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SHORT COMMUNICATION

Carpel development in a floral mutant of dioecious *Silene latifolia* producing asexual and female-like flowers

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KEYWORDS
CUP-SHAPED COTYLEDON; Dioecy; Gynoecium development; *SHOOT MERISTEMLESS*

Summary

The genes *SlSTM*1 and *SlSTM*2 (orthologs of *Arabidopsis SHOOT MERISTEMLESS*) and *SlCUC* (an ortholog of *CUP-SHAPED COTYLEDON*1 and *CUC*2) of the dioecious species *Silene latifolia* have been proposed to control the gynoecium suppression pathway in developing flowers. In a mutant of *S. latifolia* (K034) that produces no males but only asexual and imperfect female (female-like) flowers, both on the same individual, gynoecia are completely suppressed in asexual flowers and partially suppressed in female-like flowers. To determine whether these two epigenetic phenotypes in gynoecium development are caused by changes in *SlSTM* and *SlCUC* expression, we performed *in situ* hybridization with probes of *SlSTM* and *SlCUC*. We found two different pattern of gene expression in flower buds prior to the onset of phenotypic differentiation, which were similar to the reciprocal expression of the two genes described in male and female wild-type plants. In young K034 flower buds, 14.3% of developing structures showed female and the rest male determination. This ratio corresponds to the ratio of female-like to asexual flowers eventually produced by the K034 plants. The same ratio (7—16%) was not only found in the original mutants but also in the first and second backcross generations and in vegetative clones of the original mutant line. Hence, the switch-like and reciprocal *SlSTM* and *SlCUC* expression patterns in K034 correspond to the gynoecium suppression patterns in the wild type, suggesting that the mutation(s) responsible for the two mutant genotypes acts upstream of *SlSTM* and *SlCUC*.

Abbreviations: CUC, *CUP-SHAPED COTYLEDON*; DIG, Digoxigenin; STM, SHOOT MERISTEMLESS; NAC, NAM, ATAF, & CUC transcription factor.

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Introduction

SHOOT MERISTEMLESS (*STM*) and *CUP-SHAPED COTYLEDON* (*CUC*) genes in the model angiosperm *Arabidopsis thaliana* are central to shoot and flower meristem function. In *A. thaliana*, the *STM* gene, which encodes a putative transcription factor of KNOTTED1 class homeodomain proteins, is required for the maintenance of undifferentiated cells in the shoot meristem and for correct proliferation of cells in the flower meristem (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996). The *STM* gene is expressed in shoot apical, inflorescence, and flower meristems; it is also expressed in vascular tissues and in boundaries between flower whorls (Long et al., 1996). *STM* transcripts are downregulated in incipient flower primordia (Long et al., 1996).

CUC genes (*CUC*1, *CUC*2 and *CUC*3) encode members of the NAC family (*NAM*, *ATAF*, and *CUC*) of transcription factors (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003); they are involved in the establishment and maintenance of organ boundaries in shoot apical, inflorescence, and flower meristems. *CUC* gene expression occurs in one or two rows of cells that correspond to the boundaries of each organ primordium (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Breuil-Broyer et al. (2004) showed that *CUC*2 is associated with a lack of cell proliferation, particularly in whorl boundaries. Regulatory feedback loops exist between *STM* and *CUC* genes in apical meristems of the *Arabidopsis* embryo (Aida et al., 1997, 1999; Takada et al., 2001).

Zluvova et al. (2006) identified *SlSTM*1 and *SlSTM*2 (orthologs of *STM*) and *SlCUC* (an ortholog of *CUC*1 and *CUC*2) in the dioecious plant *Silene latifolia*. They performed *in situ* hybridization with *SlCUC* and *SlSTM* (*SlSTM*1 and *SlSTM*2) on young male flowers and female flowers and found differences between male and female flowers in the expression pattern of their genes. In female flowers at stage 3, *SlSTM* transcripts were expressed in the central portion of the meristem (the gynoecium region), but *SlCUC* transcripts were not. In male flowers at the same stage, *SlSTM* transcripts were absent in the gynoecium region, while *SlCUC* transcripts were present. Hence, it is likely that *SlSTM* and *SlCUC* control the *S. latifolia* gynoecium suppression pathway at an early stage in both males and females.

