

# microRNA156-targeted SPL/SBP box transcription factors regulate tomato ovary and fruit development

Geraldo Felipe Ferreira e Silva<sup>1,2</sup>, Eder Marques Silva<sup>1</sup>, Mariana da Silva Azevedo<sup>3</sup>, Mike Anderson Corazon Guivin<sup>1</sup>, Daniel Alves Ramiro<sup>2</sup>, Cassia Regina Figueiredo<sup>3</sup>, Helaine Carrer<sup>2</sup>, Lázaro Eustáquio Pereira Peres<sup>3</sup> and Fabio Tebaldi Silveira Nogueira<sup>1,2,\*</sup>

<sup>1</sup>Laboratory of Molecular Genetics of Plant Development, Department of Genetics, Instituto de Biociências, State University of Sao Paulo, Botucatu, Sao Paulo 18618–970, Brazil,

<sup>2</sup>Centro de Biotecnologia Agrícola, Escola Superior de Agricultura 'Luiz de Queiroz', University of Sao Paulo, 13418–900 Piracicaba, Sao Paulo, Brazil, and

<sup>3</sup>Laboratory of Hormonal Control of Plant Development, Department of Biological Sciences, Escola Superior de Agricultura 'Luiz de Queiroz', University of Sao Paulo, 13418–900 Piracicaba, Sao Paulo, Brazil

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\*For correspondence (e-mail ftsnogue@ibb.unesp.br).

## SUMMARY

Fruit ripening in tomato (*Solanum lycopersicum* L.) is well understood at the molecular level. However, information regarding genetic pathways associated with tomato ovary and early fruit development is still lacking. Here, we investigate the possible role(s) of the microRNA156/*SQUAMOSA* promoter-binding protein-like (SPL or SBP box) module (miR156 node) in tomato ovary development. miR156-targeted *S. lycopersicum* SBP genes were dynamically expressed in developing flowers and ovaries, and miR156 was mainly expressed in meristematic tissues of the ovary, including placenta and ovules. Transgenic tomato cv. Micro-Tom plants over-expressing the *AtMIR156b* precursor exhibited abnormal flower and fruit morphology, with fruits characterized by growth of extra carpels and ectopic structures. Scanning electron microscopy and histological analyses showed the presence of meristem-like structures inside the ovaries, which are probably responsible for the ectopic organs. Interestingly, expression of genes associated with meristem maintenance and formation of new organs, such as *LeT6/TKN2* (a KNOX-like class I gene) and *GOBLET* (a NAM/CUC-like gene), was induced in developing ovaries of transgenic plants as well as in the ovaries of the natural mutant *Mouse ear (Me)*, which also displays fruits with extra carpels. Conversely, expression of the MADS box genes *MACROCALYX (MC)* and *FUL1/TDR4*, and the *LEAFY* ortholog *FALSIFLORA*, was repressed in the developing ovaries of miR156 over-expressors, suggesting similarities with *Arabidopsis* at this point of the miR156/SPL pathway but with distinct functional consequences in reproductive development. Altogether, these observations suggest that the miR156 node is involved in maintenance of the meristematic state of ovary tissues, thereby controlling initial steps of fleshy fruit development and determinacy.

**Keywords:** microRNA156, SPL/SBPs, fruit patterning, gynoecium, ovary, *Solanum lycopersicum*.

## INTRODUCTION

Fruits are structures that are derived from a mature ovary containing seeds, and comprise a variety of tissue types (Seymour *et al.*, 2013). The gynoecium is derived from the fusion of carpels, and research on *Arabidopsis thaliana*, which produces a dry fruit (the silique), has shown that several genetic pathways regulating carpel development also have roles during leaf development (Scutt *et al.*, 2006). In fact, the *Arabidopsis* ovary is formed from the carpels as a longitudinal cylinder with medio-lateral symmetry, supporting its origin as a fused leaf-like organ

(Seymour *et al.*, 2013). Most of the patterning of the fruit is established at the onset of gynoecium development (Girin *et al.*, 2009). However, shortly after anthesis, active cell division and establishment of tissue identity are still observed in developing ovaries of most plants (Gillaspy *et al.*, 1993).

Despite centuries of intensive genetic selection of agriculturally valuable fleshy fruits, most information regarding how these fruits initially develop and which genes control this process is still lacking. While gene regulatory

networks that are involved in fruit patterning and early growth have been largely unraveled for *Arabidopsis*, genetic studies on tomato (*Solanum lycopersicum* L.), a model plant for fleshy fruits, have focused primarily on unraveling the molecular basis of fruit ripening. The identification of genes such as *MADS-RIN* (Vrebalov *et al.*, 2002) and *COLORLESS NON-RIPENING (CNR)* (Manning *et al.*, 2006) revealed the existence of complex pathways that are capable of controlling fruit ripening. More recently, Karlova *et al.* (2011) showed that the transcription factor APET-ALA2a controls tomato fruit ripening via regulation of ethylene biosynthesis and signaling. In addition to ripening, genetic pathways associated with fruit size and shape in tomato have been investigated in recent years. For instance, the *Fw2.2* gene has been shown to act as a major regulator of fruit size and to encode a fruit-specific protein that negatively regulates mitosis (Cong and Tanksley, 2006). Genes controlling fruit shape in tomato have also been identified, such as *SUN* and *OVATE* (Liu *et al.*, 2002; Xiao *et al.*, 2008), which control fruit elongation shape, *FASCIATED (FAS)*, which is associated with flat-shaped fruit and the number of locules, and *LOCULE NUMBER (LC)*, which also regulates the number of locules (Cong *et al.*, 2008; Rodriguez *et al.*, 2011).

Transcription factors that control early steps of the development of lateral organs, such as leaves, also appear to have roles in tomato flower and fruit development. The *NO APICAL MERISTEM/CUP-SHAPED COTYLEDON (NAM/CUC)* gene *GOBLET (GOB)* is expressed in local, specific stripes that mark and precede initiation of leaflets. Ectopic *GOB* expression leads to formation of additional floral organs in each whorl, resulting in fruits with extra carpels (Berger *et al.*, 2009). Similarly, mis-regulation of the KNOX class I gene *TOMATO KNOTTED2 (LeT6/TKn2)* leads to enhanced organogenic activity, altering leaf and flower development as well as the final morphology of the fruits (Parnis *et al.*, 1997; Janssen *et al.*, 1998).

Together with transcription factors, plant microRNAs (miRNAs) integrate genetic networks to regulate development and reproduction (Axtell 2013). Recently, it has been shown that several miRNAs are differentially expressed during tomato fruit development, and thus probably play roles in this developmental process (Mohorianu *et al.*, 2011). Among them, microRNA156 shows a dynamic expression pattern during early stages of fruit development (Mohorianu *et al.*, 2011). miR156 targets most members of the *SQUAMOSA* promoter-binding protein-like (SPL or SBP box) family of plant-specific transcription factors (Schwab *et al.*, 2005). miR156 and its target *SPL* genes define a regulatory module (the miR156–SPL/SBP box node or simply the miR156 node; Rubio-Somoza and Weigel, 2011) that plays important roles in diverse aspects of plant development, including phase transition, plant architecture, trichome distribution, embryonic patterning and

anthocyanin biosynthesis (Schwab *et al.*, 2005; Chuck *et al.*, 2007; Wang *et al.*, 2009; Nodine and Bartel, 2010; Yu *et al.*, 2010; Gou *et al.*, 2011). Recently, 15 *SPL* family members were identified in tomato, and are named *S. lycopersicum SBP box (SlySBP)*. The transcripts of ten of these *SlySBP* genes carry putative miR156- and miR157-binding sites (Salinas *et al.*, 2012). Some of these transcripts were experimentally confirmed as targets for miR156/miR157 (Moxon *et al.*, 2008).

SPLs were firstly described as direct regulators of the expression of *MADS* box genes at early stages of flowering in *Antirrhinum majus* (Klein *et al.*, 1996). In *Arabidopsis*, miR156-targeted SPL3 positively and directly regulates the *MADS* box genes *APETALA1 (AP1)* and *FRUITFULL (FUL)* and the central regulator of flowering *LEAFY* (Yamaguchi *et al.*, 2009). Interestingly, *FUL* is a well-characterized regulator of cell differentiation during early stages of *Arabidopsis* fruit development (Gu *et al.*, 1998). Moreover, *FUL*-like genes appear to play a role in fruit development in two basal eudicot *Papaveraceae* species by promoting normal development of the fruit wall during fruit maturation (Pabon-Mora *et al.*, 2012). Expression of the tomato *MADS* box gene *FUL1/TDR4* is induced during ripening, and it is probably an ortholog of *AtFUL* (Seymour *et al.*, 2002, 2013; Bemer *et al.*, 2012). It has been speculated that *FUL1/TDR4* may be a direct target of *CNR*, a *SlySBP* family member (Bemer *et al.*, 2012). Another possible direct target of tomato SBPs is the *MADS* box gene *MACROCALYX (MC)*, whose product controls sepal and inflorescence development, and, together with the product of the *MADS* box gene *JOINTLESS*, regulates development of the tomato fruit abscission zone (Vrebalov *et al.*, 2002; Nakano *et al.*, 2012).

