Sexual dimorphism is controlled by genes on the Y chromosome in the dioecious plant Silene latifolia. K034 is the first mutant with female flowers and asexual flowers in one individual. Its stamens are suppressed completely, and its gynoecium exhibits two suppression patterns. One gynoecium resembles a thin rod, as in wild-type males (asexual flower); the other is imperfectly suppressed, having 1–3 carpels (female-like flower). The ratio of these patterns was 9:1. To exclude the possibility of chimerism in K034, we crossed a female-like flower of K034 with a wild-type male. Progeny obtained from this crossing had asexual and female-like flowers in one individual. This two-flower-type phenotype was inherited without separating. To examine the identity of flower organs in K034, we analyzed the development of asexual and female-like flowers using scanning electron microscopy and in situ hybridization with SLM1 and SLM2 (orthologs of AGAMOUS and PISTILLATA, respectively) as probes. Mitotic spreads of root tip chromosomes from hairy root cultures showed that K034 had 25 chromosomes. Fluorescent in situ hybridization analysis, using a subtelomeric repetitive sequence (KpnI subfamily) as a probe, indicated that K034 possessed two X chromosomes and one Y chromosome (Yδ), of which Yδ had been rearranged to lose the pseudoautosomal region (PAR). PCR analysis using Y-specific sequence-tagged site (STS) markers clarified that Yδ of K034 had two other deletions in gynoecium suppressing and stamen-promoting regions. It is reasonable to suggest that these sex chromosomal abnormalities resulted in two abnormal sexual phenotypes: the asexual and imperfect female (female-like) flowers in K034.

Introduction

Sex determination systems in plants have evolved independently many times and are one of several strategies that promote outcrossing and thus help to avoid inbreeding. Despite the fact that angiosperms are defined by their distinctive flowers, which typically incorporate both male and female sex organs, approximately 10% of species produce unisexual flowers in which only stamens or carpels develop to maturity (reviewed by Dellaporta and Calderone-Urrea 1993, Grant et al. 1994). In these unisexual systems, the standard hermaphrodite flower has been modified, resulting in separate male and female flowers (reviewed by Ainsworth 2000, Matsunaga and Kawano 2001). In some dioecious plants such as Silene latifolia and Rumex acetosa, male and female flowers each initiate four whorls of organs but, in each sex, the inappropriate sets of organs arrest soon after initiation. In other plants such as Mercurialis annua and Humulus lupulus, inappropriate organs are never initiated.

In S. latifolia, unisexuality is achieved through at least two functions that modify defined stages of development (Farbos et al. 1997). In male flowers, the failure of carpel development results from a sudden arrest of cell division in the fourth whorl at the time of morphological partitioning between the third and fourth whorls. In female flowers, the cessation of stamen development occurs as an arrest of differentiation at the early sporogenous stage. The asexual mutant accumulates these two obstacles to development that separately characterize the wild-type male flower (inhibition of female organ) and the wild-type female flower (arrest of male development), whereas the hermaphrodite mutant has neither (Westergaard 1946, Westergaard 1958, Farbos et al. 1999, Lard et al. 1999,}

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**Floral Development of an Asexual and Female-Like Mutant Carrying Two Deletions in Gynoecium-Suppressing and Stamen-Promoting Functional Regions on the Y Chromosome of the Dioecious Plant Silene latifolia**

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Abbreviation: AFLP, amplified fragment length polymorphism; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; FISH, fluorescence in situ hybridization; GSF region, gynoecium-suppressing functional region; MFF region, male fertility functional region; PAR, pseudoautosomal region; SEM, scanning electron microscopy; SPF region, stamen-promoting functional region; STS, sequence-tagged site
Lebel-Hardenack et al. 2002). The existence of these mutants indicates that the sex determination system of *S. latifolia* has interesting features.