In *S. latifolia*, male plants have a pair of dimorphic sex chromosomes (X and Y), whereas females have a pair of identical sex chromosomes (XX). In male flowers, the presence of the Y chromosome leads to suppression of gynoecium development and to formation of a thin rod-like structure (rather than the five fused carpels found in female flowers; Grant et al., 1994; Farbos et al., 1997; Scutt et al., 1997). The suppression pathway of carpel development may be subject to epigenetic regulation; this was indicated by analyses of an androhermaphroditic mutant induced by 5-azacytidine (demethylating reagent) treatment (Janousek et al., 1996), and by analyses of hermaphrodite mutants obtained by deletions in the Y chromosome (Lardon et al., 1999).

The Y chromosomal deletion mutant K034 of *S. latifolia* has a dimorphic flower phenotype expressed as asexual (Figure 1a) and imperfect female (female-like) flowers (Figure 1b). Gynoecia of female-like flowers have only one to three carpels, and each carpel is normal and fertile. The K034 Y chromosome has two deletions in the gynoecium-suppressing and stamen-promoting regions. Complete stamen suppression in K034 is caused by deletion of the stamen-promoting region. Consequently, all K034 flowers lack mature stamens. Partial deletion in the K034 gynoecium-suppressing region may produce completely suppressed gynoecia in asexual flowers, and partially suppressed gynoecia in female-like flowers.

The purpose of our study was to determine whether these two epigenetic suppressions of gynoecium development are caused by changes in the expression patterns of *SlSTM* and *SlCUC* in K034 flowers. If the switch-like and reciprocal *SlSTM* and *SlCUC* expression patterns in K034 correspond to the gynoecium suppression patterns in the wild type, the mutation(s) responsible for the two mutant genotypes of K034 acts upstream of *SlSTM* and *SlCUC*.

Materials and methods

Silene latifolia Poiret (white campion) is a model dioecious plant that produces flowers of only one sex on each plant. Dioecy in *S. latifolia* is determined genetically by heteromorphic sex chromosomes (XX for females and XY for males). The inbred *S. latifolia* K line was produced by 12 generations of sibling mating; this line provides healthy plants. A K034 mutation arose spontaneously within the K line (Koizumi et al., 2007). K034 has two types of flowers, while normal forms of leaves, stems, and perianth organs. One floral phenotype was asexual; the other was an imperfect female (female-like) flower. All experiments were performed with progeny obtained from backcrosses of K034 with wild-type males. K034 flower buds >0.25 mm ($>$stage 5) in length were observed

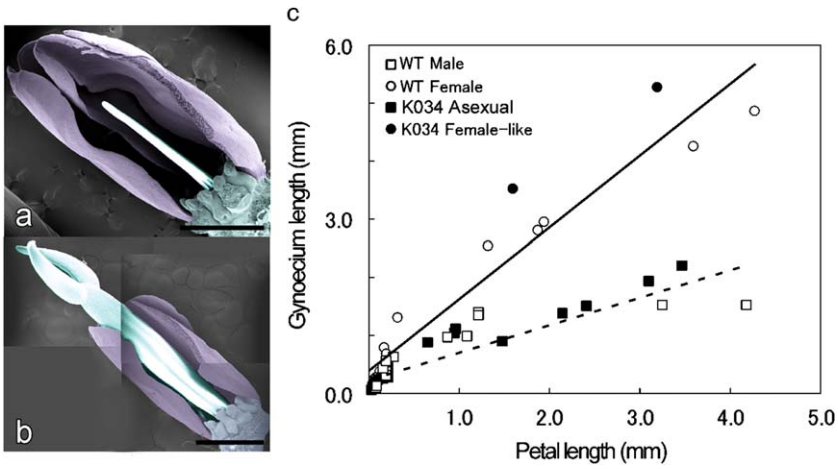

Figure 1. An asexual and female-like mutant K034 of *Silene latifolia*. Scanning electron micrographs of an asexual flower (a) and a female-like flower (b). (c) Carpel elongation patterns post stage 5 in wild-type and K034 flowers revealed by plotting gynoecium length (mm) against petal length (mm). The fitted regression line (dashed line) of wild-type male (open squares) and K034 asexual (filled squares) flowers is described by the equation $y = 0.47x+0.28$ ($r = 0.90$). The fitted regression line (solid line) of wild-type female (open circles) and K034 female-like (filled circles) flowers is described by the equation $y = 1.23x+0.42$ ($r = 0.96$).

