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The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves

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Leaves are formed at the flanks of the shoot apical meristem (SAM) and develop into a variety of forms. In tomato, prolonged leaf patterning enables the elaboration of compound leaves by reiterative initiation of leaflets with lobed margins. In *goblet* (*gob)* loss-of-function mutants, primary leaflets are often fused, secondary leaflets and marginal serrations are absent, and SAMs often terminate precociously. We show that *GOB* encodes a NAC-domain transcription factor expressed in narrow stripes at the leaf margins, flanking the distal side of future leaflet primordia, and at the boundaries between the SAM and leaf primordia. Leaf-specific overexpression of the microRNA *miR164*, a negative regulator of *GOB*-like genes, also leads to loss of secondary-leaflet initiation and to smooth leaflet margins. Plants carrying a dominant *gob* allele with an intact ORF but disrupted *miR164* binding site produce more cotyledons and floral organs, have split SAMs and, surprisingly, simpler leaves. Overexpression of a form of *GOB* with an altered *miR164* binding site in leaf primordia leads to delayed leaflet maturation, frequent, improperly timed and spaced initiation events, and a simple mature leaflet form owing to secondary-leaflet fusion. *miR164* also affects leaflet separation in *Cardamine hirsuta*, a Brassicaceae species with complex leaves. Genetic and molecular analyses suggest that *GOB* expression is intact in the simplified leaves of *entire* tomato mutants, which have a defect in a putative repressor of auxin responses. Our results show that GOB marks leaflet boundaries and that its accurate spatial, temporal and quantitative activity affects leaf elaboration in a context-dependent manner.

KEY WORDS: CUC2, NAM, Boundary, Compound leaves, miR164, Tomato

INTRODUCTION

During the generation of a complex multicellular organism, groups of cells recurrently acquire new fates, which are distinct from each other and from that of their ancestors. This often requires the specification of a unique boundary between two regions with distinct fates. In plants, new organs form and develop throughout the plant's life, requiring a mechanism for repetitive and flexible specification of new tissues and boundaries (Aida and Tasaka, 2006; Heisler et al., 2005; Sablowski, 2007).

Plant leaves are initiated from the flanks of the shoot apical meristem (SAM), and go through common developmental stages. However, very different final leaf shapes and sizes result from tuning the timing, duration and further patterning events within these stages (Barkoulas et al., 2007; Dengler and Tsukaya, 2001; Efroni et al., 2008; Kaplan, 2001; Ori et al., 2007). A major form of variation is illustrated by simple and compound leaves. In their mature form, simple leaves consist of a proximal petiole and a distal continuous blade. In compound leaves, such as those of tomato, subunits termed leaflets are attached to a central rachis through petiolules (see Fig. 1A). This pattern can be reiterated to produce further orders of the same basic pattern (Hareven et al., 1996). In addition, leaf or leaflet margins can be smooth, serrated or lobed (see Fig. 1A) (Goliber et al., 1999). However, similar final leaf shapes may result from very distinct early events. For example, a mature simple leaf can result from early arrest of leaflet initiation or from post-initiation leaflet fusion (Bharathan et al., 2002). It is therefore necessary to examine leaf ontology to define the underling developmental program.

Traditionally, leaf development has been divided into three stages: (1) initiation of the leaf at the flanks of the SAM; (2) primary morphogenesis, during which secondary structures such as serrations or leaflets are produced; and (3) histogenesis or secondary morphogenesis, in which cell expansion and final differentiation occur (Dengler and Tsukaya, 2001; Poethig, 1997). These stages are not synchronized throughout the leaf, such that different leaf regions can be at different developmental stages at the same time (Dengler, 1984; Hagemann and Gleissberg, 1996; Ori et al., 2007). Of particular importance for leaf patterning is a region at the leaf margins, the marginal blastozone, which maintains morphogenetic activity and is responsible for the initiation of secondary structures such as leaflets (Dengler and Tsukaya, 2001; Hagemann and Gleissberg, 1996; Reinhardt et al., 2007). Sufficient temporal and spatial primary morphogenesis activity at this region is required for the formation of elaborated structures such as leaflets, and thus for the formation of a compound leaf. In tomato, the *LANCEOLATE* (*LA*)-like gene family, which encodes TCP transcription factors, promotes the transition from primary morphogenesis to the histogenesis stage, defining the morphogenetic window within which leaflets can be formed (Caruso, 1968; Mathan and Jenkins, 1962; Ori et al., 2007).

Several mechanisms have been shown to act within this developmental window to promote leaf elaboration, many of which also play a role in SAM function. Class I KNOTTED1-LIKE HOMEOBOX (KNOXI) transcription factors are essential for SAM maintenance (Hake et al., 2004), and also play a central role in the modulation of compound leaves (Bharathan et al., 2002; Hareven et al., 1996; Hay and Tsiantis, 2006; Parnis et al., 1997). In some legume species, such as pea and *Medicago*, the orthologous genes

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UNIFOLIATA and *SINGLE LEAFLET*, respectively, are also involved in leaf elaboration (Hofer et al., 1997; Wang et al., 2008). Likewise, several plant hormones, such as auxin and gibberellic acid (GA), have also been implicated in leaf elaboration, either via regulation of maturation or through mediation of localized growth (Barkoulas et al., 2008; Bassel et al., 2008; Hay et al., 2002; Jasinski et al., 2008).

Leaf initiation at the flanks of the SAM is accompanied by the formation of a boundary region between the initiating leaf and the SAM. This boundary region is characterized by growth retardation and by the expression of specific boundary genes, including a group of genes encoding NAC-domain transcription factors, represented by the petunia *NO APICAL MERISTEM* (*NAM*), the *Antirrhinum CUP* and the *Arabidopsis CUC* genes (Aida et al., 1997; Souer et al., 1996; Weir et al., 2004). Single or double mutants in these genes, depending on the species, result in the failure to maintain proper structure and function of the embryonic SAM and to specify boundary regions during organ initiation and development. Several NAC-domain genes, including *Arabidopsis CUC1* and *CUC2* but not the closely related *CUC3*, are subject to negative control by the microRNA *miR164*. Analysis of the consequences of *miR164* mutations and overexpression, as well as of *miR164*-insensitive *CUC* forms, has further emphasized the importance of these genes for boundary specification, organ separation and proper plant development (Baker et al., 2005; Laufs et al., 2004; Mallory et al., 2004; Nikovics et al., 2006; Peaucelle et al., 2007; Raman et al., 2008; Sieber et al., 2007). Recently, the *Arabidopsis CUC2* gene has also been implicated in controlling the degree of elaboration of leaf serrations in its simple leaves (Nikovics et al., 2006).

Is boundary specification by NAM-related genes utilized in the process of leaflet formation during compound-leaf patterning? Here we address this question by analyzing loss- and gain-of-function tomato *goblet* (*gob*) alleles. SAMs of *gob* mutants terminate after the production of two fused cotyledons, but occasionally recover to produce plants with simpler leaves than the wild type (Brand et al., 2007). We show that *GOB* encodes a NAM homolog that is essential for the proper specification of lateral organ boundaries at the apical meristem and of leaflet boundaries in the developing compound leaf. We uncover new roles for GOB in the timing of leaf maturation, spatial and temporal positioning of leaflets, secondary-leaflet initiation and separation, and leaf margin elaboration. These processes are coordinated by a quantitative balance between GOB and *miR164* which act locally to pattern leaf development at a short distance.

