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ORIGINAL ARTICLE

Meristem maintenance and compound-leaf patterning utilize common genetic mechanisms in tomato

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Abstract Balancing shoot apical meristem (SAM) maintenance and organ formation from its flanks is essential for proper plant growth and development and for the flexibility of organ production in response to internal and external cues. Leaves are formed at the SAM flanks and display a wide variability in size and form. Tomato (*Solanum lycopersicum*) leaves are compound with lobed margins. We exploited 18 recessive tomato mutants, representing four distinct phenotypic classes and six complementation groups, to track the genetic mechanisms involved in meristem function and compound-leaf patterning in tomato. In *goblet* (*gob*) mutants, the SAM terminates following cotyledon production, but occasionally partially recovers and produces simple leaves. *expelled shoot* (*exp*) meristems terminate after the production of several leaves, and these leaves show a reduced level of compoundness. *short pedicel* (*spd*) mutants are bushy, with impaired meristem structure, compact inflorescences, short pedicels and less compound leaves. In *multi drop* (*mud*) mutants, the leaves are more compound and the SAM tends to divide into two active meristems after the production of a few leaves. The range of leaf-compoundness phenotypes observed in these mutants suggests that compound-leaf patterning involves an array of genetic factors, which act successively to elaborate leaf shape. Furthermore, the results indicate that similar mechanisms underlie SAM activity and compound-leaf patterning in tomato.

Keywords Compound leaf · Leaf shape · Mutation · Shoot apical meristem · Tomato

Abbreviations

GOB	GOBLET
EXP	Expelled shoot
SPD	Short pedicel
MUD	Multi drop
SAM	Shoot apical meristem
SEM	Scanning electron micrograph
KNOXI	Class I knotted-like homeobox

Introduction

Plant form is a dynamic feature, shaped continuously through organ generation and patterning, which take place at meristems. The shoot apical meristem (SAM) forms the aerial organs, including leaves, stems, axillary meristems and derivatives of these organs. Organ formation by the SAM is a well-controlled yet flexible process, which enables plant architecture to continually adjust to the changing developmental and environmental circumstances (Bowman and Eshed 2000; Carles and Fletcher 2003; Veit 2006). Proper SAM function requires balancing continuous organ production with the maintenance of a structured indeterminate meristem. One of the most successful approaches to revealing the mechanisms underlying this balance has been the isolation of mutants with aborted meristems and the subsequent identification of the responsible genes. This approach has led to the identification of key players in meristem maintenance (Bowman and Eshed 2000; Carles and Fletcher 2003; Veit 2006).

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Leaves are formed on the flanks of the SAM, and gradually assume their shape and become determinate. Leaf development has been roughly divided into three continuous phases: (1) leaf initiation; (2) primary morphogenesis, also called organogenesis; (3) expansion and secondary morphogenesis, also called histogenesis (Poethig 1995; Dengler and Tsukaya 2001; Holtan and Hake 2003). Leaves can be simple or compound, and numerous intermediate forms can be found (Goliber et al. 1999; Kaplan 2001; Kessler et al. 2001). A simple leaf is composed of a petiole and a single, sometimes lobed, blade. In contrast, in a compound leaf, several units, termed leaflets, each resembling a simple leaf, are connected via petiolules to a central rachis. Reiteration of leaflet initiation can lead to further orders of leaf compoundness. Simple and compound leaves are thought to have evolved from the same essential developmental process, as the two forms can be found in closely related species, and even in the same plant at different developmental stages or in reaction to environmental signals (Goliber et al. 1999; Dengler and Tsukaya 2001; Kaplan 2001; Bharathan et al. 2002). Tomato (*Solanum lycopersicum*) leaves are compound with lobed margins, and examination of natural tomato populations, tomato mutants and tomato introgression lines reveals a wide range of leaf shapes (Goliber et al. 1999; Holtan and Hake 2003; Menda et al. 2004). This flexibility in leaf shape makes tomato a useful model to study leaf patterning. Interestingly, simple and compound leaves appear similar at initiation (Dengler 1984; Holtan and Hake 2003). Leaflets are formed later, during the primary morphogenesis phase, from a region in the leaf margin termed marginal blastozone (Hagemann and Gleissberg 1996; Dengler and Tsukaya 2001). The final degree of leaf compoundness reflects the time of initiation of marginal primordia, and the relations between the growth of the marginal primordia and that of the central portion of the leaf blade (Kaplan 2001).

Recent studies have identified a role for class I *knotted1 (kn1)-like Homeobox* (*KNOXI*) genes in the control of leaf shape. *KNOXI* genes are required for SAM maintenance in various species, as revealed by loss-of-function mutants (Barton and Poethig 1993; Long et al. 1996; Vollbrecht et al. 2000). While in most species with simple leaves, such as Arabidopsis and maize, *KNOXI* genes are expressed in the SAM and downregulated in regions destined for leaf initiation, in tomato and other species with compound leaves, *KNOXI* genes are also expressed in initiating leaves (Hareven et al. 1996; Janssen et al. 1998; Bharathan et al. 2002; Hay and Tsiantis 2006). Furthermore, misexpression of *KNOXI* genes in leaves of species with simple leaves results in leaf rumpling or lobing, and their overexpression in tomato leaves causes super-compoundness (Hareven et al. 1996; Janssen et al. 1998; Reiser et al. 2000; Hake et al. 2004). In *Cardamine hirsuta*, the *KNOXI* gene *C. hirsuta STM* was shown to be required for leaflet formation, as downregulation of *C. hirsuta STM* activity through RNAi resulted in the conversion of the compound leaves into simple ones (Hay and Tsiantis 2006). These findings suggest that, in addition to their role in meristem maintenance, KNOXI proteins are involved in patterning compound leaves. However, we still do not know whether additional genetic components are involved in the differentiation between simple and compound leaves, and whether the utilization of similar mechanisms for SAM maintenance and leaf elaboration is a general phenomenon.

