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doi: 10.1093/pcp/pcw214

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Subject Areas:

[Growth and development], [Regulation of gene expression]

Number of figures and tables:

Black and white figures: 2 Color figures: 6 Tables: 2

Supplementary figures: 6 Supplementary tables: 5

SEXUAL STERILITY is essential for both male and female gametogenesis in tomato

A gene essential for gametogenesis in tomato

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Abbreviations:

DBA, day before anthesis; MMC, megaspore mother cell; PMC, pollen mother cell; SlSES, *Solanum lycopersicum* SEXUAL STERILITY; SPL/NZZ, SPOROCYTELESS/NOZZLE; WT, wild type

Abstract: Gametogenesis is a key step in the production of ovules or pollen in higher plants. The molecular aspects of gametogenesis are well characterized in the model plant Arabidopsis; however, little information is known in tomato, which is a model plant for fleshy fruit development. In this study, we characterized a tomato (*Solanum lycopersicum* L.) ɤ-ray mutant, *sexual sterility* (*Slses*), that exhibited both male and female sterility. Morphological analysis revealed that the *Slses* mutant forms incomplete ovules and wilted anthers devoid of pollen grains at the anthesis stage. Genetic and next-generation sequencing analyses revealed that the *Slses* mutant carried a 13-bp deletion within the first exon of a homologue of SPOROCYTELESS/NOZZLE (SPL/NZZ), which plays an important role in gametogenesis in Arabidopsis. Complementation analysis in which the complete *SlSES* genomic region was introduced into the *Slses* mutant fully restored normal phenotypes, demonstrating that *Solyc07g063670* is responsible for the *Slses* mutation. SlSES probably act as transcriptional repressor because of EAR motif at the C-terminal region. Gene expression levels of *WUSCHEL* (*SlWUS*) and *INNER NO OUTER* (*SlINO*), both of which are required for ovule development, were dramatically reduced in the early stages of pistil development in the *Slses* mutant, suggesting a positive regulatory role of *SlSES* in the transcription of gametogenesis genes and differences in the regulation of *INO (SlINO)* and integument development by *SPL/NZZ (SLSES)* between Arabidopsis and tomato. Taken together, our results indicate that *SlSES* is a novel tomato gametogenesis gene essential for both male and female gametogenesis.

Keywords: Anther, Gametogenesis, Mutant, Ovule, Sexual sterility

Introduction:

Tomato is one of the most important crops produced and consumed worldwide and has also been used as a model plant for studying plants in the Solanaceae family and plants bearing fleshy fruits. To date, diverse tomato studies have been conducted, especially tomato genomic studies, given the availability of the complete genome sequence (The Tomato Genome Consortium, 2012), various public databases, and mutant resources that were generated using breeding lines and model tomato lines, including a dwarf cultivar, 'Micro-Tom' (Scott and Harbaugh, 1989; Minoia et al. 2010; Saito et al. 2011).

Angiosperms form reproductive organs, such as anthers and pistils, that bear pollen grains and ovules, respectively. In this reproductive system, gametogenesis plays an important role in producing offspring. To date, several genes involved in gametogenesis have been identified in the model plant Arabidopsis, and their stage-specific roles in the regulation of reproductive development have been elucidated (Schneitz et al. 1997; Wilson and Zhang 2009; Bencivenga et al. 2011; Plackett et al. 2011).

According to Sanders et al. (1999), the development of anthers and pollen in Arabidopsis is divided into 15 stages. During stages 1 to 4, when the anther primordium emerges and establishes a bilateral structure, *EXCESS MICROSPOROCYTES1/EXTRA*

SPOROGENOUS CELLS (*EMS1/EXS*), a putative leucine-rich repeat receptor-like kinase, and *TAPETAL DETERMINANT1* (*TPD1*), a ligand for *EMS1/EXS*, both function to determine archesporial cell number. Both the Arabidopsis *ems1/exs* mutant and the *tpd1* mutant are male sterile and form extra meiocytes caused by additional L2 layer cells, and the tapetal and middle cell layers are absent in these mutants (Canales et al. 2002; Zhao et al. 2002; Yang et al. 2003). Differentiation of the endothecium, middle layer, tapetum and microspore mother cells initiate at stage 5. Then, microspore mother cells undergo meiosis between stages 5 and 7 (Sanders et al. 1999), producing microspores or pollen grains within the tapetal cell layer. *DYSFUNCTIONAL TAPETUM1* (*DYT1*) encodes a putative basic helix-loop-helix (bHLH) transcription factor that is predicted to act downstream *EMS1/EXS*, and this action is required for the normal expression of *ABORTED MICROSPORES* (*AMS*) and *MALE STERILITY1* (*MS1*), which are essential for normal tapetal development and viable pollen production during these stages (Wilson et al. 2001; Sorensen et al. 2002; Zhang et al. 2006).

On the other hand, many genes related to ovule development have also been identified in Arabidopsis (Schneitz et al. 1997; Skinner et al. 2004). Based on cytological aspects, ovule primordia are divided into 3 elements: the funiculus, chalaza and nucellus. Genes specifically expressed within each part have been identified (Schneitz et al. 1997; Sieber et al. 2004a; Kelly and Gasser 2009). The site-specific expression of the homeobox gene *WUSCHEL* (*WUS*) within the nucellus is critical for integument development and the formation of normal mature ovules

in Arabidopsis and tomato (Gross-Hardt et al. 2002; Sicard et al. 2008a). Accordingly, *WUS* expression is generally confined to the nucellus during ovule development based on the regulation by specific genes or hormones in Arabidopsis. For example, the *bell1* (*bel1*) mutant and the *corona* (*cna*)/*phabulosa* (*phb*)/*phavoluta* (*phv*) triple mutant, which showed abnormal ovule development, exhibit an aberrant *WUS* expression pattern that prevents normal integument formation (Brambilla et al. 2007; Bencivenga et al. 2012; Yamada et al. 2016). Furthermore, *WUS* expression expands into the chalaza with exogenous treatment with the plant hormone cytokinin N^6-benzylaminopurine (BAP), and the treatment results in the formation of new primordia (Bencivenga et al. 2012). In addition, precise mRNA localization of *AINTEGUMENTA* (*ANT*), which encodes an AP2 family transcription factor, and *INNER NO OUTER* (*INO*), which encodes a plant-specific YABBY family transcription factor, at the chalaza is required for normal ovule integument development (Elliott et al. 1996; Baker et al. 1997; Villanueva et al, 1999).

There are several reports that describe anther and ovule development in tomato. For example, tomato *male sterile 10*^{35} (*MS10*^{35}) has been isolated as a homologous gene of *AtDYT1*, and the *ms10*^{35} mutant exhibits dysfunctional meiosis and aberrant tapetum formation (Jeong et al. 2014). The *parthenocarpic fruit* (*pat*) mutant forms aberrant ovules caused by a failure of integument development (Mazzucato et al. 1998). In addition, the mini zinc finger gene *INHIBITOR OF MERISTEM ACTIVITY* (*IMA*) regulates ovule development by activating D-

class gene of ACBD model of flower development expression in tomato and Arabidopsis (Sicard et al. 2008ab). However, gametogenesis genes and their associated mechanisms remain largely unknown in tomato.

SPOROCYTELESS/NOZZLE (*SPL/NZZ*) plays a key role in gametogenesis, both in anther and ovule development, in Arabidopsis (Schiefthaler et al. 1999; Yang et al. 1999), and loss of function of SPL/NZZ results in abnormal germ cell production, causing strong sterility in both male and female reproductive organs. *SPL/NZZ* transcripts are observed in the stamen primordia and developing stamens as well as in ovule primordia, which is induced by AGAMOUS (AG) or brassinosteroids (Ito et al. 2004; Ye et al. 2010). *AG* is a member of MADS-box gene and it is necessary for carpel and stamen development (Pinyopich et al. 2003). Additionally, SPL/NZZ physically binds with CINCINNATA (CIN)–like TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors or YABBY family transcription factors through the N-terminal domain and the TOPLESS/TOPLESS-RELATED (TPL/TPR) co-repressor at the EAR motif (Sieber et al. 2004b; Chen et al. 2014; Wei et al. 2015). Then, SPL/NZZ functions as an adaptor-like transcriptional repressor through interaction with TPL/TPR (Wei et al. 2015). However, very little is known about the function of SPL/NZZ in tomato. In this study, we report the identification and characterization of *SlSES*, a homologue of *SPL/NZZ*, as an essential factor of gametogenesis development. This study demonstrates that

pollen and ovule development were arrested in the very early developmental stages of each reproductive organ in the *Slses* mutant.

