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ORIGINAL ARTICLE

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Characterization of two *SEPALLATA* MADS-box genes from the dioecious plant *Silene latifolia*

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Abstract Two *SEPALLATA* orthologs, *SlSEP1* and *SlSEP3*, were isolated from male flower buds of the dioecious plant *Silene latifolia*. Both genes are located on autosomes and not on the sex chromosomes. *SlSEP1* and *SlSEP3* transcripts were detected specifically in male and female flower buds. Quantitative RT-PCR and in situ hybridization revealed that both genes were expressed in young flower meristems, developing petals, male anthers, and female ovules. However, neither transcript was detected in suppressed gynoecia of male flower buds or suppressed anthers of female flower buds. The genes were differentially expressed during anther development, with the *SlSEP1* transcript accumulating in anther walls and tapetal cells, and the *SlSEP3* transcript accumulating in tetrads as well as anther walls and tapeta. Transcripts of both genes were also detected in flower buds of mutants with deletions of some parts of the Y chromosome, suggesting that neither gene is directly involved in sex determination.

Keywords Dioecious plant · *SEPALLATA* · Petal · Ovule · Pollen grains

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Introduction

The *Arabidopsis thaliana* genes *SEPALLATA 1*, *2*, and *3* (*SEP1*, *2*, and *3*) were identified as *AGAMOUS*-like genes *AGL 2*, *4*, and *9*, which are preferentially expressed in floral organs (Mandel and Yanofsky 1998; Ma et al. 1991). Analysis of triple mutants of the *SEP* genes revealed that these genes are required for the development of petals, stamens, and carpels (Pelaz et al. 2000). According to the quartet model of floral organ specification, floral organs in *A. thaliana*, including sepals, petals, stamens, and carpels, are determined by four combinations of floral homeotic proteins, most of which are known as MADS-box proteins (Theissen and Saedler 2001). The expression of class B genes and a class C gene in combination with *SEP* genes specifies stamen identity, whereas the expression of a class C gene and *SEP* genes specifies carpel identity (Honma and Goto 2001). *SEP* genes are abundantly expressed in developing petals, stamens, and carpels, and even more highly in developing ovules (Flanagan and Ma 1994; Savidge et al. 1995; Mandel and Yanofsky 1998). It has recently been shown that SEP proteins are necessary for the formation of transcription factor complexes that control ovule development (Favaro et al. 2003).

Silene latifolia is a dioecious campion that possesses sex chromosomes (Matsunaga and Kawano 2001; Negrutiu et al. 2001), with females containing two X chromosomes and males containing an X chromosome and a Y chromosome. In dioecious plants, unisexual flowers develop through the suppression or promotion of each sex primordium. The third and fourth whorls of young *S. latifolia* male and female flower meristems are indistinguishable. When the stamen and petal primordia begin to develop, the fourth whorl is larger in females than in males (Grant et al. 1994). A fourth-whorl gynoecium primordium is suppressed in the male flower and later becomes a rudimentary gynoecium in the form of a filamentous rod that lacks an ovary and pistils. In the third whorl of female flowers, the growth of stamen primordia is arrested and the tissues degenerate before the flower opens (Uchida et al. 2003). The genetic programs that determine the specific contributions of the different lineages of male and female floral organs are reflected in the sex-specific expression of floral organs. Therefore, we isolated *SEP* genes from *S. latifolia* and analyzed their expression during the formation of unisexual flowers.

Materials and methods

Plant material

We used an inbred *S. latifolia* line, K1, for the molecular experiments. Plants were grown in a temperature-controlled chamber at 22°C.

Cloning of *SEPALLATA* orthologues

Full-length cDNAs of two *SEP* homologs, *SlSEP1* and *SlSEP3*, were isolated from male *S. latifolia* flower buds using RT-PCR with degenerate primers corresponding to the MADS conserved domain, as described in Matsunaga et al. (2003).