In this study, we performed a phenotype-based screen to identify tomato mutants with compromised SAM function, altered leaf shape or both. We used these mutants to determine, via an unbiased approach, whether genes important for meristem maintenance also play a role in the control of leaf patterning. We show that many tomato mutants are affected in both SAM function and leaf elaboration, suggesting that partially overlapping genetic mechanisms are involved in these two processes.

Materials and methods

Plant material and mutant screen

Mutant screens were carried out on the tomato (*S. lycopersicum* cv. M82) mutant population described by Menda et al. (2004) (http://www.sgn.cornell.edu). Seeds were sown in a commercial nursery and grown under either natural open-air conditions (April to August), or natural daylight and regulated temperatures (15–30°C, October to March). Each family was screened several times during plant growth. The first screen was performed on 3-week-old seedlings, to identify seedling lethal phenotypes. As most mutants are infertile, self seeds from four to six siblings of each mutant were collected and scored for heritability of the phenotype. Confirmed mutants were backcrossed two to four times to the M82 determinate (*sp*) background; each backcross was performed on four to six siblings.

The genetic nature of the mutant phenotypes was assessed by scoring their segregation in self-progeny of the siblings and in F2 progeny of the backcrosses. All mutations described in this study behaved as monogenic recessive traits. In many cases, the initial segregation ratio was less than the expected 1:3 mutant to wild type. However, in advanced backcrosses, segregation ratios for all mutations approached the 1:3 ratio.

Complementation analysis

As most mutants are infertile, complementation tests were carried out by crossing four to six siblings of each mutant

examined (a total of 16–36 crosses for each allelism test). Selfed seeds of each sibling were first tested for segregation of the mutant, and subsequently, crosses in which both parents were heterozygous for the respective mutation were planted and scored for allelic relations. Two mutants were designated as allelic only after confirmation by at least two independent crosses in which mutant plants were segregated.

Phenotypic characterization

Phenotypic sorting was performed by observation at all growth steps, from germination to bloom. In tomato, leaf shape, including the degree of compoundness, varies among leaves of the same plant and with growth conditions. Therefore, all leaves of mutant and wild-type plants were observed and compared among many individuals and under several growth conditions, and photographs of the fifth leaf were taken from representative leaves and growth conditions. Leaflet number was counted on fully expanded fifth leaves from ten different mutant individuals, except in the case of *exp*, where leaflets were counted on the most compound leaf from each of ten individuals.

goblet rescue

gob seedlings were rescued by dissecting off the epicotyl tissue. Recovery rates of *gob* mutants were close to those of the wild type, and depended on the location of the incision and the growth conditions. The best recovery rate was about 90%, and was obtained when seedlings were dissected just below the cotyledon base and grown under long daylight (16/8) and low irrigation.

Scanning electron microscopy (SEM) and histology

For SEM analysis, meristem samples were taken from 12- or 20-day-old seedlings. Tissues were fixed overnight in 3% (*v/v*) glutaraldehyde, washed with NaP buffer (pH 7) and soaked in 0.5% (*v/v*) osmium-tetroxide solution overnight. After washing the tissues in an increasing gradient of ethanol (up to 100%), fixed tissues were critical-point dried, mounted on a copper plate and coated with gold. Samples were viewed using a JEOL 5410 LV microscope (Tokyo, Japan). For histological sections, tissue was fixed with FAA, sectioned (8 μm) and stained with Safranin-Fast Green as described (Rusin 1999).

RNA isolation and analysis

RNA was isolated from apices of soil-grown 20-day-old *gob* seedlings. mRNA levels of the *gob* aborted apex were compared to those of two different tissues from wild-type seedlings of similar age, as explained in Fig. 2. In the case of *exp*, *spd*, *mud* and the corresponding wild type, mRNA was extracted from shoot apices, which contained the SAM and young leaf primordia, after the removal of leaves 1 and 2. Plant tissue was ground to a fine powder with a mortar and pestle with added liquid N_2. Total RNA was isolated by a modified procedure as described previously (Logemann et al. 1987). For RT-PCR analysis, first-strand cDNA synthesis was performed on 5 μg of total RNA using Reverse-iT^{TM} (Abgene, Epsom, UK) and oligo-dT primer, according to the manufacturer's instructions. For each primer set, PCR protocols were calibrated to recover PCR products during the exponential phase. PCR fragments were separated on a 2% (*w/v*) agarose gel and visualized by ethidium bromide staining. For Semiquantitative RT-PCR, RT-PCR gels were blotted and hybridized to the respective probe. Band intensities were quantified with a Phosphoimager software (FUJIFILM FLA5000), and normalized against *TUB*. The primers used for the amplification of *TUBULIN* were: TUB-F (5′-CACATTGGTCAGGCCGGTAT-3′) and TUB-R (5′-ATCGGCCATCAGGCTGAAT-3′); for *Tkn1*: TKN1-F (5′-CTTGATCAGTTCATGGAAGCA-3′) and TKN1-R (5′-AAGGTTGCATTGGCAGAATC-3′); for *Tkn2/LeT6*: TKN2-cDNA (5′-TATCTCAATTGTCAAAAGATAGGAGC-3′) and TKN2-R (5′-GATATGCAGTTTGTTGTGATGGA-3′).

Results

Identification and genetic characterization of new meristem-maintenance mutants in tomato

With the goal of identifying genetic components responsible for SAM function, leaf elaboration or both, we screened a saturated tomato mutant population, generated with either ethyl methane sulfonate (EMS) or fast neutron in the background of the tomato inbred variety M82 (Menda et al. 2004). Eighteen mutations, featuring various degrees of SAM function and leaf-elaboration defects, were selected for further characterization. These mutations were grouped into four different phenotypic categories, based on phenotypic similarities. Complementation tests among mutants belonging to the same phenotypic group revealed that for most loci, several alleles could be identified, and that in some of the phenotypic groups, similar phenotypes were caused by mutations in distinct loci (Table 1).