MATERIALS AND METHODS

Plant material

The *Gob-4d* and *e2*, *e3* and *e4* mutants correspond to the following accession numbers in the tomato (*Solanum lycopersicum* cv. M82) mutant database (Menda et al., 2004) (http://www.sgn.cornell.edu): *Gob-4d*-e0042 and *e2*-n0741, *e3*-e0880, *e4*-e3335. *gob-1*, *2* and *3* have been described previously (Brand et al., 2007). Tomato seeds were sown in a commercial nursery and grown either during April to August in the field, or during October to March in a greenhouse under natural daylight and regulated temperature (15-30°C). Double-mutant combinations were confirmed by progeny tests in the F3 generation. *Cardamine hirsuta* plants ['Oxford strain', a kind gift of Angela Hay and Miltos Tsiantis (Hay and Tsiantis, 2006)] were grown under 18-hour cool white fluorescent light at 18-22°C. Analysis of early leaf development in tomato was performed by dissecting older leaves, exposing the appropriate developmental stage (P2-6), and observing and photographing using a dissecting scope. For each stage, at least eight seedlings were analyzed. Different plants were used for each stage.

Identification of the molecular lesions in *gob* and *entire* alleles

The *GOB* and *SlIAA9* (AJ937282) genes (including introns that lie within the coding sequence) were amplified and sequenced from genomic DNA of the respective mutants (Fig. 1B; Fig. 7A) using the primers listed in Table 1.

Isolation of tomato cDNA, plasmid construction, plant transformation and evaluation of transgenic lines

The *GOB* gene was amplified from tomato using RT-PCR with primers complementary to conserved regions of the petunia *NAM* gene. The genomic *GOB* sequence was obtained by inverse PCR. The *GOB* ORF was cloned into pTOPO (Invitrogen, Carlsbad, CA, USA) using GOBstart and GOBstop primers, which contain *Xho*I and *Bam*HI linkers, respectively. The GOB^{wt} and GOB^{4d} sequences were cloned from wild-type and *Gob-4d* cDNA, respectively, using the GOBstart and GOBstop primers (Table 1). To generate GOB^{m}, assembly PCR was used to introduce nine nucleotide substitutions into the *GOB* ORF, as described previously (Alvarez et al., 2006), using the GOBstart and GOBstop primers in conjunction with GOBmR′ and GOBmF′ primers, respectively. The *35S::miR164b* construct was generated by transcriptional fusion of a genomic fragment encompassing the *Atpre-miR164b* (Alvarez et al., 2006) in front of the 35S promoter of the ART7 vector. Constructs were subcloned into the pMLBART binary plasmid.

Tomato cotyledon transformation was performed as described (McCormick, 1991). At least three independent lines were assessed for each construct by crossing them to the same promoter line and examining the transactivated F1s. Detailed phenotypic analyses were performed with selected OP:gene responder lines that were crossed to Promoter:LhG4 driver lines as described (Lifschitz et al., 2006). Transformation into *C. hirsuta* was performed by floral dipping using the *Agrobacterium tumefaciens* GV3101 strain. *C. hirsuta* transformants were selected on soil on the basis of resistance to the herbicide BASTA.

In situ hybridization

Tissue preparation, sectioning and transcript detection were performed as described (Pekker et al., 2005), except that the fixation was performed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde). The whole-mount in situ procedure was adapted from Hejátko et al. (Hejatko et al., 2006), with fixation of plant material without heptane. The *GOB* probe was synthesized with UTP-DIG and expression of *miR164* was detected using a *miR164*-LNA probe (Exiqon, Denmark).

Table 1. Primers used in this study

Primer	Sequence (5′ to 3′)
NAMF′	TGCACTTGTTGGGATGAAGA
NAMR′	TTCTGAGTCTCCGGCACTG
gob-3 F	GCCAACTCCAAGTTTCGCTTC
gob-3 R′	GATTAGAAAATCCGCCGTGCAT
GOBstart	CTCGAGATGGAGATTTTCATCAGATGC
GOBstop	GGATCCTCAGTAGCTCCACATACAGTTCAAG
GOBmR′	AGTAGCAGTTGTACTAAAGCACGGTACATG-TTCCTTCTTG
GOBmF′	AGGAACATGTACCGTGCTTTAGTACAACTG-CTACTGCTAC
GOB RT F′	CAGGAGTTCGAAGGACGAGTGG
GOB RT R′	TTGGCTGTAGTGTATGCAAGGTG
TUBF′	CACATTGGTCAGGCCGGTAT
TUBR′	ATCTGGCCATCAGGCTGAAT
GOB RACE R′	ACGGTCCAATTAACCATCGGATTAGA
GOB RACE R′ NESTED	GATTCTCATGGAGTGATCTTAACGTT
ADAPTOR F′	GCACGAGGACACTGACATGGACTGA
ADAPTOR F′ NESTED	GGACACTGACATGGACTGAAGGAGT
ADAPTOR-RNA oligo	CGACUGGAGCACGAGGACACUG
miR164 probe	TGCACGTGCCCTGCTTCTCCA
E F′-1	GAGGAGGAGGGCCAGAGTAAT
E F′-2	GTGGCAACAAACGAGGATTTT
E R′	AATATGGATCTCATGGAGCTCCT

RNA isolation and analysis

Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hiden, Germany), and cDNA was prepared from 1 μg total RNA with a poly(A) primer, using the Verso Kit (ABgene, Epsom, UK). Relative gene expression was assayed by hybridization and quantification of RT-PCR products, as described previously (Brand et al., 2007), using a *GOB* or a *TUBULIN* probe. Intact (uncleaved) *GOB* was amplified using primers that span the *miR164* recognition site. Primer sequences, designed to exon-intron junctions, are shown in Table 1.

miR164-directed *GOB* cleavage products were mapped using RLM-RACE as described (Arazi et al., 2005). Cloned cleavage products were sequenced.

Microscopy

In situ sections were photographed with an Olympus 1X81 microscope using Cell^{R} software and whole-meristem images were captured using an Olympus SZX7 binocular microscope. Scanning electron microscopy was performed as described (Brand et al., 2007).

RESULTS

GOBLET encodes a NAC-domain transcription factor and is closely related to *NO APICAL MERISTEM*

In tomato *gob* mutants, the SAM terminates after the production of two fused cotyledons (Fig. 1E). Surgical removal of the fused cotyledons enables a few *gob* seedlings to recover and produce aberrant, less compound leaves and infertile flowers (Brand et al., 2007) (Fig. 1M,N; see Fig. S4F in the supplementary material). To study the link between the SAM and the marginal blastozone activities, we identified the affected gene in *gob* mutants. *gob* seedlings are reminiscent of the petunia *nam* mutant and the *Arabidopsis cuc1 cuc2* double mutant (Aida et al., 1997; Souer et al., 1996). A tomato *NAM* and *CUC* homolog, termed *GOB*, to which the most similar gene product in *Arabidopsis* is *CUC2* (see Fig. S1 in the supplementary material), was isolated by RT-PCR

