A SUPERMAN-like Gene is Exclusively Expressed in Female Flowers of the Dioecious Plant Silene latifolia

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To elucidate the mechanism(s) underlying dioecious flower development, the present study analyzed a SUPERMAN (SUP) homolog, SlSUP, which was identified in Silene latifolia. The sex of this plant is determined by heteromorphic X and Y sex chromosomes. It was revealed that SlSUP is a single-copy autosomal gene expressed exclusively in female flowers. Introduction of a genomic copy of SlSUP into the Arabidopsis thaliana sup (sup-2) mutant complemented the excess-stamen and infertile phenotypes of sup-2, and the overexpression of SlSUP in transgenic Arabidopsis plants resulted in reduced stamen numbers as well as the suppression of petal elongation. During the development of the female flower in S. latifolia, the expression of SlSUP is first detectable in whorls 2 and 3 when the normal expression pattern of the B-class flowering genes was already established and persisted in the stamen primordia until the ovule had matured completely. In addition, significant expression of SlSUP was detected in the ovules, suggestive of the involvement of this gene in ovule development. Furthermore, it was revealed that the de-suppression of stamen development by infection of the S. latifolia female flower with Microbotryum violaceum was accompanied by a significant reduction in SlSUP transcript levels in the induced organs. Taken together, these results demonstrate that SlSUP is a female flower-specific gene and suggest that SlSUP has a positive role in the female flower developmental pathways of S. latifolia.

Keywords: Dioecious plant • Female flower specific expression • Floral development • Silene latifolia • SUPERMAN

Abbreviations: CaMV, Cauliflower mosaic virus; NJ, neighbor-joining; RT-PCR, reverse transcription PCR; QRT-PCR, quantitative reverse transcription PCR; RACE, Rapid Amplification of cDNA Ends; SUP, SUPERMAN; TFIIIA, transcription factor IIIA; TSA, tyramide signal amplification.

The nucleotide sequence reported in this paper has been submitted to DDBJ database under accession number AB246393 (SlSUP).

Introduction

Most flowering plants are hermaphroditic, producing bisexual flowers that contain both male and female organs. However, 6% of known angiosperm species are dioecious with separate male and female plants that produce male or female flowers, respectively (Renner and Ricklefs 1995). In addition, the flowers of many dioecious species with hermaphroditic relatives tend to have rudimentary opposite-sex structures in the respective flowers of each sex, suggesting that they have recently evolved from a hermaphroditic form (Darwin 1877, Grant et al. 1994, Ainsworth et al. 1995). In some cases, the recent origins of dioecy from a cosexual state have been clearly determined (Darwin 1877, Westergaard 1946), and dioecy itself appears at a low frequency and in a

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S. latifolia is one of the best-characterized floral genes in the hermaphroditic flowers of A. thaliana and other plant species. The A. thaliana SUPERMAN (SUP) gene encodes a DNA-binding transcription factor that plays important roles mainly during two floral developmental stages (Schultz et al. 1991, Bowman et al. 1992, Gaiser et al. 1995). One is the stage that determines the boundary between floral whorls 3 and 4 in early flower development. In sup mutants, the cells of whorl 3 proliferate abnormally, resulting in a shift of this boundary toward the center of the floral meristem, which also results in an increased number of stamens. SUP expression is first detectable in part of whorl 3 adjacent to whorl 4, after the normal early expression pattern of B-class genes has been established (Sakai et al. 1995). This timing of SUP activation is in good accord with the suppression of cell proliferation around the region, and thus SUP is believed to maintain the boundary between whorls 3 and 4 (Sakai et al. 2000). The other floral developmental stage for which this gene plays a functional role is ovule development, in which cell proliferation in the outer integument on the adaxial side of the ovule is specifically repressed (Gaiser et al. 1995). In sup mutants, the outer ovule integuments exhibit symmetric growth, which results in the production of infertile ovules. A number of studies have so far demonstrated that the physiological functions of SUP in floral whorls and ovules can be attributed to its negative activity during cell proliferation. This activity requires the conserved DLELRL motif found in the C-terminal region of SUP and its P. hybrida homolog, PhSUP1 (Hiratsu et al. 2002, Hiratsu et al. 2004). In P. hybrida, the PhSUP1 gene is similar to SUP and is expressed in the boundary between whorls 3 and 4, the ovules, and the developing anthers, and its transposon-knockout mutants show abnormal morphologies in these floral organs as a general consequence of excessive cell proliferation (Nakagawa et al. 2004). The function of SUPERMAN genes has also been evaluated in ectopic expression experiments. The forced expression of SUP in an alloplasmic CMS tobacco plant caused decreases in the sizes of various organs (Bereterbide et al. 2001), and exogenous expression of either SUP or PhSUP1 in P. hybrida results in the suppression of cell expansion and a reduction of the cell number in the inflorescence stems and petal tubes (Kater et al. 2000, Nakagawa et al. 2004) (for a summary, see Supplemental Table S2). Thus, the activity of SUP against cell proliferation may be well conserved among eudicots, although the expression profile of this gene differs between plant species. Thus, it was hypothesized that a S. latifolia SUP homolog(s) may play a role in the development of sexual organs in the female and/or male plants via the control of cell proliferation.

The current study identified a S. latifolia homolog of SUP, denoted SISUP, and demonstrated that this gene is expressed only in female flowers. SISUP was found to be expressed in...
and around the stamen primordia of female flowers and in the ovules, whereas its transcripts could not be detected in male flowers. SlSUP also functionally complemented the defects of the A. thaliana sup mutant, and its overexpression in A. thaliana also resulted in suppressed development or elongation of both the stamens and petals. Based on these results, it is proposed that SlSUP plays a role in the developmental pathway of the female flower in S. latifolia.

Results

Identification of a SUP-like gene in S. latifolia

To isolate a SUP-like gene from S. latifolia, total RNA extracts from male and female flower buds were subjected to RT-PCR analysis using degenerate primers that target the conserved regions between SUP and PhSUP1 (Sakai et al. 1995, Nakagawa et al. 2004) (Supplemental Fig. S1A; Table S1). A 491-bp fragment was specifically amplified from the female flower-derived cDNAs and then sequenced. A BLAST search revealed that the putative amino acid sequence encoded by this amplicon was significantly similar to SUP and PhSUP1. The 5' and 3' extended regions of this DNA fragment were subsequently isolated using Rapid Amplification of cDNA Ends (RACE) and the full-length cDNA of the SUP-like gene in S. latifolia (SlSUP) was then obtained.

