells, the light green area described in previous paragraphs is clearly distinguishable from surrounding tissue (Figures 4a and S9a,b). *gob-3* compound leaves, which lack secondary and intercalary leaflets (Busch *et al.*, 2011; Figure 4c), formed neither ectopic shoots nor bulges on any of their DLBs (Figure 4c–e). In addition, *gob-3* mutants produced about 10-fold more stomata in DLBs than the corresponding wild type (Figure 4f–h). Occasionally, ectopic shoots developed from the rachis of *gob-3* leaves, outside of the DLB area. Such shoots were never observed on wild-type leaves (Figure S9c,d).

It has been demonstrated that *Gob* expression is co-regulated by *miRNA164* (Berger *et al.*, 2009). The *Gob-4d*

allele harbours a mutated miRNA binding site, leading to an over-accumulation of *Gob* mRNA and to a gain-of Gob function (Berger *et al.*, 2009). In contrast to *gob-3*, ectopic shoot formation from *Gob-4d* DLBs was strongly increased (Figure 4i–l). Both hetero- and homozygous *Gob-4d* plants developed multiple ectopic shoots from almost all DLBs in contrast to the corresponding wild type (cv. M82), which produced the previously described bulges from its DLBs (Figure 4i,l). In addition, *Gob-4d* homozygous plants frequently developed ectopic leaves and shoots along the rachis, which were less frequent in *Gob-4d* heterozygous plants and never observed in wild type (Figure S9c,e). Monitoring early ectopic shoot development in *Gob-4d* revealed dense chains of fused ectopic meristems, specifically in the DLB regions (Figure 4j,k). Taken together these data suggest that, in addition to *Ls*, *Gob* is required for ectopic shoot formation from DLBs.

Lateral suppressor and *Goblet* act in the same pathway

As both *Ls* and *Gob* are required for ectopic shoot formation, the question arose whether these boundary genes act independently or in a hierarchical order. To test for a genetic interaction, the *ls-1* mutant was crossed to *Gob-4d/+* heterozygotes, which, because of the partial dominant nature of the *Gob-4d* allele, express the phenotype in a slightly milder way than the homozygous *Gob-4d* mutants, which are sterile. For phenotypic analysis *ls-1/ls-1 Gob-4d/+*
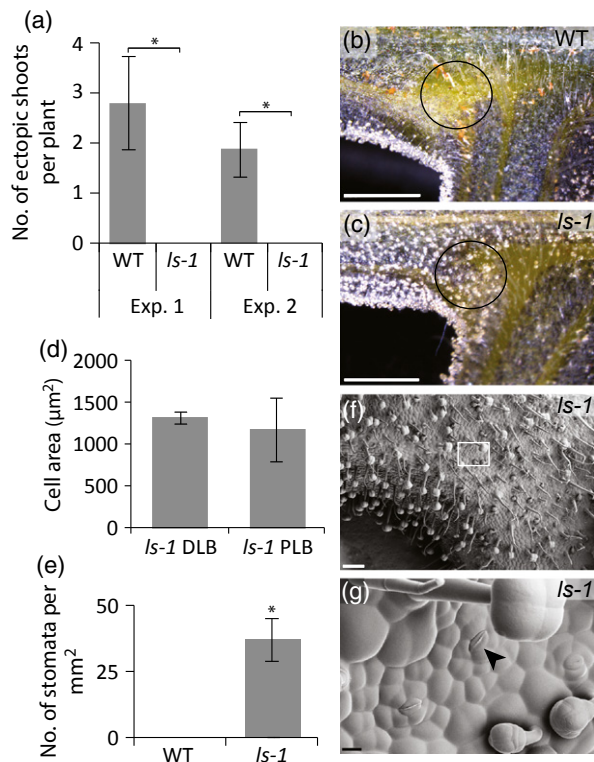
Figure 3. Lateral suppressor promotes ectopic meristem formation.
(a) Total number of ectopic shoots per plant in two independent experiments (Experiments 1 and 2) of 4-month-old wild type (WT, cv. AmB) and *ls-1*. Plants were intensively pruned and decapitated after formation of the second or third inflorescence depending on the experiments. Means ± SEM are given, $n \geq 5$. *Significant difference ($P < 0.05$, Student's t-test).
(b, c) Stereomicroscope images of a distal leaflet boundary (DLB) in WT (b) and *ls-1* (c). Circles indicate the area that differs between WT and the mutant.
(d) The surface area of epidermis cells of DLBs and PLBs in *ls-1*. Corresponding WT is shown in Figure 1(e). Given are means ± SD, $n = 120$.
(e) Comparison of number of stomata in DLBs of *ls-1* and WT. Given are means ± SD, $n \geq 5$. *Significant difference ($P < 0.01$, Student's t-test).
(f) Scanning electron micrograph (SEM) of a DLB in *ls-1* developing trichomes and stomata.
(g) Close-up of the boxed area in (e) showing stomata in *ls-1* DLB (arrowhead). Scale bars = 2 mm (b, c), 200 μm (f), 20 μm (g).

(*ls-1 Gob-4d/+*) plants were used. *Gob-4d/+* single mutant plants developed ectopic shoots from almost all DLBs. In contrast, these regions were completely barren both in *ls-1* single and in *ls-1 Gob-4d/+* double mutants (Figure 5a–d). Scanning electron microscopy demonstrated that a loss of Ls function conditions stomata development in DLBs of *ls-1 Gob-4d/+*, as in *ls-1* plants (Figure 5e,g,h), whereas almost no stomata were formed in *Gob-4d/+* DLBs (Figure 5f,h). Furthermore, ectopic meristems were initiated in the *Gob-4d/+* single mutant at a relatively early stage (Figure 5f). These data suggest that Ls function is required for ectopic shoot formation in the *Gob-4d* background.

Both *Ls* and *Gob* function are also required for the initiation of axillary meristems (Schumacher *et al.*, 1999; Busch

et al., 2011). This raises the question, how both genes interact to regulate this process. Consistent with previous results (Schumacher *et al.*, 1999), most leaf axils in *ls-1* (92.1%) did not support the formation of axillary buds (Figure 6a,b). If a side-shoot developed, it originated from the leaf axil preceding an inflorescence. Roughly 50% of these side-shoots were fused to the main stem (Figure 6c). In contrast, the *Gob-4d/+* mutants displayed enhanced branching in all leaf axils with <5% fusions to the stem (Figure 6a), and the leaf axils of *Gob-4d/+* plants were strongly swollen (Figure 6d). Furthermore, multiple accessory side-shoots were produced in almost all *Gob-4d/+* leaf axils (Figure 6d,g), but only occasionally in *ls-1 Gob-4d/+* plants. 21.9% of leaf axils were completely empty in *ls-1 Gob-4d/+*, indicating that in the *Gob-4d/+* background the formation of side-shoots is at least partially dependent on *Ls* (Figure 6a). In addition, these side-shoots were often fused to the main stem. These fusions spanned up to 30 cm in these double mutants (Figure 6f), a phenotype which was never observed in either individual mutant. Occasionally, the separation point of a side-shoot from the main axis was shifted upwards without any obvious fusion structure (Figure 6e). Taken together, the above data indicate that Gob and Ls act in one pathway.