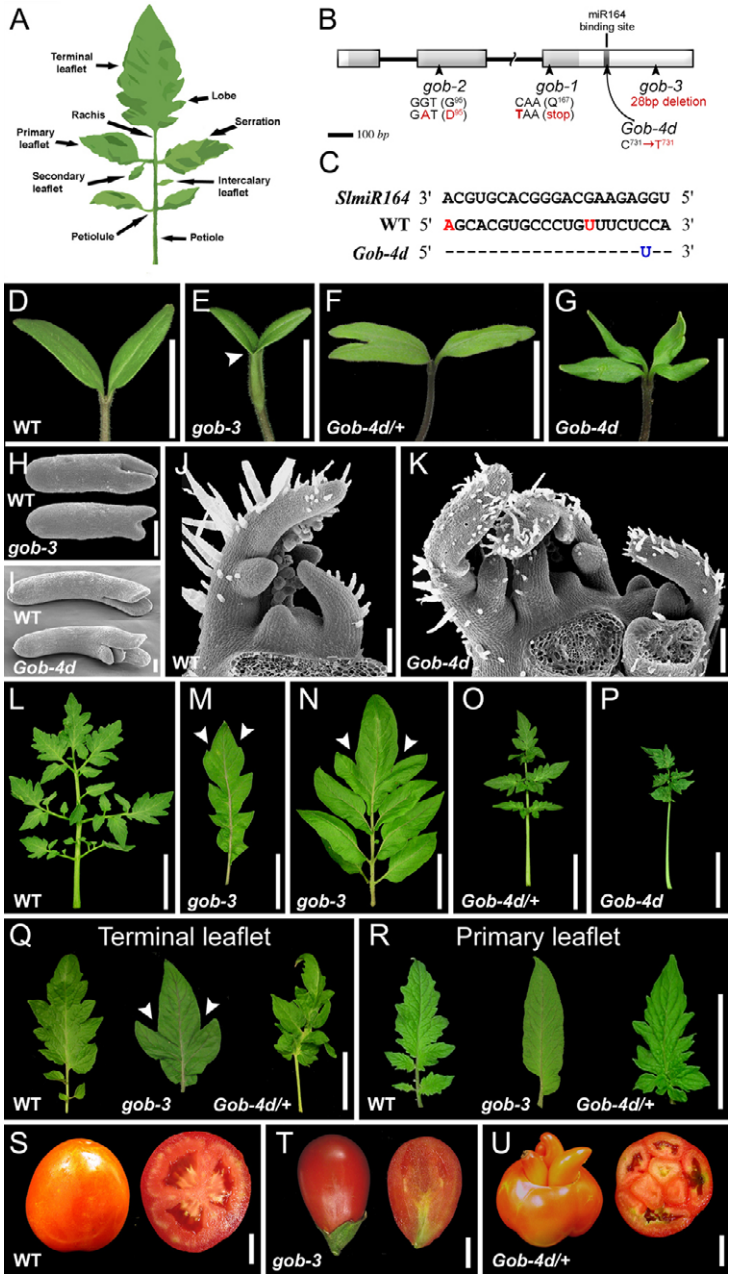

Fig. 1. GOBLET affects leaflet initiation and separation. (**A**) Diagram of the compound wild-type tomato leaf, indicating the terminology of the leaf parts. (**B**) *GOBLET* (*GOB*) gene structure, showing coding exons (rectangles), introns (black lines), the conserved NAC domain (gray box), the *miR164* recognition site, and nucleotide and amino acid changes in the *gob* loss- and gain-of-function alleles. (**C**) Sequence of *SlmiR164* (Pilcher et al., 2007) and of the *miR164* recognition site in the wild-type and *Gob-4d GOB* alleles, with mismatches represented in red and blue, respectively, and identical nucleotides represented by hyphens. (**D-G**) Nine-day-old seedlings of wild type (WT) (D), *gob-3* (E; arrowhead points to cotyledon fusion) and *Gob-4d* (F,G). (**H**,**I**) Scanning electron micrographs (SEMs) of embryos fixed 10 (H) or 18 (I) days after anthesis. (**J**,**K**) SEM of a wild-type shoot apical meristem (SAM) (J) and a split *Gob-4d* SAM (K). (**L-P**) The fifth leaf from plants of the indicated genotypes. Arrowheads point to the fusion between the *gob-3* terminal leaflet and the adjacent lateral primary leaflets. (**Q**,**R**) Terminal (Q) and primary (R) leaflets from the ninth leaf. (**S-U**) Mature fruits. *gob-3* leaves and leaflets were formed on a seedling recovered following cotyledon removal. Scale bars: 1 cm in D-G,Q,R,S-U; 100 μm in H,I; 500 μm in J,K; 5 cm in L-P.

using primers complementary to conserved regions in the *NAM* gene. *GOB* co-segregated with the *gob* mutation, its mRNA levels were dramatically reduced in *gob-3* seedlings (see Fig. S2A in the supplementary material) and sequence analysis revealed that all three *gob* alleles, which display similar phenotypes, contain lesions in this gene (Fig. 1B). These findings indicated that impaired *GOB* function underlies the *gob* phenotype. Similar to its homologs from other species, the *GOB* mRNA contains a recognition site for the microRNA *miR164*. RNA ligase-mediated (RLM)-RACE analysis confirmed the presence of *miR164*-directed *GOB* cleavage products in tomato seedlings (see Fig. S3 in the supplementary material). On this basis, we screened available semi-dominant mutants for phenotypes that might represent gain-of-function *GOB* alleles, such as the production of extra organs, ectopic meristems and altered leaf shape. This led to the identification of *Gob-4d*, which contains a point mutation in the *miR164* recognition site (Fig. 1B,C) and features elevated *GOB* mRNA levels (see Fig. S2B in the supplementary material).

GOB sets boundaries throughout plant development

To understand how GOB is utilized for boundary specification at the SAM and during compound-leaf patterning, we compared the effects of *GOB* loss- and gain-of-function mutations. In contrast to the fused cotyledons and terminated SAM observed in *gob-3* mutants, homozygous *Gob-4d* seedlings initiated more cotyledons (Fig. 1D-G). These effects were already apparent during early embryo development (Fig. 1H,I). Heterozygous *Gob-4d* seedlings showed an intermediate phenotype, with two to three cotyledons (Fig. 1F), suggesting that GOB affects cotyledon initiation, or partitioning of the embryo apical domain, in a quantitative manner. Likewise, *Gob-4d* flowers had more floral organs per whorl, whereas *gob-3* flowers produced extended and fused organs (see Fig. S4E-I in the supplementary material). In contrast to the reduced SAM activity in *gob-3* plants, *Gob-4d* plants showed increased indeterminacy throughout development. The SAM often split into two or more parallel SAMs (Fig. 1J,K) and, under certain environmental conditions, ectopic meristems and leaves were initiated from the rachis of older leaves (see Fig. S4C in the supplementary material). In *Gob-4d* fruits, ectopic carpels were often produced inside the gynoecium, whereas *gob-3* mutants produced fruits with fewer locules than the wild type (Fig. 1S-U). Plant architecture was severely altered in *Gob-4d* plants owing to variable stem elongation and the tendency of the SAM to split (see Fig. S4A,B in the supplementary material). However, at the level of leaf initiation, spiral phyllotaxis was maintained, similar to *Arabidopsis* transgenic plants overexpressing a *miR164*-resistant *CUC2* or that are mutant in all three *miR164*-coding loci (Peaucelle et al., 2007; Sieber et al., 2007).

GOB sets boundaries during compound-leaf patterning

Alterations in GOB activity dramatically affected leaf shape. Leaves of *gob-3* seedlings that recovered following cotyledon removal produced only primary leaflets with smooth margins, compared with the compound wild-type tomato leaves that have primary, secondary and intercalary leaflets and lobed leaflet margins. Moreover, primary *gob-3* leaflets were often fused (Fig. 1A,L-N,Q,R). Later leaves showed severe fusion of leaflets and petiolules, resulting in a malformed leaf (see Fig. S4D in the supplementary material). By contrast, *Gob-4d* leaflet margins were deeply lobed, and the lobe sinuses were wider than those of the wild type (Fig. 1O-R). Compared with the wild-type, leaf petioles were shorter in *gob-3* and longer in *Gob-4d*. Strikingly, the level of leaflet reiteration was reduced in both loss- and gain-of-function *gob* alleles: in both cases there was a reduction in the number of distinct secondary leaflets (Fig. 1L-R), but this resulted from different underlying causes and was manifested in very different mature leaf shapes. Whereas in *gob-3* no secondary leaflets were observed, in *Gob-4d* initiation events from the primary leaflets developed into lobes rather than secondary leaflets, which is likely to be due to their fusion (Fig. 1O-R). As a result, the *gob-3* primary leaflets were flat and simple, whereas those of *Gob-4d* were buckled and deeply lobed. Intercalary leaflets were absent and leaflet petiolules were shorter in both the loss- and gain-of-function alleles.