The full-length SlSUP cDNA encodes a protein of 222 amino acids. An alignment of the deduced SlSUP, PhSUP1, and SUP protein sequences is shown in Figure 1. Overall, SlSUP showed 38% and 37% identity with SUP and PhSUP1, respectively. The zinc-finger and leucine-zipper (LZ)-like domains were also highly conserved among these three proteins (Fig. 1). The zinc-finger motifs and flanking basic residues in SlSUP were 87% identical to those in SUP and PhSUP1, and the LZ-like domains in SlSUP, SUP, and PhSUP1 share identical residues, including the DLELRL hexapeptide (Hiratsu et al. 2004), that confer the transcriptional repression activity of SUP. SlSUP is also a member of the transcription factor IIIA (TFIIIA)-type zinc-finger proteins (Kubo et al. 1998). Phylogenetic analysis was performed for the conserved zinc-finger domain of the putative proteins from four plant species using the neighbor-joining (NJ) method (Saitou and Nei 1987). The results showed that SlSUP falls into a distinct group with SUP, PhSUP1, RBE, and AtZFP1 (Supplemental Fig. S2A). RBE and AtZFP1 are A. thaliana SUP-like proteins, and the former is required for the early development of the organ primordia of whorl 2 (Takeda et al. 2004), whilst the latter is expressed predominantly in vegetative tissues but is of unknown function (Tague et al. 1996). To clarify the relationships between these five proteins, another phylogenetic tree was constructed using the entire protein sequence (Supplemental Fig. S2B) and the results showed that SlSUP formed a distinct group with SUP and PhSUP1. Hence, it is likely that SlSUP is an ortholog of SUP.

To determine whether SlSUP is located on one of the sex chromosomes in S. latifolia, genomic Southern hybridization analysis of this gene was performed using a probe that is

Fig. 1 Alignment of the SUPERMAN and SUPERMAN-like proteins. The amino acid sequences of SlSUP, SUP, and PhSUP1 were aligned using CLUSTAL W ver. 1.8 (Thompson et al. 1994). Identical and similar amino acid residues are indicated by asterisks and dots, respectively. Shading was performed using BOXSHADE ver. 3.21 (http://www.ch.embnet.org/software/BOX_mat.html). The conserved zinc-finger and leucine zipper (LZ)-like domains are also highlighted. The DLELRL domain required for transcriptional repression activity of SUP (Hiratsu et al. 2004) is indicated by the boxed area.
complementary to the region 359-bp downstream of the conserved zinc-finger domain sequence (see Supplemental Fig. S1A). Under high-stringency conditions, the autosomal and the sex-chromosomal genes could be distinguished by their band intensities (data not shown; Nishihara et al. 2007). The resulting DNA restriction patterns were indistinguishable between males and females and their signal intensities were similar (Fig. 2), indicating that SISUP is an autosomal single-copy gene.

Genetic complementation of the Arabidopsis sup mutant by SISUP
To examine whether SISUP is functionally related to the SUP gene from A. thaliana, a 3.5-kb genomic DNA fragment containing the full-length SISUP (pgSISUP) was introduced into an A. thaliana sup mutant using Agrobacterium-mediated transformation (Supplemental Fig. S1A). Of the 74 transformants obtained, 20 lines were found to harbor the homozygous sup (sup-2) mutant allele (see Supplemental Fig. S1B), and were then further characterized. Wild-type A. thaliana flowers have four sepals, four petals, six stamens, and one pistil that comprised two carpels (Fig. 3A; Smyth et al. 1990). The sup mutants, however, develop more stamens per flower compared with wild-type plants (Fig. 3D; 8.7 ± 0.29 on average (range 7–13); n = 35, from seven independent plants) (Schultz et al. 1991, Bowman et al. 1992). Furthermore, in the sup A. thaliana mutant, normal pistil development is inhibited, which severely reduces its fertility (Fig. 3G), whereas petal formation is unaffected.

Two sup transgenic lines harboring pgSISUP (pgSISUP/sup-2, lines 1 and 2) were found to have a wild-type flower phenotype (Fig. 3B) and fewer stamens (from three to eight) with an average of 5.0 ± 0.28 and 5.8 ± 0.77 per flower in line 1 and line 2, respectively (Table 1). In addition, whereas normal pistils were rarely observed in the sup flowers, 43% of the flowers that developed in the complemented pgSISUP/sup-2 plants showed a normal pistil morphology (n = 35, from seven independent plants) (Fig. 3B), and also produced normal-looking siliques which contain viable seeds (Fig. 3H; data not shown). Most of the other pgSISUP/sup-2 lines showed a partially complemented phenotype with fewer stamens (Table 1), and either normal pistils or stamen-pistil mosaic structures (Fig. 3E). These fully and partially

Fig. 2 Southern blot analysis of the SISUP gene in male and female S. latifolia plants. Male and female genomic DNAs derived from mature leaves were digested with EcoRI (EI), EcoRV (EV), or HindIII (H), electrophoresed in a 1.0% (w/v) agarose gel, transferred to a nylon membrane, and hybridized with a genomic fragment of SISUP (see Supplemental Fig. S1A). The positions of the molecular weight standards are indicated on the left.

Fig. 3 Complementation of an A. thaliana sup mutant by SISUP. A 3.5-kb genomic DNA fragment containing the entire coding sequence of SISUP (pgSISUP, Fig. S1), or the SISUP cDNA under the control of the Arabidopsis SUP promoter (pSUP::SISUP, Fig. S1) were introduced into the sup mutant (sup-2) (Schultz et al. 1991). Flowers from the wild-type plant (A) and the sup-2 mutant (D) are shown. Transgenic plants expressing the gSISUP construct showed both complete (B) and weak (E) recovery of flower development. Transgenic plants harboring the pSUP::SISUP construct also developed both a complete (C) and weak (F) complement phenotype. Infl orescences of the sup-2 mutant (G), a pgSISUP/sup-2 plant (H) and a pSUP::SISUP/sup-2 plant (I) with recovered flower development are also shown. Bar, 1 mm (A–F); 1 cm (G–I).
complemented phenotypes of the pgSISUP/sup-2 plants were found to be associated with the hygromycin resistance conferred by the transgene and persist through successive plant generations (data not shown).