The miR156-targeted *SlySBP* gene *CNR* acts as a crucial factor controlling fruit ripening in tomato (Manning *et al.*, 2006; Moxon *et al.*, 2008), suggesting that the miR156 node may have roles during fruit development. Here, we found that several miR156-targeted *SlySBP* genes were differentially expressed in pre- and post-anthesis ovaries, indicating a dynamic regulation of these transcription factors during early stages of fruit development. Over-expression of *AtMIR156b* in tomato cultivar Micro-Tom (MT) led to down-regulation of most *SlySBP* genes in developing ovaries and an alteration of morphology, with formation of fruits containing ectopic organs such as extra carpels and leaf-like structures. Such developmental modifications may be a result of the observed mis-regulation of *LeT6/TKn2* and *GOB* genes. Furthermore, the transcript levels of *MC* and *FUL1/TDR4* as well as *FALSIFLORA (FA)*, an *LEAFY* ortholog were severely reduced in transgenic developing ovaries. These results indicate similarities with *Arabidopsis* gene regulation at this point of the miR156/SPL node, but with distinct functional consequences in tomato ovary and fruit development. Taken together, our results indicate an

additional function for miR156 and its targets in regulating fleshy fruit development.

## RESULTS

### Expression patterns of *SlySBP* genes and *miR156* in developing flowers and ovaries

Although tomato fruit development initiates with the restart of the ovary growth, induced by pollination and fertilization events, changes in fruit architecture may be determined in the ovary prior to anthesis or during the early stages of its growth (Cong *et al.*, 2008). To initially address possible functions of miR156-targeted *SlySBP* genes in fruit development, we examined their expression profiles in floral and fruit tissues by analyzing publicly available RNA-seq data. Some miR156-targeted *SlySBP* genes showed preferential expression patterns, with *SlySBP3* being the most highly expressed *SlySBP* in flower buds (Figure S1a). To investigate possible roles of the miR156 node in the early events of flower development, we evaluated the expression of miR156 and *SlySBP3* in buds of MT tomato by *in situ* hybridization. miR156 transcripts were localized in placental, ovule primordial and pollen mother cell tissues (Figure 1a,b). Similarly, *SlySBP3* mRNA was detected, but at lower levels, in the placenta and pollen mother cell tissues of young buds (Figure 1c). The absence of control probe hybridization signals (Figure 1d) confirmed the specificity of the observed patterns.

We then analyzed the expression profiles of several miR156-targeted *SlySBP* genes in pre- and post-anthesis stages of developing ovaries. All *SlySBP* genes evaluated were expressed in developing ovaries (Figure 1e). Although *SlySBP3* and *SlySBP13* transcripts accumulate at higher levels in pre-anthesis ovaries, *SlySBP2*, *SlySBP6a* and *SlySBP15* were more highly expressed in post-anthesis developing ovaries. *CNR* and *SlySBP10* genes showed slight variations in transcript levels between pre- and post-anthesis stages (Figure 1e). Interestingly, we detected comparable levels of mature miR156 transcripts in pre- and post-anthesis developing ovaries (data not shown). Similar results comparing the expression of *SlySBP* genes and miR156/miR157 have been reported during fruit ripening (Moxon *et al.*, 2008; Salinas *et al.*, 2012).

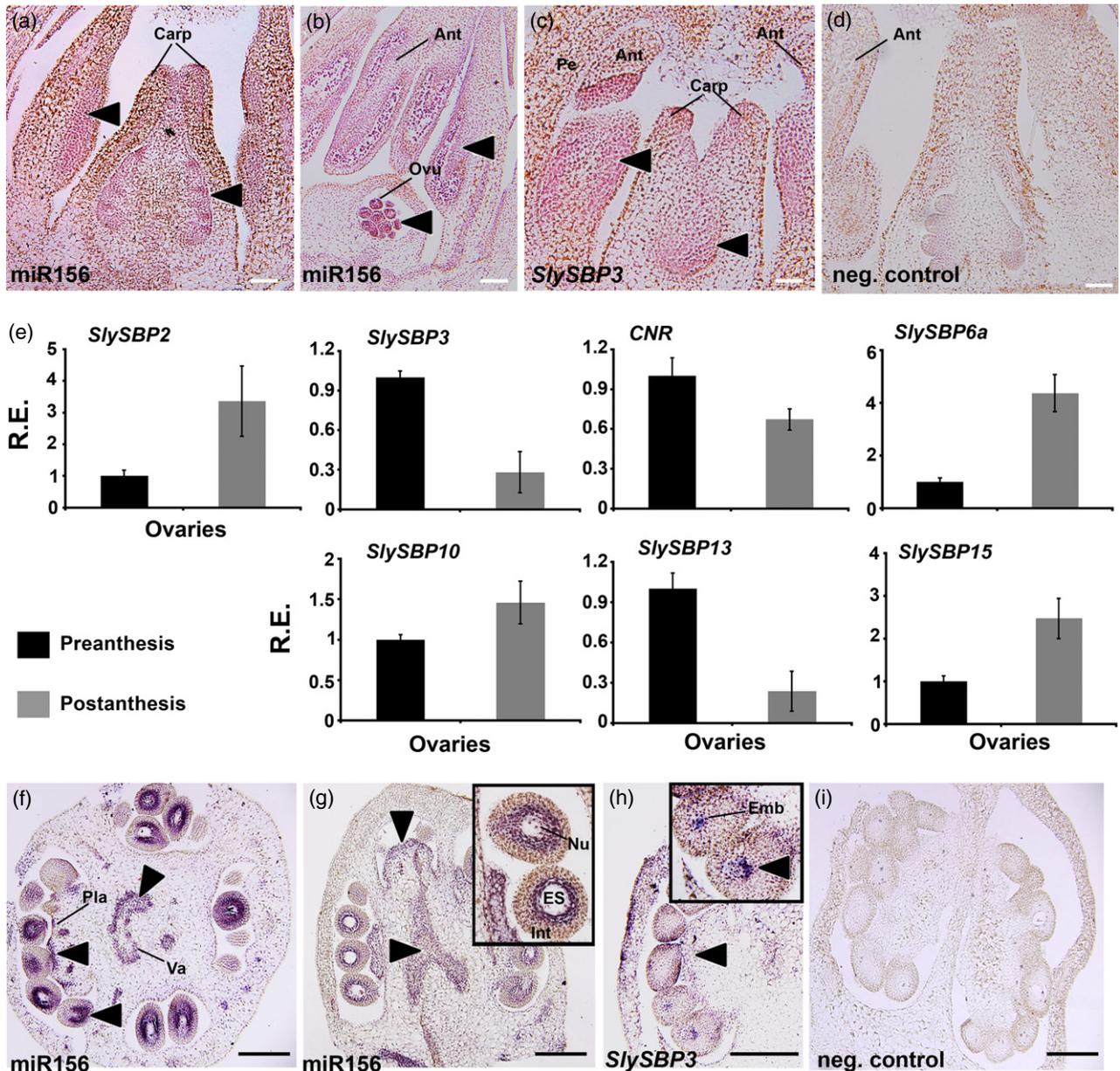
To obtain further insights into the possible roles of miR156 node during early stages of ovary and fruit development, we investigated the spatial and temporal patterns of miR156 and *SlySBP3* in developing ovaries by *in situ* hybridization. miR156 transcripts accumulated at higher levels in meristematic tissues in developing ovaries at pre- or post-anthesis, including sub-epidermal cells of the placenta and the inner part of the integument of developing ovules. miR156 transcripts were also strongly detected in the vascular bundles in the columella, with weak expression throughout other ovary tissues (Figure 1f,g). *SlySBP3*

was also weakly expressed throughout ovary tissues, including the placenta, but this expression was still consistently stronger than in the control (Figure 1h,i). In developing ovules, *SlySBP3* expression was confined to the developing embryos (Figure 1h, inset), and its transcripts were barely detected in the integument. This expression pattern is complementary to that of miR156 in developing ovules (Figure 1f,g). Together, RNA expression analyses on buds and developing ovaries suggest that miR156 and its targets are expressed in early events of ovary development, mostly in meristematic tissues of the ovules and placenta (at least for *SlySBP3*).