Goblet modulates *Lateral suppressor* expression

It was previously demonstrated that *Ls* and *Gob* have similar expression domains (Berger *et al.*, 2009; Busch *et al.*, 2011; Figure 7a). To examine, whether both transcripts co-localise, RNA *in-situ* hybridization experiments were performed using *Ls* and *Gob* probes on consecutive sections (Figure 7b–k; cv. AmB). *Ls* and *Gob* expression domains largely overlapped in both leaflet (Figure 7b–g) and axillary boundary zones (Figure 7h–k). Moreover, in the leaflet boundaries, both genes were found to be exclusively expressed in the DLB, not in the PLB (Figure 7b–g). This triggered the question, whether *Ls* expression is dependent on *Gob* activity, or vice versa. RNA *in-situ* hybridization experiments indicated that *Ls* transcripts accumulate to higher levels in both DLBs and leaf axils of *Gob-4d* plants compared to the corresponding wild type (Figure 7l, m,o,p). This observation was corroborated by qRT-PCR results showing elevated *Ls* transcript levels in leaves of hetero- and homozygous *Gob-4d* plants in a dose dependent manner (Figures 7n and S10a). In concordance, regenerated apices (including primordia P1–P4) of the *gob-3* loss-of-function mutant showed a strong down-regulation of *Ls* transcripts compared to wild type (Figures 7q and S10b). Comparison of *Gob* transcript accumulation between *ls-1* and the corresponding wild type revealed neither an alteration in localisation of the expression domains nor in expression level (Figures 7r–t and S10c). Combined these data suggest that in both processes *Ls* expression is at least partially dependent on *Gob* function.
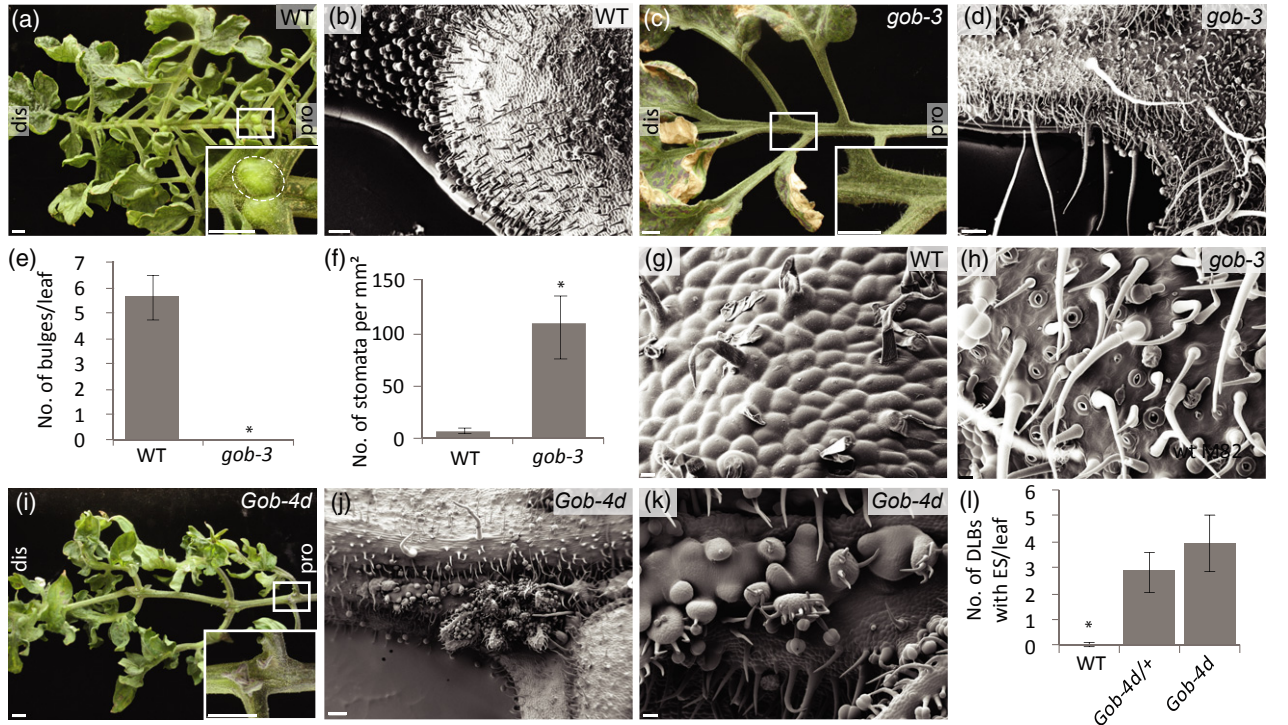
Figure 4. Goblet is required for ectopic meristem formation on tomato leaves.
(a) Wild-type (WT, cv. M82) leaf with bulges formed in DLBs (higher magnification in inset shows two bulges, upper bulge encircled).
(b) Scanning electron micrograph (SEM) of a WT DLB.
(c) Leaf of a *gob-3* loss-of-function plant with barren DLBs (higher magnification in inset).
(d) SEM picture of a *gob-3* DLB.
(e) Comparison of number of DLB bulges per leaf between WT and *gob-3*. Means ± SD are given, $n \geq 46$.
(f) Comparison of number of stomata in DLBs between WT and *gob-3*. Means ± SD are given, $n \geq 12$.
(g, h) Close-up of epidermis cells in DLBs of M82 (g) and *gob-3* (h).
(i) Leaf of *Gob-4d* gain-of-function mutant forming several ectopic shoots per DLBs.
(j) SEM picture of a *Gob-4d* DLB.
(k) Close-up of ectopic meristems formed in a *Gob-4d* DLB.
(l) Comparison of number of DLBs with ectopic shoots (ES) in WT (cv. M82), *Gob-4d*/+ and *Gob-4d*. $n \geq 33$. *Significant difference ($P < 0.01$, Student's *t*-test).
Scale bars = 1 cm (a, b, i), 200 μm (b, d, j), 20 μm (g, h), 100 μm (k).