The *goblet* mutants show severe defects in meristem maintenance and leaf patterning

The *goblet* (*gob*) mutant displays severe SAM-maintenance phenotypes. Two fused cotyledons are the only

Table 1 Phenotypic and complementation groups of the described mutants

Phenotypic group[a]	Family[b]	Mutant[c]
goblet (*gob*)	e1976-m1	*gob*^{1}
	e3883-m1	*gob*^{2}
	n5126-m1	*gob*^{3}
expelled shoot (*exp*)	e2011-m1	*exp1*^{1}
	n5661-m1	*exp1*^{2}
	n6518-m1	*exp1*^{3}
	e1891-m1	*exp2*
short pedicel (*spd*)	e1476-m1	*spd1*
	e1655-m1	*spd2*^{1}
	e2142-m1	*spd2*^{2}
	n6663-m1	*spd2*^{3}
	e9582-m1	*spd2*^{4}
	e9195-m1	*spd2*^{5}
multi drop (*mud*)	e2008-m1	*mud*^{1}
	e3410-m1	*mud*^{2}
	e9019-m1	*mud*^{3}
	e9718-m1	*mud*^{4}
	e6408-m1	*mud*^{5}

[a] Mutants in the same phenotypic groups share similar phenotypes

[b] Family name as designated by Menda et al. (2004, http://www.sgn.cornell.edu)

[c] Families confirmed to be allelic were given the same name

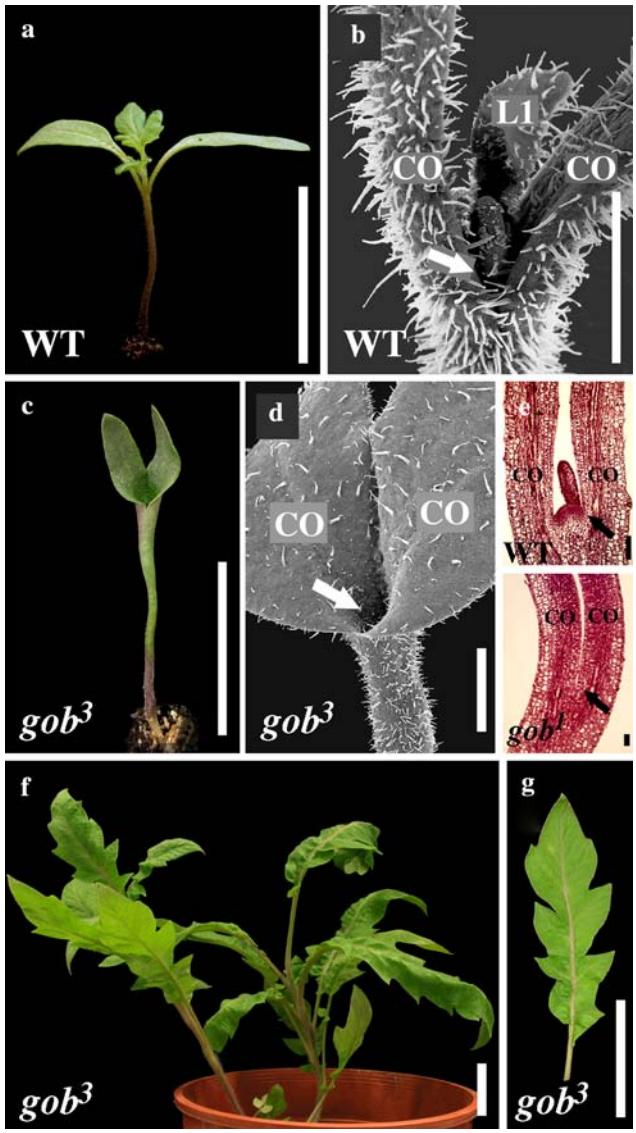

Fig. 1 Phenotypes of *goblet* (*gob*) mutants. **a**, **b** Twelve-day-old wild-type (WT) seedlings. Young leaves emerge from the active SAM (*arrow* in **b**) between the two cotyledons. **c**, **d** Twenty-day-old *gob*^{3} seedlings. The cotyledons are fused and the nonfunctional SAM fails to produce leaf primordia (*arrow* in **d**). **e** Histological sections of a WT (*top*) and *gob*^{1} (*bottom*) seedling. **f** A *gob*^{3} seedling which recovered after dissecting off its cotyledons. **g** A leaf from a recovered *gob*^{3} plant. **b**, **d** are scanning electron micrographs of shoot apices. *CO*—cotyledons, *L1*—the first leaf formed. *Scale bars*: 5 cm (**a**, **c**, **f**, **g**), 1 mm (**b**, **d**), 100 μm

organs formed by the *gob* SAM under standard growth conditions (Fig. 1). Examination by scanning electron microscope (SEM) and histological sections revealed that whereas in wild-type plants an active SAM is present between the cotyledons (Fig. 1a, b), in *gob* mutants, no functional SAM is present and no leaf primordia are formed (Fig. 1c–e). When the tips of *gob* mutants are dissected off, seedlings occasionally recover by forming ectopic meristems from the site of cotyledon removal. These meristems produce several leaves (Fig. 1f) and abnormal inflorescences with infertile flowers. Leaves produced by recovered *gob* mutants are deeply lobed, but simple (Fig. 1g), indicating that the *GOB* gene is also required for the formation of a compound leaf. Three independent *gob* mutants with very similar phenotypes were identified in the screen. Complementation tests revealed that the three mutations are allelic (Table 1).

KNOXI genes are essential for meristem maintenance in many species, and in tomato have been shown to be expressed in initiating leaves as well (Hareven et al. 1996; Janssen et al. 1998). To test whether altered *KNOXI* expression is involved in the *gob* phenotype, we compared the levels of *Tkn1* and *Tkn2/LeT6* mRNA between wild-type and *gob* seedlings. The levels of both *Tkn1* and *Tkn2/LeT6* were undetectable in *gob* mutants under our assay conditions (Fig. 2). Sequencing analysis revealed that the *Tkn1* and *Tkn2/LeT6* genes are intact in *gob* mutants (data not shown), and the *gob* mutation was mapped to chromosome 7 (not shown), while *Tkn1* and *Tkn2/LeT6* have been localized to chromosome 4 and 2, respectively (Hareven et al. 1996; Chen et al. 1997; Parnis et al. 1997). Thus, *gob* does not represent a mutation in one of these *KNOXI* genes but dramatically affects their expression.