Dioecy in *S. latifolia* is determined genetically by heteromorphic sex chromosomes (XX for female and XY for male). Sex chromosomes are much larger than autosomes in *S. latifolia*. Sexual dimorphism in *S. latifolia* is controlled by genes located on the differential arm of the active Y sex chromosome (Westergaard 1958, Van Nigtevecht 1966). The Y chromosome is divided into four basic functional regions (Westergaard 1958): (i) a region causing suppression of gynoecium development, often called the gynoecium-suppressing functional (GSF) region (Lardon et al. 1999, Lebel-Hardenack et al. 2002); (ii) a region causing stamen-promoting function (SPF) region (Farbos et al. 1999, Lebel-Hardenack et al. 2002); (iii) a region containing the gene necessary for late anther development, known as the male fertility functional (MFF) region (Westergaard 1958, Lebel-Hardenack et al. 2002); and (iv) a recombining pseudoautosomal region (PAR; Westergaard 1958). The Y chromosome map was originally constructed using 27 amplified fragment length polymorphism (AFLP) markers, two Southern hybridization-based markers and one PCR marker based on 44 X-ray-induced hermaphroditic and asexual mutants (Lebel-Hardenack et al. 2002). Lebel-Hardenack et al. (2002) looked for a strong correlation between the loss of markers and mutant phenotypes that would indicate the position of the sex-determining genes. This approach was used by Moore et al. (2003) to order three genes on the Y chromosome possessing copies on the X chromosome: *SIY1* (Delichere et al. 1999), *SIY4* (Atanassov et al. 2001) and *DD44Y* (Moore et al. 2003). The map was further refined by Zluvova et al. (2005) by adding several other markers, including three Y-linked genes: *Sls* (Filatov 2005), *SIY3* (Nicolas et al. 2005) and *SIAP3Y* (Matsunaga et al. 2003). MK17 was recently added to the map by Hobza et al. (2006). The map has revealed that the hermaphroditic and asexual mutants reported by Lebel-Hardenack et al. (2002) are derived from the male (XY); moreover, asexual mutants lack some markers on the SPF region, and hermaphroditic mutants lack markers in the GSF region. Despite such recent advances, it is still unknown what kind of flowers are produced by the mutant lacking both the GSF and SPF regions on the Y chromosome in *S. latifolia*.

We have newly isolated a spontaneous mutant, K034, that has two abnormal sexual phenotypes, expressed as asexual and imperfect female (female-like) flowers in *S. latifolia*. To clarify this abnormality, we used scanning electron microscopy (SEM) and in situ hybridization with B- and C-function gene probes. We also performed karyotype analysis and PCR analysis using Y-specific sequence-tagged site (STS) markers to reveal the genotype of K034.

**Results**

**Asexual mutant K034**

An asexual mutant, K034, was obtained as a spontaneous mutant from the inbred K line of *S. latifolia*. K034 had two types of flowers (two-flower type) with normal forms of leaves, stems and perianth organs. One flower phenotype was asexual (Fig. 1c, g); the other was an imperfect female (female-like) flower (Fig. 1d, h). Wild-type male flowers of *S. latifolia* have 10 stamens and a suppressed gynoecium as an undifferentiated rod (Fig. 1a, e). Wild-type female flowers of *S. latifolia* have a gynoecium composed of five fused carpels, and lack mature stamens (Fig. 1b, f). Asexual flowers of K034 had rudimentary stamens and a suppressed gynoecium (Fig. 1c, g). Female-like flowers of K034 were imperfect female flowers, with a slimmed ovary and two styles (Fig. 1d, h). The number of styles is normally five in wild-type female flowers. The number of styles was mainly two in the female-like flowers, but was sometimes one or three. The gynoecium of female-like flowers was similar to that of wild-type females (Fig. 1d, h).