### Over-expression of *AtMIR156b* dramatically alters tomato fruit morphology

miR156 family is one of eight highly conserved miRNA families in plants (Cuperus *et al.*, 2011). Accordingly, miR156 sequences are highly conserved between tomato and *Arabidopsis* (Figure S1b). However, the tomato precursor sequences deposited in miRbase version 20 (<http://www.mirbase.org>) (namely *SlyMIR156a-c*), as well as an expressed sequence tag (EST) used to generate transgenic tomato plants a previous study (Zhang *et al.*, 2011), are similar to *AtmiR157* sequences (Figure S1b). These similarities with miR157 include an extra 5' uracil and two internal nucleotide substitutions in at positions 12 and 15, which clearly discriminate miR156 and miR157 sequences in tomato and *Arabidopsis* (Figure S1b). The differences in sequence and expression levels between miR156 and miR157 in tomato (Salinas *et al.*, 2012) may affect their miRNA/mRNA duplex interactions in specific cell/tissue types (Palatnik *et al.*, 2007), although this has not been formally tested for these miRNAs.

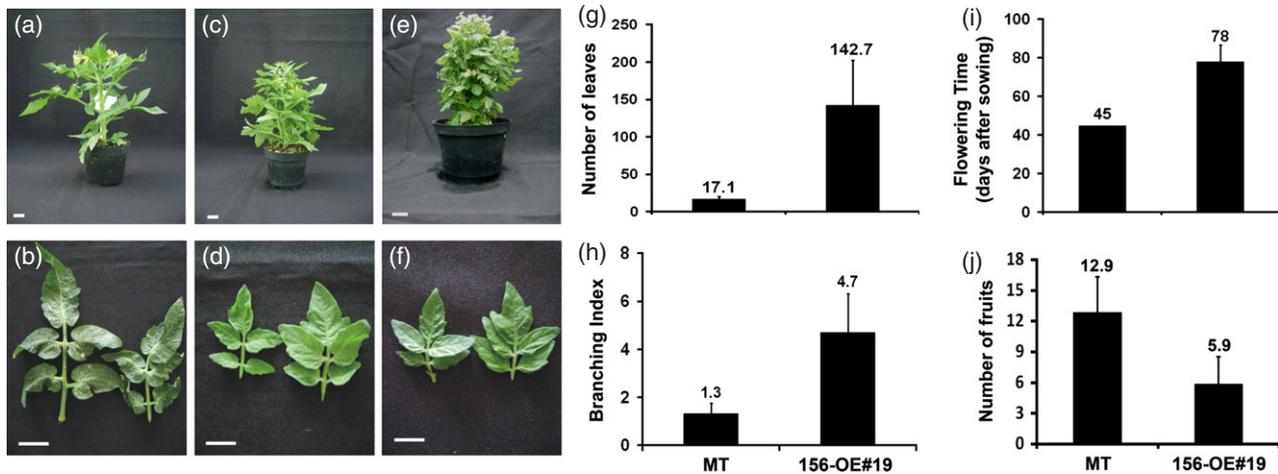
Novel miR156 precursors in tomato have recently been identified *in silico* from genomic sequences of the tomato WGS scaffold database (<http://solgenomics.net/>; Salinas *et al.*, 2012), although there has been no support for these loci to be expressed by cDNAs or ESTs in public databases so far. Over-expression of the *Arabidopsis MIR156b* gene (Schwab *et al.*, 2005) was shown to strongly reduce transcript levels of miR156-targeted *SPL* genes in distinct plant species, and led to similar vegetative and reproductive phenotypes (Wei *et al.*, 2010; Wang *et al.*, 2011). To investigate the functional role(s) of miR156-targeted *SlySBP* genes, we generated several independent transgenic tomato cv. MT plants over-expressing *AtMIR156b* (abbreviated as 156-OE). In agreement with previous reports (Schwab *et al.*, 2005), tomato 156-OE plants showed reduced levels of *SlySBP* transcripts in leaves (Figure S1c) and altered vegetative architecture (Figures 2 and S2a). Compared with MT plants, 156-OE plants produced a higher number of small, pale-green leaves (Figure 2a-g). Thus, as has been shown for other plant species (Xie *et al.*, 2012), over-expression of miR156 affects leaf development



**Figure 1.** Expression patterns of *SlySBP* genes and miR156 in flower buds and developing ovaries. (a,b) A 3'-labeled LNA-modified oligonucleotide detecting miR156 was hybridized with longitudinal sections of MT flowers at developmental stages 8 and 9. (c) A digoxigenin-labeled probe detecting *SlySBP3* transcripts was hybridized with longitudinal sections of MT flowers at developmental stage 6. (e) Comparative expression analysis of *SlySBP* genes in pre- and post-anthesis ovaries from tomato cv. MT. The quantitative RT-PCR experiments used tissues from ovaries at pre-anthesis as the reference sample (set to 1.0). Error bars indicate standard deviation of three biological samples. RE, relative expression. (f) A 3'-labeled LNA-modified oligonucleotide detecting miR156 was hybridized with transverse sections of pre-anthesis MT ovaries. (g) A 3'-labeled LNA-modified oligonucleotide detecting miR156 was hybridized with longitudinal sections of post-anthesis MT ovaries. The inset shows miR156 expression in developing ovules. (h) A digoxigenin-labeled probe detecting *SlySBP3* transcripts was hybridized with longitudinal sections of post-anthesis MT ovaries. The inset shows *SlySBP3* expression in developing embryos. (d,i) A 3'-labeled scrambled miR probe was used as a negative control. Purple staining shows probe localization (arrowheads). Scale bars = 10  $\mu$ m (a-d) and 20  $\mu$ m (f-i). Carp, carpel; Ant, anther; Pe, petal; Ovu, ovule; Pla, placenta; Va, vascular tissues; Nu, nucellar tissues; ES, embryo sac; Emb, Embryo; Int, integument.

in tomato (Figure S2b). Axillary shoots developed vigorously, and almost every leaf axil formed a new shoot (Figure 2c,e), leading to a substantial increase in the branching index in 156-OE plants compared with MT

plants (Figure 2h). Flowering time was delayed, and the number of fruits was reduced in tomato 156-OE plants (Figure 2i,j), similar to phenotypes described previously (Zhang *et al.*, 2011).



**Figure 2.** Phenotypes of tomato plants over-expressing *AtMIR156b*.

(a,b) Plants (a) and leaves (b) of tomato cv. MT at 87 days old.

(c,d) Plant (c) and leaves (d) of 156-OE line 19 at 87 days old.

(e,f) Plant (e) and leaves (f) of 156-OE line 1 at 87 days old.

(g) Number of leaves measured 87 days after sowing.

(h) Branching index measured 87 days after sowing.

(i) Flowering time.

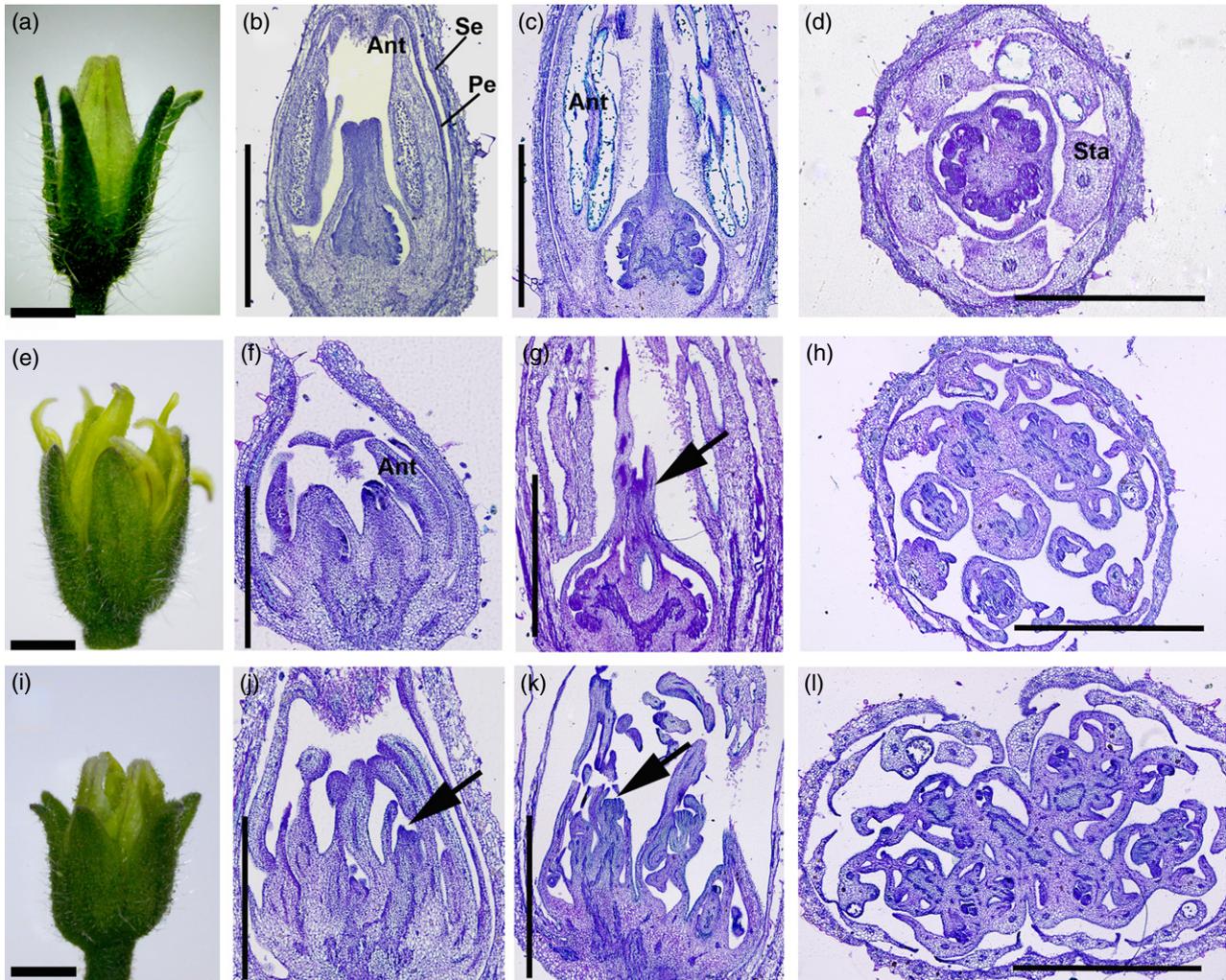
(j) Number of fruits.