Fig. 2 A dramatic reduction in *Tkn1* and *Tkn2/LeT6* expression in *gob* mutants. *Top* Scheme illustrating the tissues from which RNA was extracted. The *gob* aborted shoot and cotyledon base region was compared to two different wild-type (WT) samples, from the seedling apex (*WT-a*) and from the base of the cotyledons (WT-c). *Bottom* Levels of *Tkn1* and *Tkn2/LeT6* mRNA were assayed by RT-PCR. WT-a and WT-c represent RNA from two different WT tissues, as shown in the illustration. *TUBULIN* (*TUB*) expression was used as a control for the amounts of input RNA

Aborted meristems and less compound leaves in *expelled shoot* mutants

The *expelled shoot* (*exp*) mutant (Fig. 3) produces four to five leaves, followed by a termination of their meristems, sometimes with the production of abnormal, infertile flowers. On rare occasions, meristems recover repeatedly to produce an abnormal plant. Examination of $exp1^1$ SAMs and early leaf development showed that the SAM is flatter than that of the wild type, and that $exp1^1$ leaf primordia expand laterally and unfold at an earlier plastochron (P) than the wild type (compare Fig. 3g, h to c, d). These observations suggest that *exp* leaves either differentiate faster than the wild type, or initiate at a much slower rate than the wild type. *exp* leaves are less compound than those of the wild type (compare Fig. 3f, b). To further characterize this phenotype, the number of leaflets was compared between wild-type and *exp* leaves. Compound leaf shape has been previously characterized by five different criteria: primary, secondary, tertiary and intercalary leaflet counts, and the degree of lobing (Holtan and Hake 2003). The number of primary leaflets in mature *exp* leaves was similar to that in wild-type leaves, but secondary and intercalary leaflets were almost entirely absent in the mutants, and lobing was dramatically reduced (Figs. 3f, 4). Four independent *exp* mutants were identified in the screen. Complementation tests revealed that these represent two distinct loci (Table 1).

Reduced leaf compoundness and impaired meristem function in *short pedicel* mutants

The *short pedicel* (*spd*) mutant displays a packed and bushy overall plant architecture, short internodes and packed inflorescences, with very short pedicels (Fig. 5). The flowers are aberrant and infertile in most of the mutants of this group. Six independent *spd* mutants, which show a range of phenotypic severities, were identified in the screen.

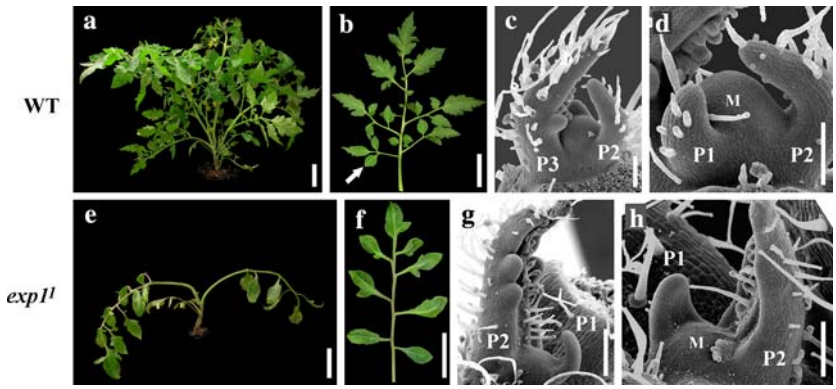

Fig. 3 Phenotypes of the *expelled shoot* (*exp*) mutants. **a** Two-month-old wild-type (WT) plant, showing continuous growth and organ production. **b** WT fifth leaf, with primary and secondary (*arrow*) leaflets. **c** Leaf production at the WT SAM, showing the structured initiation of leaf primordia, and primary leaflet initiation on the third youngest primordium. **d** Close-up of a WT SAM. **e** Two-month-old $exp1^1$ mutant plant. The meristem terminated following the formation of a few leaves. **f** $exp1^1$ fifth leaf, showing the nearly normal number of primary leaflets and the near absence of secondary leaflets. **g**, **h** Leaf production at the $exp1^1$ SAM. The SAM is flatter than that of the WT (compare **h** to **d**), and the developmental interval between two successive primordia is longer than in the WT (compare **g** to **c**). Plastochron (P) number designates the developmental stage of leaf primordia relative to the SAM, such that the latest emerging leaf is termed *P1*, the next oldest leaf *P2*, etc. *M*—SAM. **c**, **d**, **g**, **h** Scanning electron micrographs of shoot apices. *Scale bars*: 5 cm (**a**, **b**, **e**, **f**), 100 μm (**c**, **d**, **g**, **h**)

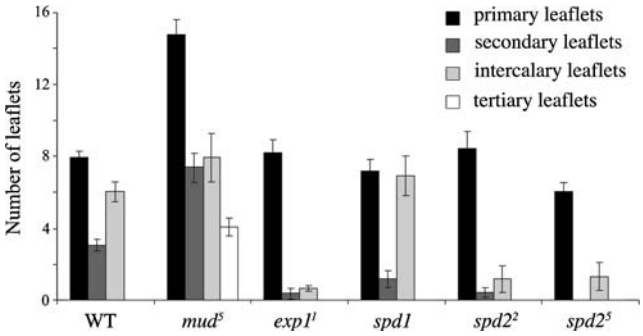

Fig. 4 Number of primary, secondary, tertiary and intercalary leaflets on leaves of the indicated genotypes. *Bars* represent means ± SE. For each genotype, leaflets were counted on the fifth leaf of ten individuals, except in *exp1* where leaflets were counted on the most compound leaf in ten individuals