**The inflorescence of K034**

Wild-type *S. latifolia* produces either male or female flowers in separate individuals (Fig. 2a, b). K034, however, produced two types of flowers in one individual. We observed the flower phenotype in cutting clones of K034 and the K034-type backcross progeny (BC1-1 and BC2-15), and these were used to examine whether the phenotype, i.e. asexual or female-like, is determined by the position of the flower in the inflorescence. In the original K034 individual, 29 flowers bloomed in 1 month; three (10%) were female-like and the rest (90%) were asexual (Table 1). BC1-1 and BC2-15 also showed the same flowering tendency (Table 1). The female-like flowers tended to be the first flowers in an inflorescence. The frequency of asexual flowers increased in later floral branches (Fig. 2c–g). To exclude the possibility of chimerism in K034, we crossed a female-like flower of K034 with a wild-type male because the female-like flowers of K034 are fertile (Table 1). We obtained 40 progeny (female: male: K034 type = 21:13:6) in the first backcross generation (BC1) and 31 progeny (female: male: K034 type = 17:11:3) in the second backcross generation (BC2), as shown in the footnote of Table 1. There were no progeny that had either asexual flowers or female-like flowers exclusively. All K034-type progeny of BC1 and BC2 had both asexual flowers and female-like flowers in one individual, as found in BC1-1 and BC2-15 (Fig. 2f, g). Thus, this K034-type phenotype was inherited and was not separated.
Early flower development of asexual and female-like flowers of K034 (through stage 6)

Sexual abnormalities in the asexual and female-like flowers of K034 only occurred in certain developmental stages. To determine when morphological abnormalities appear, SEM analysis was performed (Fig. 3, Table 2). The developmental stages of wild-type flowers have been described by Grant et al. (1994) and Farbos et al. (1997). The sex organ inhibition defining the sex of flowers occurs after stage 4, when the sex organ primordia emerge and form the organs. Stage 4 is defined by the emergence of all five sepals (whorl 1) and the pentagon meristem, which contains prospective petal (whorl 2), stamen (whorl 3) and gynoecium (whorl 4) regions (Grant et al. 1994, Farbos et al. 1997). Up to stage 4, flower development was indistinguishable between the wild type and K034 (Fig. 3a–d).

The first clear sex differences appear at stage 5 in the wild type, when the petal and stamen primordia emerge (Grant et al. 1994, Farbos et al. 1997). The central meristem of wild-type females is larger than that of wild-type males (Fig. 3e, f). The size of the central meristem in asexual and female-like flowers of K034 was similar to that in wild-type males (Fig. 3g, h). Female-like flowers of K034 had a flatter gynoecium (whorl 4) than did the asexual flowers of K034 (Fig. 3g, h).

At stage 6, the gynoecium is suppressed and the carpel is not visible in wild-type males (Fig. 3i), whereas the gynoecium is developed and carpels are visible in wild-type females (Fig. 3j). In contrast, the gynoecium in asexual flowers of K034 became a thin rod, similar to that in flowers of wild-type males (Fig. 3k). The gynoecium in female-like flowers of K034 had 1–3 carpels, as in the wild-type females (Fig. 3l). At this stage, the ratio of the diameter of the gynoecium to the whole flower is 65–75% in wild-type female flowers, and 34–40% in wild-type male flowers (Grant et al. 1994; Fig. 3i, j). We measured the diameters of gynoecia in asexual and female-like flowers of K034 and found that the gynoecium occupied only 32.8 ± 3% of the

Fig. 1 Photographs of wild-type and K034 flowers in S. latifolia. Longitudinal views (a, b, c, d) and sections (e, f, g, h) of male (a, e) and female (b, f) flowers of the wild-type and asexual (c, g) and female-like (d, h) flowers of K034 are shown. The wild-type male flower (a, e) possesses yellow stamens (white arrowheads). The wild-type female (b, f) and K034 female-like (d, h) flowers are shown with ovaries (o) and styles (double white arrowheads). The K034 asexual flower (c, g) lacks mature stamens and styles (white arrows). Bar = 1.0 cm.
whole flower in these flowers. This was almost the same as that found in the wild-type male flowers (Fig. 3i, k, l).

Middle and late flower development of K034 (stages 6–7 and stages 8–9)

In stage 7, stamen primordia separate into anthers and anther filaments in wild-type males (Fig. 4a). Anthers and anther filaments in wild-type females do not develop fully (Fig. 4b). The gynoecium of females begins to elongate and forms five cylinder-like carpels (Fig. 4b). However, both the asexual and female-like flowers of K034 had stamens that were separated into anthers and anther filaments (Fig. 4c, d). In the female-like flower of K034, the gynoecium continued to elongate and formed a carpel (Fig. 4d). One to three carpels were also observed. The female-like flowers of K034 were consistently imperfect because their carpel number was not restored to five, as in wild-type female flowers (Fig. 4d, p, one-carpel type; Fig. 5d, two-carpel type).