Ectopic meristem formation beyond tomato

The observed capacity of tomato leaves to form ectopic meristems in the distal boundary zone of leaflets raises the question: Is this a peculiarity of tomato leaf development, or the result of a more widespread developmental mechanism? Literature searches revealed that ectopic shoot formation has been reported for the Brassicaceae species *C. pratensis*, which produces whole plantlets on its leaves under wet conditions (Salisbury, 1965). From which part of the leaf do these plantlets develop? Using scanning electron microscopy we observed that bud development in *C. pratensis* is initiated by the formation of meristems in the DLBs of leaflets (Figure 8a–d), very similar to tomato. Different from tomato, adventitious roots are immediately formed (Figure 8e,f). These observations led to the question: Is ectopic meristem formation in *C. pratensis* also correlated with specific expression of *Ls* and *Gob* homologous genes in DLBs? RNA *in-situ* hybridization experiments showed that *CpLAS* as well as *CpCUC2* and *CpCUC3*, co-orthologues of the tomato *Gob* gene, are expressed in those domains of the DLB that give rise to the formation of ectopic meristems (Figure 8g–i). Furthermore, mRNAs of these three genes also accumulated in the boundary between a young leaf primordia and the SAM (Figure 8j–l), recapitulating the expression pattern of these boundary genes in tomato. These results suggest that Ls/Gob-correlated ectopic meristem formation from DLBs follows a developmental program established in different dicotyledonous plant species.

DISCUSSION

Distal leaflet boundaries harbour pluripotent cells

In plants, boundary regions are required to separate different organs or tissues from each other. This separation is achieved by a local repression of growth, which constitutes
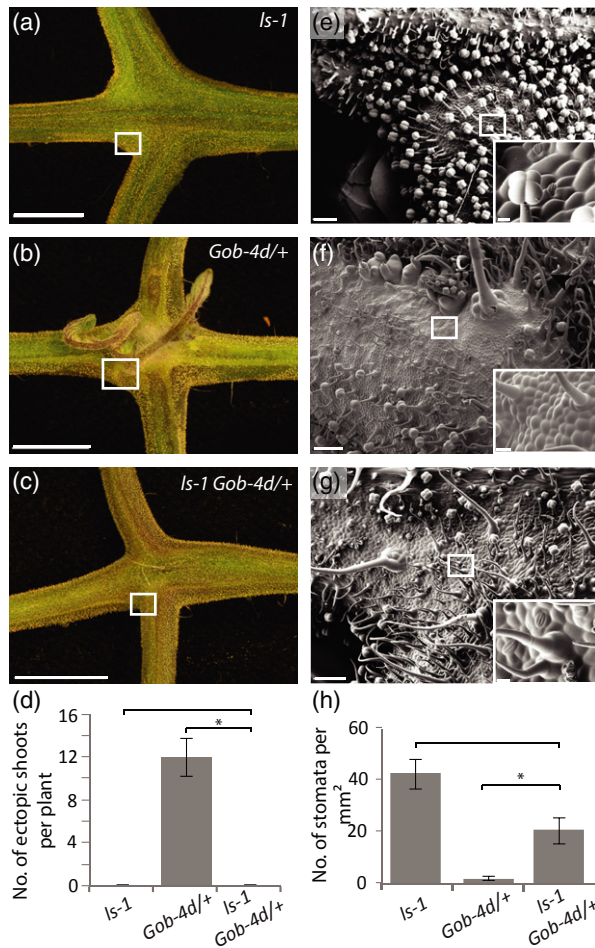
Figure 5. *ls-1* acts down-stream of *Gob-4d* with respect to ectopic shoot formation.

(a–c) Close-ups of leaflet-to-rachis junctions of indicated genotypes. Ectopic shoots are formed in *Gob-4d*/+ (b), but are completely lacking in *ls-1* (a) and *ls-1 Gob-4d*/+ (c) mutants.

(d) Comparison of ectopic shoot formation in 4-month-old *ls-1*, *Gob-4d*/+, *ls-1 Gob-4d*/+ mutants. Means ± SEM are given, *n* = 12.

(e–g) Scanning electron microscopy images of DLB of *ls-1* (e), *Gob-4d*/+ (f) and *ls-1 Gob-4d*/+ (g), shown at higher magnification in the insets.

(h) Comparison of stomata density in DLBs of *ls-1*, *Gob-4d*/+ and *ls-1 Gob-4d*/+. Means ± SEM are given, *n* ≥ 7. *Significant difference (*P* < 0.01, Student's *t*-test). Scale bars = 1 cm (a–c), 200 μm (e–g), 20 μm (insets of e–g).

Figure 6. Shoot branching phenotype of *ls-1 Gob-4d*/+ double mutants.

(a) Percentage of barren leaf axils, displaced side-shoots, fused side-shoots and normal side-shoots in leaf axils per plant in *ls-1*, *Gob-4d*/+ and *ls-1 Gob-4d*/+ mutants. Means ± SEM of two independent experiments are given, *n* ≥ 12.

(b) Barren leaf axils in *ls-1*. Similar phenotype was also observed in *ls-1 Gob-4d*/+.

(c) A pruned side-shoot (asterisk) fused to the stem in *ls-1*.

(d) Pruned primary side-shoot (asterisk) and accessory side-shoot (arrow) in a *Gob-4d*/+ mutant.

(e) Side-shoot (arrow) separating from the main axis at a distal position in *ls-1 Gob-4d*/+.

(f) Fused side-shoots of *ls-1 Gob-4d*/+ with pruning sites marked by asterisks. Side-shoots fused with the stem are indicated by brackets.

(g) Number of accessory side-shoots per leaf axil in *ls-1*, *Gob-4d*/+ and *ls-1 Gob-4d*/+. Means ± SEM of two independent experiments are given, *n* ≥ 10. Scale bars = 2 cm (b–f).