Results:

The identification of the sterile Slses mutant

To uncover the regulatory mechanism underlying reproductive organ development, we screened for mutants that were defective in normal reproductive development. From the tomato mutant populations that were generated by gamma ray-irradiated lines (Saito et al. 2011), one mutant producing seedless fruits was isolated. To characterize the cytological aspects of the mutant, we first counted the number of each type of flower organ. In both the WT and mutant, the inflorescence was typically composed of 5 sepals, petals and anthers and a single pistil (Table 1). Although the WT formed an inflorescence with swollen anthers containing mature viable pollen grains at the anthesis stage, the mutant formed clearly defective anthers; they appeared wilted and small, and no pollen grains were visible at this time (Fig. 1A-D). To confirm the fertility of the ovules and pollen of the mutant, we crossed WT pollen to the mutant stigma or mutant pollen to the WT stigma and examined whether viable seeds were produced. No pollen was visually observed in the mutant. As the result, no viable seeds were produced from either type of cross-pollination; however, the mutant occasionally produced seedless fruit (data not shown). These results indicated that the mutant showed both male and female sterility.

Based on this phenotype, the mutant was designated as *sexual sterility* (*Slses*).

Then, to clarify genetic nature of the *Slses* mutation, we conducted crossing analysis with wild type Micro-Tom. Given that the *Slses* homozygous mutant exhibited both complete male and female sterility, heterozygous *Slses* plants were crossed with WT, and the resulting F_1 seeds and the following F_2 offspring were obtained (Supplementary Fig. S1). The morphological phenotype and fertility of 17 F_1 plants were almost equivalent to those of WT, indicating that the *Slses* mutation was recessive (Table 2). The phenotypes of the F_2 offspring segregated to 118 (phenotype; WT) : 40 (phenotype; *Slses*), which approximately fit the theoretical ratio of the WT and *Slses* phenotypes 3 : 1, suggesting that the *Slses* mutation was monogenic (Table 2).

Next, time course analysis of cross-sections during anther development was conducted. In this analysis, flower buds at different developmental stages, which were defined by the size of flower buds, were obtained, and cross-sections of the anther structure were examined under light microscopy. There was no obvious difference in timing of flowering and flower development between WT and the *Slses* mutant. At the stage when the flower buds were 1 to 2 mm and 2 to 3 mm, normal formation of the vascular bundle (VB), epidermis (Ep), endothecium (En), middle layer (ML), tapetum (T), and pollen mother cells (PMC, 1-2 mm bud) or meiotic cells (MC, 2-3 mm bud) were observed in WT (Fig. 2E, F). In contrast, the formation of the En, ML, T, PMC and MC were not clearly evident, and we only observed the vascular bundle and

epidermis at the stage of 1 to 2 mm and 2 to 3 mm buds in the *Slses* mutant (Fig. 2I, J). Microspores (Msp) were observed at the bud stage of 4 to 5 mm in WT but not in the *Slses* mutant (Fig. 2G, K). At anthesis stage, when the bud size is 6 to 7 mm, many mature pollen grains were clearly observed in the anther locules in the WT, whereas the *Slses* mutant never underwent pollen grain formation inside the anther (Fig. 2A-D, H, L). The anther structure appeared wilted and less colored in the mutant compared with the WT (Fig. 1A, B, Fig. 2A-D).

Next, we observed the pistil in the WT and the *Slses* mutant (Fig. 1E-I). The size of the ovary at anthesis in the *Slses* mutant was clearly smaller than that in the WT (Fig. 1I). At the 1 to 2 mm bud size stage, we observed ovule primordia around the placenta in both the WT and the *Slses* mutant (Fig. 3E, I). At the 2 to 3 mm and 4 to 5 mm bud stages, ovule integuments and megaspore mother cells (MMCs) were observed, and the ovule primordia enlarged as the stages proceeded in WT. We could not observe the ovule integuments and MMC, which are essential for complete ovule development, and the enlargement level of ovule primordia was quite low in the *Slses* mutant (Fig. 3 F, G, J, K, M-O). From this stage, ovule development was arrested in the *Slses* mutant. We observed cross-sections of mature ovules at the anthesis stage in WT. The ovules were smaller, and their structure appeared disorganized in the *Slses* mutant (Fig. 3A-D, H, L).

The Slses mutant produces seedless fruit

Despite male and female sterility, the *Slses* mutant occasionally bore red mature fruits and the size and weight of these fruits were significantly reduced compared with WT fruits (Fig. 4A-D). Most of mature fruits produced in the WT contained 3 locules (average 3.2 locules per fruit), whereas those in the *Slses* mutant contained 2 locules (average 2.2 locules per fruit), indicating that the number of locules was significantly reduced in the *Slses* mutant (Fig. 4E). The mature red fruits produced in the *Slses* mutant were completely seedless, indicating that the *Slses* mutant induces parthenocarpy. However, molecular mechanism of how the *Slses* mutation induces parthenocarpy is still unclear.

SlSES encodes SPL/NZZ

To map the *Slses* mutation, positional cloning was performed using SolCap infinium carrying a large number of SNPs that could discriminate tomato genotypes (Sim et al. 2012), with 2098 markers available for distinguishing between ‘Micro-Tom’ and ‘Ailsa Craig’. The SolCap array coupled with 28 F_2 mapping populations that were derived from crosses between *Slses* heterozygous plants and Ailsa Craig allowed for narrowing down the candidate area to a 2.3 Mbp region corresponding to the physical position between 62,465,682 base pairs (bps) to 64,759,097 bps on chromosome 7 based on the protein annotation extracted from version SL2.40 from the SGN database (Fig. 5A). The 2.3 Mbp region spans 334 open reading frames (ORFs), and none of these ORFs are involved in tomato gametogenesis

(http://www.kazusa.or.jp/tomato_sbm/). Then, we obtained the whole genome sequence of the *Slses* mutant using Illumina HiSeq 2000 next-generation sequencing, and its genome information was compared with the tomato reference genome sequence according to Ariizumi et al. (2014). Given that the *Slses* mutant was a monogenic recessive mutation, we exclusively focused on homozygous mutations specifically found in the *Slses* mutant. The genome sequence analysis identified 94 homozygous mutations consisting of 9 point mutations and 85 insertion and deletion (indel) mutations within the 2.3 Mbp region. The 9 point mutations included 1 nonsynonymous, 1 synonymous, and 7 intergenic mutations. The 85 indels included 2 mutations within exon causing modification of amino acid sequence of putative ORFs and 19 mutations within intron or UTR regions. In addition, 64 mutations were present within the intergenic region (Supplementary Table S1). Therefore, 1 missense and 1 deletion mutation, which were found in *Solyc07g063670*, and 1 insertion mutation, which was found in *Solyc07g065700*, were considered candidates for the *SlSES* gene. Among them, one deletion mutation, which caused a 13-bp deletion from the first exon of *Solyc07g063670*, was primarily selected because the *Slses* mutant was produced by gamma-ray irradiation and caused massive flame shift rather than the insertion mutation. Gamma-ray irradiation often causes genome insertion or deletion rather than SNPs, and we often obtain flame shift caused mutant. The deletion caused a frame shift at the 123rd nucleotide position from the start codon, which was considered the 1st position. As the results of frame shift the putative amino acid was shortened

from 353 to 57 in the *Slses* mutant (Fig. 5B, C).

Genetic linkage analysis using a DNA marker (*SlSES* marker-1; Supplementary Table S2) distinguishes this deletion and the F_2 populations derived from a cross between the WT Micro-Tom and the *Slses* mutant, revealing a perfect linkage between the mutation and the phenotypes (Supplementary Fig. S2). The candidate gene of the *Slses* mutant, *Solyc07g063670*, encodes a domain of plant transcription factor NOZZLE (29-215 a.a.; NCBI Conserved Domain Search; Fig. 5C). This protein shares 26.4% identity (39.2% similarity) with the SPL/NZZ protein and 21.1% identity (31.8% similarity) with the TCP interactor containing EAR motif 3 (TIE3) protein of Arabidopsis (Fig. 5D; Supplementary Table S3). The candidate protein also had an EAR motif (LxLxL; Kagale and Rozwadowski 2011) at the C-terminal region (345-351 a.a.) similar to that found in the SPL/NZZ and TIE proteins of Arabidopsis (Fig. 5E). Further sequence analysis indicated that no paralogues of the candidate gene existed in the tomato genome (Fig. 5D), suggesting that *SlSES* exists as a single gene and plays a non-redundant role in the tomato genome.

Complementation experiments of the Slses mutant

To confirm whether the candidate gene *Solyc07g063670* was responsible for the *Slses* mutation, the genomic region of the candidate gene, including the 2.5-kb promoter region, 1,310-bp coding sequence, and 700-bp terminator region, were introduced into the offspring of

heterozygous *Slses* plants (Supplementary Fig. S3) such that the transgene randomly integrated into plants that were homozygous, heterozygous or azygous for the 13-bp nucleotide deletion within *Solyc07g063670*. We developed a PCR genotyping method that allowed for discrimination of 13-bp nucleotide deletion such that we selected the transgenic plants that carried both the transgene and the endogenous homozygous 13-bp nucleotide deletion (*SlSES* marker-2; Supplementary Table S2; Supplementary Fig. S4). Three such transgenic plants were obtained. The plants exhibited swollen anthers, producing fertile pollen grains and fertile ovules; thus, self-pollination produced viable offspring seeds that germinated thereafter (Supplementary Fig. S5), indicating that the phenotypes in the complementation lines were fully restored. These results indicated that the 13-bp deletion found in *Solyc07g063670* was responsible for the *Slses* mutation and that the Arabidopsis *SPL/NZZ* homologue in tomato (*SlSES*) functions to regulate normal reproductive organ development.