Phylogenetic analysis

Published sequences were obtained from the nr and dbEST data sets at NCBI using the programs BLASTX or BLASTP (version 2.0.10) (Altschul et al. 1997). Clustering and calculation of bootstrap values were performed using the program CLUSTAL X (Thompson et al. 1997). Genetic distances were estimated by the neighbor-joining method (Saitou and Nei 1987) as described previously (Matsunaga et al. 2002).

Genomic distribution

Flow-sorted chromosomes were collected as described in Kejnovsky et al. (2001). The sets of oligonucleotide primers used for the 311-bp *SlSEP1* fragment were SlSEP1-F: 5′-AAGACCCAGTCGATGCTCGATCAAC-3′ and SlSEP1-R: 5′-ACTTGAGCATGAGATGTCCCAG-CAG-3′, and for the 389-bp *SlSEP3* fragment SlSEP3-F: 5′-CACTAAGCAATGAAGCAAACATTAC-3′ and SlSEP3-R: 5′-CTCACTGAATTAAAGTTGGCAAATC-3′. Genomic DNA for Southern hybridization was isolated from young leaves using an automatic DNA isolation system PI50 (Kurabo, Osaka, Japan).

Expression analyses

RNA isolation and northern hybridization were performed according to the method of Matsunaga et al. (1996). Total RNA was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Amersham). RT-PCR was performed using Ready-to-go RT-PCR beads (Amersham) with cDNA. Quantitative RT-PCR was performed according to the method of Matsunaga et al. (2003). The gene for the GTPase beta subunit (*SlGb*), which is expressed constitutively in all organs (S. Matsunaga, unpublished data), was used as an internal standard to estimate the relative expression of mRNA. The relative expression of mRNA for a given tested gene was defined as the mean value for that gene divided by the mean for *SlGb*, with the same cDNA used as template. Relative expression values and corresponding standard deviations for the transcripts were calculated from three to four experimental replicates. The oligonucleotide primer sets used for quantitative RT-PCR were as follows: 5′-GACATGGTGACAGCCATAG-CAACA-3′ and 5′-TCACGAGAAGCAGAGAC-TATCTGT-3′ for *SlGb*; SlSEP1-F and SlSEP1-R for *SlSEP1*; SlSEP3-2F: 5′-GAAGCGGATCAGGTAAGTT-CAGCTC-3′ and SlSEP3-2R: 5′-CCGGCTGCTGTAACGTTCATCTGCT-3′ for *SlSEP3*. Preparation of biotin-labeled probes for *SlSEP1*, *SlSEP3* and histone H4 (*SlH4*), was performed according to the method of Matsunaga et al. (2004). In situ hybridization was performed as described previously (Matsunaga et al. 2003), with an automatic ISH robot AIH-101B (Aloka, Tokyo, Japan) using tyramide amplification in the GenPoint system (Dako, Kyoto, Japan).

Results and discussion

Full-length cDNAs of two *SEP* homologs, *SlSEP1* and *SlSEP3*, were isolated from male *S. latifolia* flower buds using RT-PCR. *SlSEP1* (accession number AB162019) and *SlSEP3* (accession number AB162020) are 1,222 and 1,161 bp in length and encode proteins of 256 and 244 amino acids, respectively. *SlSEP1* and *SlSEP3* are 64% and 71% identical at the amino acid level to *SEP1* and *SEP3* of *A. thaliana*, respectively. Evolutionary relationships were examined by neighbor-joining phylogenetic analysis of 15 amino acid sequences of MADS-box proteins including *APETALA1*, *AGL6* and *AGL13* as the outer group (Fig. 1). This analysis showed that *SlSEP1* and *SlSEP3* are closely related to the *SEP* genes of *A. thaliana*