an important characteristic of boundary regions (Hussey, 1971). During compound leaf development two boundary zones are established, one at the distal side (DLB) and another one at the proximal side (PLB) of a leaflet. At first glance these boundaries appear to be rather similar. However, at the microscopic level we observed that known differentiation markers (e.g. trichomes, stomata and enlarged epidermal cells) (Poethig and Sussex, 1985; Hagemann and Gleissberg, 1996; Bergmann and Sack, 2007) were less frequent at the adaxial edge of the DLB compared to the PLB (Figure 2). In tomato and *C. pratensis*, cells in the DLB that express *Ls* and *Gob* homologous genes are smaller compared to cells in the PLB, which express neither of these
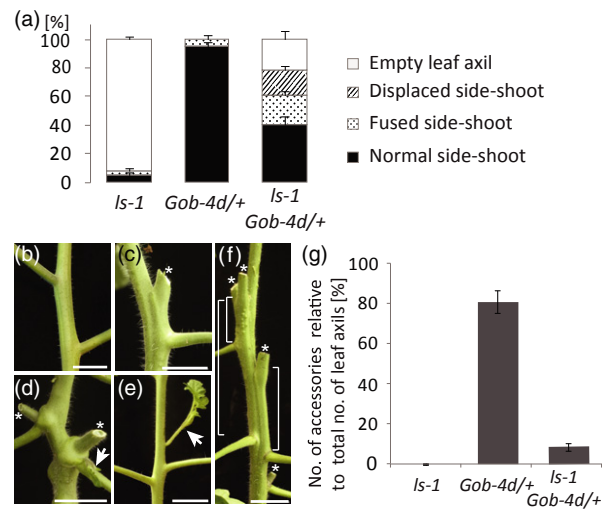
genes (Figures 2, 7 and 8). These results are in line with the observations that tomato plants harbouring a miRNA resistant version of *Gob* or plants over-expressing *Ls* exhibit a repression of growth (Berger *et al.*, 2009; Busch *et al.*, 2011). In addition, stomata development and enlarged epidermal cells were only observed in DLBs of the loss-of-function mutants *ls-1* and *gob-3*, but not in their corresponding wild types (Figures 3 and 4). These results indicate that a specific cell group in the DLBs of both mutants has lost its low differentiation level. *Histone H4*, a marker for cell divisions, was shown to be down-regulated in domains, where members of the *NAM/CUC3* gene family are expressed (Blein *et al.*, 2008). We observed a similar complementary expression between *Histone H4* and *Ls*, both in leaf axils and in DLBs (Figure S7). In summary, DLBs and PLBs show similarities and differences. Both are functional boundary zones that contribute to the separation of a leaflet from its neighbours. However, only the DLBs, expressing both *Ls* and *Gob*, support the formation of new meristems. Ls and Gob functions seem to be needed to repress differentiation in a group of DLB cells that is essential for meristem initiation.
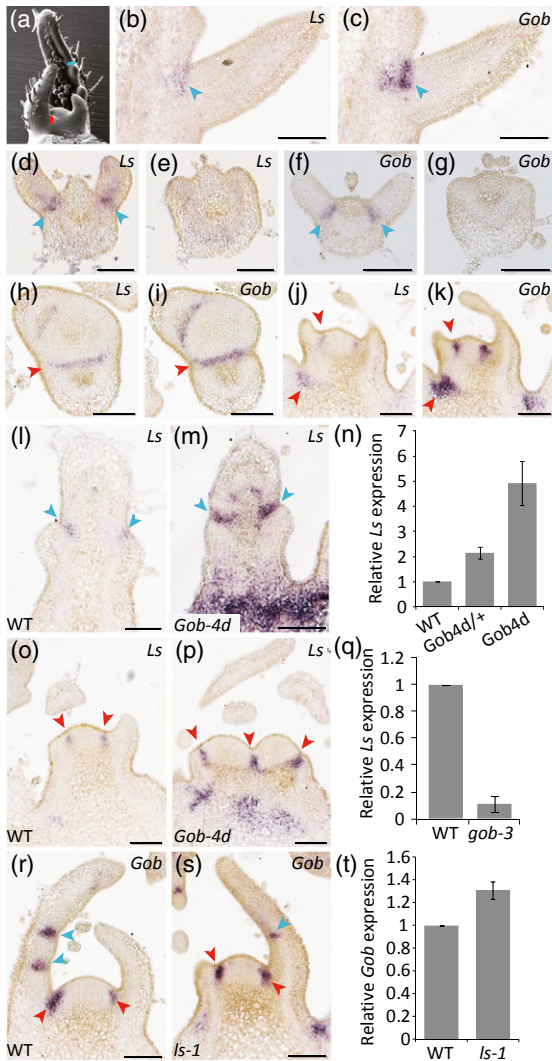
Figure 7. Goblet regulates *Ls* expression.
(a) Scanning electron micrograph (SEM) of a wild-type (WT) tomato shoot apex (cv. AmB) with co-localising expression domains of *Ls* and *Gob*, as indicated in DLB (blue) and leaf axil (red).
(b–k) Longitudinal (b, c, j, k) and transverse (d–i) sections through wild type shoot apices were hybridized to *Ls* or *Gob* antisense probes (indicated in upper right corner). (b, c, h–k) show consecutive sections (6 µm). Arrowheads point to expression domains in DLBs (in blue) and leaf axils (in red).
(d–g) Two cross-sections (separated by 72 µm) through a P6 (d, e) and a P5 (f, g) leaf primordium show *Ls* (d, e) and *Gob* (f, g) transcript accumulation in distal (d, f) but not in proximal (e, g) leaflet boundaries.
(l, m) *Ls* expression in leaves of *Gob-4d* (m) compared to WT (cv. M82) (l).
(n) Relative *Ls* transcript accumulation in P3-P6 leaf primordia of 16-day-old WT (cv. M82), *Gob-4d*/+ and homozygous *Gob-4d* plants, measured by quantitative RT-PCR.
(o, p) Comparison of *Ls* expression in apices of *Gob-4d* (p) and WT (cv. M82) (o).
(q) Comparison of *Ls* transcript accumulation in apices, including P1–P4 leaf primordia, of regenerated *gob-3* and WT (cv. M82) plants.
(r, s) *Gob* expression in leaves and leaf axils of *ls-1* (s) compared with WT (cv. AmB; r).
(t) Comparison of *Gob* mRNA accumulation in apices, including P1–P4 leaf primordia, of *ls-1* and WT (cv. AmB). For quantitative RT-PCR, transcript level of each replicate was normalized to the reference gene *TIP41* and represents the ratio to WT (set to 1) (n, q, t). Means of three biological replicates are given, ±SEM, *n* = 3. Scale bars = 100 µm.
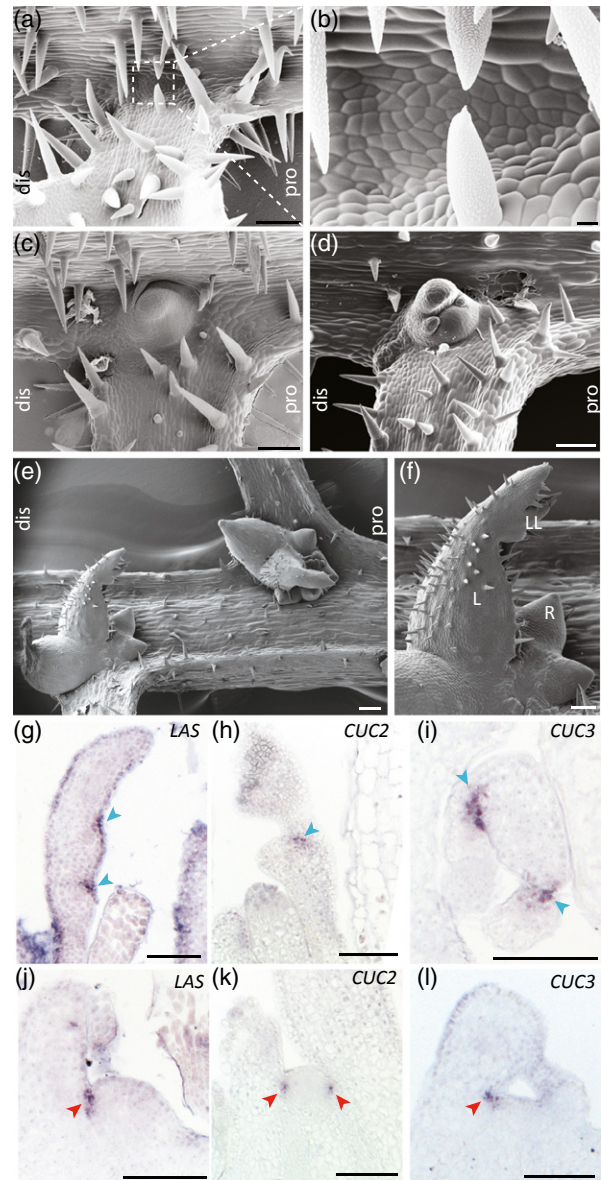