SlSES is expressed at early stages of reproductive development and regulates SlWUS and SlINO expression

qRT-PCR analysis was conducted to examine the expression pattern of the *SlSES* gene (Fig. 6). mRNA was extracted from flower buds 1 mm in size; pistils from developmental stages when the flower bud is 3, 4.5 and 6 mm in size; pistils 1 day before anthesis (DBA); and pistils at the anthesis stage. In this analysis, several genes related to anther or ovule development in

tomato or Arabidopsis were also examined regarding the effect of the *Slses* mutation on the transcriptional regulation of gametogenesis genes. *SlSES* mRNA was highly expressed in 1 mm flower buds and 3 mm pistils. However, mRNA levels decreased along with pistil development and were barely detectable in 6 mm pistils and 1 DBA buds in WT (Fig. 6). mRNA expression of *Male sterile 10*^{35} (*MS10*^{35}), which is involved in both meiosis and programmed cell death of the tapetum during microsporogenesis in tomato (Jeong et al. 2014), was evident in 1 mm WT buds, whereas its mRNA was minimally detectable in the *Slses* mutant (Fig. 7A). mRNA expression of *BARELY ANY MERISTEM1* and *2-like* (*BAM1/2-like*) and *EMS1/EXS-like*, which are homologues of Arabidopsis *BAM1/2* and *EMS1/EXS*, respectively, and are essential for early anther development (Zhao et al. 2002; Hord et al. 2006), were significantly downregulated in the *Slses* mutant (Fig. 7B, C). mRNA expression of *TOMATO AGAMOUS1* (*TAG1*), a homologue of Arabidopsis *AG1*, which is involved in the transcriptional regulation of *SPL/NZZ*, was decreased in the *Slses* mutant compared with the WT (Fig. 7D). In addition, mRNA expression of *WUSCHEL* (*WUS*) and *INNER NO OUTER* (*INO*), which are the important genes for ovule formation, and *GOBLET* (*GOB*), a homologue of Arabidopsis *CUP-SHAPED COTYLEDONS1* (*CUC1*) and *CUC2*, which are essential for the formation of carpel margin meristems (CMMs) (Hendelman et al. 2013; Kamiuchi et al. 2014), was decreased in the *Slses* mutant compared with WT (Fig. 7E, F, M). The expression of *AINTEGUMENTA-like* (*ANT-like*), *PIN-FORMED1-like* (*PIN1-like*), *BELL1-like* (*BEL1-like*), *REVOLUTA* (*SlREV*),

CORONA-like (*CNA-like*) and *PHABULOSA/PHAVOLUTA-like* (*PHB/PHV-like*), which are homologues of Arabidopsis and are required for ovule formation, were downregulated in the pistil from 4.5 mm and buds in the *Slses* mutant (Fig. 7G-L).

Discussion:

We identified a 13-bp deletion in the *SlSES* CDS in the *Slses* mutant, and the deletion caused a frame shift, resulting in the loss of the last 296 amino acids. The plant transcription factor NOZZLE motif and EAR motif are completely lost in the *Slses* mutant (Fig. 5B, C). In contrast, complementation experiments demonstrated that introduction of the genomic region of *Solyc07g063670* with its native promoter and terminator into the *Slses* mutant fully restored these phenotypes (Supplementary Fig. S5). These results indicate that *SlSES* (*Solyc07g063670*) is the gene responsible for the *Slses* mutant, and the *SlSES* loss-of-function mutation had a drastic effect on the development of reproductive organs.

The SlSES protein sequence exhibits significant homology with the SPL/NZZ (Schiefthaler et al. 1999; Yang *et al*. 1999) and TIE proteins (Tao et al. 2013) of Arabidopsis (Fig. 5D). These proteins carry an EAR motif and act as transcriptional repressors due to interaction with TOPLESS through the EAR motif. TOPLESS can repress transcription in vivo (Long et al. 2006; Pan et al. 2010; Causier et al. 2012; Tao et al. 2013; Wei et al. 2015). The putative amino acid sequence of SlSES exhibited a highly conserved EAR motif at the C-

terminus (Fig. 5E), suggesting that the tomato SlSES protein may interact with TOPLESS and act as transcriptional repressor.

The Arabidopsis homologous gene *SPL/NZZ* is one of the key genes involved in anther development, especially in the regulation of sporocyte formation in Arabidopsis (Yang et al. 1999; Plackett et al. 2011). En, ML, T, PCM and MC development were observed at the 1 to 3 mm bud stages in WT but not in the *Slses* mutant (Fig. 2E, F, I, J). These tissues differentiate from the L2 layer of initial stages of anther development, indicating that differentiation from the L2 layer was aborted in the *Slses* mutant. As a result, the *Slses* mutant exhibited wilted anthers and produced no mature pollen grains (Fig. 2C, D). The phenotypes of the *Slses* mutant were similar to those of the Arabidopsis *spl/nzz* mutant (Yang et al. 1999), indicating that *SlSES* is essential for anther primordia formation in tomato. We detected minimal mRNA expression of *MS10*^{35} and *EMS1/EXS-like*, which are homologues of Arabidopsis *DYT1* and *EMS1/EXS*, respectively, in the 1 mm bud of the *Slses* mutant (Fig. 7A, C). Given that both the tomato *ms10*^{35} mutant and the Arabidopsis *dyt1* mutant exhibit normal PMC development and abnormal tapetum development during pollen production in the anther (Zhang et al. 2006; Jeong et al. 2014) and that *DYT1* acts downstream of *SPL/NZZ* in Arabidopsis (Zhang et al. 2006), it is suggested that *SlSES* also acts upstream of *MS10*^{35}. In addition, *MS10*^{35} mRNA expression might be required for normal PMC development in tomato. Additionally, *EMS1/EXS* acts upstream of *DYT1* and downstream of *SPL/NZZ*. The *ems1/exs* mutant produces anthers lacking

the tapetum but with extra PMCs in Arabidopsis (Zhao et al. 2002). This finding may suggest that *EMS1/EXS-like* (*Solyc09g098420*) acts downstream of *SlSES* and has similar functions as its homologue in Arabidopsis.

The locule number and ovary size were significantly reduced in the *Slses* mutant compared with the WT (Fig. 1I, 4E), indicating that SlSES is partly involved in determining carpel number and ovary development. Although SPL/NZZ is essential for ovule primordia and nucellus development, integuments develop normally in Arabidopsis (Schiefthaler et al. 1999; Sieber et al. 2004a; Bencivenga et al. 2011). Interestingly, the *Slses* mutant formed a pistil (carpel) with ovules whose development arrested at the 2 to 3 mm bud stages, which was associated with a failure in nucellus (MMC) and integument development (Fig. 3I, J), indicating that SlSES functions in nucellus development, similar to Arabidopsis and integument development in tomato. INO plays a key role in the formation of the outer integuments in Arabidopsis (Baker et al. 1997; Villanueva et al. 1999). *INO* expression is not affected in the *spl/nzz* mutant compared with WT in Arabidopsis; therefore, the *spl/nzz* mutant forms integuments normally (Balasubramanian and Schneitz 2002; Sieber et al. 2004a). In tomato, the *Slses* mutant did not exhibit any integument development (Fig. 3O), and *SlINO* expression in the mutant is minimally detectable in the pistils of the 4.5 mm bud and anthesis stages (Fig. 7D), suggesting that the molecular regulation between SPL/NZZ and INO in Arabidopsis and SlSES and SlINO in tomato differs. SlSES function is necessary for *SlINO* expression in tomato.

Arabidopsis forms ovules from two integuments (inner and outer integuments and bitegmic ovules), whereas tomato forms ovules from a single integument (unitegmic ovules) (Wang and Ren 2008; Skinner et al. 2016). Our results indicate that the characteristics of the single integument in tomato are similar to that of the outer integument in Arabidopsis.