under a stereomicroscope (MZ16; Leica Imaging Systems, Cambridge, UK), and the lengths of gynoecia and petals were measured in wild-type males, wild-type females, and asexual and female-like flowers. Total RNA was extracted from young flower buds (<1 mm) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (100 ng) was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The probes used were genes *SlCUC* and *SlSTM*2 with *SlCUC*-specific primers (*SlCUC*F2, 5′-CTTCTTCAGCTTGCGAGACC-3′ and *SlCUC*R1, 5′-AGTCATCGGGATCGTCAAAG-3′) and *SlSTM*2-specific primers (*SlSTM*2F1, 5′-CAGAAGCGGAAGCGGTAG 3′ and *SlSTM*1_2_R1, 5′-ATTGCTGACGGGCTTCTTTA-3′) without the conserved homeodomains. The amplified insert was used to produce digoxigenin (DIG)-labeled sense and antisense RNA probes with a DIG RNA Labeling Kit SP6/T7 (Roche Applied Science, Indianapolis, USA). Flower buds were immediately fixed in FAA solution (3.7% [v/v] formaldehyde, 50% [v/v] ethanol, 5% [v/v] acetic acid) at 4 °C. The fixed buds were dehydrated in an ascending ethanol series (25, 50, 75, and 100%, each step for 20 min at 4 °C) and stored in 100% ethanol overnight. The samples were embedded in HISTOSEC (Merck). Sections (8 μm) were cut with a microtome and mounted on slides at 37 °C overnight. The protocols of Kazama et al. (2005) were used (with some modifications) for *in situ* hybridization. Hybridization was performed in a moist chamber at 55 °C overnight.

Results

Wild-type male flowers of *S. latifolia* have 10 stamens and a suppressed gynoecium in the form of an undifferentiated rod. Wild-type female flowers of *S. latifolia* have a gynoecium composed of five fused carpels and lack mature stamens. K034 is the first mutant described in *S. latifolia* to have asexual flowers and imperfect female flowers in one individual. Mature asexual flowers of K034 had rudimentary stamens and a suppressed gynoecium (Figure 1a). Female-like flowers had one to three (mostly two) styles; there are normally five in wild-type female flowers. Carpels of female-like flowers were similar to those of wild-type females (Figure 1b). K034 gynoecia had two suppression patterns, while all stamens were completely suppressed. The gynoecia of the first flowers in the inflorescence tended to be partially suppressed, having a reduced ovary and two styles (female-like flowers). The frequency of totally suppressed gynoecia (gynoecia of asexual flowers) increased in flowers on later inflorescence branches.

We measured gynoecium lengths in wild-type and K034 flower buds after stage 5, (when petal and stamen primordia emerge) and plotted them against petal length (Figure 1c), because petal length in flowers correlates with the size of other flower organs (Farbos et al., 1997). We observed a positive correlation between gynoecium length and petal length in both wild-type and K034 post stage 5 flower buds. In wild-type individuals, carpel elongation resulted in male and female types.

In stage 9 or later wild-type females, carpel elongation accelerated in flowers with petals more than 0.2 mm long. At that time, wild-type female gynoecia produced styles from carpel tips. In K034, we observed two types of the carpel elongation pattern, similar to those in wild-type males or females. The first morphological sex differences appear at stage 5 in *S. latifolia* (Grant et al., 1994; Farbos et al., 1997). It is likely that K034 already had two types of carpels at stage 5, when the morphological sex differences appeared.