Next, the full-length SISUP gene under control of the upstream genomic sequence of A. thaliana SUP (Ito et al. 2003) was introduced into a sup-2 mutant. A total of 19 lines harboring the homozygous sup (sup-2) mutant allele were obtained, and seven randomly selected lines were then characterized. In this experiment, the sup mutants developed more stamens than in the experiments described above (Table 2; 9.6 ± 0.25 on average (range 6–12); n = 35, from seven independent plants), but four lines (pSUP::SISUP/sup-2, lines 1, 2, 4, and 6) showed an obvious wild-type flower phenotype (Fig. 3C) with the correct number of stamens (Table 2), and developed normal-looking siliques (Fig. 3I). Two lines (3 and 5) showed a partially complemented phenotype (Fig. 3F), and line 7 was found to be similar to the sup-2 mutant (Table 2). Unlike pgSISUP/sup-2, even the weakly complemented plants tended to have normal-looking pistils (data not shown). Taken together, these experiments indicated that SISUP can genetically complement the defects of floral development in the sup mutant, and that SISUP encodes a protein with properties that are conserved with SUP.

**Suppression of petal and stamen development caused by overexpression of SISUP in A. thaliana**

To further investigate the role of SISUP in A. thaliana, overexpression analyses were performed. The full-length SISUP cDNA was inserted downstream of the Cauliflower mosaic virus 35S gene (CaMV35S) promoter and the resulting transgene (35S::SISUP) was introduced into wild-type A. thaliana. A total of 13 transgenic lines were subsequently obtained, and seven of these were randomly selected for further characterization. The 35S::SISUP-transformed plants (35S::SISUP/wt) only showed morphological changes in their flowers and no deleterious effects on vegetative organs could be observed (data not shown). In contrast to the A. thaliana wild-type flower with six stamens (Fig. 3A and Fig. 4A and B), the 35S::SISUP/wt transgenic flowers developed an average of only 4.7 ± 0.12 stamens (n = 35, from seven plants; Table 3), and 20% showed elongated pistils and failed to open fully (Fig. 4C). They also typically produced comparatively shorter petals, although the number of petals was never reduced (Fig. 4C).

Dissection of these unopened flowers revealed stunted stamens (Fig. 4D and E). Figures 4F and 4G show scanning electron micrographs of a 35S::SISUP/wt flower bud. These images revealed a severely stunted stamen during the early stages of its development, which could have resulted in the reduction seen in the stamen numbers in the mature flowers of the 35S::SISUP/wt plants (Fig. 4E). On the other hand, both silique elongation and seed production were perturbed concomitantly in a population of the overexpressor flowers with the most severe phenotypes (data not shown), although these are likely to be indirect consequences. These observations showed that the CaMV35S promoter-driven SISUP affected both stamen development and petal elongation. Taken together with the results of the present complementation analysis (Fig. 3), these data suggest that SISUP functions via the suppression of cell proliferation in A. thaliana to control the development of floral organs.

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**Table 1** Average stamen number per flower in A. thaliana wild-type, sup-2, and transgenic sup-2 plants expressing SISUP.

<table>
<thead>
<tr>
<th>Plant (control)</th>
<th>Stamen number</th>
<th>Plant (transgenic)</th>
<th>Stamen number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.0 ± 0.0</td>
<td>pgSISUP/sup-2</td>
<td>5.0 ± 0.28</td>
</tr>
<tr>
<td>sup-2</td>
<td>8.7 ± 0.29</td>
<td>line 1</td>
<td>5.0 ± 0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 2</td>
<td>5.8 ± 0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 3</td>
<td>7.2 ± 0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 4</td>
<td>7.0 ± 0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 5</td>
<td>8.6 ± 0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 6</td>
<td>6.4 ± 0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 7</td>
<td>7.2 ± 0.82</td>
</tr>
<tr>
<td>Average</td>
<td>6.7 ± 0.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values shown are the means ± SE of 35 flowers from wild-type and sup-2, or 5 flowers from SISUP transgenic plants (pgSISUP/sup-2). For SISUP transgenic plants, 7 independent plants were arbitrarily selected and examined.

**Table 2** Average stamen number per flower in A. thaliana wild-type, sup-2, and transgenic sup-2 plants expressing SISUP under the control of the Arabidopsis SUP promoter.

<table>
<thead>
<tr>
<th>Plant (control)</th>
<th>Stamen number</th>
<th>Plant (transgenic)</th>
<th>Stamen number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.0 ± 0.0</td>
<td>pSUP::SISUP/sup-2</td>
<td>5.8 ± 0.18</td>
</tr>
<tr>
<td>sup-2</td>
<td>9.6 ± 0.25</td>
<td>line 1</td>
<td>5.8 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 2</td>
<td>5.8 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 3</td>
<td>8.2 ± 0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 4</td>
<td>6.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 5</td>
<td>7.4 ± 0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 6</td>
<td>6.0 ± 0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 7</td>
<td>10.0 ± 0.28</td>
</tr>
<tr>
<td>Average</td>
<td>7.0 ± 0.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values shown are the means ± SE of 35 flowers from wild-type and sup-2, or 5 flowers from SISUP transgenic plants (pSUP::SISUP/sup-2). For SISUP transgenic plants, 7 independent plants were arbitrarily selected and examined.

*Significant differences found between sup-2 mutants and transgenic plants (P < 0.05).
To ascertain whether the SlSUP gene is differentially expressed between male and female flowers in *S. latifolia*, RT-PCR analyses were performed (Fig. 5). Total RNA was isolated from male and female leaves, open flowers, and from flower buds corresponding to stages 1–8, 9–11.2, 11.3–11.4, and 11.5–11.7. The results showed that SlSUP was expressed in the female flower at all of the stages examined, but not in leaves or in male flowers (Fig. 5). Repeat experiments confirmed the lack of any detectable SlSUP transcripts in male flowers (data not shown) suggesting that SlSUP has a female flower-specific role.

To gain further insights into the temporal and spatial regulation of SlSUP expression during floral sex differentiation, in situ hybridization analyses were performed (Fig. 5). Total RNA was isolated from male and female leaves, open flowers, and from flower buds corresponding to stages 1–8, 9–11.2, 11.3–11.4, and 11.5–11.7. The results showed that SlSUP was expressed in the female flower at all of the stages examined, but not in leaves or in male flowers (Fig. 5). Repeat experiments confirmed the lack of any detectable SlSUP transcripts in male flowers (data not shown) suggesting that SlSUP has a female flower-specific role.