Figure 8. Ectopic shoot formation in *Cardamine pratensis*.
(a–f) Scanning electron micrograph (SEM) pictures illustrating sequential steps in development of ectopic meristems in *C. pratensis* DLBs. Distal (dis) and proximal (pro) side of the junction is indicated. Close-up of the boxed area in (a) is shown in (b). (f) Close-up of a root (R) and a leaf (L) with leaflets (LL).
(g–l) Longitudinal (g, h, j–l) and transverse (i) sections through ectopic shoots of *C. pratensis* were hybridized to *CpLas* and *Cardamine hirsuta* (*Ch*) *CUC2-3* antisense probes (indicated in upper right corner). Arrowheads point to expression domains in DLBs (g–i, blue arrowheads) and leaf axils (j–l, red arrowheads). Scale bars: 200 µm (e) 100 µm (a, c, d, f–l), 50 µm (b).

Where do these groups of pluripotent cells originate from? Our results suggest that meristems at the DLB of tomato leaflets develop from cells that previously co-expressed *Ls* and *Gob* (Figure 7), which is supported by the fact that in both loss-of-function mutants ectopic meristems are missing (Figures 3 and 4). Similarly, secondary

meristems develop from *Ls/Gob*-positive cells in leaf axils (Schumacher *et al.*, 1999; Busch *et al.*, 2011). Probably, in both positions of the plant body pluripotent cell groups are derived from established meristematic regions (i.e. the marginal blastozone and the SAM, respectively) and this cell lineage is maintained during subsequent development. This view is in line with the detached meristem concept outlined by Wardlaw (1943), who showed that in ferns axillary meristems develop from pluripotent cell groups tracing back to the SAM.

Gob co-regulates *Ls* transcript accumulation

Ls and *Gob* play important roles in the formation of axillary meristems (Schumacher *et al.*, 1999; Busch *et al.*, 2011) and their expression territories in the boundary zone between the SAM and leaf primordia match very well (Figure 7). We showed that *Ls* mRNA accumulation is strongly down-regulated in *gob-3* and up-regulated in the *Gob-4d* gain-of-function mutant (Figure 7). Interestingly, the *Gob-4d* mutation considerably enhances ectopic meristem formation only from DLBs, but not from PLBs, demonstrating the robustness of this phenotype. In contrast with *Gob-4d*, *ls-1 Gob-4d*/+ double mutants did not form any ectopic shoots, similar to *ls-1* single mutants. Combined, these data suggest that both genes are required to form ectopic meristems on leaves and that *Ls* acts downstream of *Gob*.

Recently, the promoter of the *Ls*-orthologous *LAS* gene in Arabidopsis was identified as a region that is bound by many transcription factors (Tian *et al.*, 2014). Yeast-one-hybrid and chromatin immunoprecipitation assays (Tian *et al.*, 2014) demonstrated that *CUC2*, one of the co-orthologues of *Gob* in Arabidopsis, interacts with different regulatory sequences identified upstream and downstream of the *LAS* coding region (Raatz *et al.*, 2011). These results combined with the results of our genetic experiments and the finding that *Ls* mRNA accumulation is strongly reduced in *gob-3* mutants suggest that *Gob* directly binds to the *Ls* promoter and activates transcription.

Furthermore, *ls-1 Gob-4d*/+ double mutants formed less primary side-shoots than *Gob-4d* and only very few accessories (Figures 5 and 6), suggesting that Ls activity is also required in leaf axils to fully activate the *Gob* pathway. About 75% of primary side-shoots developed in *ls-1 Gob-4d*/+ plants, indicating that Gob can modulate axillary meristem formation also in an Ls-independent manner. However, such side-shoots were often extensively fused to the stem (Figure 6), suggesting that axillary boundary zone establishment and organ separation is severely compromised in this double mutant.

Homology between simple leaves and compound leaves

Different hypotheses have been proposed to explain the relationship between simple and compound leaves. One hypothesis assumes that the leaflets of compound leaves are individual units resembling simple leaves, and that these leaves have a partially indeterminate shoot-like structure (Sattler and Rutishauser, 1992; Champagne and Sinha, 2004). Efroni *et al.* (2010) emphasise that leaves are different from leaflets, leaflets from lobes and lobes from serrations. In their view, development of different appendages of compound leaves follows distinct genetic programs. An alternative hypothesis states that an increasing dissection of the leaf margin results in serrations, lobes and finally leaflets, describing the whole spectrum of leaf forms as a continuum (Kaplan, 2001; Blein *et al.*, 2008; Canales *et al.*, 2010). In the course of this debate, leaflets were repeatedly described as being devoid of axillary meristems at their base (e.g. Berg, 2007; Efroni *et al.*, 2010). However, formation of ectopic meristems from DLBs, which we observed and studied in detail in tomato and *C. pratensis*, can be regarded as the equivalent of axillary meristem formation in the leaf axil, even though ectopic shoots did not form in the center of the leaflet axils, but shifted to the adaxial (dorsal) side of the leaf. Although the phenomenon of ectopic shoot development on tomato leaves has been mentioned already more than one and a half centuries ago (Duchartre, 1853) so far nobody had characterized it in detail. Several other compound leaved angiosperms, like *Sorbus aucuparia* (Lall *et al.*, 2006) and *Fraxinus excelsior* (Hammatt, 1994), develop adventitious shoots from the base of leaflets. These findings seem to strengthen the similarity between compound leaves and shoots. However, also simple-leaved plants, like *Kalanchoë daigremontiana*, develop meristems in the sinuses of their serrated leaves (Howe, 1931; Yarbrough, 1932; Batygina *et al.*, 1996; Garcês and Sinha, 2009). We conclude that the formation of ectopic meristems is not a unique feature of compound leaves and, therefore, does not favour the first hypothesis outlined above. In tomato, *C. pratensis* and *Kalanchoë daigremontiana*, ectopic meristems initiate from boundary zones established during leaf development. These data suggest that indentations of the leaf margin have similar properties as the base of leaflets and, therefore, supports the view that serrations, lobes and leaflets are generated by similar mechanisms (Kaplan, 2001; Blein *et al.*, 2008; Canales *et al.*, 2010). Defects in boundary zone establishment, due to mutations in the *Gob* and *Potato leaf* (C) genes, convert the compound tomato leaf into a simple leaf (Busch *et al.*, 2011), supporting the view that compound and simple leaf development is following similar blueprints.