To determine whether these two epigenetic phenotypes of gynoecium development are caused by changes in the expression patterns of *SlSTM*1, *SlSTM*2, and *SlCUC* in K034, we analyzed the expression of *SlSTM*1, *SlSTM*2, and *SlCUC* by *in situ* hybridization in young flowers before stage 5. The developmental stages of wild-type flowers are described by Grant et al. (1994) and Farbos et al. (1997). At stage 2, no flower primordia are visible, and outermost primordia form two bracts. At stage 3, sepal primordia appear on flanks of the flower meristem. Stage 4 is defined by the emergence of all five sepals, before the appearance of stamens and petals. At stage 5, all flower organ primordia are formed.

In wild-type flowers at stage 2, *SlSTM* (*SlSTM*1 and *SlSTM*2) was clearly expressed in the center of the flower meristem and was downregulated in incipient medial sepal primordia (Figure 2a and b). In wild-type male flowers, *SlSTM*-negative regions arose within dark-staining masses of cells at the positions expected for the gynoecium primordia from stage 3 onward (Figure 2e, i, m). In contrast, *SlSTM* continued to be expressed in the central portions of the wild-type female flower meristem (Figure 2f, j, n). After initiation of all flower organ primordia, we still found their residual expression at the bases of organ primordia in wild-type male and female flowers (Figure 2m and n). We also observed their expression in the central region of the developing wild-type female flower gynoecium (Figure 2n). Ovules developed from the flank of this region.

In stage 2 K034 flowers, *SlSTM* was expressed in the center of the flower meristem and was

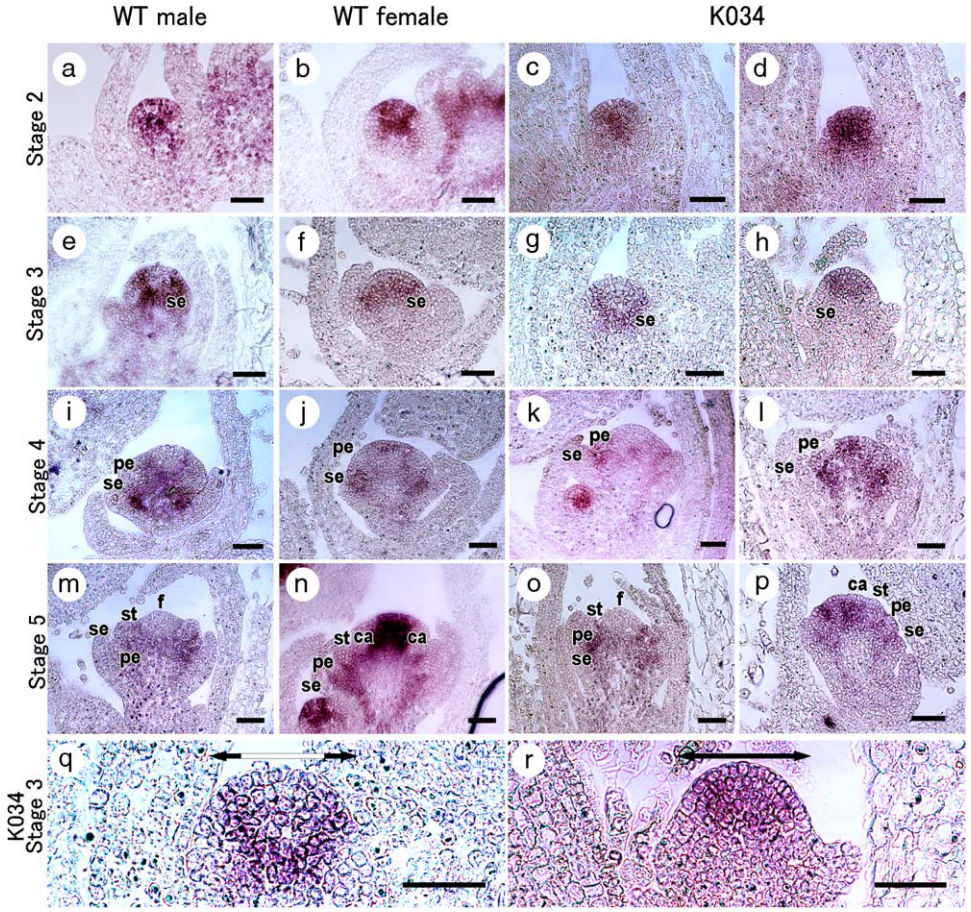

Figure 2. *SlSTM* expression in developing wild-type and K034 flowers. Wild-type male (a, e, i, m) and female (b, f, j, n) flowers; K034 male-type (c, g, k, o) and female-type (d, h, l, p) flowers. K034 flowers showed two *SlSTM1* and *SlSTM*2 expression patterns from stage 3 onward, similar to those in wild-type males and females (q and r [enlargements of the images in g and h, respectively]). *Se*, sepal; *pe*, petal; *st*, stamen; ca, carpel. Bars = 100 μm.