### Table 3 Reduced stamen numbers per flower in SlSUP-overexpressing transgenic *A. thaliana.*

<table>
<thead>
<tr>
<th>Plant (control)</th>
<th>Stamen number</th>
<th>Plant (transgenic)</th>
<th>Stamen number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.0 ± 0.0</td>
<td>35S::SlSUP/wt</td>
<td>4.6 ± 0.22a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 1</td>
<td>4.6 ± 0.22a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 2</td>
<td>5.0 ± 0.28a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 3</td>
<td>4.8 ± 0.18a</td>
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<tr>
<td></td>
<td></td>
<td>line 4</td>
<td>4.8 ± 0.44a</td>
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<tr>
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<td></td>
<td>line 5</td>
<td>5.0 ± 0.18a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 6</td>
<td>4.2 ± 0.28a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>line 7</td>
<td>4.8 ± 0.28a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>4.7 ± 0.12</td>
</tr>
</tbody>
</table>

The values shown are the means ± SE of 35 flowers from wild-type, or 5 flowers from SlSUP transgenic plants (35S::SlSUP/wt). For SlSUP transgenic plants, 7 independent plants were arbitrarily selected and examined.

*Significant differences found between wild-type and transgenic plants (*P* < 0.05).
In addition, at the base of the sup-

However, hybridization with the antisense probes revealed that SISUP was not expressed at any stage in the male flower (Fig. 6K and L) or in the female flower up to stage 3 (Fig. 6E), but could be detected from stage 4 in female flowers (Fig. 6F).

After stage 4 in the female flower, accumulation of the SISUP transcripts was found to be limited to the whorl-2 and whorl-3 regions, from which the petal and stamen primordia develop at later stages, respectively (Fig. 6F). SISUP continued to be expressed in whorls 2 and 3 until stage 5, at which time the petal and stamen primordia emerged (Fig. 6G). At stage 8, the accumulation of SISUP transcripts was observed to be limited to the bases of the stamen primordia and was seemingly associated with the suppression of their development (Fig. 6H and I). In addition, at the base of the suppressed stamens, marginal SISUP signals could be detected near the base of the petals (Fig. 6I). At stage 12, the expression of SISUP was detectable in suppressed stamens (Fig. 6J and M). Furthermore, it was found that SISUP transcript accumulated in the ovules. These observations indicate that the SISUP gene is expressed in the developing female flowers of *S. latifolia*, particularly in the stamen primordia and ovules. A Y chromosome-dependent suppression of the autosomal SISUP gene expression is thus implied.

**A reduction in SISUP transcript levels in the developing stamens of female flowers is induced by smut fungus infection**

The current data and previous results (Sakai et al. 1995, Nakagawa et al. 2004) suggest a trend towards the local activation of SUP and SUP-like genes in the floral tissues, particularly the stamen primordia where a strict coordination of cell proliferation control is required. It is currently difficult to assess the SISUP functions in stamen development directly because molecular genetic techniques have not been established in *S. latifolia*. In an attempt to resolve this, a smut fungus (*Microbotryum violaceum*) infection system was utilized that can biologically induce stamen development in the female flowers of *S. latifolia*. The smut infected-female flower seemed morphologically to be hermaphroditic, and thus it was expected that it would be a useful system to elucidate any correlations between floral gene expression and stamen development (Fig. 6N and O) (Westergaard et al. 2003).

In situ hybridizations were performed on smut-infected and uninfected flowers in the same manner. The expression of SISUP was maintained at stage 5 but reduced at (and until) stage 6 in developing stamens of the infected hermaphroditic-like flower (Fig. 6P and Q). These expression patterns were suggestive of a positive relationship between the SISUP expression levels and the suppression of stamen development. To further elucidate this, quantitative RT-PCR (QRT-PCR) analyses were performed (Fig. 7). Total RNA was extracted from flower buds of male and female plants corresponding to stages 1–8 and 9–11.2, and from female gynoecia at stages 9–11 for both infected and uninfected flowers. The SISUP transcript levels, relative to the 18S rRNA control, were calculated from three independent experiments. In this analysis, the SISUP transcript levels could not be measured directly in de-suppressed stamens, as the stamens at stages 1–11 were too small to be isolated intact. Instead, the relative transcript levels of SISUP in whole flower buds at each stage were investigated. The results showed that in uninfected female plants the SISUP transcripts levels were dramatically increased by more than 5-fold at stages 9–11 compared with stages 1–8. However, this activation was not observed in the infected flowers (Fig. 7A). Notably, the SISUP transcript levels in the female gynoecium in infected flowers were about two-fold higher compared with this same organ in uninfected female flowers. The primary cause for the suppressed SISUP activation in the female flower thus appeared to occur in the stamens. Use of a control SLM2 gene, a homolog of Arabidopsis *PISTILLATA*, which underwent de-suppression in the repressed female flower stamens following infection (Kazama et al. 2005) validated the present experimental conditions for fungal infection and QRT-PCR (Supplemental Fig. S3).

No SISUP mRNA could be detected in male flowers, with or without fungal infection, by QRT-PCR (Fig. 7A). To further understand the transcriptional changes of SISUP before ovule development occurs, the SISUP mRNA levels were measured at individual stages from 4 to 7 in uninfected and infected female flower buds (Fig. 7B). SISUP transcript levels were detected at all of the stages tested, but were found to be increased remarkably at stage 7 in the uninfected flowers.