Complementation tests indicated that these represent two independent loci, designated *spd1* and *spd2* (Table 1). The *spd2* complementation group consists of five alleles, which display a gradient of phenotypic severities. The *spd2*^{5} mutant shows the most severe phenotype. The plant is very short and bushy (Fig. 5l), due to the development of several independent shoots with reduced apical dominance. Inflorescences are packed due to extremely short pedicels and aberrant phylotaxis (Fig. 5n). Flowers are abnormal, with aberrant carpels and sometimes with abnormal carpeloid stamens (Fig. 5n, inset). Ectopic inflorescences are occasionally formed on the leaf petiole (Fig. 5o). Phenotypes of the *spd2*^{2} allele are similar to, but less severe than those of *spd2*^{5} (Fig. 5h–k). *spd1* is comprised of a single allele, and displays much milder phenotypes. *spd1* plants are bushy with packed inflorescences and short pedicels (Fig. 5e–g), but produce normal flowers and are fertile. SEM examination of *spd2*^{2} and *spd2*^{5} SAMs revealed an impaired, flat, structure (Fig. 5k, p). The SAM seems to abort after the production of several leaves, which is followed by the initiation of a new meristem from the periphery of the flat terminated meristem (Fig. 5k, p, arrows). This leads to the bushy, disorganized appearance of *spd* plants, in which many adjacent shoots develop simultaneously (Fig. 5h, l).

All *spd* mutants display extremely reduced or completely eliminated leaflet lobing, as well as a dramatic reduction in the number of secondary leaflets (Figs. 4, 5f, i, m), with no secondary leaflets in the extreme case of *spd2*^{5} (Figs. 4, 5m). Primary leaflet number is slightly reduced in *spd1* plants (Figs. 4, 5f), whereas in *spd2*^{5} leaves the proximal pair of primary leaflets is usually missing and the leaf petiole is much longer (Figs. 4, 5m). The number of intercalary leaflets is similar to the wild type in *spd1* mutants but

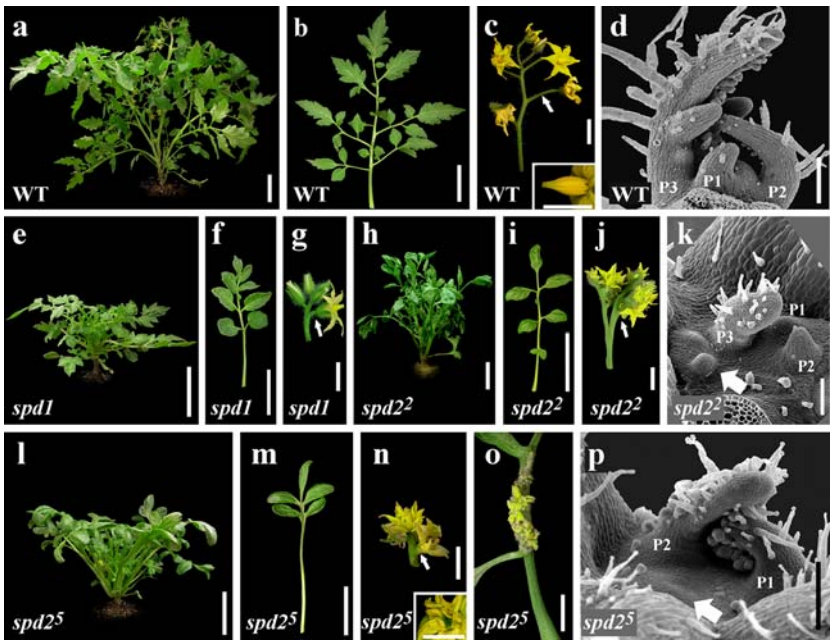

Fig. 5 The *short pedicel* (*spd*) mutants. **a-d** Wild-type (WT) whole plant (**a**), leaf (**b**), inflorescence (**c**), flower (**c**, *inset*) and SAM (**d**). Internode elongation results in an upright stature (**a**). The flower is positioned on a long pedicel (*arrow* in **c**). **e-p** Whole plants (**e**, **h**, **k**), leaves (**f**, **i**, **m**), inflorescences (**g**, **j**, **n**), flower (**n**, *inset*), leaf petiole (**o**) and SAM (**k**, **p**) of the different *spd* mutants, as indicated. *spd* plants are bushy with packed, disorganized, inflorescences and short pedicels (*arrows* in **g**, **j**, **n**). Leaves show a reduced level of compoundness. The *spd2*^{2} and *spd2*^{5} SAMs (**k**, **p**) are wide and flat, and abort after the production of a few leaves, followed by initiation of a new meristem from its periphery (*arrows*). *spd2*^{5} flowers are abnormal, with aberrant carpels and abnormal, sometimes carpeloid, stamens (**n**, *inset*). Ectopic inflorescences are occasionally formed on the *spd2*^{5} leaf petiole (**o**). **d**, **k**, **p** Scanning electron micrographs of shoot apices. *Scale bars*: 5 cm (**a**, **b**, **e**, **f**, **h**, **i**, **l**, **m**), 1 cm (**c**, **g**, **j**, **n**, **o**), 100 μm (**d**, **k**, **p**)

is reduced in *spd2*^{2} and *spd2*^{5} (Fig. 4). *spd* leaves are thus mainly affected in the higher orders of leaf compoundness.

The *multi drop* mutants display super-compound leaves

The striking correlation between meristem-maintenance and leaf-shape phenotypes prompted us to screen for mutants with more compound leaves. *multi drop* (*mud*) leaves have about twice as many primary and secondary leaflets as the wild type. The number of intercalary leaflets is slightly higher as well, and in contrast to wild-type leaves, *mud* leaves also show a third reiteration of leaflet formation (Figs. 4, 6b, d). In contrast to the striking increase in leaflet number, *mud* leaflets are rounder, and their margins less lobed than in the wild type (compare Fig. 6c, d). Examination of the *mud* SAM revealed that it tends to divide into two adjacent active meristems (compare Fig. 6e–g), leading to a bushy plant (Fig. 6i). Thus, the increase in the degree of leaf compoundness is correlated with increased and less controlled meristematic activity. Five independent *mud* alleles were identified in the screen, all showing similar phenotypes.