The numbers above each bar indicate the mean values. Error bars indicate standard deviation;  $n = 10$  (g–i) or 30 (j). Scale bars = 2 cm.

Strikingly, fruits from all 156-OE transgenic lines displayed an altered morphology in which fruit-like structures emerged from the styler end of the main fruit. Additionally, several lines showed formation of vegetative-like organs that protruded from the fruits (Figure S3a). These data suggest that 156-OE gynoecia and fruits present variable degrees of abnormal growth and indeterminacy, which are correlated with the levels of miR156 transcripts in developing ovaries as well as in immature fruits of 156-OE plants (Figure S3b). The phenotypes of the fruits suggested abnormal growth of 156-OE flowers. Similarly to an *Arabidopsis* miR156b over-expressor (Schwab *et al.*, 2005), tomato 156-OE plants had flowers with a squashed appearance (Figure 3e,i) compared with MT (Figure 3a). However, they also displayed additional morphological modifications that are not observed in flowers of MT plants (Figure 3b–d). Such modifications include the presence of extra whorls and meristem-like structures, which are already present in early developmental stages of buds (stage 6, Figure 3f,j). Later on in development (stage 12), only flowers from lines weakly over-expressing miR156 were able to form ovary-like structures with few ovules (Figure 3g,h). Lines moderately over-expressing miR156 displayed ectopic structures in place of ovary and ovules, leading to indetermination of floral structures (Figure 3k,l). Thus, over-expression of miR156 in tomato MT appeared to enhance the phase of floral meristem proliferation in relation to organ initiation, such that when organs finally formed, the meristem was able to produce more floral whorls than normal.

These flower modifications correlate with formation of additional partially fused carpels in 156-OE plants (Figure 4b,c), but not in MT plants (Figure 4a). Abnormal carpel structures probably account for the appearance of 156-OE fruits (Figure 4h,i). The carpel of cultivated tomato usually comprises several ovule-containing locules arranged side by side (Hayward, 1938), which give rise to a rounded fruit (Figure 4g). A longitudinal section of MT tomato carpels revealed one or two locules (depending on the orientation of the section) that contain several ovules (Figure 4d). In weak 156-OE lines (represented by line 19), the partially fused extra carpels showed one or two locules containing few ovules with occasional ectopic meristem-like structures (Figure 4e), which may give rise to ectopic fruits (Figure 4h). Moderate 156-OE lines (represented by line 1) often displayed indeterminate carpels with no noticeable locules and the presence of at least one ectopic meristem-like structure per carpel (Figure 4f). Similar defects were observed for strong 156-OE lines (Figure S3a).

Whereas most tomato MT fruits displayed three locules in transverse slices (Figure 5a,d), fruits of weak 156-OE lines showed a higher and more variable number of locules (Figure 5b,e), with fewer but viable seeds (Figure 5f). The small number of seeds observed in the fruits of line 19 was probably due to the smaller number of ovules produced by the placenta. Fruits from moderate and strong 156-OE lines are seedless (Figure 5c). Scanning electron microscopy analysis of MT (Figure 5g) and 156-OE carpels confirmed the development of partially fused extra carpels in individual flowers of weak 156-OE lines. In



**Figure 3.** Flower phenotypes of tomato plants over-expressing *AtMIR156b*.

Flower phenotypes of MT plants (a–d), 156–OE line 19 plants (e–h) and 156–OE line 1 plants (i–l). (b,f,j) Longitudinal sections of flowers at developmental stages 6–8 from MT (b), 156–OE line 19 (f) and 156–OE line 1 (j). (c,g,k) Longitudinal sections of flowers at developmental stages 11–12 from MT (c), 156–OE line 19 (g) and 156–OE line 1 (k). (d,h,l) Transverse sections of flowers from MT (d), 156–OE line 19 (h) and 156–OE line 1 (l) harvested between developmental stages 11 and 12. Arrows indicate meristem-like structures. Se, sepal; Pe, petal; Ant, anther; Sta, stamen. Scale bars = 2 mm (a,e,i) and 50  $\mu$ m (b–d,f–h,j–l).

general, two of three carpels were enlarged (Figure 5h), indicating proper development of these carpels only. Additionally, formation of ectopic structures was often observed in carpels of the moderate 156–OE line (Figure 5i). Occasionally, gynoecea of weak 156–OE lines also give rise to partially fused leaf-like organs whose epidermal surface was covered by several developing trichomes (Figure 5j), as commonly found in leaf primordia, stem and sepals of cultivated tomato.

Although the tomato cultivar MT is useful for research purposes because of its size and relatively short generation time, it harbors four independent mutations (Marti *et al.*, 2006) that may influence tomato development. To investigate whether the phenotypes observed in 156–OE plants were reproducible in a non-dwarf cultivar, we crossed the

weak transgenic lines 18 and 19 with plants of the non-dwarf cultivar ‘Santa Clara’ (a commercial cultivar that is widely planted in Brazil), and determined the vegetative and reproductive phenotypes of the  $F_1$  offspring in which the recessive mutations of MT are in the heterozygous form. The wild-type progeny from the cross showed normal vegetative architecture, but those expressing the 35S:*AtMIR156b* transgene had a bushy appearance with a higher number of smaller leaves (Figure S4), similar to the 156–OE lines (Figure 2). Most importantly, fruit phenotypes in the large-fruited hybrid offspring were comparable to those from MT weak transgenic lines harboring the 35S:*AtMIR156b* construct (Figure 4 h), indicating that miR156 over-expression was effective in altering fruit development in the hybrid background (Figure S4).



**Figure 4.** Reproductive phenotypes of tomato plants over-expressing *AtMIR156b*.

(a) Carpels of MT plants.

(b) Carpels of 156-OE line 19 plants.

(c) Carpels of 156-OE line 1 plants.

(d–f) Longitudinal sections of ovaries of MT (d), 156-OE line 19 (e) and 156-OE line 1 (f). Arrows indicate meristem-like structures. The inset in (e) shows an ectopic meristem formed in the styler end of a representative ovary.

(g) MT fruit.

(h, i) Fruits of 156-OE line 19 and 156-OE line 1 plants, respectively, which contain fruit-like structures protruding from their styler end.

Scale bars = 2 mm (a–c) and 20  $\mu\text{m}$  (d–f).

#### Mis-regulation of *LeT6/TKn2* and *GOBLET* genes in developing ovaries of 156-OE and homozygous Mouse ear mutant plants

Most *SlySBP* genes were strongly down-regulated at variable levels in developing ovaries of 156-OE plants and young fruits (Figures 6 and S5). Over-expression of miR156 in tomato altered the temporal expression patterns of miR156-targeted *SlySBP* genes observed during ovary development (Figure 1e), and probably affected their spatial expression as well (Figure 6).

This disruption of temporal expression of miR156-targeted *SlySBP* genes in 156-OE ovary tissues may affect the expression of downstream targets of the miR156 node. The aforementioned phenotypic alterations in flowers and ovaries suggested that 156-OE lines are defective, at least in part, in proper expression of tomato KNOX genes such

as *LeT6/TKn2*. To determine whether *LeT6/TKn2* expression is directly or indirectly regulated by the miR156 node, we performed quantitative RT-PCR analyses in developing ovaries of representative weak and moderate 156-OE lines, as well as the near-isogenic *Me* homozygous mutant (*Me/Me*) (Lombardi-Crestana *et al.*, 2012). The *Me* mutation is a gene fusion that causes ectopic over-expression of *LeT6/TKn2* and leads to abnormal flower and fruit development (Parnis *et al.*, 1997).