Ectopic meristem formation on leaves opens the route to vegetative propagation

In tomato, we observed that a complete removal of shoot meristems (i.e. SAM and all AMs), by decapitation and intensive pruning, increased the number of ectopic

meristems initiated at the leaflet DLBs (Figure 1). This indicates that tomato plants are capable of sensing the loss of shoot meristems and induce ectopic meristem formation as an alternative route. Decapitation mimics the loss of the SAM, due to abiotic stress or animal attack, a condition which plants -to varying degrees- can compensate for by the activation of AMs. However, the availability of AMs is limited and, therefore, the possibility to initiate ectopic meristems considerably increases the size of a species bud bank (Klimešová and Klimeš, 2007). In turn, this increased bud bank can be an advantage for successful survival and, if buds or shoots are detached from the mother plant, may be used for vegetative propagation. In *C. pratensis*, meristem formation at the DLBs is the crucial step in a fully implemented developmental program of vegetative reproduction, especially under wet conditions (Salisbury, 1965). These examples suggest that ectopic meristem formation at leaflet DLBs is an important asset for survival and vegetative reproduction of dicotyledonous plants.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The tomato (*Solanum lycopersicum*) genotypes used in this study were either ordered from the Tomato Genetic Resource Center (TGRC, http://tgrc.ucdavis.edu/) or kindly provided by the laboratory which first described them. Lines are Antimold-B (AmB, LA3244), *ls-1* (AmB, LA0329) (Schumacher *et al.*, 1999), M82 (LA3475), Lukullus (Lu, LA0534), Moneymaker (MM, LA2706), Ailsa Craig (AC, LA2838A), *ls-1* (Craigella, LA3761) (Taylor, 1979), Craigella (CG, LA3247), Rheinlands Ruhm (RR, LA0535). *gob-3* (n5126-m1), *Gob-4d* (e0042) and M82 cultivar were from http://zamir.sgn.cornell.edu/mutants (Menda *et al.*, 2004) and seeds were kindly provided by Naomi Ori (Hebrew University of Jerusalem, Rehovot, Israel).

All seeds were pretreated with Na_3PO_4, thoroughly rinsed with sterile water and kept in water for 48 h at 25°C in darkness prior to sowing. Plants were grown under standard greenhouse conditions with 16 h photoperiod. In the decapitation experiments, plants were pruned and the SAM removed after either the second or third inflorescence, depending on the experiment. Seedlings of *gob-3* and the corresponding wild type (cv. M82) were regenerated from wound-induced callus on hypocotyls (Brand *et al.*, 2007).

C. pratensis plants were kindly provided by Franziska Turck (MPIPZ, Germany).

In-situ hybridization

Sample preparations and *in situ* hybridizations were performed as previously described by Müller *et al.* (2006). For tomato 2-week-old apices were used for fixation, whereas for *C. pratensis* ectopic shoots of mature leaves were used. Antisense probes were synthesized from PCR products with the T7 promoter sequence included in the reverse primer. All primers and templates used are listed in Table S1.

C. pratensis 454 data kindly provided by Martin Lysak (Mandáková *et al.*, 2013) were mapped against the Arabidopsis genome (TAIR10) using CLC genomics workbench. A 694-bp read mapped to the nucleotides +404 to +1098 bp relative to the ATG of *AtLAS*. Sequence information of this read was used for primer design of *CpLAS* antisense probe. *C. pratensis CUC2* and *CUC3* transcripts were detected using *C. hirsuta* antisense probes as described in Blein *et al.* (2008).

qRT-PCR

Vegetative shoot apices or leaf primordia were harvested as indicated. RNA was extracted using the RNeasy plant Micro Kit (Qiagen, http://www.qiagen.com/), including a subsequent DNase I treatment (Roche Diagnostics, https://lifescience.roche.com/shop/home). One microgram of RNA was used for cDNA synthesis with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, http://www.thermoscientificbio.com/fermentas/). qPCR was performed using the PowerSYBR-Green PCR Master Mix (Applied Biosystems, http://www.appliedbiosystems.com/absite/us/en/home.html). Primers are described in Table S1. Relative quantification was done using internal standard curves and correcting by the use of the reference gene *TIP41* (Exposito-Rodriguez *et al.*, 2008).

Microscopy

In-situ sections were imaged using bright-field microscopy on an Axioplan 2 (Zeiss). Scanning electron microscopy (SEM) was performed on a Supra 40 VP with a GEMINI column (Zeiss, http://www.zeiss.com/microscopy/en_de/home.html). Fresh tissue was first frozen in liquid nitrogen and transferred to an Emitech K1250X (Emitech, http://www.quorumtech.com/home) for sublimation and subsequently coated with gold palladium before imaging. All images were obtained and processed using the SMARTSEN software. Cell sizes were assessed using IMAGEJ software (Abramoff *et al.*, 2004).

Statistical analysis

When appropriate, data were subjected to Student's *t*-test (Microsoft Excel).

AUTHOR CONTRIBUTIONS

S.R., W.K., A.H. and K.T. designed research and analysed data; S.R., W.K. and A.H. performed research; S.R., W.K., A.H and K.T. wrote the article.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Ectopic shoot formation in different tomato cultivars.

Figure S2. Ectopic shoot distribution in different tomato cultivars.

Figure S3. Ectopic shoots are missing on lower leaves independent of decapitation position.

Figure S4. Distal leaflet boundaries are characterized by reduced chlorophyll levels in subepidermal cell layers.

Figure S5. Ectopic meristem development on tomato leaves.

Figure S6. Ectopic shoot development from callus.

Figure S7. *Histone H4* and *Lateral suppressor* show complementary expression.

Figure S8. *ls* mutants do not form ectopic shoots from distal leaflet boundaries.

Figure S9. Formation of bulges at distal leaflet boundaries.

Figure S10. Relative expression of *Ls* and *Gob* in different tissues.

Table S1. Primers.

REFERENCES

Abramoff, M.D., Magalhães, P.J. and Ram, S.J. (2004) Image processing with ImageJ. *Biophotonics Int.* **11**, 36–42.

Aida, M. and Tasaka, M. (2006) Morphogenesis and patterning at the organ boundaries in the higher plant shoot apex. *Plant Mol. Biol.* **60**, 915–928.