ABC functional genes determine the identity of flower organs (Coen and Meyerowitz 1991, Weigel and Meyerowitz 1994). To examine the identity of flower organs in K034, we analyzed the expression of SLM1 and SLM2 (orthologs of AGAMOUS and PISTILLATA; Hardenack et al. 1994) by in situ hybridization. In the middle stages (stages 6–7), SLM1 expression in both female and male flowers is limited to the center of the floral meristem (whorls 3 and 4) (Fig. 4e, f). Similar expression patterns were observed in asexual and female-like flowers of K034 (Fig. 4g, h). SLM2 was expressed in stamens (whorl 3) and petals (whorl 2) of K034 flowers, as also observed in the wild type (Fig. 4i–l).

In stage 8, stamens begin to elongate in wild-type male flowers (Fig. 4m). Stamens do not develop in wild-type female flowers (Fig. 4n). In contrast, stamens in asexual flowers and female-like flowers of K034 were suppressed.

Table 1  Asexual and female-like flowers in cutting clones and backcrosses with the wild type

<table>
<thead>
<tr>
<th>Class</th>
<th>Asexual flower</th>
<th>Female-like flower</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>K034 original</td>
<td>43 (93%)</td>
<td>3 (7%)</td>
<td>46 (100%)</td>
</tr>
<tr>
<td>Cutting clones of K034</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K034-1</td>
<td>26 (89%)</td>
<td>3 (11%)</td>
<td>27 (100%)</td>
</tr>
<tr>
<td>K034-2</td>
<td>85 (84%)</td>
<td>16 (16%)</td>
<td>101 (100%)</td>
</tr>
<tr>
<td>Progeny of K034 × K12&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC1-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 (92%)</td>
<td>4 (8%)</td>
<td>47 (100%)</td>
</tr>
<tr>
<td>Progeny of B1-1 × K12&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC2-15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32 (92%)</td>
<td>3 (8%)</td>
<td>36 (100%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>K12, which was produced from 12 generations of sibling mating, was used as the wild-type male.

<sup>b</sup>BC1-1 is a representative K034-type progeny of 40 progeny (female : male : K034 type = 21 : 13 : 6) obtained in the first backcross generation.

<sup>c</sup>BC2-15 is a representative K034-type progeny of 31 progeny (female : male : K034 type = 17 : 11 : 3) obtained in the second backcross generation.
and showed no change from observations in stage 7 (Fig. 4o, p). At the late stages (stages 8–9), SLM1 transcripts were detected in developing stamens and the suppressed gynoecium in the wild-type male (Fig. 4q). In wild-type females, the developing gynoecium had SLM1 signals, but the stamen primordia did not (Fig. 4r). These stamen primordia do not develop, and anther filaments do not elongate. SLM1 expression in the stamens was also suppressed in asexual and female-like flowers of K034, similar to that in wild-type females (Fig. 4s, t). The developing or suppressed gynoecia had SLM1 signals in asexual and female-like flowers of K034 (Fig. 4s, t). At stages 8–9, SLM2 continues to be expressed in developing stamens and petals in wild-type male flowers (Kazama et al. 2005; Fig. 4u). In the wild-type female, the developing petals have SLM2 signals, but the stamen primordia do not (Kazama et al. 2005; Fig. 4v). SLM2 expression in the stamen primordia was suppressed in asexual and female-like flowers of K034, similar to that in wild-type females (Fig. 4w, x). The SLM2 expression pattern in the stamen was similar to the SLM1 expression pattern in K034 and in wild-type female flowers (Fig. 4r–t, v–x).

Late flower development of K034 (stages 9–10)

In stage 9, stamens of wild-type males continue to elongate, whereas those of wild-type females are suppressed (Fig. 5a, b). The gynoecium of females elongates and develops five styles (Fig. 5b). The asexual and female-like flowers of K034 had suppressed stamens, which were similar to those of wild-type females (Fig. 5c, d). The gynoecium of the female-like flower of K034 elongated, similar to that of wild-type females, and developed two styles (Fig. 5d). The number of styles corresponded to the number of carpels.