downregulated at its flanks (Figure 2c and d). The downregulation appeared to precede the outgrowth of sepal primordia, similar to wild types (Figure 2a–d). At stage 3, most K034 flowers had *SlSTM*-negative regions in flower meristem central portions (Figure 2g, q), although some did not (Figure 2h, r). Subsequently, we observed two K034 expression patterns (Figure 2g, h, k, l, o, p). In total 32 flowers examined, 27 flowers were classified into a male type (Figure 2e, i, m) whereas 5 flowers were classified into a female type (Figure 2f, j, n). *STM* in *A. thaliana* is required for proper proliferation of cells in the flower meristem. It is reasonable to propose that K034 flower gynoecia without *SlSTM* expression become suppressed (appearing as rods), while gynoecia with *SlSTM* expression become mature gynoecia with one to three carpels.

In young wild-type flowers, *SlCUC* transcripts are detectable at the boundaries between whorls of primordia (Figure 3a, b, d, e, g, h, j, k). In stage 3 male flowers, *SlCUC* transcripts were detectable in the flower meristem central region and at the boundaries between sepal primordia and the flower meristem central dome (Figure 3d). In stage 3 female flowers, *SlCUC* transcripts were detectable at whorl boundaries, but they were not detected at the meristem summit (Figure 3e). In the flowers at stage 4, the transcripts were detectable between sepal and petal primordia and between petal and stamen primordia (Figure 3g, h). At stage 5, the male–female sexual organ border was clearly visible because *SlCUC* was detected at the boundaries between the third and fourth whorls of wild-type male and female flowers (Figure 3j, k). In stage 5 female flowers, *SlCUC* mRNA was also found in some of the prospective ovules (Figure 3k). These results in wild-type male and female flowers were almost identical to those of Zluvova et al. (2006).

In K034 flowers, *SlCUC* signal expression delimited domains corresponding to sepal, petal, stamen, and gynoecium whorls (Figure 3c, f, i, l). *SlCUC* expression patterns appeared almost identical in both the flower meristems of wild-type and K034 flowers at all stages examined except stage 3. K034 flowers had two *SlCUC* expression patterns similar to those in wild-type males and females at stage 3 (Figure 3f, m, n). This switch-like change of *SlCUC* expression patterns would attribute to the male-type and female-type *SlSTM* expressions in the ratio of 27:5.

Discussion

Wild-type males and females show two morphological differences in the gynoecium; one is the existence of carpels, and the other is the size of the fourth whorl. *SlSTM* and *SlCUC* seem to be involved in this differentiation, because the expression patterns of these genes in gynoecia are different between wild-type males and females (Zluvova et al., 2006). Thus, the expression patterns of *SlSTM* and *SlCUC* may determine the existence of carpels or the size of the fourth whorl. On the other hand, we showed that K034 produces asexual flowers (no carpel) and female-like flowers (one to three carpels) even though all K034 flowers have small fourth whorls, similar to those of wild-type males (Koizumi et al., 2007). Moreover, K034 had two kinds of *SlSTM* and *SlCUC* expression patterns. Therefore, the *SlSTM* and *SlCUC* pathway is involved in the development of carpels and not in the size of the fourth whorl.

Since *SlSTM*1 and *SlSTM*2 share approximately 80% amino acid identity and show similar patterns of expression (Zluvova et al., 2006), we performed *in situ* hybridization using a *SlSTM*2 probe that hybridized to both *SlSTM*1 and *SlSTM*2. Our results obtained in wild-type male and female flowers support the findings of Zluvova et al. (2006) except for their observation of *SlSTM* expression in the central region of the developing gynoecium of wild-type female flowers at stage 5. This difference between these studies may be attributable to differences in probes. We used the *SlSTM*2 probe whereas they used the *SlSTM*1 probe (Zluvova et al. 2006).