GOB expression marks leaflet boundaries in the leaf margin

gob phenotypes suggest that the precise timing and location of GOB activity might be required for proper formation and separation of lateral organs and leaflets. We therefore analyzed the spatial distribution of *GOB* mRNA by in situ hybridization. Similar to its petunia and *Arabidopsis* orthologs, *GOB* mRNA was expressed in stripes at the boundaries between the SAM and initiating organs (Fig. 2A,D). Stripes of *GOB* mRNA expression additionally marked the flanks of initiating primary and secondary leaflets (Fig. 2D,G,J,K). Initially, *GOB* expression appeared in a single band at the margin of an early P3 (P, plastochron number) leaf primordium, prior to visible leaflet initiation (Fig. 2D,J). Following leaflet initiation, two bands could be detected on each side of the initiating leaflet (Fig. 2J). The presence of *miR164*-directed *GOB* cleavage products in wild-type plants and the phenotypes of *Gob-4d* indicated that *miR164* is a negative regulator of *GOB*. We examined the spatial distribution of *miR164* in wild-type tomato plants. *miR164* expression was observed at the flanks of the SAM and in young leaf primordia, but was downregulated at the boundary between the initiating leaves and the SAM (Fig. 2C,F). Strong *miR164* expression appeared in leaflets shortly after their initiation, and its expression was downregulated between initiating leaflets (Fig. 2F,I,L). Thus, the *miR164* and *GOB* expression domains are largely complementary.

To further understand *GOB* expression and function, we examined how the *Gob-4d* mutation affects *GOB* spatial expression. In *Gob-4d* seedlings, *GOB* expression marked the SAM leaf and the leaflet boundaries as in the wild type, but was also expanded to include part of the SAM peripheral zone and the leaf base (Fig. 2B,E). Within the developing leaf, the edges of the *GOB* expression domain were more diffuse than in the wild type (Fig. 2E,H). In addition, *GOB* levels appeared higher in *Gob-4d*, and patches of ectopic expression were occasionally observed. These results imply that *GOB* expression is controlled both transcriptionally and post-transcriptionally, and that *miR164* spatially and quantitatively sharpens and tunes the *GOB* expression domain. This is compatible with the emerging picture of the combined quantitative effects of transcriptional control and *miR164* action on the spatial and temporal activity of the *Arabidopsis CUC1* and *CUC2* genes (Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007). The importance of accurate expression of *GOB* is revealed by the dramatic phenotypic changes that are caused by the expansion and blurring of the *GOB* expression domain in *Gob-4d*.

The combined observed effects of the *Gob-4d* mutation on *GOB* expression and leaf development imply that proper leaflet separation requires a sharp boundary between high *GOB* expression and no *GOB* expression in adjacent leaf marginal regions. *gob*-3 and *Gob-4d* both impair the sharpness of this boundary, and this might underlie their common effects in simplifying the leaf.

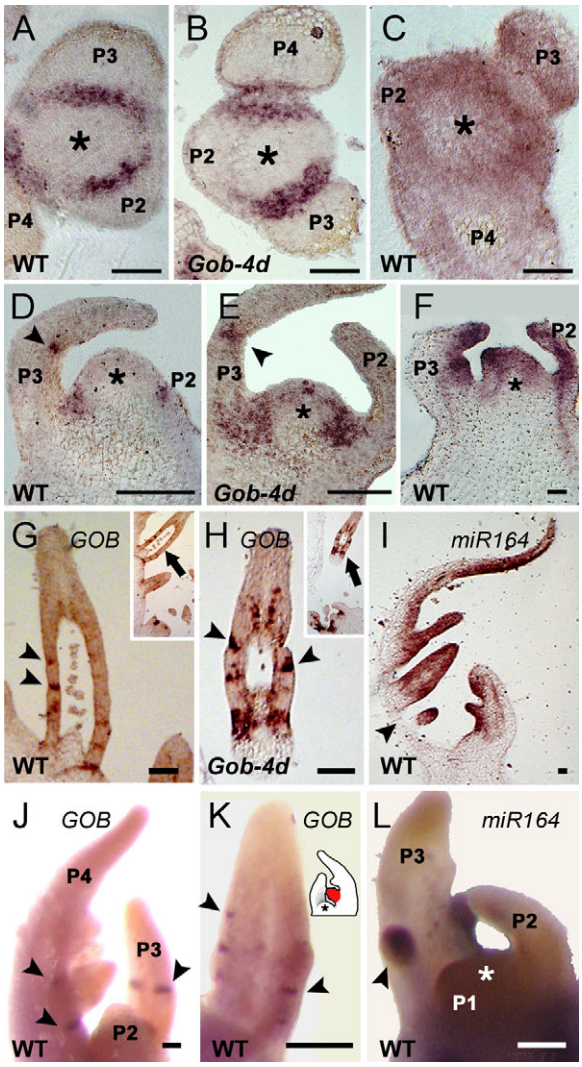

Fig. 2. *GOB* is expressed in tomato leaf and leaflet boundaries in a complementary pattern with *miR164*. In situ hybridization with *GOB* or *miR164* probes. Probes are indicated at the top right corner and genotypes at the bottom left corner of each panel. (**A-C**) Transverse sections of the SAM. (**D-F**) Longitudinal sections of the SAM. (**G**,**H**) Longitudinal sections of primary leaflets. Insets indicate the context of the portion shown within the leaf primordia, and arrows point to the leaflet shown in the enlarged section. (**I**) Longitudinal sections of leaf primordia. (**J-L**) Whole-mount in situ hybridization of the SAM (J,L) or a primary leaflet (K). The inset in K indicates the context of the primary leaflet, and the red area marks the leaflet shown. Asterisks mark the SAM, P indicates the plastochron number, and arrowheads point to stripes of *GOB* expression (D,E,G,H,J,K), reduced *miR164* expression between leaflets (I) or elevated *miR164* expression in initiating leaflets (L). Scale bars: 100 μm.

miR164 overexpression eliminates secondary leaflets and lobes

The complementary expression of *GOB* and *miR164*, and the effect of *Gob-4d* on *GOB* expression and leaf structure, suggested that *GOB* is negatively regulated by *miR164*. In agreement with this, strong constitutive overexpression of an *Arabidopsis miR164* precursor in tomato resulted in a *gob*-like phenotype (Alvarez et al., 2006). The simpler leaf phenotype of *gob-3* could be an indirect consequence of the dramatic SAM function defect or might

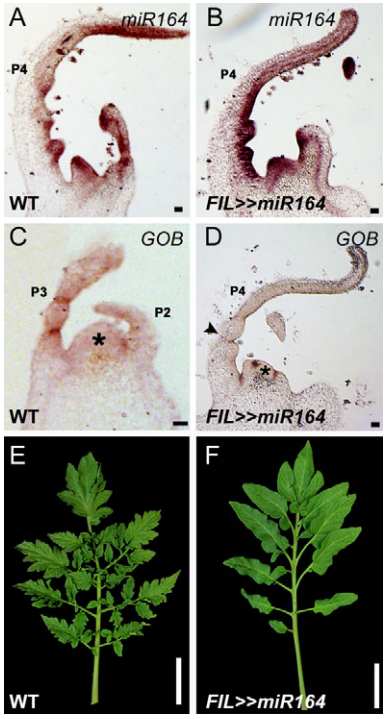

Fig. 3. Leaf-specific overexpression of *miR164* results in simple leaflets. (**A-D**) In situ hybridization of longitudinal leaf primordia sections. Probes are indicated at the top right corner and genotypes at the bottom left corner of each panel. Asterisk, SAM; P, plastochron number; arrowhead, stripe of *GOB* expression. (**E**,**F**) Fully expanded fifth leaves. Scale bars: 100 μm in A-D; 5 cm in E,F.