Previous reports showed that *LeT6/TKn2* expression in tomato wild-type ovaries is severely reduced at the post-anthesis stage (Parnis *et al.*, 1997; Avivi *et al.*, 2000). Conversely, *LeT6/TKn2* was highly expressed in both pre- and post-anthesis ovaries of 156-OE lines and *Me* homozygous plants (Figure 7a). *LeT6/TKn2* mis-expression may account for the abnormal development of 156-OE fruits, as similar

**Figure 5.** Immature fruit phenotypes and scanning electron micrographs of ovaries from tomato cv. MT and plants over-expressing *AtMIR156b*.

(a–c) Transverse slices of immature fruits from MT (a), 156-OE line 19 (b) and 156-OE line 1 (c).

(d,e) Percentages of fruits with various numbers of locules (LN) in MT (d) and 156-OE line 19 (e) ( $n = 30$  plants).

(f) Mean seed number. Error bars indicate standard deviation ( $n = 30$  plants).

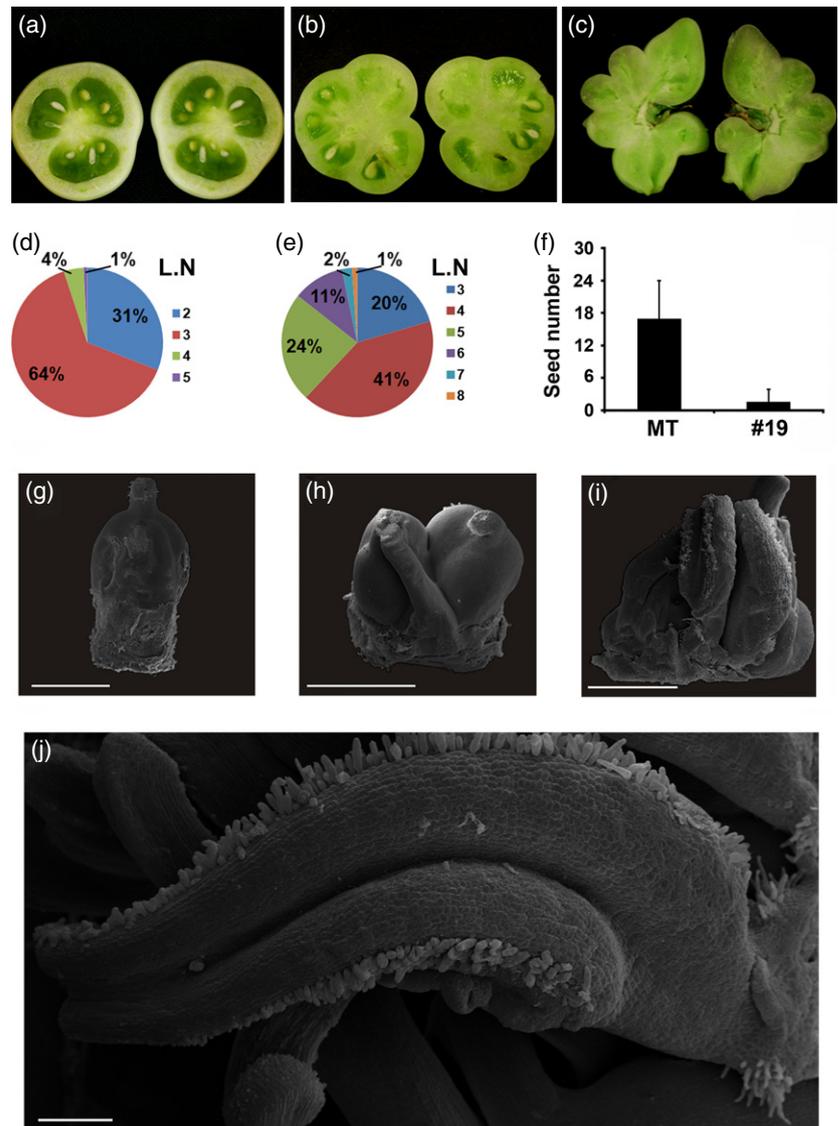
(g) MT gynoecium, showing one ovary per flower.

(h) Gynoecium of 156-OE line 19 with extra carpels.

(i) Gynoecium of 156-OE line 1 showing ectopic structures.

(j) Occasional leaf-like structures with developing trichomes growing from the gynoecium of 156-OE line 19 gynoecium.

Scale bars = 200  $\mu$ m (j) and 1 mm (g–i).

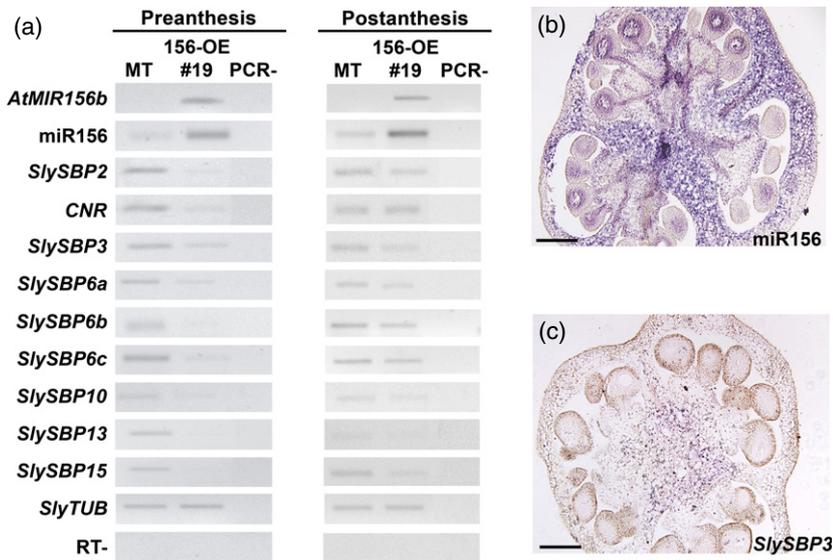


developmental defects were often observed in *Me* homozygous plants (Figure 7b,c). A recessive tomato mutant, *clausa* (*clau*), also mis-expresses *LeT6/TKn2* in post-anthesis ovaries, and frequently displays ectopic meristems in place of ovules (Avivi *et al.*, 2000). It is possible that up-regulation of *LeT6/TKn2* expression in developing 156-OE ovaries also leads to formation of the observed ectopic meristems (Figures 3 and 4).

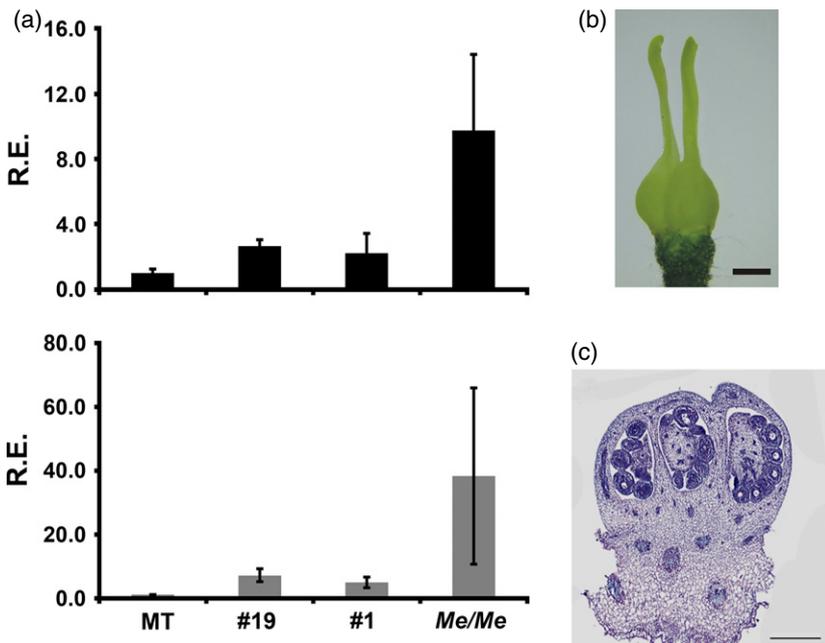
*GOBLET* (*GOB*) encodes a NAM/CUC homolog that is necessary for the proper specification of organ boundaries throughout tomato development. To obtain further insight into the possible roles of *GOB* in gynoecia development, we performed RNA *in situ* hybridizations with *GOB* antisense probe in longitudinal and transverse sections of MT flower buds at stages 6–12 (Figure 8a,b). In young buds (stage 6), *GOB* transcripts were detected at the boundaries of placental tissues and at the boundaries between inner

and outer regions of the anthers (Figure 8a,b). Later in development, *GOB* was also expressed in megasporocytes (Figure 8b).