Leaves of the dominant *Mouse ears* (*Me*) mutation, in which *Tkn2/LeT6* is misexpressed, show an increased number of primary, secondary and tertiary leaflets with smooth leaflet margins, similar to *mud* leaves (Chen et al. 1997; Parnis et al. 1997) (Fig. 7b). To test whether *mud* and *Me* affect leaf compoundness through common or distinct mechanisms, we generated a double mutant among these mutations. Leaves of *mud Me* double mutants showed phenotypic characteristics of both single mutants, but were not more compound than either of the single mutants (Fig. 7a–c). Thus, *MUD* and *Tkn2/LeT6* likely affect leaf shape through a common mechanism.

Effect on *KNOXI* gene expression

To determine whether altered expression of *KNOXI* genes is involved in the leaf phenotypes of *exp*, *spd* and *mud* mutants, we used semi-quantitative RT-PCR to analyze the expression levels of *Tkn1* and *LeT6/Tkn2* in shoot apices, containing the SAM and young leaf primordia, of wild-type and mutant seedlings. *Tkn2/LeT6* mRNA levels were elevated relative to the wild type in *exp1*^{1} *spd2*^{5} and *spd1* mutants (Fig. 8), despite the aberrant SAM structure and reduced leaf compoundness. This elevation could be secondary to the developmental defects observed in these mutants. *Tkn1* levels were essentially unchanged in *exp1*^{1} *spd2*^{5} and *spd1* mutants. Thus, *KNOXI* expression is not always correlated with the degree of leaf compoundness, suggesting that additional, *KNOXI*-independent pathways are involved in this process. The *mud*^{5} mutant showed slightly elevated *Tkn1* and *Tkn2/LeT6* levels relative to the wild type. As a control, we tested the levels of these genes in *Me* seedlings, which misexpress *Tkn2/LeT6* due to a mutation in the *Tkn2/LeT6* locus (Chen et al. 1997; Parnis

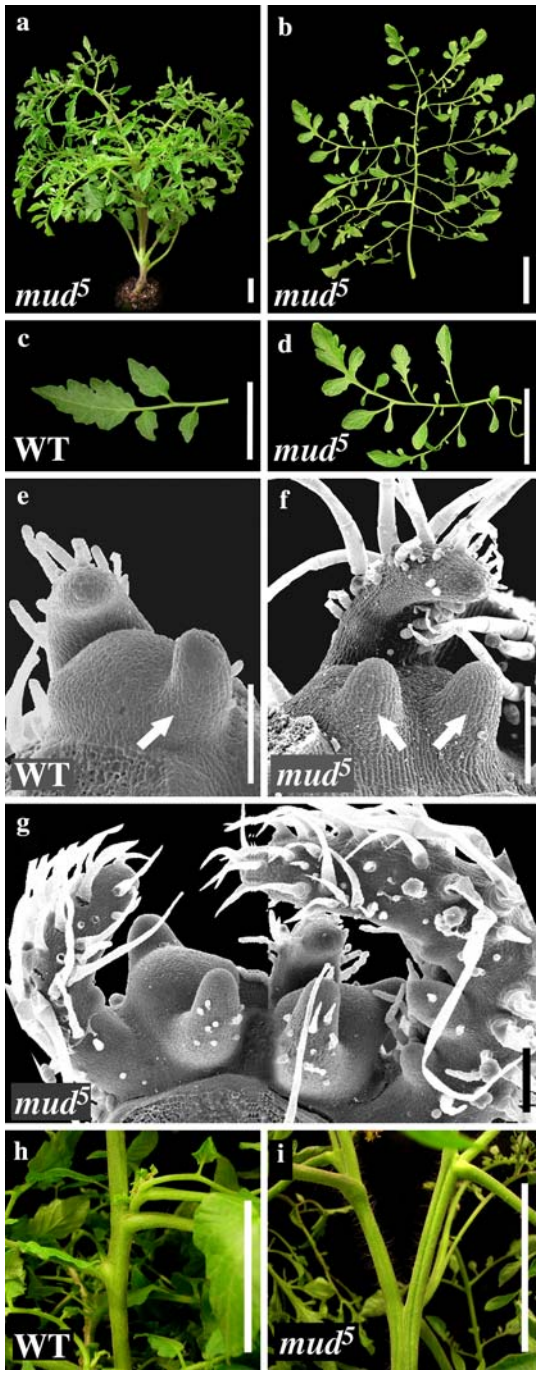

Fig. 6 Phenotypes of *multi drop* (*mud*) mutants. **a**, **b** *mud*^{5} whole plant and a leaf, respectively. **c**, **d** Wild-type (WT) (**c**) and *mud*^{5} (**d**) primary leaflets. **e-g** WT (**e**) and *mud*^{5} (**f**, **g**) SAMs. *Arrows* point to the P1 primordium in the WT (**e**), and to two simultaneously initiating P1 primordia in *mud*^{5} (**f**), representing SAM bifurcation (**g**). **h**, **i** WT and *mud*^{5} stems, respectively, showing the point of stem bifurcation in *mud*^{5}. **e–g** Scanning electron micrographs of shoot apices. *Scale bars*, 5 cm (**a–d**, **h**, **i**), 100 μm (**e–g**)

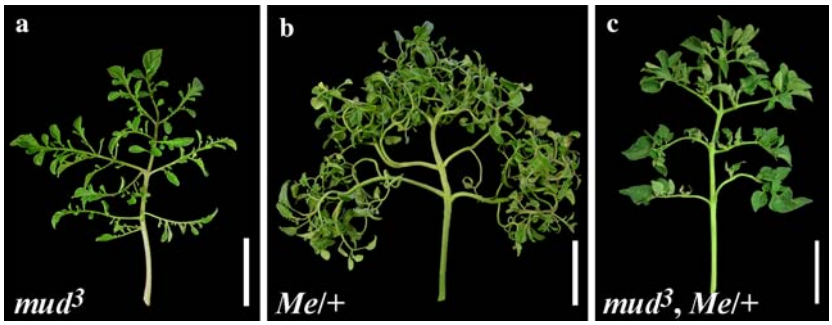

Fig. 7 Leaf phenotype of *Mouse ears* (*Me*) *mud* double mutants. Leaves of both single mutants (**a**, **b**) are more compound than the wild-type leaves. Leaves of the double mutant (**c**) show phenotypic characteristics of both single mutants but are not more compound than either of the single mutants