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**Fig. 5 RT-PCR analysis of SISUP expression in male and female *S. latifolia* plants. Total RNAs from leaves, flower buds (stages 1–8, 9–11.2, 11.3–11.4, and 11.5–11.7), and open flowers were analyzed by RT-PCR using the SISUP_F2 and SISUP_R primers, which exclusively amplify SISUP transcripts. The stages were determined by the lengths of the flower buds (<1 mm for stages 1–8; 2–5 mm for stages 9–11.2; 6–10 mm for stages 11.3–11.4; 11–15 mm for stages 11.5–11.7), as previously described (Grant et al. 1994; Farbos et al. 1997). SISUP expression is detectable in female plants only. An actin gene was amplified as a control as described previously (Sugiyama et al. 2003).**

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**A reduction in SISUP transcript levels in the developing stamens of female flowers is induced by smut fungus infection**

The current data and previous results (Sakai et al. 1995, Nakagawa et al. 2004) suggest a trend towards the local activation of SUP and SUP-like genes in the floral tissues, particularly the stamen primordia where a strict coordination of cell proliferation control is required. It is currently difficult to assess the SISUP functions in stamen development directly because molecular genetic techniques have not been established in *S. latifolia*. In an attempt to resolve this, a smut fungus (*Microbotryum violaceum*) infection system was utilized that can biologically induce stamen development in the female flowers of *S. latifolia*. The smut infected-female flower seemed morphologically to be hermaphroditic, and thus it was expected that it would be a useful system to elucidate any correlations between floral gene expression and stamen development (Fig. 6N and O) (Westergaard et al. 2003).

In situ hybridizations were performed on smut-infected and uninfected flowers in the same manner. The expression of SISUP was maintained at stage 5 but reduced at (and until) stage 6 in developing stamens of the infected hermaphroditic-like flower (Fig. 6P and Q). These expression patterns were suggestive of a positive relationship between the SISUP expression levels and the suppression of stamen development. To further elucidate this, quantitative RT-PCR (QRT-PCR) analyses were performed (Fig. 7). Total RNA was extracted from flower buds of male and female plants corresponding to stages 1–8 and 9–11.2, and from female gynoecia at stages 9–11 for both infected and uninfected flowers. The SISUP transcript levels, relative to the 18S rRNA control, were calculated from three independent experiments. In this analysis, the SISUP transcript levels could not be measured directly in de-suppressed stamens, as the stamens at stages 1–11 were too small to be isolated intact. Instead, the relative transcript levels of SISUP in whole flower buds at each stage were investigated. The results showed that in uninfected female plants the SISUP transcripts levels were dramatically increased by more than 5-fold at stages 9–11 compared with stages 1–8. However, this activation was not observed in the infected flowers (Fig. 7A). Notably, the SISUP transcript levels in the female gynoecium in infected flowers were about two-fold higher compared with this same organ in uninfected female flowers. The primary cause for the suppressed SISUP activation in the female flower thus appeared to occur in the stamens. Use of a control SLM2 gene, a homolog of Arabidopsis *PISTILLATA*, which underwent de-suppression in the repressed female flower stamens following infection (Kazama et al. 2005) validated the present experimental conditions for fungal infection and QRT-PCR (Supplemental Fig. S3).

No SISUP mRNA could be detected in male flowers, with or without fungal infection, by QRT-PCR (Fig. 7A). To further understand the transcriptional changes of SISUP before ovule development occurs, the SISUP mRNA levels were measured at individual stages from 4 to 7 in uninfected and infected female flower buds (Fig. 7B). SISUP transcript levels were detected at all of the stages tested, but were found to be increased remarkably at stage 7 in the uninfected flowers.
Fig. 6 In situ hybridization analysis of SISUP mRNA in S. latifolia flowers. The male flower (A) has ten stamens and a suppressed carpel. The female flower (B) has a gynoecium and suppressed stamens. Suppression of the gynoecium in the male flower (C) and stamens in the female flower (D) can be observed at stage 8 using scanning electron microscopy. To investigate the expression patterns of SISUP, longitudinal serial sections were made at each stage of female (E–J, M) and male (K, L) flower development, and then a selected central sections were subjected to in situ hybridization with a digoxigenin-labeled SISUP antisense RNA probe. (E) Female flower at stage 3. SISUP transcripts were not observed. (F) Female flower at stage 4. SISUP transcripts are visible in whorls 2 and 3; closed arrowheads indicate the boundary between whorls 3 and 4 in (F) and (P). (G) Female flower at stage 5. SISUP expression was detectable in the stamen and near the petal primordia. (H) Female flower at stage 8; the boxed area is shown at a higher magnification in (I) and reveals SISUP expression in the basal region of a suppressed stamen. (J) Female flower at stage 12; the boxed area in (J) is shown at higher magnification in (M) and indicates positive SISUP expression in a suppressed stamen and ovules. (K) Male flower at stage 4. (L) Male flower at stage 6. SISUP expression was not detectable in male flowers at any stage. (N) A healthy female flower with its sepals and petals removed. When the female plant of S. latifolia is infected with M. violaceum, stamens develop in the female flowers, which then appear morphologically to be hermaphroditic (O). SISUP expression was weakly detected in the stamens of the infected female flower buds at stage 5 (P). SISUP expression could not be detected in developing stamens at stage 8 (Q). cp, carpel primordia; ds, developing stamen; g, gynoecium; o, ovules; p, petal; pp, petal primordium; sc, suppressed carpel; sp, stamen primordium; ss, suppressed stamen. Bars, 100 µm (single line), 1.0 cm (double line).
Female specific expression of SUPERMAN-like gene

It has been well documented that dioecious plants evolved from hermaphroditic ancestors and that *S. latifolia* is an excellent model for the analysis of the mechanisms of dioecy (Charlesworth 1985, Ainsworth 2000, Matsunaga and Kawano 2001, Charlesworth 2002). Indeed, the floral whorls explained by the original ABC model are well conserved between *A. thaliana* and *S. latifolia* (Hardenack et al. 1994). Because the structure of male *S. latifolia* flowers may be similar to that of the *Arabidopsis superman* mutant, SUP was proposed as a candidate gene for involvement in sexual flower development (Lebel-Hardenack and Grant 1997, Matsunaga et al. 2004b). In relation to this, whether (i) this candidate gene restricts the stamen number in male flowers, (ii) suppresses stamens and/or promotes ovule development in female flowers, (iii) defines the floral boundary in either or both sexes, or (iv) fulfills these roles in a redundant way, has been a subject of much interest. It is alternatively possible that SUP homologs, if they exist, have distinct roles given the evolutionarily divergence of *S. latifolia* from *A. thaliana* (Charlesworth 2002). This possibility was examined experimentally in the current study by investigating the SISUP gene expression profile in *S. latifolia* flowers and by characterizing the function of SISUP in transgenic *Arabidopsis* plants.