represent a separate role for GOB in compound-leaf patterning. To distinguish between these possibilities, and to test the combined role of *miR164* targets in leaf development, we expressed an *Arabidopsis miR164* precursor via the *FIL* promoter, which directs expression specifically in lateral organs and is not expressed in the SAM (Lifschitz et al., 2006; Ori et al., 2007). As expected, *FIL>>miR164* leaves showed expanded *miR164* expression and a dramatic reduction of *GOB* expression in developing leaves, but not in the SAM (Fig. 3A-D; see Fig. S2B in the supplementary material). *FIL>>miR164* transgenic plants had normal SAM function (not shown) but simpler leaves (Fig. 3E,F) that lacked secondary leaflets and had smooth margins, similar to *gob-3* leaves. Thus, GOB expression, either before or during leaflet initiation, is necessary for the development of secondary leaflets.

Ectopic *GOB* expression dramatically affects compound-leaf patterning

To further understand how GOB affects leaf development, we generated two *GOB* mutant forms with decreased levels of complementarity to *miR164*. GOB^{4d} contains the same mutation as the *gob-4d* allele, whereas GOB^{m} carries nine silent mutations in the *miR164* binding site (see Fig. S5 in the supplementary material). These forms were expressed specifically in developing lateral organs using the *FIL* promoter. In contrast to the narrow stripes of *GOB* expression observed in wild-type leaves, *FIL>>*GOB^{m} primordia expressed *GOB* throughout the leaf primordia, whereas the expression in the SAM, where *FIL* is not expressed, remained similar to that in the wild type (Fig. 4A-D). *FIL>>*GOB^{wt}, *FIL>>*GOB^{4d} and *FIL>>*GOB^{m} leaves showed a gradual increase in phenotypic

severity, leading to smaller leaves that lacked distinct secondary leaflets (Fig. 4E-L). *FIL*-driven expression of any of the *GOB* forms often resulted in serrated cotyledons and petals (Fig. 4M; see Fig. S6 in the supplementary material). In contrast to *FIL>>miR164* primary leaflets, which were elongated, flat and smooth, those of *FIL>>GOB*^{m} were extremely rounded, rumpled and deeply lobed. This suggests that the failure to elaborate distinct secondary leaflets resulted from different causes in these two genotypes.

In contrast to the leaf-specific effects of *FIL>>GOB*^{4d}, overexpressing *GOB*^{4d} via the strong ubiquitous promoter 35S (*35S>>GOB*^{4d}) resulted in complete loss of ordered organ initiation by the embryonic SAM, and in the formation of numerous meristems at the seedling apex and on the cotyledons (Fig. 4N,O), similar to findings for CUC genes in *Arabidopsis* (Takada et al., 2001). These results imply that balancing indeterminate and determinate fates requires restriction of GOB function.

GOB affects the rate of leaf maturation, leaflet elaboration and secondary-leaflet initiation

A final leaf shape can result from different early events (Champagne and Sinha, 2004). We followed early leaf development in backgrounds with altered GOB activity to further understand the role of GOB in the ontogeny of the tomato compound leaf (Fig. 5). In the wild-type tomato leaf, primary leaflets were initiated at the P3 stage from the marginal blastozone, which was characterized by distinct meristem-like cell morphology and the lack of trichomes. In this manner, leaflet initiation from the leaf margin resembled leaf initiation from the flanks of the SAM (Fig. 5A,D,G). At the P5 stage, secondary leaflets were initiated from the marginal blastozone of the primary leaflet, and, slightly later, intercalary leaflets were initiated along the rachis (Fig. 5J,K,Q). The initiation of primary leaflets appeared normal in *FIL>>miR164* leaves; however, no initiation of secondary leaflets and intercalary leaflets was observed (Fig. 5C,F,I,N,O,R). Moreover, basal primary leaflets arose at an earlier developmental stage than in the wild type. Early leaf development in recovered *gob-3* mutants was similar to that of *FIL>>miR164*, although an accurate assignment of a developmental stage to these mutants was impossible owing to the abnormal leaf initiation (see Fig. S7F,I in the supplementary material). The marginal blastozone of primary *FIL>>miR164* and *gob-3* leaflets was visible, but narrower than that of the wild type. Developing *FIL>>GOB*^{m} leaf primordia failed to properly expand at their distal end. Overall, these leaves showed prolonged blastozone activity and appeared younger than wild-type leaves of the same developmental stage, as judged by delayed trichome formation and chlorophyll accumulation (Fig. 5B,E,H,L,M). In addition, *FIL>>GOB*^{m} failed to retain proper spatial and temporal spacing between initiation events. At the P3 stage, these primordia were shorter than those of the wild type, and had already initiated three or more primary leaflets, in comparison to a single one in the wild type (Fig. 5D,E). Soon after their initiation, primary *FIL>>GOB*^{m} leaflets initiated numerous secondary outgrowths, which in turn precociously initiated additional, tertiary outgrowths (Fig. 5H,L,M). All these structures remained connected and developed into lobes rather than distinct leaflets. Thus, the final *FIL>>GOB*^{m} leaf shape is a combination of inhibition of leaf differentiation, ectopic or precocious lateral initiation events and improper spatial and temporal spacing between them. Young *Gob-4d* leaves initiated fewer primary leaflets than the wild type, in agreement with their final leaf shape (see Fig. S7A-E in the supplementary material). Initiating primary leaflets were shorter and wider than in the wild type, and precocious initiation events from the margins of their terminal leaflet were observed, possibly reflecting the more diffuse GOB expression. Secondary outgrowths were clearly initiated from the primary leaflet margin (see Fig. S7G,H in the supplementary material).