Female and male-type flowers made up 14.3% and 85.7% of young K034 flower buds (total of 32 flowers), respectively, among those we analyzed by *in situ* hybridization with *SlSTM* and *SlCUC*. Analysis of the carpel elongation patterns in all flower buds in Figure 1c at a fixed point in time revealed three female-like flowers (13.0%) and 23 asexual flowers (87.0%). This ratio is almost identical to those in female-like flowers of original K034 plants that had bloomed for 1 month, in cutting clones, and in K034-type progeny in the first and second backcross generations (7–16%; Koizumi et al., 2007). Hence, *SlSTM* and *SlCUC* expression patterns correspond to the carpel suppression patterns in K034.

In K034, we discerned two kinds of *SlSTM* and *SlCUC* expression patterns (prior to any morphological differentiation), which were similar to those in wild-type males or females. The frequency of flower types in K034-type progeny obtained from backcrosses of K034 with wild-type males was almost identical to that of the original K034. These results preclude the possibility of chimerism in K034. The two kinds of *SlSTM* and *SlCUC* expression patterns in K034 flowers are not thought to result from cellular differences between K034 flowers. We did not observe any intersexual phenotype between

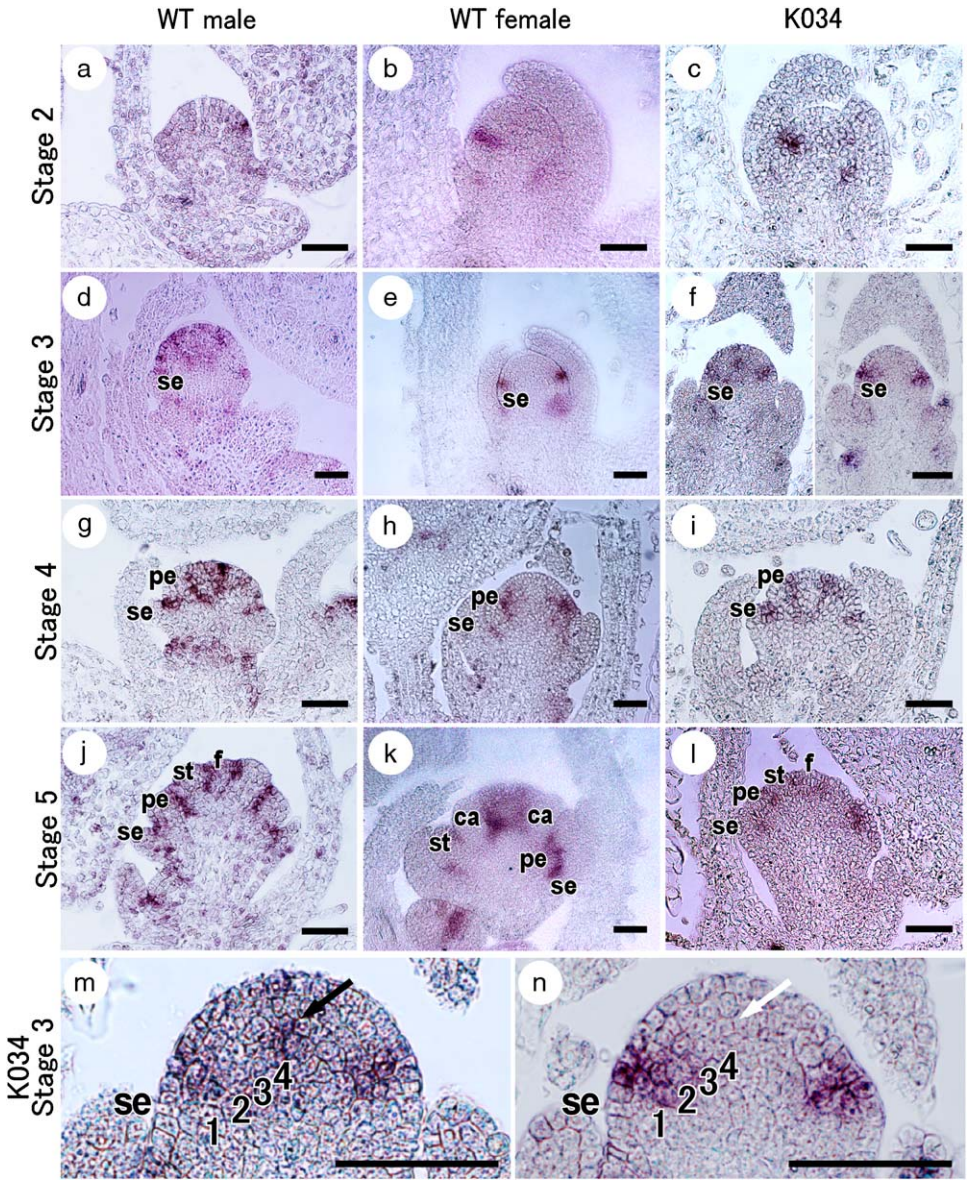

Figure 3. *SlCUC* expression in developing wild-type and K034 flowers. Wild-type male (a, d, g, j) and female (b, e, h, k) flowers; K034 male-type and female-type (c, f, i, l) flowers. K034 flowers contained two types of *SlCUC* expression at stage 3 (m and n [enlargements of right and left images in f]). One type showed *SlCUC* expression in the central portion of the flower meristem (*black arrows* in m), while the other did not (*white arrows* in n). *Se*, sepal; *pe*, petal; *st*, stamen; ca, carpel. Numbers in the image (m) and (n) indicate prospective flower whorls. Bars = 100 μm.