Conserved and divergent functions of the SISUP protein

Importantly for the increased understanding of the basic protein function of SISUP among the SUPERMAN family, the SISUP gene rescued the pleiotropic floral phenotypes of the sup mutant (Fig. 3). This suggests that SISUP functions similarly to SUP in *A. thaliana*, when the transgene is expressed appropriately. Despite the low sequence similarity between SISUP and SUP (Fig. 1), the zinc-finger motif and the LZ-like domains are highly conserved, suggesting a conservation of some transcription factor functions. Presumably, the DNA-binding activity and the transcriptional repression mechanisms of SUP and SISUP in *A. thaliana* cells are similar, though the target genes remain to be identified.

On the other hand, some differences between the activities of SISUP and SUP were also implied by the current data. The present observations regarding SISUP overexpression in transgenic *Arabidopsis* plants, which showed defects in both stamen and petal formation (Fig. 4), were in contrast to the reported findings of a previous study showing that the overexpression of SUP under the control of the CaMV35S promoter resulted in severe dwarfism and a size reduction of all organs examined (Hiratsu et al. 2002, see Supplemental Table S2). This difference may be attributable to the relatively low amino acid sequence conservation, including the degeneration of the zinc-finger domain, which would largely affect the developmental functions of SUP or SISUP. SISUP might not control some of the SUP target genes in *Arabidopsis* sepal and carpel development. Nonetheless, the overexpression of SUP and SISUP in *A. thaliana* suppressed stamen and petal development in common (Fig. 4; Yun et al. 2002, Hiratsu et al. 2002), indicating that SUP and SISUP exerted similar,

![Quantitative RT-PCR (QRT-PCR) analysis of the SISUP gene in male and female flowers of *S. latifolia* with or without *Microbotryum violaceum* infection of the flower buds. (A) Total RNAs extracted from gynoecia and flower buds (20 organs in individual pools) corresponding to stages 1–8 and 9–11 were analyzed by QRT-PCR using SISUPF4 and SISUPR3 primers (Supplemental Table S1), which specifically amplified SISUP mRNA. (B) Total RNAs were extracted from individual stages (4–7) of uninfected or infected female flower buds, respectively, and analyzed by QRT-PCR. The seeming difference of data obtained in (A) and (B) should be due to differences in sample collection.](https://academic.oup.com/pcp/article-abstract/50/6/1127/1920297?redirectedfrom=ref)
if marginally different, effects during floral tissue development in *A. thaliana*.

**Functions of SISUP in *S. latifolia***

Although the SISUP-transgenic *A. thaliana* lines provided useful information on the activity of this gene in relation to its homolog, SUP, such heterologous experiments do not address the true function of SISUP in the *S. latifolia* plants per se. The present analyses using in situ hybridization (Fig. 6) and QRT-PCR (Fig. 7) assessed the physiological role of this gene. Unexpectedly, based upon some initial hypotheses (Lebel-Hardenack and Grant 1997, Matsunaga et al. 2004b), SISUP was found to be expressed exclusively in female flowers. The SISUP transcripts accumulated in floral regions where tight cell proliferation control is required (Fig. 6). This is a common trend for the three conserved SUP-like genes, SISUP, PhSUP1, and SISUP, in their endogenous plants. In light of the fact that PhSUP1 showed some distinct features from SUP with respect to gene expression pattern and mutant phenotype other than the *A. thaliana* sup-like organ phenotypes, i.e. additional tissue formation around the stamen filament, an unusual anther morphology, and an abnormal cell size around the vascular bundle of the anther (Nakagawa et al. 2004), at least these SUPERMAN-like genes might share roles in the control of cell proliferation during the development of certain organs related to flower structure.

As supported by the current QRT-PCR and in situ hybridization data, as well as previous results with a smut fungal infection system (Westergaard 1958, Uchida et al. 2003), it is likely that SISUP has a role in the suppression of stamen development in the female flower of *S. latifolia*. On the other hand, accumulation of the SISUP transcripts in the ovule and near the petal primordia also raised the possibility of the involvement of this gene in ovule and petal development. In *A. thaliana*, the outer integument on the adaxial side of the ovule specifically elongates, and it has been found that SUP is involved in the asymmetric growth of the outer integument (Gaiser et al. 1995). Thus, the suppressive function of SUP is needed to repress ectopic cell proliferation in the outer integument of the ovule. SISUP transcripts were also detectable in the ovules after stage 8 in the current experiments, similar to SUP. The current complementation analysis showed that some of 35S::SISUP/WT flowers had normal-looking pistils. It is therefore likely that the SISUP gene might be involved in ovule development, although more detailed analysis is needed to clarify the function of SISUP in ovule development.

Normal petal development in *A. thaliana* does not require SUP and the SUP expression regions are actually distant from the differentiation regions of the petal (Sakai et al. 1995). In contrast, SISUP transcripts were detectable in the vicinity of the petal primordia of female flowers in *S. latifolia* (Fig. 6G), although it remains to be determined whether SISUP is actually expressed at petal primordia itself. Petal formation occurs in both male and female flowers of *S. latifolia*, and this is currently believed to be a process mediated by a common mechanism (Grant et al. 1994). If it is assumed that SISUP and SUP have analogous functions, this pattern of SISUP expression is possibly an unexpected outcome. SISUP may not affect petal development, because it does not appear to be expressed in the male petals. Alternatively, it might be the case that SISUP is somehow involved in petal formation. Taken together, the available evidence indicates that the physiological functions of SISUP are to some degree divergent from those of SUP.

The female specific expression of SISUP necessarily raises many questions, not least of which is the nature of the controlling mechanism for the stamen number in *S. latifolia* male flowers. To explain this, another SUPERMAN-like gene might participate in this process, although the present Southern analysis did not provide any evidence of this (Fig. 2). Therefore, it is equally possible that a “SUPERMAN-like gene”-independent mechanism works to determine the stamen number in male flowers. The fact that whorl 4 of the male flowers is smaller than that of female flowers at early developmental stages (stages 2–3) (Hardenack et al. 1994, Kazama et al. 2005, Koizumi et al. 2007), which indicates a differential regulation of male and female flower meristems, lends some support to this additional possibility.