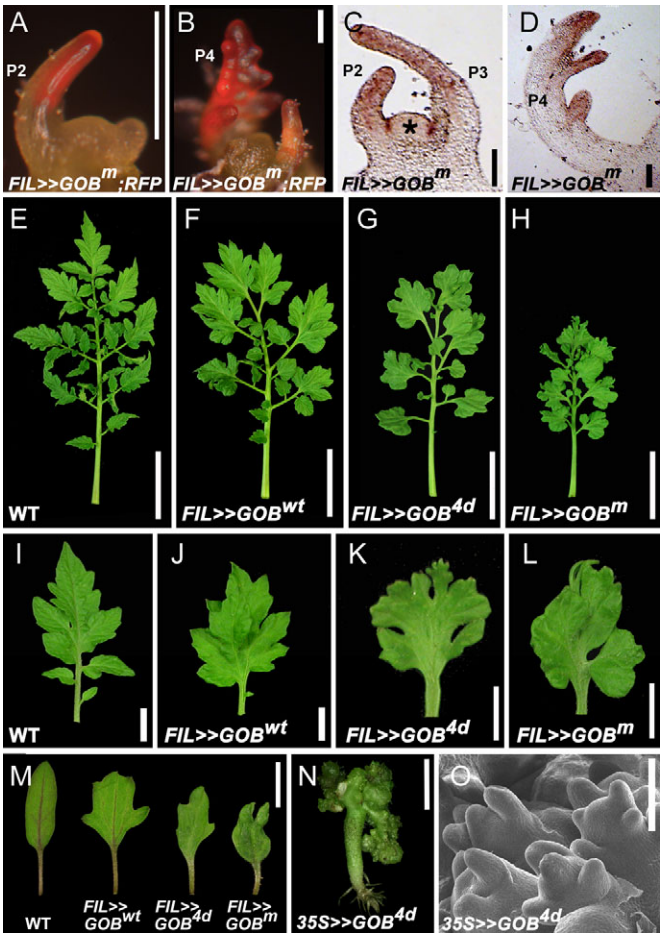

Fig. 4. Dramatic leaf shape alterations are caused by ectopic *GOB* expression. (**A**,**B**) Expression pattern of the *FIL* promoter in the *FIL>>GOB*^{m} SAM (A) and leaf primordia (B), using an *op:RFP* reporter. (**C**,**D**) In situ hybridization performed on longitudinal sections of a *FIL>>GOB*^{m} SAM (C) and leaf primordia (D) with the *GOB* probe. (**E-M**) Mature fifth leaf (E-H), terminal leaflet from the ninth leaf (I-L) and cotyledons (M) of tomato plants expressing the different *GOB* versions through the *FIL* promoter. (**N**,**O**) A *35S>>GOB*^{4d} seedling (N) and SEM of ectopic meristems developed on a *35S>>GOB*^{4d} seedling apex (O). Asterisk, SAM; P, plastochron number. Scale bars: 200 μm in C,D,O; 500 μm in A,B; 1 cm in I-N; 5 cm in E-H.

These results demonstrate that precise regulation of spatial and temporal GOB activity affects the progression of leaf maturation, the location and timing of leaflet initiation sites and leaflet separation. The elaboration of higher order initiation events into distinct leaflets appears to require a sharp boundary between adjacent regions that feature high versus no *GOB* expression.

Conserved role for *miR164* in leaf elaboration

To investigate the effect of overexpressing the *GOB* regulator *miR164* on leaflet formation in a different species with complex leaves, we ectopically overexpressed *miR164* in *Cardamine hirsuta* plants. *C. hirsuta* leaves become gradually divided with age, owing to significant heteroblasty in primary leaflet number and shape (Fig. 6A). *35S::AtmiR164b C. hirsuta* transformants exhibited extensive

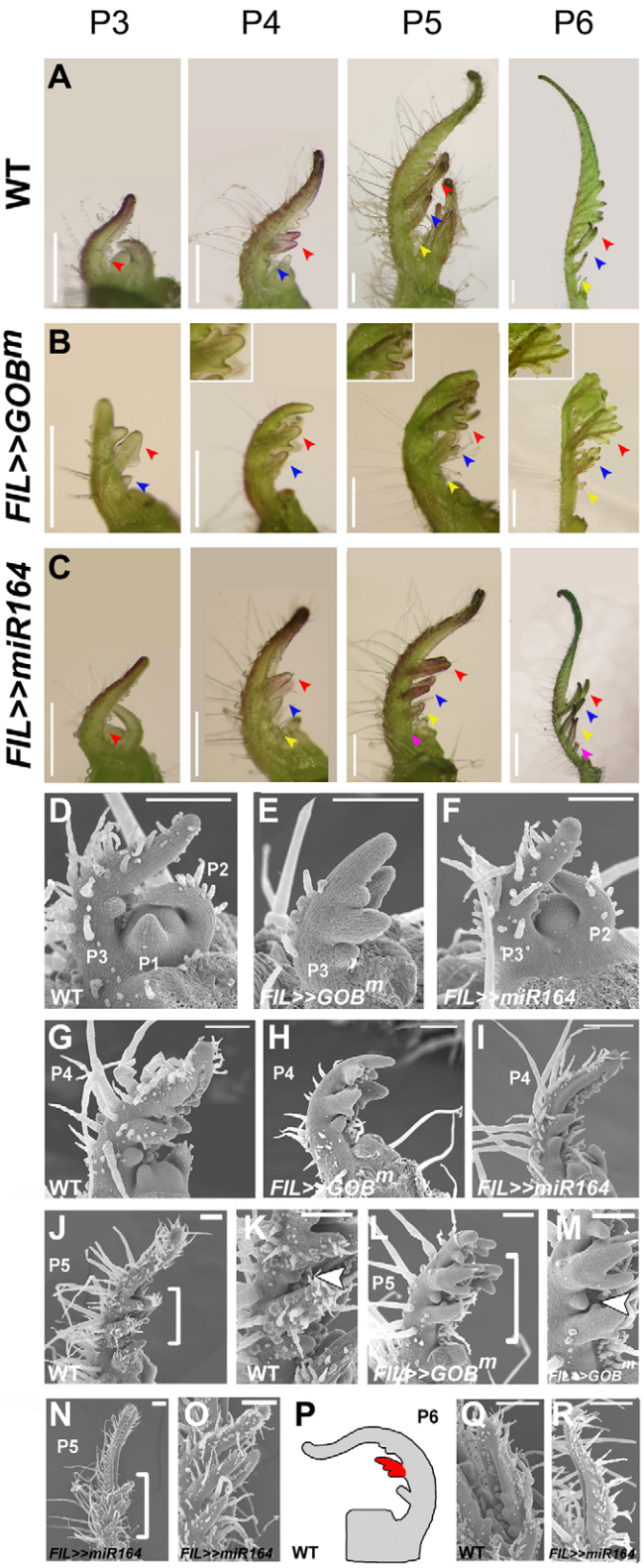

Fig. 5. The effect of altering GOB expression on early leaf and leaflet development. (**A-C**) Early leaf development (P3-6) in wild-type (A), *FIL>>GOB*^{m} (B) and *FIL>>miR164* (C) tomato plants. Arrowheads point to primary leaflet primordia according to the order of their initiation: red, blue, yellow and pink, respectively. (**D-O**) SEMs of P3 (D-F), P4 (G-I) and P5 (J-O). K, M and O are magnifications of J, L and N, respectively, demonstrating the appearance of intercalary leaflets. (**P**) Schematic illustrating the context of the secondary leaflet shown in the SEM image in Q and R. The red area marks the leaflet shown. (**Q**,**R**) SEM of the secondary leaflet of wild type (Q) and *FIL>>miR164* (R). Scale bars: 500 μm in A-C; 200 μm in D-O,Q,R.

fusion phenotypes, which are characteristic of equivalent transformants in *Arabidopsis* (Laufs et al., 2004), including cotyledon fusion and a loss of the embryonic apical meristem in strong lines, and fusion between leaves, leaflets and same-whorl floral organs in less severe lines (see Fig. S8 in the supplementary material). As in tomato, *AtmiR164* overexpression resulted in extensive leaflet fusion and the smoothing of serrated margins, a phenotype that was particularly prominent in cauline leaves (Fig. 6B,C). Unlike in tomato leaves, a primary effect of *miR164* overexpression on *C. hirsuta* leaves was a reduction in the number of primary leaflets (Fig. 6).