It has been demonstrated that exclusive and timely *WUS* expression in the nucellus is critical for normal ovule development in Arabidopsis (Groß-Hardt et al. 2002). In the *Slses* mutant, *SlWUS* mRNA expression was considerably downregulated compared with WT (Fig. 7E). In Arabidopsis, *WUS* expression in the nucellus was reduced in the *spl/nzz* mutants same as our study (Sieber et al. 2004a), and this information suggests that *SlSES* expression is required to induce *SlWUS* mRNA transcription in tomato. *ANT-like* and *BEL1-like* mRNA expression was downregulated in the pistils of the 4.5 mm bud in the *Slses* mutant compared with the WT (Fig. 7G, I). These genes also play key roles in the development of ovules and integuments in Arabidopsis (Elliott et al. 1996; Schneitz et al. 1997; Brambilla et al. 2007). *ANT* is expressed within the developing integuments and funiculus. *BEL1* is strongly expressed within the developing integuments, and the *spl/nzz* mutant exhibits ectopic expression of *ANT* and *BEL1* within the ovule primordia in Arabidopsis (Balasubramanian and Schneitz 2000; Bencivenga et al. 2012). *ANT* and *BEL1* expression are required for the ovule integument development in Arabidopsis (Schneitz et al. 1997). Ovule development, especially the ovule integument (Fig. 3O), in the *Slses* mutant was arrested, suggesting reduced expression of *ANT*-

like and *BEL1-like* genes, which was actually in accordance with our results. PIN1 is an auxin efflux facilitator, and the *pin1-5* mutant exhibits abnormal ovule development. Therefore, *SPL/NZZ* expression in the ovule is required to induce *PIN1* transcription in Arabidopsis (Bencivenga et al. 2012). Moreover, the class III homeodomain leucine zipper (HD-ZIPIII) genes *CNA*, *PHB*, *PHV* and *REV* are expressed within the chalaza and restrict *WUS* and *INO* expression to the nucellus and gynobasal side of the ovule in Arabidopsis (Kelley et al. 2009ab; Yamada et al. 2016). *PIN1*, *CNA*, *PHB*, *PHV* and *REV* are required for precise spatiotemporal expression for correct ovule development. In tomato, *PIN1-like*, *CNA-like*, *PHB/PHV-like* and *SlREV* expression was reduced in the pistils of the 4.5 mm bud and/or anthesis flower, but the reduction was comparatively small. Nevertheless, ovule development was arrested in the *Slses* mutant (Fig. 7H, J-L). Given that the precise spatial expression of these genes is essential to successfully form ovules, the control of the spatial expression and expression level of these genes were collapsed in the *Slses* mutant.

SPL/NZZ expression is positively regulated by *AG* in Arabidopsis (Ito et al. 2004). On the other hand, the highest mRNA expression point of *SlSES* and *TAG1* was in the 1 mm bud and the pistils of 4.5 mm bud, respectively (Fig. 6, 7D). This may represent that *TAG1* doesn't regulate *SlSES* expression or they need other factor to regulate the mRNA expression of *SlSES*. In any case, we could not judge there is such a relationship between *SlSES* and *TAG1* in our study. In order to clarify, we need to do further experiments.

In this study, we identified a key gene, *SlSES*, that is responsible for both male and female gametogenesis in tomato. SlSES is a homologue of SPL/NZZ in Arabidopsis, and the loss-of-function *SlSES* mutant exhibits sexual sterility caused by a failure to form pollen and ovules. Possible roles of *SlSES* in ovule and anther development are proposed in Fig. 8 and are compared with *SPL/NZZ* of Arabidopsis. In ovule development, *SPL/NZZ* positively regulates *PIN1* and *WUS* expression and plays a key role in nucellus development in Arabidopsis (Sieber et al. 2004a; Bencivenga et al. 2012). On the other hand, *SlSES* is required to express *SlWUS* and *SlINO*. Moreover, we demonstrate that *SlSES* has a role in nucellus and integument development via controlling *SlINO* expression in tomato (Fig. 8). In addition, in anther development, *SPL/NZZ* positively regulates *BAM1/2* and *EMS1/EXS* expression and is essential for archesporial cell development in Arabidopsis (Yang et al. 1999; Zhao et al, 2002; Hord et al. 2006; Ma et al. 2012). *SlSES* positively regulates *EMS1/EXS-like* expression and plays a key role in archesporial cell development in tomato (Fig. 8). Isolation of the *SlSES* gene may positively impact gametogenesis research in tomato.

Materials and Methods:

Plant materials and growth conditions

The miniature tomato ‘Micro-Tom’ and the cultivated species ‘Ailsa Craig’ were used in this study. The *Slses* mutant was obtained from gamma ray-irradiated Micro-Tom mutant

populations produced by the National BioResource Project (NBRP, http://www.nbrp.jp/) (Saito et al. 2011). All lines were grown under a regimen of 16-h days and 8-h nights at a constant temperature of 25°C under fluorescent lights at 100 to 150 $\mu Em^{-2}s^{-1}$.

Histological and microscopic analysis

Whole anthesis flowers and flower buds were collected and fixed in FAA (formalin : acetic acid : 70% EtOH = 1 : 1 : 18), vacuum-infiltrated twice for 10 min each, and left overnight. The fixed tissues were dehydrated with a *t*-butyl alcohol series from No. 1 to 6, which contained *t*-butyl alcohol, ethanol and DW (Supplementary Table S4). Each step required a greater than 6-hour incubation at room temperature, and the No. 6 solution was used twice. The tissues in the No. 6 solution were incubated at 60°C for 2 hours. Then, melted paraffin was added, and the tissues were stored at 60°C overnight. The embedded tissues were sliced into 10-μm sections using a microtome (MICROM HM325, Leica, http://www2.leicabiosystems.com/). The sections were stained with 0.05% toluidine blue (pH 7.0). Paraffin was removed with xylene, and the sections were mounted with entellan new (Merck Millipore). Then, sections were observed under an optical microscope (Olympus BX53, http://www.olympus-lifescience.com/).

Map-based cloning using DNA markers

To conduct SolCAP analysis (http://solcap.msu.edu/index.shtml) for rough mapping, an F_2 mapping population was constructed from a cross between *S. lycopersicum* 'Ailsa Craig' and the *Slses* heterozygous mutant in the 'Micro-Tom' background. Then, we used 28 F_2 plants that exhibited the *Slses* mutation for this analysis. In total, 7,720 markers were used for this experiment, and 2,098 markers showed polymorphism to distinguish between 'Micro-Tom' and 'Ailsa Craig'. Then, the region of the responsible gene was narrowed down.

Genome re-sequencing and identification of responsible gene

The genome sequence of the *Slses* mutant was obtained by Illumina HiSeq 2000. The sequences of 'Heinz 1706' and 'Micro-Tom' Japan were used as reference genomes (Ariizumi et al. 2014; Kobayashi et al. 2014). Linkage analysis was conducted with F_2 plants which obtained crossed with the *ses* mutant and 'Micro-Tom', WT or 'Ailsa Craig'. The marker (*SlSES* marker-1, Supplementary Table S2) was designed to amplify the 13-bp deletion region. The amplicon sizes were different in the *ses* mutant and WT.

Characterization of the identified gene

The genome sequences and amino acid sequences were obtained from the Sol Genomics Network (SGN) (https://solgenomics.net/). The estimation of the SlSES protein domains was conducted using the Conserved Domain Database (CDD)

(http://www.ncbi.nlm.nih.gov/cdd). The homologous genes from Arabidopsis and Tomato were searched using The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/) and SGN, respectively. We selected the homologous gene of which e-value is under '0.1'. The phylogenetic tree was constructed with multiple sequence alignment using CLUSTALW (http://www.genome.jp/tools/clustalw/). The amino acid homology of SlSES and SPL/NZZ was analyzed using the EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) in the European Bioinformatics Institute (http://www.ebi.ac.uk/).

Creation of transgenic plants

The binary vector pIG121Hm was used for the creation of transgenic plants. The complete *SlSES* gene, which included 2563 bp of upstream sequence and 763 bp of downstream sequence, was amplified with gene-specific primers (whole *SlSES* gene primer, Supplementary table S2) using the In-Fusion technique. The amplified fragments of the complete genomic region were inserted into pIG121Hm digested by *Hind*III and *Sal*I using the In-Fusion technique (TAKARA) (Supplementary Fig. S3). Then, the construct was transformed into *Agrobacterium tumefaciens* GV2260 by electroporation, and transformants were selected on LB plates containing kanamycin (50 mg L^{-1}). The transformation construct was transformed into 'Micro-Tom' WT and plants heterozygous or homozygous for the *Slses* mutation using an *Agrobacterium*-mediated transformation method (Sun et al. 2006). The transgenic plants were

selected on MS plates containing kanamycin (100 mg L^{-1}). Ploidy and transgene copy number transgene were assessed by flow cytometry and Southern blotting analysis, respectively, in the T0 generation. Plants that were both diploid and single-copy transgenic were selected, and their T1 or T2 generations that harbored the *Slses* homozygous mutation were used for further analysis. The *Slses* homozygous mutants were selected using "*SlSES* marker-2" and the restriction enzyme *Bsl* I, we could distinguish among homozygous, heterozygous and WT by electrophoresis band pattern (Supplementary Table S2; Supplementary Fig. S4). The presence of transgene was confirmed by detecting "*NPT* II marker" using PCR (Supplementary Table S2).