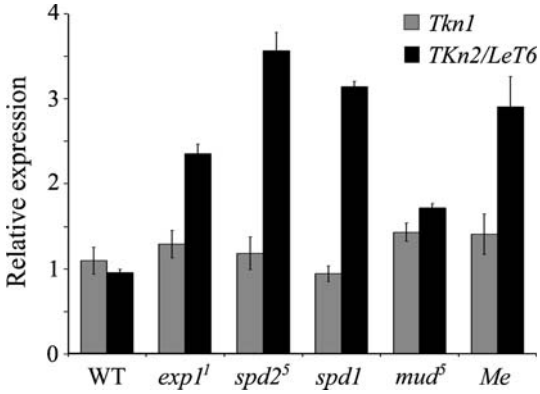

Fig. 8 *Tkn1* and *Tkn2/LeT6* mRNA levels in wild-type (WT) and mutant shoot apices. mRNA levels were assayed by RT-PCR. Expression was normalized relative to the *TUBULIN* (*TUB*) gene. Each bar represents the average ± standard error of three biological replicates

et al. 1997). As expected, *Me* mutants showed elevated *Tkn2/LeT6* levels.

Discussion

The presented results show that impaired SAM function is often accompanied by a respective alteration in the degree of leaf compoundness. These results imply that tomato employs common genetic mechanisms for SAM maintenance and leaf shaping. The described mutants affect differential aspects of leaf compoundness, indicating that the affected genes act successively to elaborate leaf shape.

Several tomato meristem-maintenance mutants have been described previously. The *defective embryo and meristems* (*dem*) tomato mutant shows variable cotyledon number and impaired SAM and root apical meristem structures (Keddie et al. 1998). The *DEM* gene encodes a protein with unknown function, and only one *dem* allele has been described. We did not recover any similar mutant in our screen, which could be because of the different genetic background, a different screening strategy or *dem* representing a unique event. The role of the *dem* gene in leaf patterning cannot be assessed due to SAM abortion at the seedling stage.

The tomato *polycotyledon* (*poc*) mutant is also affected in both meristem maintenance and leaf elaboration (Al-Hammadi et al. 2003). *spd* and *poc* mutants share some phenotypic characteristics, including short stature, packed inflorescences, smooth leaflet margins and the occasional ectopic meristems on the leaf rachis. However, there are also several differences, including the extremely short pedicels of the *spd* mutants, and the infertility of *spd2*. While *spd1* and *spd2*$^{1-4}$ seedlings have a normal number of cotyledons, most *spd2*^{5} seedlings produce three or four of them (not shown).

The partially dominant tomato *Lanceolate* (*La*) mutation has also been shown to affect both leaf patterning and meristem maintenance. *La* leaves are simple, and homozygous *La* plants show severe SAM-maintenance defects (Mathan and Jenkins 1960; Mathan and Jenkins 1962). We recently identified the *LA* gene and showed that its precocious expression in *La* mutant leaves leads to early differentiation of the leaf marginal blastozone (Ori et al. 2007).

Similarities and differences among species

A comparison with known mutants from Arabidopsis reveals that some of the mutants described here are very similar to known Arabidopsis mutants, whereas others are unique. Double mutants among genes from the *CUP SHAPED COTYLEDON* (*CUC*) family of Arabidopsis, or single mutants in the *NO APICAL MERISTEM* (*NAM*) from petunia show aborted meristems and fused cotyledons (Souer et al. 1996; Aida et al. 1997; Vroemen et al. 2003). These genes encode transcription factors from the NAC domain class. The phenotype of the *gob* mutant is very similar to that of *nam* and *cuc1 cuc2*. Similarities include the aborted meristem and the fused cotyledons and leaves, as well as the smooth leaflet margins (Nikovics et al. 2006). *spd* mutants share some characteristics with Arabidopsis *bp* mutants, including short pedicels and short internodes. However, there are also some marked differences, such as the near sterility of most *spd* mutants. The *exp* phenotypes are different from known Arabidopsis mutants, demonstrating that mutant screens in different

species may help identify new components that are required for SAM function, but have not been identified through mutant screens in Arabidopsis due to lethality or redundancy.

Patterning a compound leaf is a multistep developmental process

Comparison of the different phenotypes of the described mutants implies that the corresponding genes affect distinct aspects of the compound leaf's development (Fig. 9). While *GOB* affects the development of primary leaflets, *SPD* and *EXP* are not involved in primary leaflet development but are required for the generation of further levels of reiteration, as well as for the formation of lobed leaflet margins. Interestingly, *gob* meristems also abort much earlier than those of *exp* and *spd*.

Similar to meristems, where a balance of positive and negative factors acts to maintain a constant SAM structure, it seems that opposing genetic factors act to balance the degree of leaf compoundness (Fig. 9). In *mud* mutants, fractionation events at both the SAM and the developing leaf are less controlled, indicating that *MUD* acts to negatively control these processes. *MUD* affects both the number of primary leaflets and the degree of reiteration. In contrast, *mud* leaf margins are less lobed. This could result from different mechanisms affecting these processes or from some form of negative feedback.