**Regulation of SISUP expression in *S. latifolia***

The sex-specific expression of SISUP suggests that its expression depends on the presence or absence of the Y chromosome. X-chromosome dosage dependency may also be involved, but studies on sex expression in experimentally produced polyploids demonstrated that the presence or absence of the Y chromosome was only related to sex differentiation (Ono 1939). A recent study has shown an accelerated rate of sex chromosome evolution (Marais et al. 2008), which implies that remarkable alterations of gene expression regulation occur to govern sexual flower differentiation, and that the controlling mechanism for SISUP expression also differs considerably from that of hermaphrodite plants. On the basis of previous studies of deletion mutants (Westergaard 1946, Lardon et al. 1999, Farbos et al. 1999, Zulvova et al. 2007), the suppressed SISUP expression in male plants might well be governed by the ‘stamen promoting region’ of the Y chromosome. In this regard, it would be interesting to investigate the SISUP expression profiles in a series of Y chromosome mutants in combination with fungal infection experiments in future studies. Some of the interesting data generated by the current study may relate to the reduction in SISUP transcripts by smut fungus-mediated stamen de-suppression (Fig. 7). Given that the temporal and spatial expression patterns of SISUP upon infection were very consistent with the de-suppression of
the stamens, it might be that the de-suppression involves a bypassing or modification of the Y chromosome-dependent floral developmental pathway and a weakening of the positive regulation of SISUP expression in this pathway. Nevertheless, the nature of the de-suppression mechanism remains completely unknown. The possibility that the de-suppression of cell proliferation in stamen primordia and subsequent cell elongation take place without any involvement of SISUP in S. latifolia cannot be excluded. To elucidate the relationship between SISUP and fungus-infection in the control of stamen development, additional analyses will be required.

In summary, the present study has for the first time identified a SUP homolog in a dioecious plant. Because this gene was not located on the Y chromosome, SISUP is not a sex-determination gene but may function in the sex (female) flower differentiation pathway in S. latifolia. SISUP expression is probably controlled by a sex determination gene(s) on the Y chromosome in S. latifolia. Further comparative studies of SUP homologs in Silene species, including hermaphroditic Silene, and other dioecious plants could greatly contribute to the knowledge of the function of SUP homologs and also understanding of the evolutionary differentiation of dioecy.

**Materials and Methods**

**Plant materials**

An inbred S. latifolia line, the K line (Kazama et al. 2003), was used in all of the molecular experiments. Plants were grown from seeds in pots in a regulated chamber at 23°C in a 16 h light/8 h dark cycle. The leaves and flower buds were frozen in liquid nitrogen and stored at –80°C prior to DNA and RNA extraction. The Columbia (Col) ecotype of A. thaliana was used as the wild-type control in the transgenic analyses. The sup mutant used in this study harbors the sup-2 (flo10) allele (Schultz et al. 1991) and was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University). Arabidopsis seeds were grown in either soil or 0.7% (w/v) agar-containing Murashige-Skoog (MS) medium (Wako-junyaku) supplemented with 2% (w/v) sucrose and Gamborg’s B5 vitamins at 23°C on a 16 h light/8 h dark cycle (Fujiwara et al. 2004).

**Isolation of SISUP**

Total RNA was extracted from male and female flower buds using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 3 mg of total RNA using Superscript II reverse transcriptase (Invitrogen). A fraction of the cDNA preparation was then subjected to 30 cycles of PCR amplification (94°C for 1 min, 50°C for 1 min, and 70°C for 1 min) with the degenerate primers, QALGGH (Nakagawa et al. 2004) and kSUPR1 (Supplemental Table S1), which correspond to conserved sequences in the zinc-finger domain and the C-terminal LZ domain of SUP, respectively. The PCR products were then subcloned into the pGEM-T Easy vector (Promega) and sequenced. To determine the sequence of the full-length SISUP cDNA, both 5’ and 3' RACE were performed using GeneRacer (Invitrogen). Primers for the 5’ and 3’ regions of the isolated fragment were designated as SISUP_SRACER1 and SISUP_3RACEF1, respectively (Supplemental Table S1). PCR conditions were 94°C for 2 min, five cycles of 94°C for 30 s and 72°C for 1 min, five cycles of 94°C for 30 s and 70°C for 1 min, 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, then 72°C for 10 min. Nested PCR with SISUP_SRACER2 and SISUP_3RACEF2 primers (Supplemental Table S1) was carried out using the same PCR conditions. The PCR products were subcloned into the pGEM-T Easy vector and sequenced. A genomic DNA fragment containing the 5’-upstream region (2.8 kb) and the complete coding region of SISUP (0.7 kb) was then obtained by inverse PCR with the SISUP_SRACER2 and SISUP_3RACEF2 primers (Supplemental Table S1). HindIII-digested genomic DNA was self-ligated using T4 DNA ligase (Promega) and used as a template for the inverse PCR. The resulting 3.8-kb fragment was then cloned and sequenced.

**Phylogenetic analysis**

The amino acid sequences of the zinc-finger domains of plant TFIIIA-type proteins were aligned using CLUSTAL X version 1.81 (Thompson et al. 1997). To compare the zinc-finger motifs of SISUP and other TFIIIA-type proteins, 31 amino acid residues of the zinc-finger motifs were aligned. For proteins, such as ZPT2, with more than two zinc-finger motifs, each motif was theoretically isolated and named ZPT2-1, ZPT2-2, etc. The CLUSTAL X alignment was used in a neighbor-joining analysis (Saitou and Nei 1987) in MEGA version 2.1 (Kumar et al. 2001). The robustness of the resulting tree was tested using bootstrap analysis with 1000 replications, using the MEGA program.

**Southern blotting analysis**

Genomic DNA was extracted from S. latifolia leaves using a Nucleon PhytoPure Genomic DNA Extraction Kit (Amersham Biosciences). Genomic DNA (15 µg) was digested with EcoRI, EcoRV, or HindIII for 12 h. The concentrations of the digests were measured and loaded equally onto a 1.0% (w/v) agarose gel and then transferred to an Immobilon-Ny+ membrane (Millipore). Hybridization and signal detection were performed using the Gene Images AlkPhos Direct Labeling and Detection System (Amersham Biosciences) and a SISUP-specific probe amplified by PCR between SISUP_F2 and SISUP_R (Supplemental Table S1). Post-hybridization washes were carried out under relatively high stringency conditions at 65°C for 2 × 10 min (Nishihara et al. 2007). The hybridized membranes were visualized using Hyperfilm ECL (Amersham Biosciences) at room temperature with appropriate exposure times.
Transformation of A. thaliana

A 3.5-kb genomic fragment corresponding to the 5'-upstream region (2.8 kb) and coding regions of SlSUP was amplified by PCR using the primers gSlSUP_Hind_F and gSlSUP_SucR (Supplemental Table S1). The resulting fragment was digested with HindIII and SacI and fused into the pSMAH621 binary vector, which was kindly provided by Dr. H. Ichikawa (National Institute of Agrobiological Sciences, Tsukuba, Japan), to generate pgSlSUP. This pgSlSUP construct was then introduced into Agrobacterium tumefaciens strain C58, and the resulting bacteria were used to transform heterozygous sup-2 plants using the floral-dip method (Clough and Bent 1998). The genotype of the SUP locus in hygromycin-resistant T1 plants was analyzed by examining the Ncol restriction pattern of the SUP locus after amplification with SUP-specific primers AtSUPF and AtSUPR (Supplemental Table S1). Ncol cleaved the wild-type SUP sequence but not the sup-2 allele. The phenotypes of 6-week-old T1 plants harboring the homozygous sup-2 allele were then observed.