Gene expression analysis

mRNA expression levels were analyzed by quantitative RT-PCR (qRT-PCR) using SYBR Premix Ex Taq II (TAKARA). The template cDNAs were synthesized from total RNA extracted from 1 mm whole buds or pistils of unopened buds classified by bud length (3 mm, 4.5 mm, 6 mm, 1 day before anthesis) and anthesis flowers. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, http://www.qiagen.com/), and cDNA was synthesized with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, http://www.lifetechnologies.com/). SGN-U316474 (*SAND*) mRNA was used as an internal control (Expósito-Rodríguez et al. 2008). The expression level was calculated according to Pfaffl 2001. The primers were designed with

[Primer3Plus] (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Supplementary Table S5).

Statistical analysis

We used Student's *t* test (P = 0.05 and 0.01) to show the significance between two values, WT and the *Slses* mutant. Then, Chi-squared (χ^2) test was performed to examine the correctness of fit between the expected Mendelian ratios and segregation data for the *Slses* mutation. We calculated χ^2 value on below conditions; degrees of freedom, 1; the expected Mendelian ratios, WT : *Slses* =3 : 1.

Funding

This work was supported by Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, Japan (grant no. 26013A) to HE. Seeds of Micro-Tom (TOMJPF00001), Ailsa Craig (TOMJPF0004), and *Slses* were obtained from the National BioResource Project, Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

Disclosures:

The authors have no conflicts of interest to declare.

Acknowledgements:

We thank all of the members of our laboratory for helpful discussions throughout the work.

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Tables:

Table 1. Comparison of the numbers of flower organs between the *Slses* mutant and WT.

	Flower organ			
	Sepal	Petal	Anther	Pistil
WT	5.20 ± 0.09	5.05 ± 0.09	5.15 ± 0.08	1.00 ± 0.00
Slses	5.30 ± 0.11	5.25 ± 0.10	5.30 ± 0.11	1.00 ± 0.00

Values are means ± standard error. Statistical significance was analyzed using the Student's *t* test.

Table 2. Comparison of the phenotypes of F_1 and F_2 plants obtained by crossing the *Slses* mutant (heterozygous) and WT plants.

Cross	Generation	Phenotype		χ^2 value	P value
		WT	*Slses*		
Slses (Heterozygous) × WT	F_1	17	0	-	-
	F_2 (A)	118	40	0.008	0.927
	F_2 (B)	149	0	-	-

Two patterns of F_2 population were observed: segregated (A) and non-segregated (B).
See supplementary Fig. S1.
The χ^2 value was calculated from the F_2 populations (A)

Figure legends

Figure 1.

The floral morphology of the *Slses* mutant at the anthesis stage. (A, B) The flower, (C, D) the anther and pollen grains, (E-H) the pistil. (A, C, E, G) WT, (B, D, F, H) *Slses* mutant. Bar = 1 mm. (I) The diameter and length of the ovary. Statistical analysis was performed using the Student's *t*-test. **P<0.01; *P<0.05, n = 10, error bar = SE.

Figure 2.

Cross-sections of developing anthers in WT and the *Slses* mutant. (A, C) Cross sections of anthers at the anthesis stage, (B, D) enlarged view of a piece of anther at the anthesis stage, (E, I) 1 to 2 mm bud, (F, J) 2 to 3 mm bud, (G, K) 4 to 5 mm bud, (H, L) 6 to 7 mm bud. (A, B, E-H) WT, (C, D, I-L) the *Slses* mutant. En, endothecium; Ep, epidermis; MC, meiotic cell; ML, middle layer; Msp, microspore; P, pollen; PMC, pollen mother cell; S, style; T, tapetum; VB, vascular bundle. Bar = 500 μm.

Figure 3.

Sections of developing pistils in WT and the *Slses* mutant. (A, C) Cross-section, (B, D) longitudinal section at the anthesis stage. (E, I) 1 to 2 mm bud, (F, J) 2 to 3 mm bud, (G, K) 4 to 5 mm bud, (H, L) 6 to 7 mm bud. Bar = 500 μm. (M) Ovule primordia of 2 to 3 mm bud, (N,

O) ovule primordia of 4 to 5 mm bud. Bar = 50 μm. (A, B, E-H, M, N) WT, (C, D, I-L, O) the *Slses* mutant. MMC, megaspore mother cell; O, ovule; OI, ovule integument; OP, ovule primordia; P, placenta.

Figure 4.

Morphology of ripe red fruits in the WT and the *Slses* mutant. (A) Appearance of red ripe fruits, (B) cross-section of the red fruits. Bar = 1 cm. (C) Fruit weight, (D) fruit size and (E) the number of locules per fruit in the WT and the *Slses* mutant. n = 20, error bar = SE, statistical analysis was performed using the Student's *t*-test, **P<0.01.

Figure 5.

Gene structure of *Solyc07g063670*. (A) The location and gene structure of *Solyc07g063670*. The blue bar indicates chromosome 7, the gray box indicates the UTR, white boxes indicate exons, and regions between the boxes indicate introns. (B) Genome and amino acid sequences of WT and the *Slses* mutant at the mutation point. The upper black letters indicate genome sequences, and the lower red letters indicate putative amino acids. Hyphens indicate the 13-bp deletion. (C) The putative amino acid length and domain of the SlSES protein. The putative amino acid length and domain region were exhibited under and upper of SlSES protein models respectively. The domain is indicated with yellow and red boxes. * = stop codon. (D)

Phylogenetic tree of *SlSES* and its homologous proteins in Arabidopsis. (E) The EAR motif of SlSES and SPL/NZZ at the C-terminal region. The red frame indicates the EAR motif.

Figure 6.

Expression analysis of *SlSES* in the WT by qRT-PCR. *SlSES* expression level in the WT in the 1 mm bud and in pistils from the 3 mm, 4.5 mm, 6 mm, 1 day before anthesis (DBA) and anthesis stages. n = 3.

Figure 7.

Expression analysis of gametogenesis genes in the WT and the *Slses* mutant by qRT-PCR. (A) *Male Sterile 10*^{35} (*MS10*^{35}), (B) *BARELY ANY MERISTEM1 and 2-like* (*BAM1/2-like*), (C) *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS-like* (*EMS1/EXS-like*), (D) *Tomato AGAMOUS1* (*TAG1*), (E) *WUSCHEL* (*SlWUS*), (F) *INNER NO OUTER* (*SlINO*), (G) *AINTEGUMENTA-like* (*ANT-like*), (H) *PIN-FORMED1-like* (*PIN1-like*), (I) *BELL1-like* (*BEL1-like*), (J) *REVOLUTA* (*SlREV*), (K) *CORONA-like* (*CNA-like*), (L) *PHABULOSA/PHAVOLUTA-like* (*PHB/PHV-like*), (M) *GOBLET* (*GOB*). mRNA expression was analyzed at the 1 mm bud stage and in pistils from the 4.5 mm bud and anthesis stages. n = 3, error bar = SE. Statistical analysis was performed using the Student's *t*-test. **P<0.01; *P<0.05.

Figure 8.

A model for ovule (left) and anther (right) development regulated by *SlSES* (*SPL/NZZ*) in tomato and Arabidopsis. Solid arrows represent positive regulation, and dotted arrows indicate gene function (red = tomato, blue = *Arabidopsis*). The gray box shows the model of integument development in Arabidopsis (left) and tomato (right). I = integument, II = inner integument, OI = outer integument, PMC = pollen mother cells, PPC = primary parietal cells, PSC = primary sporogenous cells, SPC = secondary parietal cells.