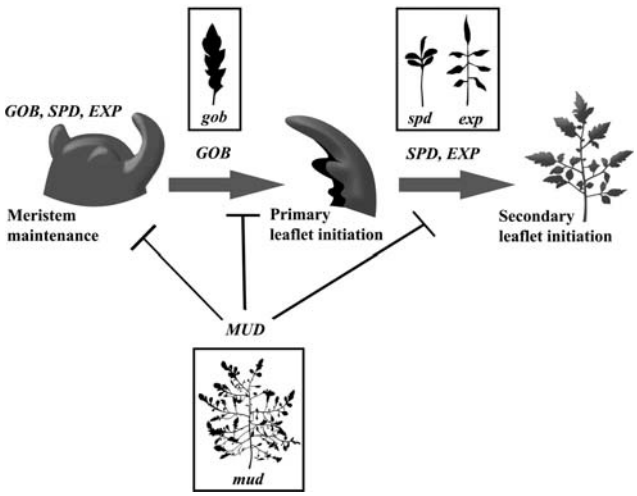

Fig. 9 A proposed model of the involvement of the described genes in SAM maintenance and compound-leaf patterning. *GOB*, *EXP* and *SPD* positively regulate SAM activity and leaf patterning, while *MUD* acts to negatively balance these processes. Patterning in the compound tomato leaf is a multistep process, in which each step involves different genetic factors. *GOB* is required for primary leaflet development, while *EXP* and *SPD* are involved in further degrees of leaf dissection. A schematic drawing of the respective mutant leaf phenotypes is shown for each of the genes

Common genetic mechanisms are involved in the function of the SAM and the leaf marginal blastozone

The link between SAM and leaf-shape phenotypes is in agreement with previous findings indicating a function for *KNOXI* genes in patterning compound leaves (Hareven et al. 1996; Bharathan et al. 2002; Tsiantis and Hay 2003; Kessler and Sinha 2004; Hay and Tsiantis 2006). This study extends these findings and suggests that along with *KNOXI* genes, additional genes are involved in both processes. Moreover, the finding that relative *Tkn2/LeT6* and *Tkn1* mRNA levels are not consistently correlated with the degree of leaf compoundness in a subset of the mutants described in this study implies that the corresponding genes affect SAM maintenance and leaf shape independently of *KNOXI*. However, further experiments will be required to understand the relationship between *KNOXI* genes and these mutants. In Arabidopsis, the *CUC2* gene, known to be essential for SAM maintenance, has been recently shown to be involved in the control of serration at the leaf margin as well (Nikovics et al. 2006). However, because Arabidopsis leaves are simple, the ability to use this plant to uncover the full extent of the effect of such factors on leaf shape is limited.

The correlation between SAM function and the degree of leaf compoundness is intriguing with respect to models suggesting that the process of leaflet initiation in compound leaves shares mechanistic similarities with leaf initiation on the flanks of meristems (Sachs 1969; Hagemann and Gleissberg 1996; Goliber et al. 1999; Hofer et al. 2001). According to Hagemann and Gleissberg (1996), a leaf initiates at the flanks of the SAM, by fractionation of the SAM into apical and lateral portions. The lateral portion retains organogenetic competence in its marginal blastozone (also called marginal meristem) during primary morphogenesis. Organogenetic competence is lost as the leaf undergoes histological differentiation at the expansion stage. These authors describe leaflet initiation in compound leaves as a reiteration of a similar fractionation process, generated by the marginal blastozone. In simple leaves such as those of tobacco, histological differentiation follows the growth of the marginal blastozone closely, such that it does not reach the required length for fractionation. Thus, the leaf's marginal blastozone has some common characteristics with the SAM (Hagemann and Gleissberg 1996). It is therefore intriguing that some genetic mechanisms are utilized by both the SAM and the leaf marginal blastozone. Indeed, our results suggest that in mutants with early SAM termination, the activity of the leaf marginal blastozone also terminates precociously. It should be borne in mind, however, that leaf and leaflet initiations differ in several ways. First, initiating leaves are very different from the SAM from which they are formed,

in both shape and function, while leaflets and leaves may have similar shapes and functions. Related to this, an axillary meristem is formed in the axils of leaves but not in the axils of leaflets. It is interesting to note, in this respect, that when *KNOXI* genes are misexpressed in Arabidopsis leaves, lobed leaves are formed and ectopic meristems sometimes form in the sinuses between lobes (Chuck et al. 1996; Ori et al. 2000). Thus, while normally no axillary meristems are formed in the axils of leaflets or lobes, these regions may have some common characteristics with the boundaries between the SAM and initiating leaves.

It should be borne in mind that since leaves are initiated from the SAM, the effect of the described mutations on leaf shape could be the result of aberrations in SAM structure, rather than the two phenotypes stemming from independent activities of the mutated genes. For example, the change in meristem structure or size observed in all mutants shown could affect the number of cells allocated for leaf initiation, and thus affect leaf development and leaf shape. In addition, the first few tomato leaves, as well as the last few leaves before flowering, are simpler than the rest of the leaves. The difference in leaf shape could thus result, for example, from the fewer leaves generated in the case of *exp*, or SAM reiteration in the case of *spd*. In the case of *spd* this is less likely, as leaves formed after the transition to flowering are also less compound than the wild-type leaves. While in most of the mutants described in this study both SAM maintenance and leaf shape were affected, some known mutants with simpler leaves, such as *entire* (*en*), *potato leaf* (*c*) *procera* and *solanifolia* (*sf*), have no obvious SAM abnormalities (Dengler 1984; Chandra-Sekhar 1990; Goliber et al. 1999; Peeters et al. 2002). Thus, some genes play unique roles in leaf dissection and are not required for SAM function. Alternatively, these genes may act redundantly in the SAM.

The dual roles of some genes in tomato SAM maintenance and leaf patterning may be useful for identifying additional genetic factors important for SAM balance. For example, genes with redundant roles in the SAM may be identified through mutants with altered leaf shape. Furthermore, mutants with increased leaf dissection may represent misexpression of genes required for both processes, and may serve to identify the misexpressed genes and/or their negative regulators (Chen et al. 1997; Goodrich et al. 1997; Parnis et al. 1997; Timmermans et al. 1999; Tsiantis et al. 1999; Ori et al. 2000; Byrne et al. 2002).

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