In stage 10, anthers in wild-type males continued to mature into a butterfly-like shape, whereas those in wild-type females degenerated (Fig. 5e, f). In contrast, stamens in asexual and female-like flowers of K034 did not degenerate (Fig. 5g, h). Because the MFF region on the Y chromosome is involved in the maintenance of formed stamens, it is unsurprising that stamens at stage 10 differed between wild-type females and K034.

Karyotype analysis of K034

Root tips from hairy root cultures of K034, induced by Agrobacterium rhizogenes strain 15834, were used as a source of chromosomes for mitotic spreads. K034 had 25 chromosomes (Fig. 6). We also confirmed that all six K034-type progeny of BC1 had 25 chromosomes. Three of the 25 chromosomes were sex chromosomes because they were much larger than the other 22 chromosomes. We measured the arm ratio (q/p ± SD) of K034...
sex chromosomes; two of the three were submeta-
centric (1.25 ± 0.12) and the other was a subtelocentric
chromosome (2.15 ± 0.09). In wild-type \textit{S. latifolia}, the
X chromosome is submetacentric (arm ratio = 1.44 ± 0.15,
Ciupercescu et al. 1990; 1.20 ± 0.13, Lardon et al. 1999),
whereas the Y chromosome is metacentric (arm ratio = 1.09 ± 0.04, Ciupercescu et al. 1990; 1.15 ± 0.09, Lardon et al. 1999). Thus, the two large submetacentric
chromosomes of K034 were thought to be X chromosomes.

Fluorescence in situ hybridization (FISH), using the
KpnI subfamily (the subtelomeric repetitive sequence with the
8k b KpnI subfamily genomic clone) as a probe, gives
fluorescent hybridization signals in the distal regions of
both arms of the X chromosome and in one arm of the
Y chromosome (Kazama et al. 2003; Fig. 6a). In K034, the
KpnI probe also hybridized to both arms of the two
metacentric sex chromosomes, but did not hybridize to
the one subtelocentric sex chromosome (Fig. 6b). These results
suggest that K034 has two submetacentric X chromosomes
and one sex chromosome that was rearranged and had lost
the PAR (probably Y). We also confirmed that all six K034-
type progeny of BC1 had two X chromosomes and one
rearranged and deleted chromosome.

Deletions in the Y chromosome of K034

To determine whether K034 had a Y chromosome,
PCR analysis using Y-specific STS markers was performed.
First, we selected STS markers which are normally
detectable in the K line (the genetic background of K034).
Because 27 AFLP markers reported by Lebel-Hardenack
et al. (2002) amplified non-specific bands in the K line
(data not shown), AFLP markers were not used. Based on
the SlssY and Bgl16 sequences, we designed PCR primers
that would amplify a male-specific band. We finally selected
13 markers on the Y chromosome (MK17, SlssY, Bgl10,
DD44Y, Bgl16, MS4, ScD05, SlAP3Y, ScQ14, MS7, MS2