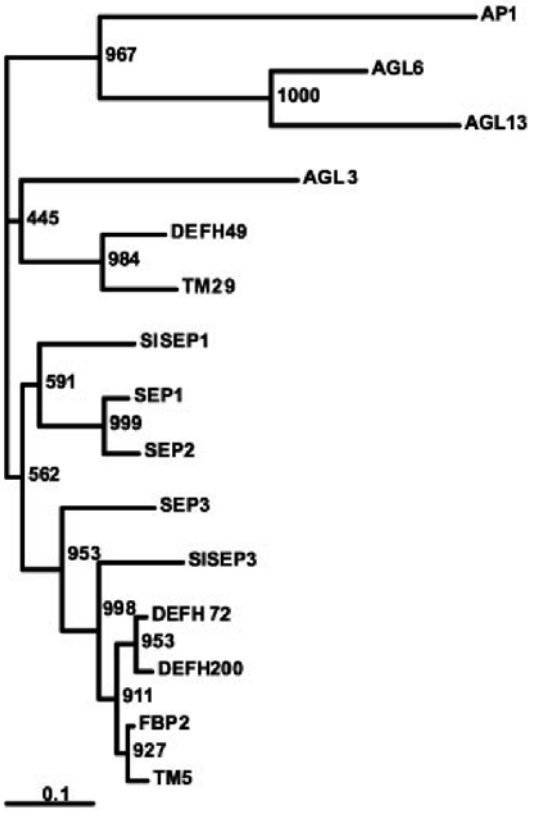

Fig. 1 Phylogenetic analysis of amino-acids sequences of *SEPALLATA*-like MADS-box proteins. Branch lengths are proportional to genetic distances as estimated by the neighbor-joining method. Numbers on the branches are the bootstrap percentages for 1,000 replicates. *AP1*, *AGL3*, *AGL6*, *AGL13*, *SEP1*, *SEP2* and *SEP3* from *Arabidopsis thaliana* (acc. nos. S27109, P29383, P29386, Q38837, P29382, P29384 and O22456; Mandel et al. 1992; Ma et al. 1991; Rounsley et al 1995; Mandel and Yanofsky 1998), *DEFH49*, *DEFH72* and *DEFH200* from *Antirrhinum majus* (S78015, S71756 and S71757; Davies et al. 1996), *FBP2* from *Petunia hybrida* (AAA86854; Angenent et al. 1992), *TM5* and *TM29* from *Lycopersicon esculentum* (Q42464 and CAC83066; Pnueli et al. 1991; Ampomah-Dwamena et al. 2002)

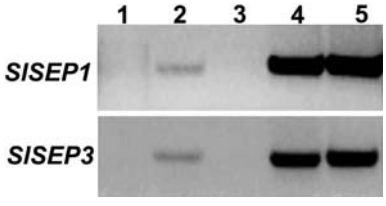

Fig. 2 Analysis of the chromosomal localization of the *SlSEP* genes using flow-sorted chromosomes and genomic DNA. The upper and lower panels show electrophoretically separated PCR products amplified with primers specific for *SlSEP1* or *SlSEP3*, respectively, with the templates indicated. Lanes: *1* No chromosomes, *2* flow-sorted autosomes, *3* X chromosomes, *4* female genomic DNA, *5* male genomic DNA