Such expression patterns suggest that *GOB* is mainly implicated in whorl boundary formation. Indeed, flowers of the gain-of-function *GOB* mutant *Gob-4d*, which ectopically expresses *GOB*, have more floral organs per whorl, and ectopic carpels are often produced inside the fruits (Berger *et al.*, 2009). These abnormal features of flowers and fruits resembled those observed in our tomato 156-OE lines and in the homozygous *Me* mutant, and *GOB* expression is indeed up-regulated in pre-anthesis ovaries of 156-OE lines and *Me* plants (Figure 8d). As Arabidopsis KNOX and NAM/CUC genes are required for proper carpel and ovule development (Ishida *et al.*, 2000; Pautot *et al.*, 2001; Scofield *et al.*, 2007), it is reasonable to assume that their orthologs have conserved functions in tomato ovary



**Figure 6.** Expression of miR156-targeted *SlySBP* genes in developing ovaries of MT and 156-OE line 19 plants. (a) Detection of the *AtMIR156b* precursor, miR156 and miR156-targeted *SlySBP* transcripts by RT-PCR in ovaries at pre- and post-anthesis stages. (b) A 3'-labeled LNA-modified oligonucleotide detecting miR156 was hybridized with transverse sections of post-anthesis ovaries of 156-OE line 19. (c) A digoxigenin-labeled probe detecting *SlySBP3* transcripts was hybridized with transverse sections of post-anthesis ovaries of 156-OE line 19. Scale bars = 20 μm.



**Figure 7.** Expression of *LeT6/TKn2* in developing ovaries of MT, *AtMIR156b* over-expressing plants, and the homozygous *Me* mutant. (a) Detection of *LeT6/TKn2* transcripts by quantitative RT-PCR in pre-anthesis ovaries (top) and post-anthesis ovaries (bottom). (b,c) Extra carpels (b) and longitudinal section (c) of a post-anthesis ovary of the *Me* homozygous mutant. Scale bars = 2 mm (b) and 20 μm (c).

development. Thus, mis-expression of both *LeT6/TKn2* and *GOB* may be partly responsible for the formation of extra carpels and ectopic meristem activity observed in 156-OE ovaries and fruits.

The regulation of *GOB* expression by miR156-targeted *SlySBP* genes may be indirect, as *GOB* is post-transcriptionally regulated by miRNA164 (Berger *et al.*, 2009). Interestingly, tomato miR164 is down-regulated in 156-OE pre-anthesis ovaries (Figure 8e), which suggests that the miR156 node may indirectly regulate *GOB* expression via miR164. Recently, expression of miR164 was shown to be repressed and that of its targets was up-regulated in devel-

oping leaves of rice (*Oryza sativa*) transgenic plants that over-express miR156 (Xie *et al.*, 2012).

To examine the possible roles of the miR164/*GOB* pathway in MT fruit development, we generated transgenic plants harboring a *35S:AtMIR164a* construct (abbreviated as 164-OE, Spinelli *et al.*, 2011). *GOB* is down-regulated in pre-anthesis ovaries of 164-OE plants, and the styler ends of transgenic fruits are fused (Figure 8f,g). In addition, most fruits from 164-OE plants (80%, *n* = 30) had only two locules (Figure 8 h), as opposed to three to four locules as observed for MT or four to seven locules as observed in transverse slices of 156-OE fruits (Figure 5). These

**Figure 8.** Expression of *GOB* in flower buds and developing ovaries.

(a,b) A digoxigenin-labeled probe detecting *GOB* transcripts was hybridized with longitudinal (a) and transverse (b) sections of MT flowers at developmental stages 6 and 12, respectively. Arrowheads indicate hybridization signals. Scale bars = 10  $\mu$ m. Carp, carpel; Ant, anther; Pe, petal; Ovu, ovule.

(c) A 3'-labeled scrambled miR probe was used as a negative control.

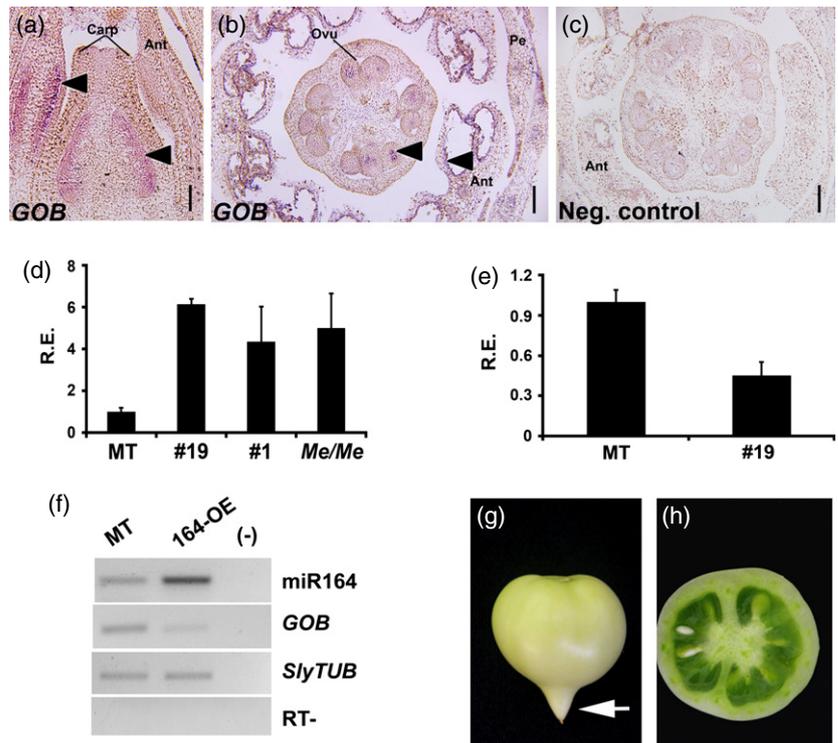
(d) Detection of *GOB* transcripts by quantitative RT-PCR in pre-anthesis ovaries of MT, *AtMIR156b* over-expressing plants, and *Me/Me* plants.

(e) Detection of miR164 transcripts by quantitative RT-PCR in pre-anthesis ovaries of MT and 156-OE line 19 plants.

The quantitative RT-PCR experiments used ovary tissues from MT as the reference sample (set to 1.0). Error bars indicate the standard deviation of three biological samples. RE, relative expression.

(f) Expression patterns of miR164 and *GOB* in MT and 164-OE pre-anthesis ovaries.

(g,h) Immature fruits from 164-OE transgenic plants. The arrow indicates the fused stylar end.



phenotypes contrast with those observed in 156-OE fruits, and indicate that miR164 over-expression in MT leads to inter-whorl fusions, as previously reported (Hendelman *et al.*, 2013). Moreover, the defects found in fruits of the *Gob-4d* mutant are caused by expression of a miR164-resistant version of the *GOB* gene (Berger *et al.*, 2009). Together with the evidence that *GOB* is central to formation of tomato floral organ boundaries (Berger *et al.*, 2009), our data indicate that miR156-targeted *SlySBP* genes may positively regulate miR164 levels in floral organs to maintain low expression levels of *GOB* during tomato reproductive development.

#### Expression of the MADS box genes *MC* and *FUL1/TDR4* as well as *FALSIFLORA* is repressed in developing ovaries of 156-OE plants

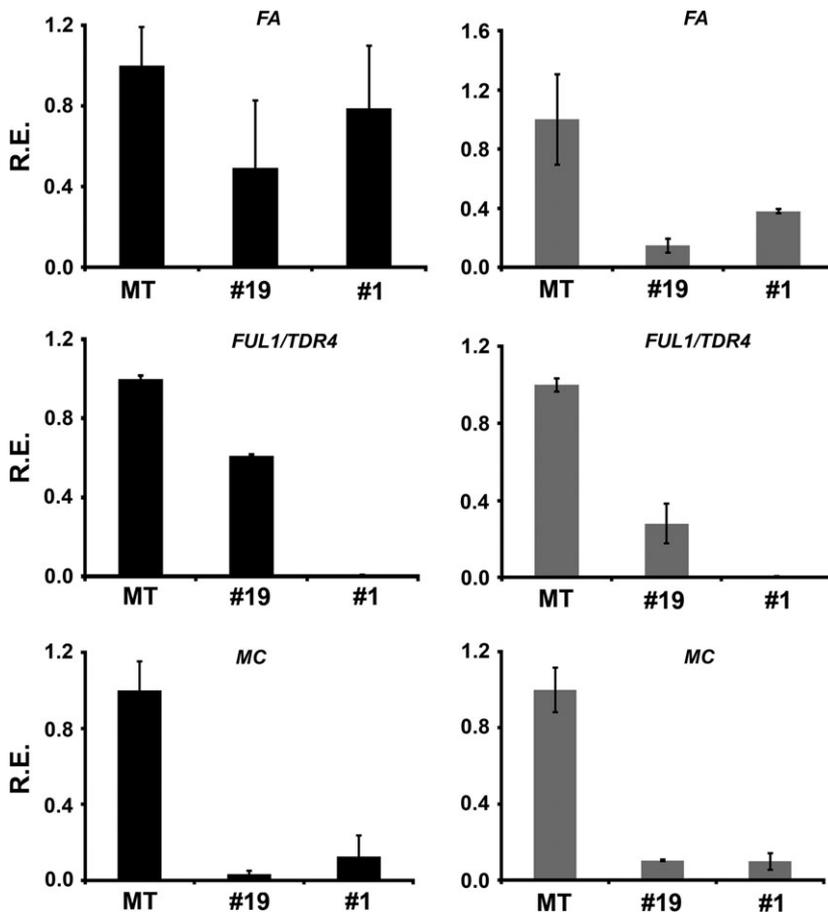
In *Arabidopsis*, the MADS box genes *AP1* and *FUL*, as well as the central regulator of flowering *LEAFY*, are direct targets of the miR156 node (Yamaguchi *et al.*, 2009). To investigate whether expression of their putative orthologs is regulated by the tomato miR156 node during early fruit development, we evaluated the expression patterns of the MADS box genes *MC* and *FUL1/TDR4* and the *LEAFY* ortholog *FALSIFLORA* (*FA*) in developing ovaries of 156-OE plants (Figure 9). *FA* was similarly expressed in pre-anthesis ovaries of MT and 156-OE plants, but it was strongly repressed at post-anthesis (Figure 9, upper panels). Conversely, expression of *FUL1/TDR4* and *MC* was repressed in both pre- and post-anthesis developmental stages of