Interaction between *entire* and *gob*

It was recently shown that the auxin response marks initiating leaflet primordia in *C. hirsuta* leaves and promotes their elaboration (Barkoulas et al., 2008). In agreement, mutations in the tomato putative auxin-response repressor *ENTIRE* (*E*) (also known as *SlIAA9*) were shown to cause a severe reduction in leaflet separation (Wang et al., 2005; Zhang et al., 2007). Several new *e* alleles have been identified in a screen of a mutant population generated in the same genetic background as *gob-1* to *-4* (Menda et al., 2004) (http://zamir.sgn.cornell.edu/mutants). Of these, we identified the mutation in two alleles of the *E* gene (Fig. 7A), and confirmed allelism with *E* for two additional alleles. Leaves of *e* and recovered *gob* mutants show a striking resemblance (compare Fig. 1M with Fig. 7B). Moreover, in *Arabidopsis*, auxin has been proposed to negatively regulate *CUC2*, possibly via positive regulation of *miR164* (Furutani et al., 2004; Guo et al., 2005; Heisler et al., 2005; Vernoux et al., 2000). To test whether this is the basis for the phenotypic similarity between *gob-3* and *e*, we examined the genetic and molecular interaction between *e* and *gob*. If the *e* leaf phenotype results from upregulation of *miR164*, the *Gob-4d* phenotype is expected to be epistatic to the *e* phenotype. However, leaves of *e Gob-4d* double mutants appeared very similar to those of *e*, although some features of the *Gob-4d* phenotype were evident (Fig. 7B-D). In agreement, in situ hybridization revealed no obvious change in *GOB* expression in *e* leaves (Fig. 7G-I), arguing against E as a positive regulator of GOB expression. Finally, *FIL>>miR164* or a reduction in *GOB* copy number (in *e gob-3/+*) enhanced the *e* phenotype (Fig. 7E,F). These results suggest that E and GOB might act through partially independent pathways, However, as no mutation was identified in the *E* coding region in *e-2* mutants, the possibility remains that it represents a weak allele that is enhanced by *gob-3/+*, in which case both genes might act in the same pathway. The relationship of both regulators with auxin-mediated leaf elaboration warrants more detailed examination.

DISCUSSION

The multiple functions of GOB in the development of the tomato compound leaf

The development of a compound leaf requires a prolonged maturation process, during which leaflets are reiteratively initiated from regions at the leaf margin. In the present study, we have

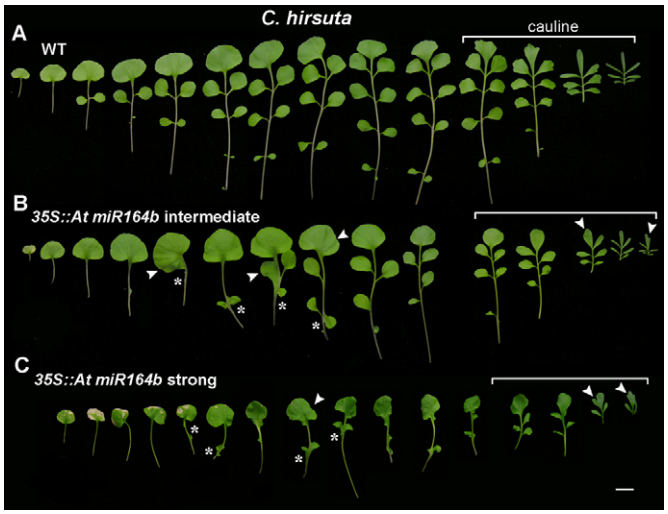

Fig. 6. Effect of *miR164* overexpression on *Cardamine hirsuta* compound-leaf development. (**A**) Leaves removed from a wild-type *Cardamine hirsuta* plant and displayed in acropetal sequence from left to right. Cauline leaves formed following the transition to flowering are bracketed. (**B**) Leaves removed from an intermediate line of *35S::AtmiR164b C. hirsuta* and arranged in acropetal sequence from left to right. (**C**) Leaves dissected from a strong line of *35S::AtmiR164b C. hirsuta* and placed in acropetal sequence from left to right. Probable fusion events between adjacent leaves are marked by arrowheads; regions of the rachis where torn leaf tissue exists from a disrupted congenital fusion event are marked with an asterisk. Scale bar: 1 cm.

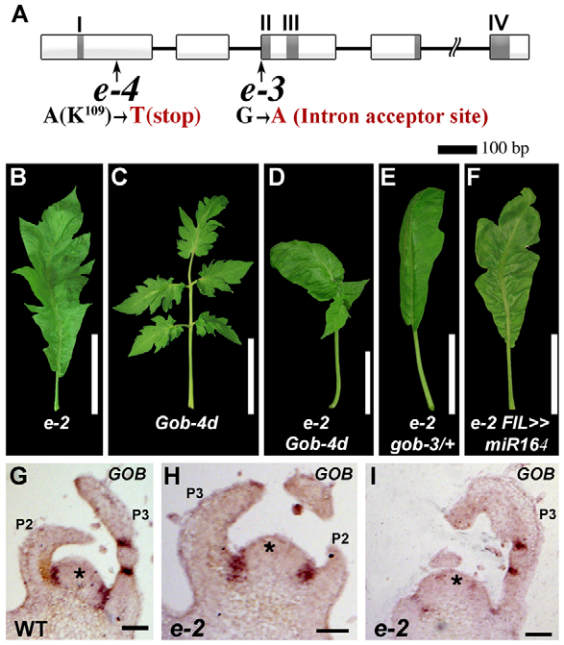

Fig. 7. Interaction between *GOB* and *ENTIRE*. (**A**) Structure of the tomato *ENTIRE* (*E*) gene. Exons (white boxes), introns (black lines), conserved IAA domains I-IV (gray stripes), and the nucleotide and amino acid changes in the *e-3* and *e-4* alleles are indicated. The *e-2* mutant, used for the genetic interaction and in situ hybridization shown in B and C, was confirmed as an *e* allele but sequence analysis of the genomic region spanning the entire ORF could not identify the causative mutation. (**B-F**) A fifth leaf from each of the indicated single and double mutants. (**G-I**) In situ hybridization of longitudinal SAM sections with the *GOB* probe. Genotypes are indicated at the bottom left corner. Scale bars: 5 cm in B-F; 100 μm in G-I.

uncovered novel roles for GOB in inhibiting leaf maturation and in the spatial and temporal positioning of leaflets, secondary-leaflet initiation and separation, and leaf margin elaboration (Fig. 8A). As these processes are interrelated, it is currently impossible to conclude whether the requirement for GOB for the later events, such as secondary-leaflet initiation and separation and margin elaboration, represent a primary role or a secondary consequence of its effect on leaf maturation. For example, extended marginal blastozone activity has been shown to cause extended marginal initiation events (Ori et al., 2007). The absence of any secondary-leaflet initiation in *gob-3* and *FIL>>miR164* leaves in spite of the maintenance of an undifferentiated marginal blastozone (Fig. 5R; see Fig. S7I in the supplementary material) might indicate that these are distinct effects.

Reduction and elevation of GOB activity both lead to a lack of distinct secondary leaflets (Fig. 1; Fig. 5; Fig. 8B,C). Examination of early leaf development reveals that whereas in *gob-3* and *FIL>>miR164* this results from the absence of secondary leaflets, in *Gob-4d* and *FIL>>GOB*^{m} secondary leaflets are initiated but fail to properly separate, leading to the formation of lobes rather than distinct leaflets (Figs 4 and 5). We propose that the development of separate secondary leaflets with petiolules requires a sharp boundary between a leaf marginal region with relatively high *GOB* expression and an adjacent *GOB*-less region (Fig. 8C). The absence of such a sharp boundary in *Gob-4d* leaf primordia, owing to elevated and more diffuse *GOB* expression, leads to secondary-leaflet fusion and a lobed primary leaflet (Fig. 1; Fig. 8C). This leaflet fusion is further enhanced in *FIL>>GOB*^{m} leaves, which are subjected to continuous high-level *GOB* expression (Fig. 4). Leaflet fusion is also likely to be affected by the alteration in the timing and location of leaflet initiation in these genotypes. That similar initiation events can lead to either distinct leaflets or to lobes, depending on their position relative to other leaflets, suggests that these forms are homologous.