asexual flowers and female-like flowers except in carpel number variation. Moreover, we did not observe any intersexual *SlSTM* and *SlCUC* expression pattern in K034. This suggests that mutation(s) responsible for two mutant genotypes in K034 acts upstream of the pathway involved in *SlSTM* and *SlCUC*. On the K034 Y chromosome, both the gynoecium-suppressing region and the stamen-promoting region have deletions (Koizumi et al., 2007). We believe that only the deletion in the gynoecium-suppressing region is involved in the dimorphic gynoecium phenotype of K034. To uncover the epigenetic mutation in gynoecia of *S. latifolia*, further studies of gene loss in K034 are necessary.

Acknowledgments

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References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. Plant Cell 1997;9:841–57.

Aida M, Ishida T, Tasaka M. Development 1999;126: 1563–70.

Barton MK, Poethig RS. Development 1993;119:823–31.

Breuil-Broyer S, Morel P, de Almeida-Engler J, Coustham V, Negrutiu I, Trehin C. Plant J 2004;38:182–92.

Clark SE, Jacobsen SE, Levin JZ, Meyerowitz EM. Development 1996;122:1567–75.

Endrizzi K, Moussian B, Haecker A, Levin JZ, Laux T. Plant J 1996;10:967–79.

Farbos I, Oliveira M, Negrutiu I, Mouras A. Sex Plant Reprod 1997;10:155–67.

Grant SR, Hunkirchen B, Saedler H. Plant J 1994;6: 471–80.

Janousek B, Siroky J, Vyskot B. Mol Gen Genet 1996; 250:483–90.

Kazama Y, Koizumi A, Uchida W, Ageez A, Kawano S. Plant Cell Physiol 2005;46:806–11.

Koizumi A, Amanai Y, Ishii K, Nishihara K, Kazama Y, Uchida W, Kawano S. Plant Cell Physiol 2007;48:1450–61.

Lardon A, Georgiev S, Aghmir A, Le Merrer G, Negrutiu I. Genetics 1999;151:1173–85.

Long JA, Moan EI, Medford JI, Barton MK. Nature 1996; 379:66–9.

Scutt CP, Li Y, Robertson SE, Willis ME, Gilmartin PM. Plant Physiol 1997;114:969–79.

Takada S, Hibara K, Ishida T, Tasaka M. Development 2001;128:1127–35.

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MA, de Vries SC. Plant Cell 2003;15:1563–77.

Zluvova J, Nicolas M, Berger A, Negrutiu I, Monéger F. Proc Natl Acad Sci USA 2006;103:18854–9.