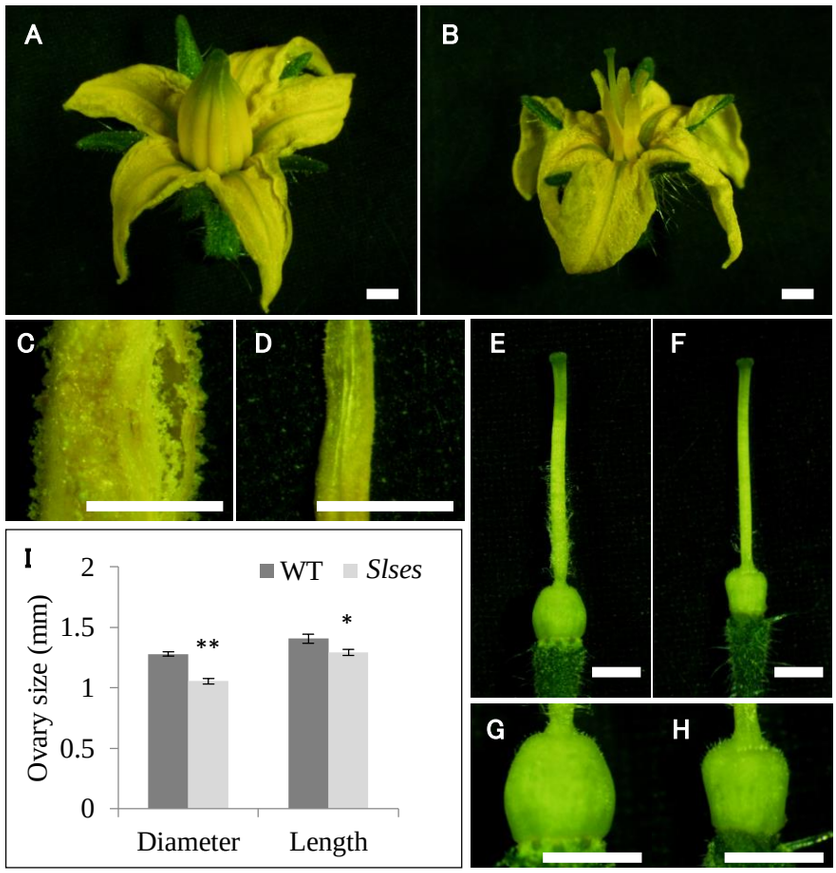

Figure 1

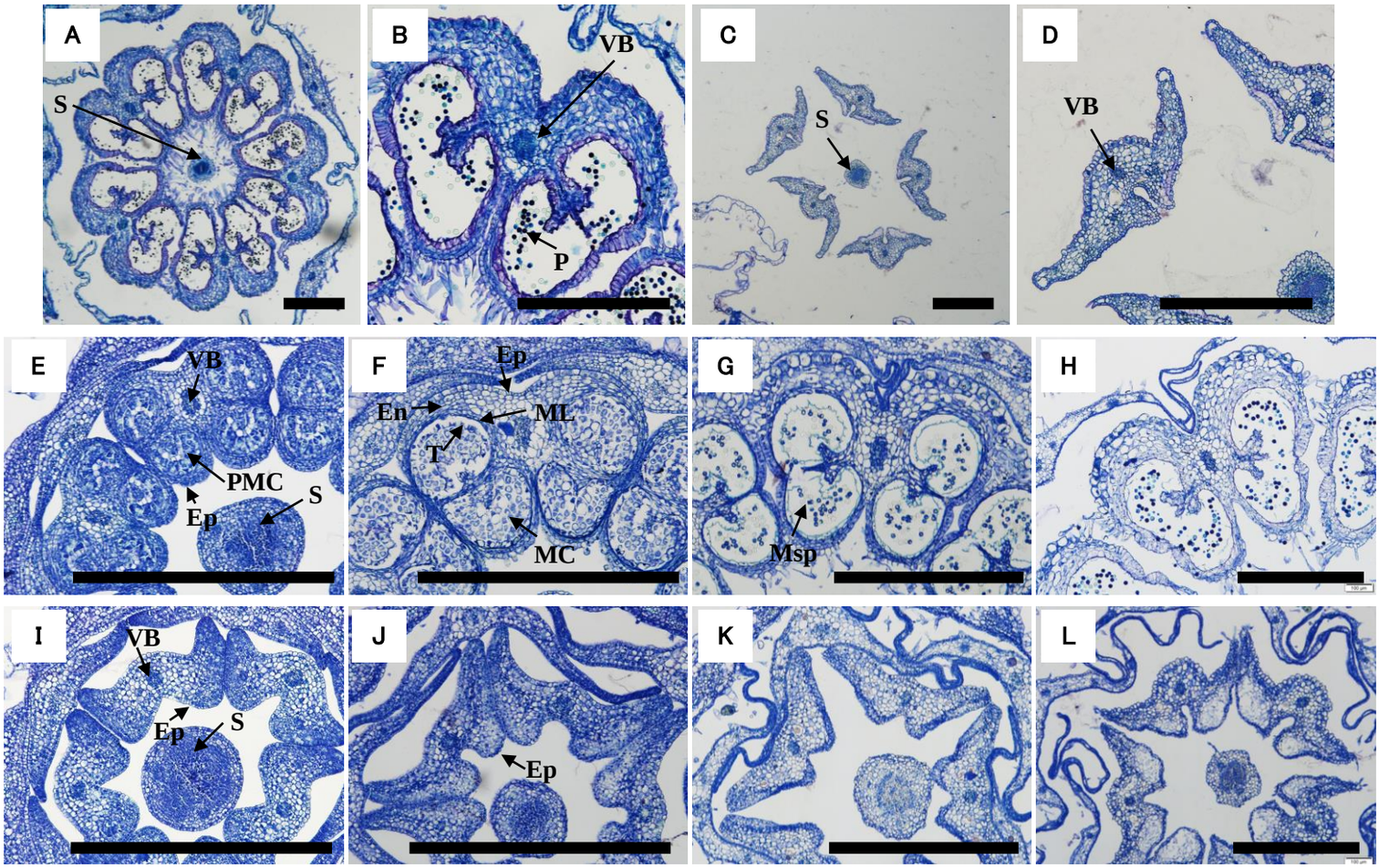

Figure 2

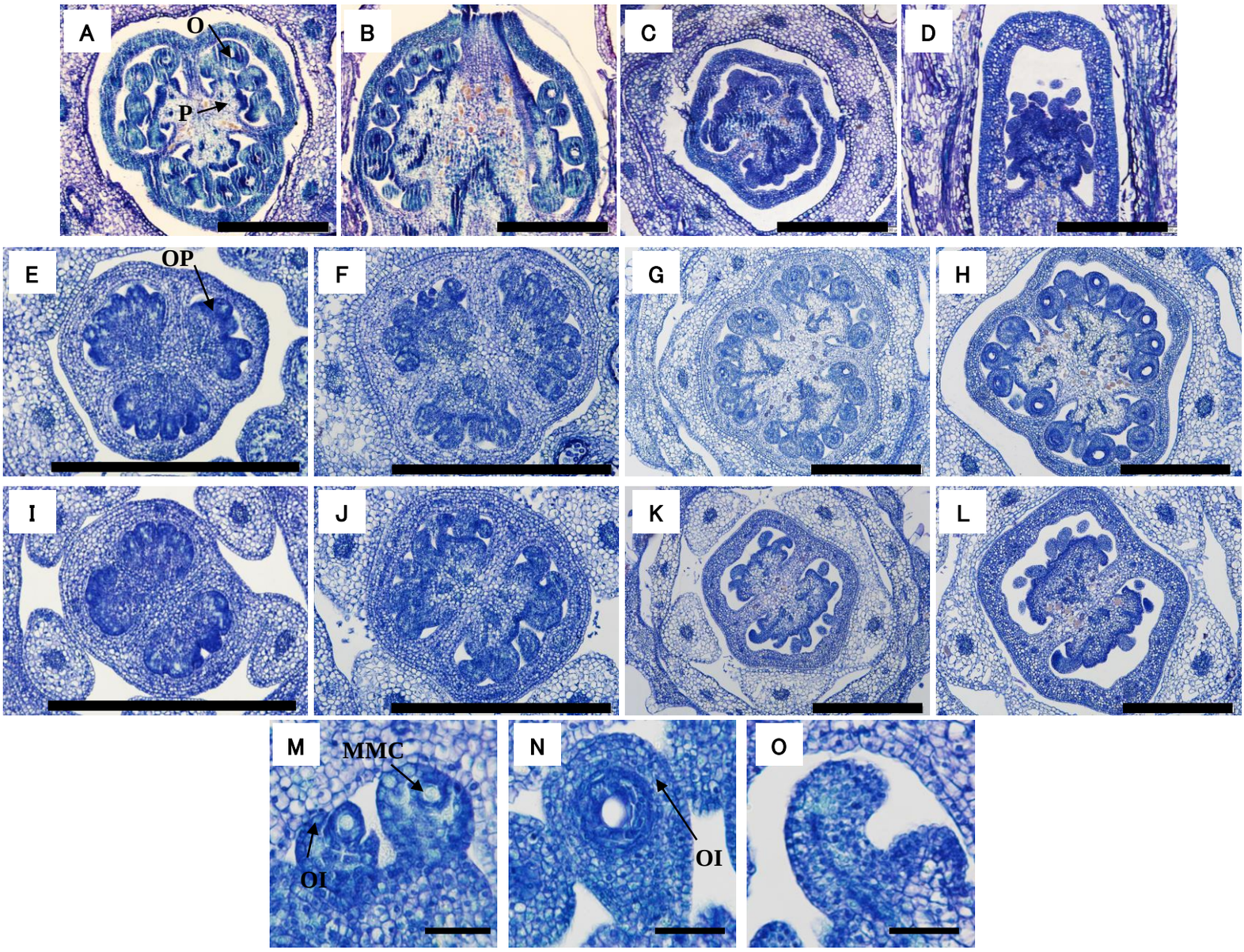

Figure 3

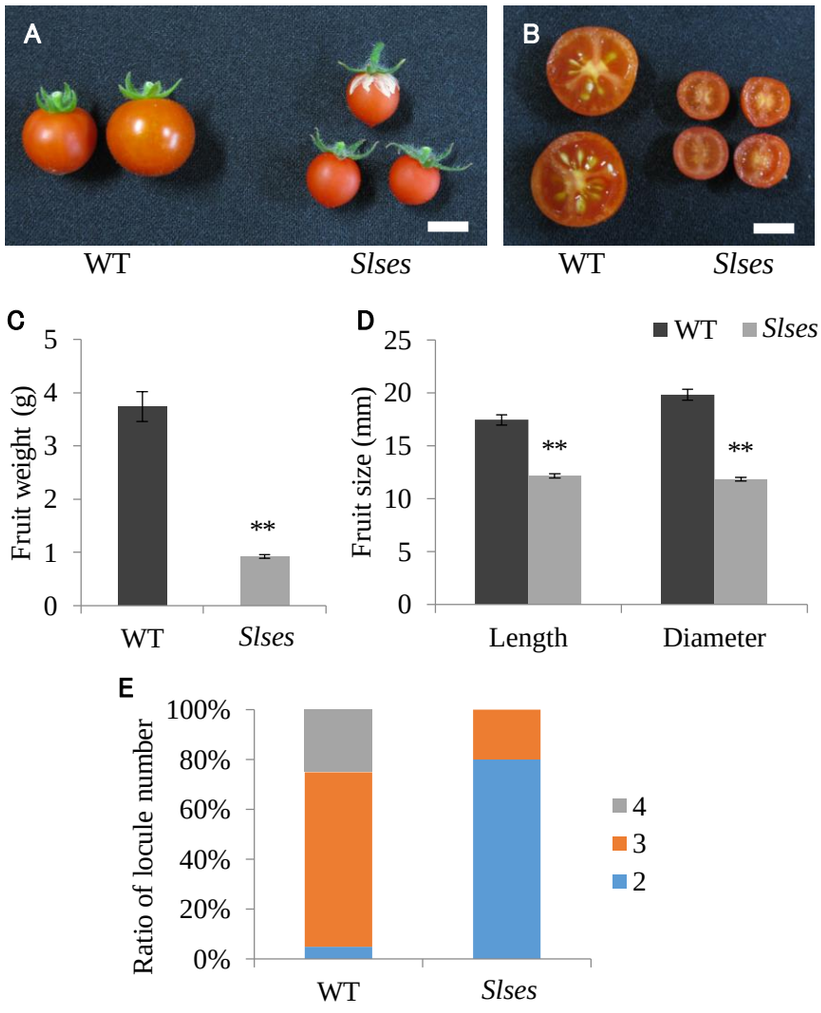
A
B
WT
Slses
WT
Slses
C
Fruit weight (g)
0
1
2
3
4
5
**
WT
Slses
D
WT
Slses
Fruit size (mm)
0
5
10
15
20
25
**
**
Length
Diameter
E
Ratio of locule number
0%
20%
40%
60%
80%
100%
4
3
2
WT
Slses

Figure 4

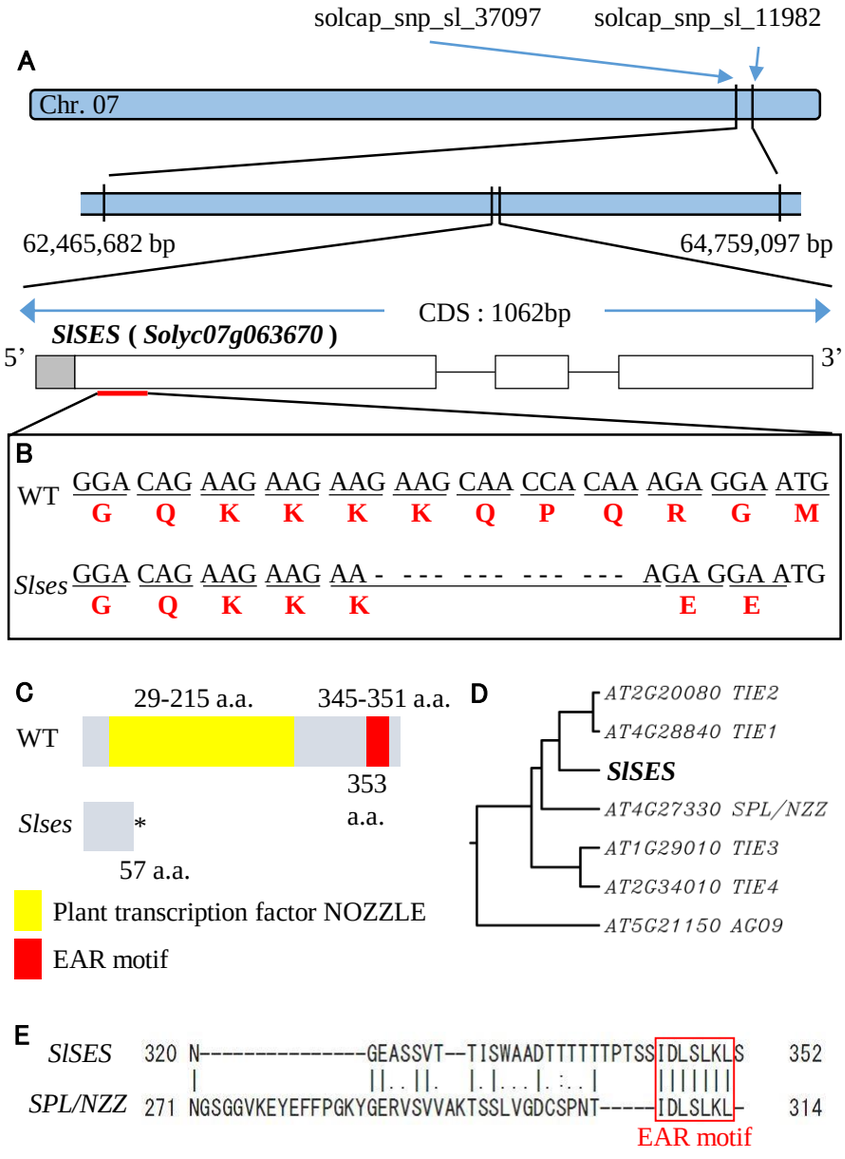
solcap_snp_sl_37097
solcap_snp_sl_11982
A
Chr. 07
62,465,682 bp
64,759,097 bp
CDS : 1062bp
SlSES (Solyc07g063670)
5'
3'
B
WT GGA CAG AAG AAG AAG AAG CAA CCA CAA AGA GGA ATG