Context-dependent effect of GOB on leaf shape

The terminal, distal-most leaflet of the compound tomato leaf is initiated directly from the flanks of the SAM. As such, it is largely equivalent to a simple leaf. Initiation of the terminal leaflet is largely insensitive to GOB expression, but GOB is required for elaboration of its marginal serrations and to prevent its fusion with the two lateral primary leaflets formed subsequently (Fig. 1M,N). Similarly, many simple lateral organs in the tomato, such as the cotyledons or sepals, require GOB to inhibit their congenital fusion with primordia in the same whorl. The requirement for GOB in leaflet separation is reduced in the context of the other primary tomato leaflets (Fig. 8B). The response of leaflets of different order to GOB further emphasizes the distinct nature of the different leaf units: whereas the effect of impaired GOB-like function on the initiation of primary leaflets is relatively mild, it completely abolishes the initiation of secondary and higher order leaflets. Finally, GOB is essential in all leaflets for the elaboration of lamina margins. GOB activity thus helps to define the unique properties of the different leaf units, and illustrates how individual leaflets are independently regulated within a compound leaf.

The context-dependent role of GOB in leaf patterning is further exemplified by comparing the effects of altered NAM-like activity in species with different leaf shapes. Such a comparison might hint at the relationship between these different leaf forms. The role of CUC2 in the formation of leaf serrations in *Arabidopsis* (Nikovics et al., 2006) resembles the effect of GOB on leaflet margins, suggesting that leaflets and simple leaves are at least partially equivalent structures.

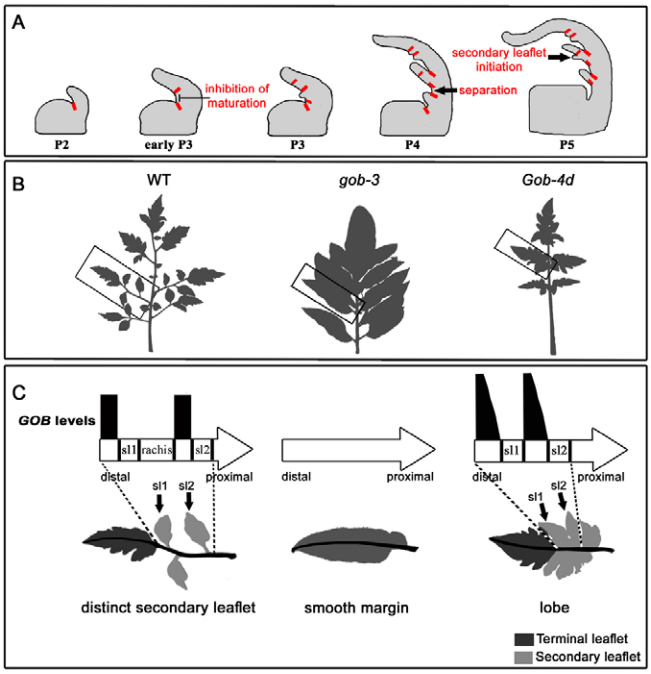

Fig. 8. A proposed model of GOB function. (**A**) The roles of GOB in early stages of tomato leaf development. The GOB expression pattern is indicated in red. In early P3 primordia, GOB is expressed at the leaf margin prior to leaflet initiation and inhibits maturation in the adjacent area, enabling future leaflet initiation. During primary leaflet formation, restricted GOB expression in space and time allows proper leaflet separation. In the terminal leaflet, GOB expression enables the development of lobes and serrations. In plastochron 5 (P5), stripes of GOB expression in the primary leaflet flanks enable initiation and separation of secondary leaflets. (**B**) Diagrams of the fifth leaf of wild-type, *gob-3* and *Gob-4d* plants, demonstrating the relatively minor effect of alterations in GOB activity on primary leaflet initiation. The rectangle indicates the primary leaflets shown in C. (**C**) The effect of fluctuations in *GOB* expression on secondary-leaflet initiation and separation. (Top) Schematics of relative spatial *GOB* expression in the developing leaflets of wild type and *Gob-4d*, and of GOB activity in *gob-3*. (Bottom) Diagrams of the resulting primary lateral leaflet. In the wild type, a sharp boundary between a narrow region of high *GOB* expression and an adjacent region with no expression enables initiation and development of a distinct secondary leaflet (sl1, sl2, arrows). In *gob-3*, elimination of *GOB* activity results in a faliure to form a boundary and in the lack of initiation of secondary leaflets, leading to the development of a primary leaflet with smooth margins. In *Gob-4d*, *GOB* expression is elevated and expanded relative to the wild type. The boundary is less sharp, and adjacent GOB expression domains are closer to each other than in the wild type. This results in lobed primary leaflets owing to fusion of adjacent leaflet primordia (sl1, sl2, arrows), and the lack of distinct secondary leaflets with petiolules.

C. hirsuta leaves lack higher order leaflets, and the formation of its primary leaflets was more susceptible to *miR164* overexpression than in tomato, suggesting that primary leaflets in these two species represent partially distinct structures. Together, these observations suggest that GOB is utilized in leaf patterning in a context-dependent manner. The degree of leaf elaboration appears to be determined by both GOB-dependent and GOB-independent mechanisms. The potentially redundant involvement of other NAM/CUC transcription factors, such as the orthologs of *Arabidopsis* CUC3 (Aida et al., 1997; Hibara et al., 2006; Vroemen et al., 2003), in compound-leaf patterning remains to be determined.

In light of the proposed role for NAM/CUC transcription factors in restricting growth (Aida and Tasaka, 2006; Nikovics et al., 2006), the punctuate GOB expression pattern and its role in boundary specification (Fig. 8), it is somewhat surprising that relatively uniform *GOB* expression in developing leaves did not inhibit leaflet initiation altogether. This implies that GOB acts in the context of additional factors that function in the laminar GOB-less region to specify proper leaflet initiation and growth. Such factors might include growth and differentiation factors such as LANCEOLATE or the tomato orthologs of genes such as FILAMENTOUS FLOWER, as well as auxin. Auxin has been implicated in the development of serrations, lobes and leaflets in different species (Avasarala et al., 1996; Barkoulas et al., 2008; Hay et al., 2006; Wang et al., 2005; Zhang et al., 2007). The enhancement of *FIL>>miR164* by *e* might imply that GOB and auxin interact in leaflet positioning.

Similarities and differences between leaf and leaflet initiation

Morphological and genetic evidence point to striking similarities between leaf initiation from the flanks of the SAM and leaflet initiation from the leaf margin (Barkoulas et al., 2008; Brand et al., 2007; Hagemann and Gleissberg, 1996; Mathan and Jenkins, 1962; Ori et al., 2007; Sachs, 1969). Here we show that *NAM*-like genes, shown to be involved in boundary specification during lateral organ formation from the SAM, are also utilized for boundary specification at the site of leaflet initiation. However, leaflet initiation is also very different from leaf initiation, both morphologically and in molecular terms. Whereas the SAM continues to form many more leaves, the marginal blastozone forms a finite number of leaflets. In addition, whereas in the SAM *GOB* expression marks the boundary between the adaxial side of the leaf and the SAM, during leaflet initiation it first marks the distal lateral domain of the leaflet (Fig. 2). This implies a conserved but flexible role for GOB in boundary specification.

Our results demonstrate how plants adapt a common boundary-specification program to modulate different developmental processes in a context-specific manner. It remains to be seen how the boundary specification by GOB interacts with additional factors in the context of compound-leaf patterning.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/5/823/DC1

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