The 5.1-kb fragment harboring the endogenous promoter region of Arabidopsis SUP (Ito et al. 2003) was amplified using the primers AtSUP_p1F_Smal and AtSUP_p1R_Smal (Supplemental Table S1). The resulting product was digested with Smal, while pSMAH621 was cut by HindIII and SacI and blunt-ended using KOD polymerase (TOYOBO). These fragments were then fused to generate pSUP:SlSUP. This construct was used to transform heterozygous sup-2 plants and the phenotypes of 19 6-week-old T1 plants harboring homozygous sup-2 allele were then observed.

For the overexpression of SUP, an Xhol-Sacl fragment containing the complete cDNA was amplified using the primers cSlSUPF and gSlSUP_SucR (Supplemental Table S1), and fused into the pSMAH621 vector between the CaMV35S promoter and the nos terminator (35S::SlSUP) (Supplemental Fig. S1A). This construct was then used to transform wild-type A. thaliana as described above. The phenotypes of 13 hygromycin-resistant T1 plants were then observed.

Scanning electron microscopy

Flower buds were dissected and fixed overnight in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. After three washes with distilled water, the fixed buds were dehydrated in an ethanol series (50, 70, 80, 90, and 95% (v/v), each step for 1 h at 4°C) and stored in 100% (v/v) ethanol overnight at 4°C. The ethanol was replaced with isopentyl acetate, and the buds were dried with a critical-point dryer (HCP-2; Hitachi) and sputter-coated with carbon using an ion sputter (E-1010; Hitachi). The buds were then examined in a 5-3000 N scanning electron microscope (Hitachi) operating at 15 kV in a high-vacuum mode.

RT-PCR

RNA purification and cDNA synthesis were performed as described above. The SlSUP-specific forward and reverse primers, SlSUP_F2 and SlSUP_R (Supplemental Table S1) were used to amplify a fragment corresponding to the region beginning downstream of the conserved zinc-finger domain of SlSUP. Primers specific for the S. latifolia actin gene, SLactin01 and SLactin02 (Supplemental Table S1), were also used as a control. The PCR parameters used were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The resulting 359-bp fragment of the SlSUP gene and 611-bp fragment of the actin gene were ligated into pGEM-T Easy vectors and sequenced to verify the amplified products.

Quantitative RT-PCR (QRT-PCR) was performed using an Applied Biosystems 7900HT real-time PCR system with SYBR Green detection chemistry (Applied Biosystems). Primers used to amplify 18S rRNA (Sl18SF1 and Sl18SR1), SlSUP (SlSUPF4 and SlSUPR3), and SLM2 (SLM2F1 and SLM2R1) are listed in Supplemental Table S1, and were confirmed experimentally for their specificity. The product lengths for the 18S RNA, SlSUP, and SLM2 amplified fragments were 148 bp, 201 bp, and 201 bp, respectively, and all were confirmed by sequencing. 18S rRNA was used as a reference control by performing QRT-PCR with 100-fold cDNA dilutions as templates in each sample. The QRT-PCR data shown are the average relative quantities ± SE from at least three biological replicates.

In situ hybridization

The 359-bp insert of SlSUP in the pGEM-T Easy vector was amplified using universal vector primers and then used to produce digoxigenin (DIG)-labeled sense and antisense RNA probes with a DIG RNA Labeling Kit (Roche Diagnostics Corp.). Flower buds were vacuum-infiltrated with FAA (50% (v/v) ethanol, 5% (v/v) acetic acid, and 3.7% (v/v) formaldehyde) and fixed overnight at 4°C. The fixed buds were dehydrated in an ethanol series and then embedded in Histosec (Merck). The embedded buds were then sectioned to a thickness of 8 µm, affixed to microscope slides by overnight incubation at 42°C, and used for in situ hybridization. In situ hybridizations were performed using an adaptation of the methods described in the previous report (Kazama et al. 2005). Briefly, the probes were dissolved at 1 µg ml⁻¹ in mRNA in situ Hybridization Solution (Dako). Hybridizations were performed overnight in a moist chamber at 65°C, followed by washing in 0.2×SSC at 65°C for 2 h, 0.2×SSC at room temperature for 5 min, and 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl (NT) at room temperature for 5 min. Signal detection was performed using the TSA Biotin System (PerkinElmer). Slides were then treated with 1% blocking reagent in NT (TNB) for 30 min, and anti-DIG antibody coupled with
horseradish peroxidase (HRP; 1:100 dilution; Roche Diagnostics Corp.) was then applied for 30 min. The slides were then rinsed three times in NT with 0.05% (v/v) Tween 20 (NTT) for 5 min and in NT for 5 min. The slides were treated with biotinyl tyramide diluted 1:50 in 1 × Amplification Reagent (PerkinElmer) for 7 min, and then washed three times each in NTT for 5 min and in NT for 5 min. Streptavidin-HRP diluted 1:100 in TNB was then added to each slide. After 30 min, the slides were washed as described above. A detection buffer containing diaminobenzidine (DAB; Nacalai Tesque Inc.) was applied to each slide, and color was allowed to develop for up to 10 min. After development, the slides were rinsed in distilled water. Photographs were taken using an Olympus BX52 microscope (Olympus).

**Statistics**

Statistically significant differences between data sets were assessed using Student’s t-test. Differences at the level of \( P < 0.05 \) were regarded as significant.

**Supplementary data**

Supplemental data are available at PCP online.

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