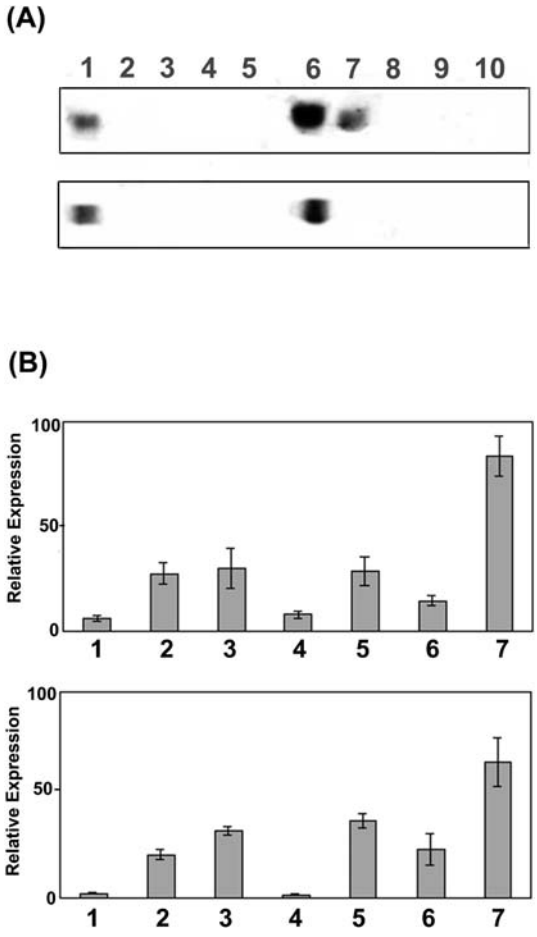

Fig. 3A,B Analysis of *SlSEP* gene expression. **A** Northern hybridization analysis of *SlSEP* genes. The upper and lower panels show fragments hybridized with *SlSEP1* and *SlSEP3* as probes, respectively. Each lane contained 20 μg total RNA. Lanes: *1* Male flower buds, *2* male flowers, *3* male leaves, *4* male stems, *5* male roots, *6* female flower buds, *7* female flowers, *8* female leaves, *9* female stems, *10* female roots. **B** Quantitative analysis of *SlSEP* transcripts. The upper and lower panels show values representing the relative expression of the *SlSEP1* and *SlSEP3* transcripts standardized based on expression of the GTPase β subunit, which is expressed constitutively in all organs (Matsunaga et al. 2003). Bars *1–7* represent the relative expression levels in male sepals, male petals, male stamens, female sepals, female petals, female styles, and female ovaries, respectively. Data are expressed as mean ±SEM (n=4)

and belong to the *AGL2* family (Becker and Theissen 2003).

To determine the chromosomal locations of *SlSEP1* and *SlSEP3*, we performed PCR analyses with genomic DNA, flow-sorted X chromosomes, and autosomes, using primers specific for these genes (Fig. 2). After PCR, only a single fragment was observed in agarose gel electrophoresis. Sequencing analyses confirmed that the fragments were derived from *SlSEP1* and *SlSEP3*, respectively. Specific fragments for both *SlSEP1* and *SlSEP3* were amplified from male and female genomic DNAs and autosomes, indicating that both genes are linked to autosomes and not to sex chromosomes.

The expression of *SlSEP1* and *SlSEP3* was examined by northern hybridization using total RNA from male and female reproductive organs and vegetative organs according to the method of Matsunaga et al. (1996) (Fig. 3A). *SlSEP1* and *SlSEP3* transcripts were detected in both male and female flower buds; moreover, expression of *SlSEP1* was also found in fully developed female flowers. This

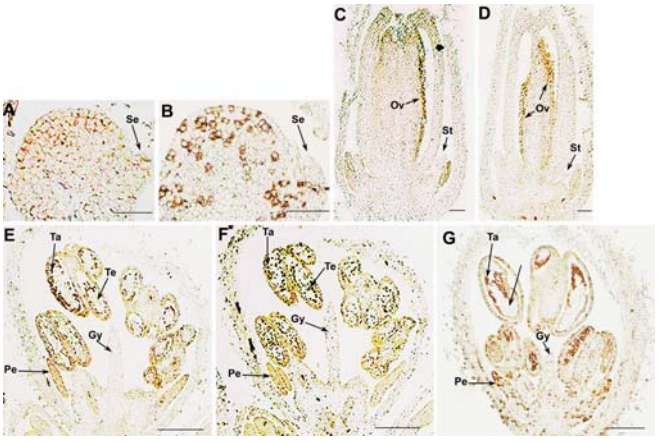

Fig. 4A–G In situ hybridization of *SlSEP* genes. Longitudinal sections of young female and male flower buds are shown. Hybridization signals appear brown. Sections were allowed to hybridize with antisense probes of *SlSEP1* (**E**), *SlSEP3* (**A**, **C**, **F**) and *SlH4* (**B**, **G**). **A**, **B** Male flower buds at stage 4. **C**, **D** Female flower buds at stage 10. **E**–**G** Male flower buds at stage 10. *Gy* Suppressed gynoecium *Ov* ovules, *Pe* petal, *Se* sepal, *St* suppressed stamen, *Ta* tapetum, *Te* tetrad. *Bars* **A**, **B** 50 μm; **C**– **G** 150 μm