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H. and Tasaka, M. (1997) Genes involved in organ separation in Arabidopsis: an analysis of the *cup-shaped cotyledon* mutant. *Plant Cell*, **9**, 841–857.

Aida, M., Ishida, T. and Tasaka, M. (1999) Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. *Development*, **126**, 1563–1570.

Barkoulas, M., Hay, A., Kougioumoutzi, E. and Tsiantis, M. (2008) A developmental framework for dissected leaf formation in the Arabidopsis relative *Cardamine hirsuta*. *Nat. Genet.* **40**, 1136–1141.

Batygina, T.B., Bragina, E.A. and Titova, G.E. (1996) Morphogenesis of propagules in viviparous species *Bryophyllum daigremontianum* and *B. calycinum*. *Acta Soc. Bot. Pol.* **65**, 127–133.

Bell, E.M., Lin, W.-C., Husbands, A.Y., Yu, L., Jaganatha, V., Jablonska, B., Mangeon, A., Neff, M.M., Girke, T. and Springer, P.S. (2012) Arabidopsis LATERAL ORGAN BOUNDARIES negatively regulates brassinosteroid accumulation to limit growth in organ boundaries. *Proc. Natl Acad. Sci. USA*, **109**, 21146–21151.

Ben-Gera, H., Shwartz, I., Shao, M.R., Shani, E., Estelle, M. and Ori, N. (2012) ENTIRE and GOBLET promote leaflet development in tomato by modulating auxin response. *Plant J.* **70**, 903–915.

Berg, L. (2007) *Introductory Botany: Plants, People, and the Environment*. Belmont, CA: Cengage Learning.

Berger, Y., Harpaz-Saad, S., Brand, A., Melnik, H., Sirding, N., Alvarez, J.P., Zinder, M., Samach, A., Eshed, Y. and Ori, N. (2009) The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves. *Development*, **136**, 823–832.

Bergmann, D.C. and Sack, F.D. (2007) Stomatal development. *Annu. Rev. Plant Biol.* **58**, 163–181.

Blein, T., Pulido, A., Vialette-Guiraud, A., Nikovics, K., Morin, H., Hay, A., Johansen, I.E., Tsiantis, M. and Laufs, P. (2008) A conserved molecular framework for compound leaf development. *Science*, **322**, 1835–1839.

Brand, A., Shirding, N., Shleizer, S. and Ori, N. (2007) Meristem maintenance and compound-leaf patterning utilize common genetic mechanisms in tomato. *Planta*, **226**, 941–951.

Breuil-Broyer, S., Morel, P., De Almeida-Engler, J., Coustham, V., Negrutiu, I. and Trehin, C. (2004) High-resolution boundary analysis during *Arabidopsis thaliana* flower development. *Plant J.* **38**, 182–192.

Busch, B.L., Schmitz, G., Rossmann, S., Piron, F., Ding, J., Bendahmane, A. and Theres, K. (2011) Shoot branching and leaf dissection in tomato are regulated by homologous gene modules. *Plant Cell*, **23**, 3595–3609.

Canales, C., Barkoulas, M., Galinha, C. and Tsiantis, M. (2010) Weeds of change: *Cardamine hirsuta* as a new model system for studying dissected leaf development. *J. Plant. Res.* **123**, 25–33.

Champagne, C. and Sinha, N. (2004) Compound leaves: equal to the sum of their parts? *Development*, **131**, 4401–4412.

Dickinson, T.A. (1978) Epiphylly in angiosperms. *Bot. Rev.* **44**, 181–232.

Duchartre, P. (1853) Notes sur des feuilles ramifères de tomates. *Ann. Sc. nat (Bot.) Paris, 3ᵉ série* **19**, 241–251.

Efroni, I., Eshed, Y. and Lifschitz, E. (2010) Morphogenesis of simple and compound leaves: a critical review. *Plant Cell*, **22**, 1019–1032.

Exposito-Rodriguez, M., Borges, A., Borges-Perez, A. and Perez, J. (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biol.* **8**, 131.

Gallavotti, A., Zhao, Q., Kyozuka, J., Meeley, R.B., Ritter, M.K., Doebley, J.F., Enrico Pe, M. and Schmidt, R.J. (2004) The role of *barren stalk1* in the architecture of maize. *Nature*, **432**, 630–635.

Garcès, H. and Sinha, N. (2009) The 'Mother of Thousands' (*Kalanchoë daigremontiana*): a plant model for asexual reproduction and CAM studies. *Emerging Model Organisms: A Laboratory Manual*. New York, NY: Cold Spring Harb. Protoc.

Gendron, J.M., Liu, J.-S., Fan, M., Bai, M.-Y., Wenkel, S., Springer, P.S., Barton, M.K. and Wang, Z.-Y. (2012) Brassinosteroids regulate organ boundary formation in the shoot apical meristem of Arabidopsis. *Proc. Natl Acad. Sci. USA*, **109**, 21152–21157.

Greb, T., Clarenz, O., Schäfer, E., Müller, D., Herrero, R., Schmitz, G. and Theres, K. (2003) Molecular analysis of the *LATERAL SUPPRESSOR* gene in Arabidopsis reveals a conserved control mechanism for axillary meristem formation. *Genes Dev.* **17**, 1175–1187.

Hagemann, W. and Gleissberg, S. (1996) Organogenetic capacity of leaves: the significance of marginal blastozones in angiosperms. *Plant Syst. Evol.* **199**, 121–152.

Hammatt, N. (1994) Shoot initiation in the leaflet axils of compound leaves from micropropagated shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). *J. Exp. Bot.* **45**, 871–875.

Heisler, M.G., Hamant, O., Krupinski, P., Uyttewaal, M., Ohno, C., Jönsson, H., Traas, J. and Meyerowitz, E.M. (2010) Alignment between PIN1 polarity and microtubule orientation in the shoot apical meristem reveals a tight coupling between morphogenesis and auxin transport. *PLoS Biol.* **8**, e1000516.

Hendelman, A., Stav, R., Zemach, H. and Arazi, T. (2013) The tomato NAC transcription factor *SlNAM2* is involved in flower-boundary morphogenesis. *J. Exp. Bot.* **64**, 5497–5507.

Hibara, K., Karim, M.R., Takada, S., Taoka, K., Furutani, M., Aida, M. and Tasaka, M. (2006) Arabidopsis *CUP-SHAPED COTYLEDON3* regulates postembryonic shoot meristem and organ boundary formation. *Plant Cell*, **18**, 2946–2957.

Howe, M.D. (1931) A morphological study of the leaf notches of *Bryophyllum calycinum*. *Am. J. Bot.* **18**, 387–390.

Huang, T., López-Giráldez, F., Townsend, J.P. and Irish, V.F. (2012) RBE controls microRNA164 expression to effect floral organogenesis. *Development*, **139**, 2161–2169.