156-OE ovaries (Figure 9, middle and lower panels, respectively). The *MC*, *FUL1/TDR4* and *FA* loci have several putative binding sites for *SlySBPs* (GTAC; Birkenbihl *et al.*, 2005) (Figure S6), suggesting they are possible conserved direct targets of the miR156 node in tomato. Nonetheless, they may have acquired additional roles during gynoecium and ovary development when integrated into tomato miR156/*SlySBP*-associated regulatory networks.

*TOMATO AGAMOUS1* (*TAG1*), a MADS box gene belonging to the *AGAMOUS* (*AG*) sub-lineage, and *TM29* (a tomato MADS box *SEPALLATA* homolog), have been implicated in floral organ development, carpel development and maintenance of the floral meristem (Ampomah-Dwamena *et al.*, 2002). We therefore analyzed whether these genes were mis-regulated in the developing ovaries of miR156 over-expressors. Based on our quantitative RT-PCR data (Figure S7), *TAG1* and *TM29* were similarly expressed in the developing ovaries of 156-OE and MT plants, suggesting that these MADS box genes are not regulated by the miR156 node during early fruit development. Conversely, they probably function in miR156-targeted *SlySBP*-independent pathways to regulate carpel and fruit development.

#### DISCUSSION

In this study, we found that over-expression of miR156 has major effects on tomato reproductive development, including formation of ectopic structures in flowers and fruits. Such modifications appear to be due to the



**Figure 9.** Expression of the MADS box genes (*FUL1/TDR4* and *MC*) and *FA* in developing ovaries of MT and *AtMIR156b* over-expressing plants.

Black bars indicate gene expression in pre-anthesis ovaries; gray bars indicate gene expression in post-anthesis ovaries. Quantitative RT-PCR experiments used ovary tissues from MT as the reference sample (set to 1.0). Error bars indicate the standard deviation of three biological samples. RE, relative expression.

mis-regulation of key genes involved in development of lateral organs and meristem identity and maintenance.

#### Expression patterns of *miR156* and *SlySBP* genes in MT developing ovaries

Several *SlySBP* genes and *miR156/miR157* are expressed in tomato carpels and developing fruits (Moxon *et al.*, 2008; Salinas *et al.*, 2012). Here, we showed that at least five *miR156*-targeted *SlySBP* genes were differentially expressed in pre- and post-anthesis ovaries (Figure 1e). Such temporal modulation of *SlySBP* expression may be crucial for early tomato fruit patterning, as, shortly after anthesis, fruit development still involves active cell division and establishment of tissue identity (Seymour *et al.*, 2013). In fact, regardless of the expression patterns in the MT ovaries, the magnitudes of the temporal changes of *SlySBP* genes were altered in 156-OE plants (Figure 6a), which may lead to abnormal tissue/organ growth.

Cells of the carpel margin have meristematic characteristics, and give rise to the placenta and ovules. Moreover, it has been proposed that ovules represent meristematic axes with their own type of lateral determinate organs (integuments), reminiscent of vegetative shoot meristems (Mathews and Kramer, 2012). Tomato *miR156* accumulated

mainly in meristematic tissues (placenta and ovules) of developing flowers and ovaries (Figure 1), and may potentially regulate its targets in these tissues, similar to its activity in shoot meristem and leaf primordia (Wang *et al.*, 2008). The expression patterns of *miR156* in tissues containing secondary meristems (i.e. meristems that produce new cells for a predetermined period and form specialized organs and tissues) may be crucial for *miR156*-targeted *SlySBP* genes to regulate the meristematic state of such specialized tissues (ovules and their integuments) in the tomato ovary to allow the organ to modulate its proper growth during flower and fruit development.

#### *miR156*-targeted *SlySBP* genes regulate gynoecia and fruit development

Tomato *miR156* over-expressors exhibited flowers with extra whorls, which led to altered carpel and fruit morphology (Figures 3 and 4). This phenotype is more severe than that observed by Zhang *et al.* (2011), possibly due to the high levels of *miR156* accumulation observed in 156-OE ovaries and fruits (Figure S3b). It has recently been shown that introducing the *35S:AtMIR156b* transgene into an *Arabidopsis sp18-1* mutant background (which harbors a non-functional allele of the *miR156* non-targeted gene

*SPL8*) leads to phenotypic changes in gynoecia morphology (Xing *et al.*, 2013), although these are dissimilar to those observed in tomato 156-OE plants (Figures 3–5). As that neither *35S:AtMIR156b* nor *spl8-1* plants exhibit abnormal gynoecia development, it is most likely that miR156-targeted and non-targeted *SPL* genes share redundant functions in Arabidopsis reproductive development. Although we cannot exclude the possibility that additional miR156-targeted genes may exist in tomato, this is unlikely as we did not find any additional targets by computational analysis, and Karlova *et al.* (2013) only identified *SlySBP* genes as miR156/miR157 targets during tomato fruit development using high-throughput sequencing and degradome analysis. Therefore, it is reasonable to presume that miR156-targeted *SlySBP* genes are crucial to regulate tomato gynoecia and initial fruit development, probably through pathways that are not shared with dry fruited species such as Arabidopsis.

#### The miR156 node is required for the proper expression of key transcription factors during tomato ovary development

Histological and scanning electron microscopy analyses demonstrated the presence of partially fused extra carpels as well as ectopic meristem- and leaf-like structures in tomato 156-OE gynoecia (Figures 3–5). Such phenotypes may be interpreted as representing indeterminate growth of the flower and fruit, and are reminiscent of the effects of mis-regulation of homeobox genes such as *LeT6/TKn2*. KNOX domain-containing transcription factors function in the establishment and maintenance of the shoot apical meristem, and some have essential roles in Arabidopsis carpel development (Pautot *et al.*, 2001; Scofield *et al.*, 2007). In wild-type carpels, *LeT6/TKn2* is expressed in vascular tissues and in the inner part of the ovule integument, as well as in developing embryos (Avivi *et al.*, 2000). Such expression patterns are similar to those observed for miR156 and *SlySBP3* in developing wild-type ovules and embryos (Figure 1), suggesting that miR156-targeted *SlySBP* genes and *LeT6/TKn2* share similar genetic pathways. This possibility is supported by the observations that *LeT6/TKn2* is up-regulated in developing ovaries of 156-OE plants, and that *Me/Me* gynoecia display extra fused carpels (Figure 7). In addition, similar carpel and fruit phenotypes are observed in transgenic *35S:LeT6/TKn2* tomato plants (Janssen *et al.*, 1998). Although induction of the *LeT6/TKn2* ortholog *SHOOT MERISTEMLESS (STM)* also causes formation of ectopic carpels (Scofield *et al.*, 2007), it is currently unknown whether the miR156 node regulates or interacts with *STM* or other KNOX class I genes in Arabidopsis.

The NAM/CUC gene *GOBLET* was also localized in the meristematic tissues of developing ovaries, similar to miR156 and its target *SlySBP3* (Figures 1 and 8). Moreover,

*GOB* was up-regulated in the developing ovaries of 156-OE plants (Figure 8d), indicating that its normal expression requires expression of miR156-targeted *SlySBP* genes. This regulatory requirement may be indirect, as miR156 over-expression also affects the accumulation of miR164 in developing ovaries (Figure 8e). Together, our data suggest that miR156-targeted *SlySBP* genes, *LeT6/TKn2* and *GOB* genes function in the same pathway when regulating tomato ovary and fruit development. It is possible that *SlySBP* represses the expression of *LeT6/TKn2* and *GOB* genes in MT to control the phase of floral meristem maintenance and cell proliferation (mainly in the whorl/whorl boundaries) in relation to organ initiation and differentiation, allowing proper carpel and ovule development. Interestingly, interactions between KNOX and NAM/CUC genes have been demonstrated during vegetative and reproductive development in Arabidopsis (Ishida *et al.*, 2000; Spinelli *et al.*, 2011). *GOB* was also up-regulated in *Me/Me* developing ovaries (Figure 8d), which suggests a conserved link between *LeT6/TKn2* and *GOB* genes during tomato ovary development. It will be interesting to determine whether *LeT6/TKn2* directly or indirectly regulates *GOB* expression during particular developmental contexts in tomato, and whether such regulation is dependent on miR156-targeted *SlySBP* genes.