result indicates that both genes are expressed exclusively in both male and female flowers. Next, analysis of the expression of these genes in floral organs (male and female sepals and petals, male stamens, female styles and ovaries) was examined using quantitative RT-PCR (Fig. 3B). Both transcripts were expressed preferentially in male and female petals, male stamens, and female styles and ovaries, with strong expression in the ovaries. In contrast, the expression of both genes was very low in male and female sepals. This result suggests that both genes are expressed in the inner three whorls during the mature stages of flower buds. In situ hybridization was performed for expression analysis of the *SlSEP* genes and a histone H4 gene of *S. latifolia*, *SlH4* (Matsunaga et al. 2004), during flower development (Fig. 4). The biotin-labeled probe was detected using tyramide amplification and the signal appeared brown. Expression of both *SlSEP1* and *SlSEP3* was not detected in floral meristems and first detected in young flower buds of males and females at stage 3 of Grant et al. (1994). At stage 4, when sepal primordia are clearly established, *SlSEP3* transcripts were detected in all whorls in both female and male flower buds, including sepals and stamen primordia (Fig. 4A). A similar expression pattern was observed until stage 7. The expression patterns of *SlSEP3* in female flower buds and *SlSEP1* in both female and male flower buds during the development of young flower buds were similar to that of *SlSEP3* in male flower buds (data not shown). Depending on the maturation stage, the accumulation of both transcripts dramatically decreased in sepals and suppressed organs, including the male filamentous gynoecium and female stamens. The accumulation of *SlH4* transcripts also decreased in the filamentous gynoecium of male flower buds (Fig. 4G). At stage 10, transcripts of neither *SlSEP1* nor *SlSEP3* were detected in the suppressed organs. This suppression is consistent with the behavior of other MADS-box genes (Hardenack et al. 1994; Matsunaga et al. 2003), a homeodomain leucine zipper gene (Ageez et al. 2003) and cell-cycle specific genes (Matsunaga et al. 2004), which are not expressed in the suppressed organs. Transcripts of both genes continued to be expressed in developing petals of male and female flower buds. At stage 10 of female flower buds, the *SlSEP1* transcript levels were higher than those of *SlSEP3* in developing styles and ovules (Fig. 4C, D). *SEP* orthologs from other plant species have been reported to be similarly expressed in developing petals and ovules, suggesting that these genes function in petal and ovule development. There were remarkable differences between the transcripts in developing male stamens. At stage 10 of male flower buds, the *SlH4* transcript accumulated in dividing tapetal cells, tetrads, developing sepals, petals, filaments, anther walls (Fig. 4G). The *SlSEP1* transcript accumulated in anther walls and tapeta but not in tetrads (Fig. 4E), but the *SlSEP3* transcript accumulated in tetrads in addition to anther walls and tapeta (Fig. 4F). This is the first report of the expression profiles of *SEP1* and *SEP3* orthologs differing during male anther development. *SlSEP* genes may have a function not only in female development but also in male development.

Finally, we examined *SlSEP* expression in flower buds from 22 mutants (9 hermaphrodites, 2 asexual, and 11 male sterile mutants) possessing different deletions on the Y chromosome (Lardon et al. 1999; Farbos et al. 1999). A part of the results of the RT-PCR analysis of these mutants is available in the electronic supplementary material. RT-PCR using specific primers for both genes with total RNA from flower buds showed that both genes were expressed in all mutants. Flowers of asexual and male sterile mutants did not form mature pistils and stamens, therefore the expression was analyzed in developing and mature petals. This result suggests that the expression of *SlSEP* genes is not directly involved in sex determination and that regulation of their expression is independent of the pathways of sex determination in *S. latifolia*.

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