\begin{table}
\centering
\caption{Landmark events in stages of wild-type and K034 flowers in \textit{Silene latifolia}}
\begin{tabular}{lll}
\hline
Stage & Description & Size (mm) \\
\hline
1 & Male (WT), female (WT), asexual (K034) and female-like (K034) & \\
2 & Apical inflorescence meristems begin to elongate & 0.08 \\
3 & One floral meristem and lateral inflorescence meristems are clearly visible & 0.10 \\
4 & Sepal primordia emerge & 0.15 \\
5 & Well-established sepal and pentagon-shaped meristem inside are visible & 0.20 \\
6 & Petal and stamen primordia arise. First differences between male and female appear & 0.25 \\
7 & Male (WT) and asexual (K034) Size (M and A) & \\
8 & Female (WT) and female-like (K034) Size (F and FL) & \\
6 & Single suppressed gynoecium is visible & 0.45 \\
7 & Stamen primordia separate into anthers and anther filaments & 0.60 \\
8 & Male & 0.80 \\
9 & Stamens continue to develop; \textit{SLM1} and \textit{SML2} expression continues & \\
10 & Single suppressed gynoecium is visible & 0.55 \\
11 & Stamen primordia separate into anthers and anther filaments & 0.85 \\
12 & Male and female-like & 1.45 \\
13 & The cessation of stamen development appears; \textit{SLM1} and \textit{SML2} expression is suppressed at arrested stamens & \\
14 & Female and female-like & \\
15 & Gynoecium becomes constricted at the tip & 2.00 \\
16 & Stamen primordia separate into anthers and anther filaments & 4.87 \\
17 & Gynoecium tube closes and styles grow from carpel tips & 10.0 \\
18 & Tapetum degenerates & 6.00 \\
19 & Stigma forms along inside ridges of styles & 28.0 \\
20 & Stamen filaments elongate and pollen matures & 25.5 \\
21 & Integuments surround ovules & 28.0 \\
\hline
\end{tabular}
\end{table}
and ScX11), four markers on the X chromosome (SlsX, SIX1, SIX4 and DD44X) and one autosomal marker (Sl-actin), which were amplified male-specifically in the K line (Fig. 7). Twelve of the 13 markers on the Y chromosome are mapped on the Y chromosome map, but one (ScX11) has not previously been mapped (Zluvova et al. 2005). MK17, SlsY, Bgl10, DD44Y and Bgl16 are in the same linkage group as the GSF linkage on the p arm of the Y chromosome [LOD (log of odds) scores >3; Lebel-Hardenack et al. 2002, Moore et al. 2003,
Zluvova et al. (2005, Hobza et al. 2006). MS4, ScD05 and SlAP3Y are also predicted to be near the GSF linkage (Zluvova et al. 2005). ScQ14 must be closely linked to the stamen-promoting locus because it is absent in all asexual mutants (Zluvova et al. 2005). MS7 and MS2 are associated with the MFF linkage, as is SlY4. As for MS7, we analyzed the map position using 22 X-ray-induced sexual phenotype mutants (kind gifts from Dr. S. Grant, UNC) reported by Lebel-Hardenack et al. (2002).

K034 had the Y-linked sequences, which were amplified by the markers of MK17, SlY4, SlAP3Y, SlssY, ScD05, MS2, MS7 and ScX11. However, the MS4 (443 bp male-specific band), Bgl10, DD44Y, Bgl16 and ScQ14 Y-specific primers did not amplify any bands from K034. The Sl-actin marker (Sugiyama et al. 2003) was amplified in the wild types and K034 as a control. The results indicated that K034, which has two flower phenotypes, possessed a Y chromosome carrying two deletions in the GSF and SPF regions (Yd). We also confirmed that the K034-type progeny of BC1 had this Yd chromosome.

**Discussion**

**Mutations in K034**

In *S. latifolia*, it is a rare phenotype where one individual has two types of flowers (Heslop-Harrison 1963, Ruddat et al. 1991, Law et al. 2002). Besides K034, only three mutants in *S. latifolia* have been reported (Westergaard 1946, Janousek et al. 1996, Lardon et al. 1999). All three mutants are androhermaphrodites, with both bisexual and male flowers. K034 is the first mutant...
with female flowers and asexual flowers in one individual with a deleted Y chromosome. All stamens of K034 were completely suppressed, and its gynoecium exhibited two suppression patterns (Fig. 1c, d, g, h). One pattern resembled a thin rod, as in wild-type males (asexual flower); the other was imperfectly suppressed, having 1–3 carpels (female-like flower). When the female-like flowers of K034 were crossed with wild-type males, six of 40 progeny had both asexual flowers and female-like flowers (K034 type), the same as the maternal line, K034. None of the backcross progeny, however, possessed only asexual flowers within a single individual or only female-like flowers within a single individual. This K034-type phenotype was inherited in the progeny without separation. The two suppression patterns in the gynoecium of K034 were not separated from the complete stamen suppression. Thus, autosomes were not involved in flower sex variation in K034.