Hussey, G. (1971) Cell division and expansion and resultant tissue tensions in the shoot apex during the formation of a leaf primordium in the tomato. *J. Exp. Bot.* **22**, 702–714.

Kaplan, D.R. (2001) Fundamental concepts of leaf morphology and morphogenesis: a contribution to the interpretation of molecular genetic mutants. *Int. J. Plant Sci.* **162**, 465–474.

Keller, T., Abbott, J., Moritz, T. and Doerner, P. (2006) Arabidopsis *REGULATOR OF AXILLARY MERISTEMS1* controls a leaf axil stem cell niche and modulates vegetative development. *Plant Cell*, **18**, 598–611.

Klimešová, J. and Klimeš, L. (2007) Bud banks and their role in vegetative regeneration–a literature review and proposal for simple classification and assessment. *Perspect. Plant Ecol. Evol. Syst.* **8**, 115–129.

Koenig, D. and Sinha, N. (2010) Evolution of leaf shape: a pattern emerges. *Curr. Top. Dev. Biol.* **91**, 169–183.

Komatsu, K., Maekawa, M., Ujiie, S., Satake, Y., Furutani, I., Okamoto, H., Shimamoto, K. and Kyozuka, J. (2003) *LAX* and *SPA:* major regulators of shoot branching in rice. *Proc. Natl Acad. Sci. USA*, **100**, 11765–11770.

Lall, S., Mandegaran, Z. and Roberts, A.V. (2006) Shoot multiplication and adventitious regeneration in *Sorbus aucuparia*. *Plant Cell Tissue Organ Cult.* **85**, 23–29.

Lampugnani, E.R., Kilinc, A. and Smyth, D.R. (2012) *PETAL LOSS* is a boundary gene that inhibits growth between developing sepals in *Arabidopsis thaliana*. *Plant J.* **71**, 724–735.

Laufs, P., Peaucelle, A., Morin, H. and Traas, J. (2004) MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. *Development*, **131**, 4311–4322.

Li, X., Qian, Q., Fu, Z., Wang, Y., Xiong, G., Zeng, D., Wang, X., Liu, X., Teng, S. and Hiroshi, F. (2003) Control of tillering in rice. *Nature*, **422**, 618–621.

Mandáková, T., Kovařík, A., Zozomová-Lihová, J., Shimizu-Inatsugi, R., Shimizu, K.K., Mummenhoff, K., Marhold, K. and Lysak, M.A. (2013) The more the merrier: recent hybridization and polyploidy in *Cardamine*. *Plant Cell*, **25**, 3280–3295.

Menda, N., Semel, Y., Peled, D., Eshed, Y. and Zamir, D. (2004) *In silico* screening of a saturated mutation library of tomato. *Plant J.* **38**, 861–872.

Müller, D., Schmitz, G. and Theres, K. (2006) *Blind* homologous *R2R3 Myb* genes control the pattern of lateral meristem initiation in Arabidopsis. *Plant Cell*, **18**, 586–597.

Poethig, R.S. and Sussex, I.M. (1985) The developmental morphology and growth dynamics of the tobacco leaf. *Planta*, **165**, 158–169.

Raatz, B., Eicker, A., Schmitz, G., Fuss, E., Müller, D., Rossmann, S. and Theres, K. (2011) Specific expression of *LATERAL SUPPRESSOR* is controlled by an evolutionarily conserved 3′ enhancer. *Plant J.* **68**, 400–412.

Raman, S., Greb, T., Peaucelle, A., Blein, T., Laufs, P. and Theres, K. (2008) Interplay of miR164, *CUP-SHAPED COTYLEDON* genes and *LATERAL SUPPRESSOR* controls axillary meristem formation in *Arabidopsis thaliana*. *Plant J.* **55**, 65–76.

Salisbury, E. (1965) The reproduction of *Cardamine pratensis* L. and *Cardamine palustris* Peterman particularly in relation to their specialized foliar vivipary, and its deflexion of the constraints of natural selection. *Proc. R. Soc. Lond. B Biol. Sci.* **163**, 321–342.

Sattler, R. and Rutishauser, R. (1992) Partial homology of pinnate leaves and shoots. Orientation of leaflet inception. *Bot. Jahrb. Syst.* **114**, 61–79.

Schmitz, G., Tillmann, E., Carriero, F., Fiore, C., Cellini, F. and Theres, K. (2002) The tomato *Blind* gene encodes a MYB transcription factor that controls the formation of lateral meristems. *Proc. Natl Acad. Sci. USA*, **99**, 1064–1069.

Schumacher, K., Schmitt, T., Rossberg, M., Schmitz, G. and Theres, K. (1999) The *Lateral suppressor* (*Ls*) gene of tomato encodes a new member of the VHIID protein family. *Proc. Natl Acad. Sci. USA*, **96**, 290–295.

Steeves, T.A. and Sussex, I.M. (1989) *Patterns in Plant Development*. Cambridge: Cambridge University Press.

Taylor, I.B. (1979) The effect of the lateral suppressor gene on seed germination in the tomato. *Euphytica*, **28**, 93–97.

Tian, C., Zhang, X., He, J. et al. (2014) An organ boundary-enriched gene regulatory network uncovers regulatory hierarchies underlying axillary meristem initiation. *Mol. Syst. Biol.* **10**, 755.

Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A. and de Vries, S.C. (2003) The *CUP-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in Arabidopsis. *Plant Cell*, **15**, 1563–1577.

Wang, Q., Kohlen, W., Rossmann, S., Vernoux, T. and Theres, K. (2014a) Auxin depletion from the leaf axil conditions competence for axillary meristem formation in Arabidopsis and tomato. *Plant Cell*, **26**, 2068–2079.

Wang, Y., Wang, J., Shi, B., Yu, T., Qi, J., Meyerowitz, E.M. and Jiao, Y. (2014b) The stem cell niche in leaf axils is established by auxin and cytokinin in Arabidopsis. *Plant Cell*, **26**, 2055–2067.

Wardlaw, C. (1943) Experimental and analytical studies of Pteridophytes I. Preliminary observations on the development of buds on the rhizome of the ostrich fern (*Matteuccia struthiopteris* Tod.). *Ann. Bot.* **7**, 171–184.

Yang, F., Wang, Q., Schmitz, G., Müller, D. and Theres, K. (2012) The bHLH protein ROX acts in concert with RAX1 and LAS to modulate axillary meristem formation in Arabidopsis. *Plant J.* **71**, 61–70.

Yarbrough, J.A. (1932) Anatomical and developmental studies of the foliar embryos of *Bryophyllum calycinum*. *Am. J. Bot.* **19**, 443–453.

Žádníková, P. and Simon, R. (2014) How boundaries control plant development. *Curr. Opin. Plant Biol.* **17**, 116–125.