G Q K K K K Q P Q R G M
Slses GGA CAG AAG AAG AA - --- --- --- --- AGA GGA ATG
G Q K K K E E
C
29-215 a.a.
345-351 a.a.
WT
353
a.a.
Slses
*
57 a.a.
Plant transcription factor NOZZLE
EAR motif
D
AT2G20080 TIE2
AT4G28840 TIE1
SlSES
AT4G27330 SPL/NZZ
AT1G29010 TIE3
AT2G34010 TIE4
AT5G21150 AG09
E
SlSES 320 N----------GEASSVT--TISWAADTTTTTTPTSSIDLSLKLS 352
SPL/NZZ 271 NGSGGVKEYEFFPGKYGERVSVVAKTSSLVGDCSPNT-----IDLSLKL- 314
EAR motif

Figure 5

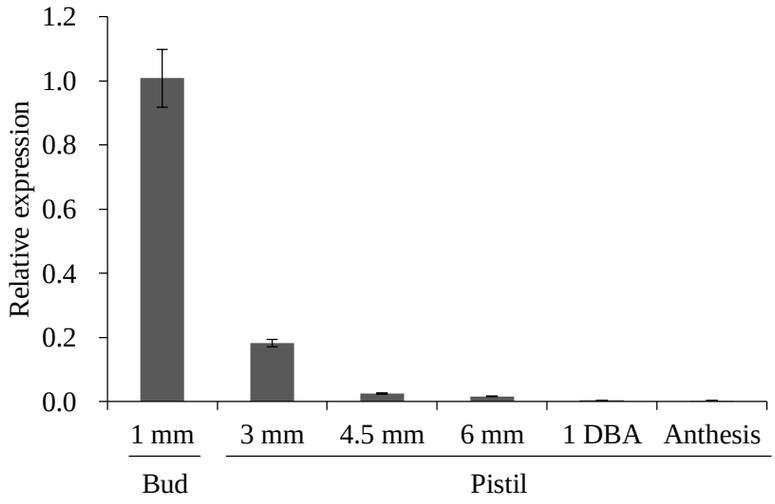
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 mm
3 mm
4.5 mm
6 mm
1 DBA
Anthesis
Bud
Pistil