Unlike *LeT6/TKn2* and *GOB* genes, the MADS box genes *MC* and *FUL1/TDR4* and *FA* were down-regulated to various extents in the developing ovaries of 156-OE plants (Figure 9). Their regulation by the miR156-targeted *SlySBP* genes may be important for age-dependent tomato flowering, similarly to Arabidopsis (Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). However, these genes may have acquired additional roles during tomato ovary and fruit development. Indeed, *MC* apparently has additional functions in fruit development, and *FUL1/TDR4* has recently been shown to control fruit ripening in an ethylene-independent manner (Bemer *et al.*, 2012; Nakano *et al.*, 2012). In addition to their roles in determination of flowering time, we speculate that *MC* and *FA* act in conjunction with *FUL1/TDR4* in a miR156/*SlySBP*-dependent developmental context to regulate floral meristem identity and specification of the floral organ whorls, leading to proper fruit development and determinacy. However, the genetic interactions among *SlySBP* genes and *MC*, *FA* and *FUL1/TDR4* genes during gynoecium and fruit development remain to be determined.

Recently, the miR156/*SPL* module has been implicated in developmental timing in evolutionarily distant species (Cho *et al.*, 2012), demonstrating its crucial importance for the plant kingdom. Here, we showed that this network also has role(s) in ovary and fleshy fruit development, presumably by controlling the rate of cell differentiation and tissue identity. It will be interesting to determine whether functions of miR156-targeted *SPL/SBP* interact with specific

hormones, such as auxin and gibberellin, during fruit development in tomato and other fleshy-fruited species.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

*Solanum lycopersicum* cv. Micro-Tom (MT) was used as the wild-type, and transgenic plants were in the MT background. The *Mouse ear* (*Me*) homozygous mutant was also used. Leaves were harvested from 15-day-old MT and transgenic plants. Ovary and flower tissues were collected based on flower developmental stages (Brukhin *et al.*, 2003). Flowers were collected between stages 6 and 12 of development. Ovaries at pre-anthesis were harvested between stages 13 and 16 of flower development. Post-anthesis ovaries were harvested at stage 20. Immature fruits with a diameter between 1.4 and 1.6 cm were collected. Tomato MT plants were grown as described by Lombardi-Crestana *et al.* (2012).

### Generation of transgenic MT plants over-expressing the *AtMIR156b* precursor

A 1600 bp fragment encompassing the *AtMIR156b* precursor (Schwab *et al.*, 2005) was amplified from genomic DNA of *Arabidopsis thaliana* ecotype Columbia. The PCR product was subcloned into TOPO TA (Invitrogen, <http://www.lifetechnologies.com>) and sequenced. The confirmed *AtMIR156b* precursor was digested using the *Bam*HI and *Sac*I restriction enzymes, and subsequently cloned into binary vector pBI121 (Chen, 2003) under the control of the CaMV 35S promoter. The binary vector was transformed into *Agrobacterium tumefaciens* strain EHA105. Stable genetic transformation of tomato cv. MT plants was performed as described by Pino *et al.* (2010). Twenty independent kanamycin-resistant transgenic lines were selected for transgene integration by PCR, and subsequently examined for transgene expression levels (data not shown). Further analyses were performed with selected lines, one of which (line 19) was in the T<sub>4</sub> generation. The 35S:*AtMIR164a* transgene (Spinelli *et al.*, 2011) was introduced into tomato cv. MT plants as described above. Ten independent kanamycin-resistant transgenic lines were selected, and one was used for further analyses in the T<sub>1</sub> generation.

### *In situ* hybridization

Pre- and post-anthesis developing ovaries from MT and transgenic plants were used for *in situ* hybridization as described by Javelle and Timmermans (2012). Locked nucleic acid (LNA) probes with sequences complementary to miR156 (5'-GTGCTCACTCTCTCTGTCA-3') and a negative control (scrambled miR, 5'-GTGTAACACGTCTATACGCCCA-3') were synthesized by Exiqon (<http://www.exiqon.com/>), and digoxigenin-labeled using a DIG oligonucleotide 3' end labeling kit (Roche Applied Science, <https://www.roche-applied-science.com>). Ten picomoles of each probe were used for each slide. Probes for *SlySBP3* (Solyc10g009080, positions 360–844 of the coding sequence) and *GOBLET* (Solyc07g062840, positions 111–840 of the coding sequence) were used as described by Javelle and Timmermans (2012) at 0.5 ng/μL/kb or 0.5 ng μL<sup>-1</sup>kb<sup>-1</sup>. Hybridization and washing steps were performed at 55°C.

### Analyses of branching index, total number of leaves, and flowering time

The branching index (ratio between the total length of lateral ramification and the length of the main plant axis; Morris *et al.*, 2001)

was estimated 87 days after sowing ( $n = 10$ ). The total number of leaves was measured at the same time point ( $n = 10$ ). The flowering time of MT and transgenic plants ( $n = 10$ ) was evaluated as the day on which the first bud flowering became visible. The total number of fruits was determined 100 days after sowing ( $n = 30$ ).

### RNA extraction and stem-loop pulsed RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and treated with DNase I (Invitrogen). DNase I-treated RNA (2.0 μg) was reverse-transcribed to generate first-strand cDNA, as described previously (Varkonyi-Gasic *et al.*, 2007). An oligo(dT) primer was also added to the reaction to detect target mRNAs and internal controls. cDNAs were used for PCR reactions as follows: 1.0 μl cDNA, 1.5 mM magnesium sulfate, 0.25 mM of each dNTP, 10 pmol of each primer, and 1 unit of Taq DNA polymerase (Promega, <https://www.promega.com>). The reactions were performed under the following conditions: 94°C for 2 min, and the appropriate number of cycles of 94°C for 20 sec, 60°C for 30 sec and 72°C for 45 sec. All reactions were repeated twice with two biological samples. Primer sequences are listed in Table S1.

### Stem-loop pulsed quantitative RT-PCR

First-strand cDNA was transcribed as described above. SYBR Green PCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems, [www.appliedbiosystems.com](http://www.appliedbiosystems.com)). Briefly, 5 μl of 1:80 v/v cDNA dilutions were added to 12.5 μl of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 3 pmol of each primer, and ddH<sub>2</sub>O to a final volume of 25 μl. The reactions were amplified for 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The tomato *Tubulin* gene (Solyc04g081490) was used as an internal control (Mounet *et al.*, 2009). PCR products for each primer set were subjected to melt-curve analysis, confirming the presence of only one peak on thermal dissociation generated by the thermal denaturing protocol. Three technical replicates were analyzed for each of three biological samples (each comprising at least ten ovaries or immature fruits from five plants), together with template-free reactions as negative controls. The threshold cycle ( $C_T$ ) was determined automatically by the instrument, and fold changes for each gene were calculated using the equation  $2^{-\Delta\Delta C_T}$  (Livak and Schmittgen, 2001). Primer sequences are listed in Table S1.

### Histological analysis and scanning electron microscopy

Samples of flower buds and ovaries were fixed in Karnovsky solution (Karnovsky, 1965), dehydrated in an increasing ethanol series (10–100%), and subsequently infiltrated into synthetic resin using a HistoResin embedding kit (Leica, [www.leica-microsystems.com](http://www.leica-microsystems.com)), according to the manufacturer's instructions. Tissue sections were obtained using a rotary microtome (Leica) and stained with toluidine blue 0.05% (Sakai, 1973). Permanent slides were mounted with synthetic resin (Entellan<sup>®</sup>, Merck, [www.merck.com](http://www.merck.com)). For scanning electron microscopy, samples were fixed, mounted and analyzed as described by Bharathan *et al.* (2002).

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## SUPPORTING INFORMATION

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

**Figure S1.** miRNA156 and *SPL/SBP* genes are conserved in tomato.

**Figure S2.** miR156-regulated *SlySBP* genes modulate vegetative development.

**Figure S3.** miR156 over-expression affects tomato fruit development.

**Figure S4.** Vegetative and fruit phenotypes of weak 156-OE lines in the non-dwarf ‘Santa Clara’ background.

**Figure S5.** Comparative expression analysis of representative *SlySBP* genes in immature fruit tissues from tomato cv. MT and 156-OE lines.

**Figure S6.** Computational analysis of putative binding sites for SPL/SBP proteins in tomato genomic sequences.

**Figure S7.** *TAG1* and *TM29* expression patterns in pre-anthesis and post-anthesis ovaries of tomato cv. MT and 156-OE lines.

**Table S1.** Oligonucleotide sequences used in this work.

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