Figure 6

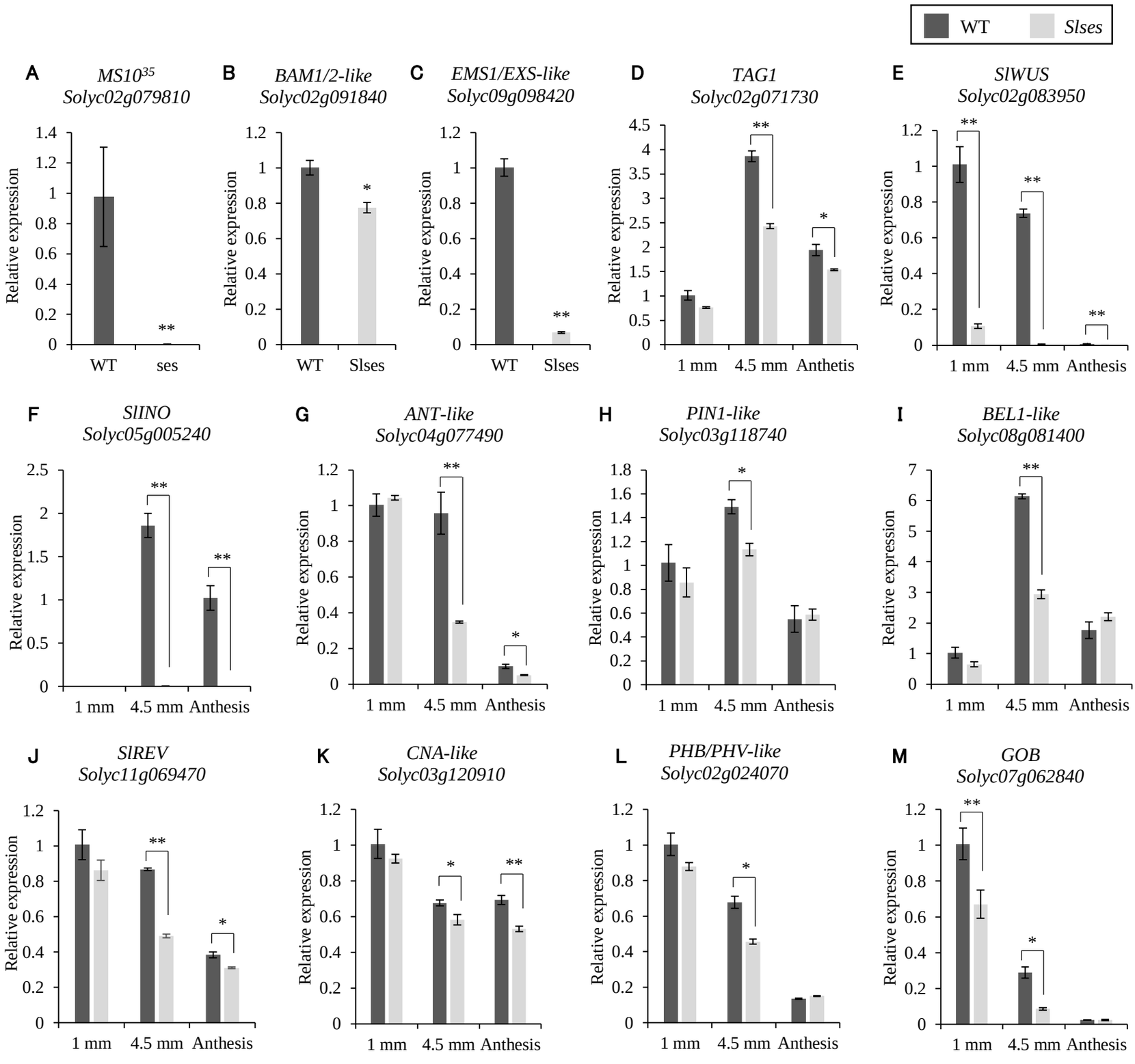
WT
Slses
A
MS10^35
Solyc02g079810
Relative expression
WT
ses
B
BAM1/2-like
Solyc02g091840
WT
Slses
C
EMS1/EXS-like
Solyc09g098420
WT
Slses
D
TAG1
Solyc02g071730
1 mm
4.5 mm
Anthetis
E
SlWUS
Solyc02g083950
1 mm
4.5 mm
Anthesis
F
SlINO
Solyc05g005240
1 mm
4.5 mm
Anthesis
G
ANT-like
Solyc04g077490
1 mm
4.5 mm
Anthesis
H
PIN1-like
Solyc03g118740
1 mm
4.5 mm
Anthesis
I
BEL1-like
Solyc08g081400
1 mm
4.5 mm
Anthesis
J
SlREV
Solyc11g069470
1 mm
4.5 mm
Anthesis
K
CNA-like
Solyc03g120910
1 mm
4.5 mm
Anthesis
L
PHB/PHV-like
Solyc02g024070
1 mm
4.5 mm
Anthesis
M
GOB
Solyc07g062840
1 mm
4.5 mm
Anthesis

Figure 7

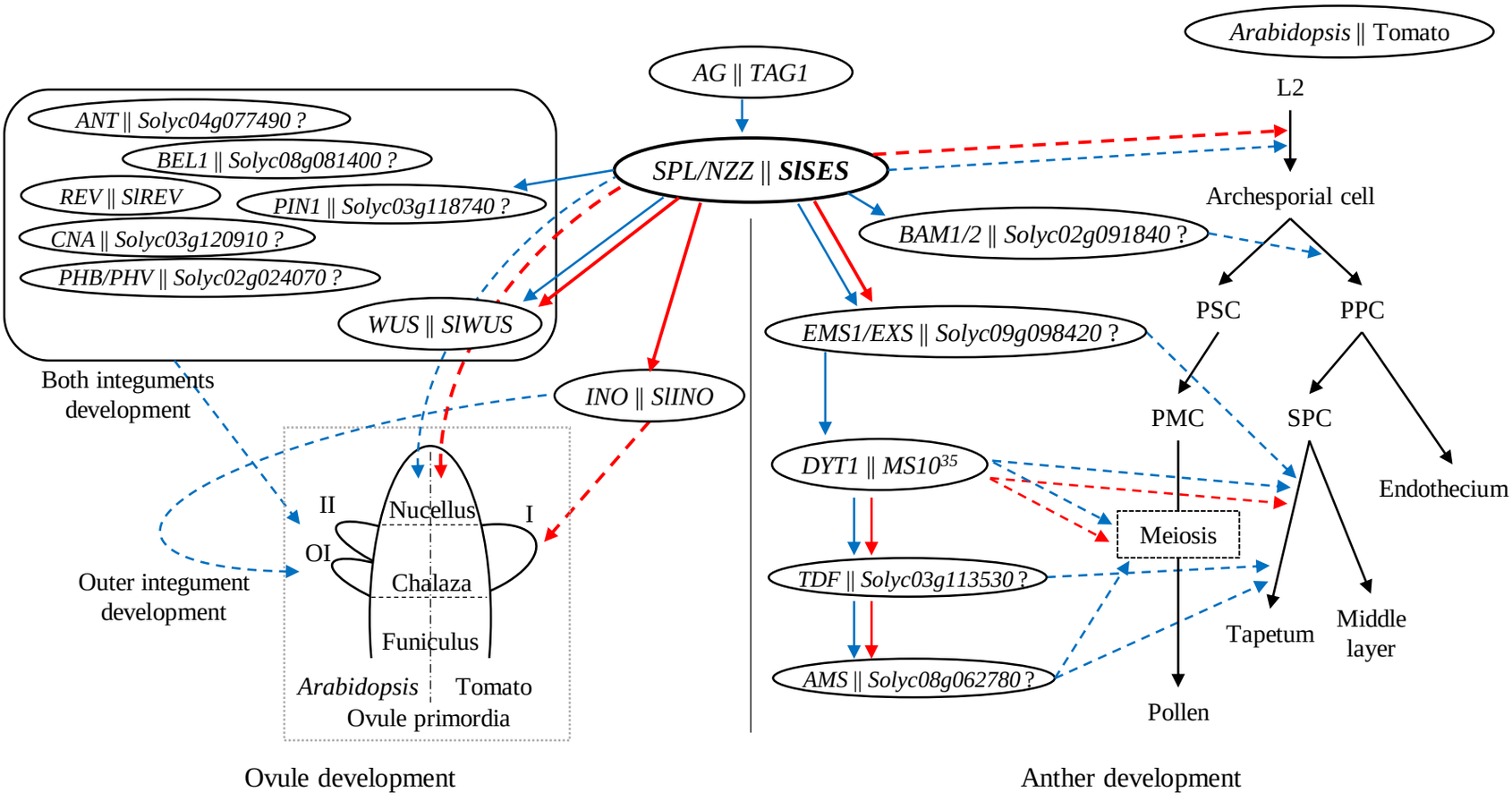
Arabidopsis || Tomato
AG || TAG1
L2
ANT || Solyc04g077490 ?
BEL1 || Solyc08g081400 ?
REV || SlREV
PIN1 || Solyc03g118740 ?
CNA || Solyc03g120910 ?
PHB/PHV || Solyc02g024070 ?
SPL/NZZ || SlSES
Archesporial cell
BAM1/2 || Solyc02g091840 ?
PSC
PPC
WUS || SlWUS
EMS1/EXS || Solyc09g098420 ?
Both integuments development
INO || SlINO
PMC
SPC
DYT1 || MS10^35
Endothecium
II
Nucellus
I
OI
Meiosis
Outer integument development
TDF || Solyc03g113530 ?
Chalaza
Tapetum
Middle layer
Funiculus
AMS || Solyc08g062780 ?
Arabidopsis
Tomato
Ovule primordia
Pollen
Ovule development
Anther development

Figure 8