Karyotype analysis showed K034 had three sex chromosomes, two X chromosomes and one rearranged Y chromosome. (Figs. 6b, 7B). To examine if these sex chromosomal abnormalities (two X chromosomes and one rearranged Y chromosome) were the cause of the floral defects, we checked the phenotype and karyotype of the progeny (female : male : K034 type = 21 : 13 : 6; Table 1) obtained in the first backcross generation (BC1). Karyotype analysis showed that the K034-type progeny possessed 25 chromosomes and suggested that the K034-type progeny of BC1 had two X chromosomes and one rearranged and deleted chromosome. PCR analysis using Y-specific STS markers confirmed that the K034-type progeny of BC1 had the Yd chromosome of K034. We thus confirmed the co-segregation of the floral defect phenotype and XXYd.

Westergaard (1946) reported that a triploid mutant with XXY chromosomes showed a normal male phenotype.

Fig. 7  PCR amplification (A) of sequence-tagged sites (STSs) using genomic DNA isolated from K034, wild-type male (M) and wild-type female (F). A schematic representation of the entire Y chromosome of K034 (B) expected from FISH analysis (Fig. 6) and PCR analysis of STS markers (A). The map (B) is arranged according to Lebel-Hardenack et al. (2002), Moore et al. (2003) and Zluvova et al. (2005). The open and solid arrowheads indicate the 596 bp bands derived from the autosome and the 443 bp male-specific band, respectively (A). GSF, gynoecium-suppressing functional region; SPF, stamen-promoting functional region; MFF, male fertility functional region; PAR, pseudoautosomal region.
K034 is an XXYd mutant (Figs. 6b, 7B). We thus considered that the two-flower-type (K034 type) phenotype was not due to the double X chromosomes, but rather to the rearranged Y chromosome in K034.

**Two deletions on the Y chromosome**

K034 lacked the markers on the gynoecium-suppressing region and the stamen-promoting region (Fig. 7). It is assumed that the two-flower-type (K034 type) phenotype is attributed to deletions in the stamen-promoting region and the gynoecium-suppressing region on the Y chromosome. Lebel-Hardenack et al. (2002) identified 44 X-ray-induced hermaphroditic and asexual mutants in *S. latifolia*. Zluvova et al. (2005) analyzed 22 of these mutants (13 hermaphroditic mutants and nine asexual mutants). K034 lacked the ScQ14 marker on the stamen-promoting region (Fig. 7). Similarly, five of the mutants lack this marker, and all of these mutants are asexual (Zluvova et al. 2005). We confirmed that complete stamen suppression in K034 was caused by the deletion of the stamen-promoting region on the Y chromosome.

K034 also lacked Bgl10, DD44Y, Bgl16 and MS4 markers on the gynoecium-suppressing region (Fig. 7), although none of the 22 mutants lacked just these markers. To date, the role of this gynoecium-suppressing region deletion in K034 has not been identified. Because K034 is a novel gynoecium-suppressing mutant, this deleted region is likely to be involved in the defect in gynoecium suppression in *S. latifolia*.

**Double X chromosome mutant with the deleted Y chromosome**

We obtained 21 females, 13 males and six K034-type progeny in the first backcross generation (BC1, see Table 1). Regarding the 21 females and the 13 males of BC1, they had 24 chromosomes with XX and with XY, respectively, as far as we examined (data not shown). On the other hand, the six K034-type progeny of BC1 did not have 24 chromosomes with XYd but rather 25 chromosomes with XXYd. The PAR-lacking Y chromosome of K034 seemed to be inherited by BC1 progeny because of the two X chromosomes. Although the segregation ratio was expected to be 1:1 (female: K034), it was actually 21:6 (Table 1). This suggests that the Yd chromosome is not always accompanied by one of the two X chromosomes during meiosis. The PAR, which is missing in K034, is necessary for sex chromosome pairing. Yd without the PAR may occasionally not be transmitted properly to gametes when two X chromosomes segregate.

**Suppression of the gynoecium and stamens**

Gynoecia of K034 are divided into male and female types. The imperfect female-type gynoecia of K034 were developed and elongated, whereas male-type gynoecia of K034 were suppressed as undifferentiated rods. The male-type gynoecia still continued to elongate even though they were suppressed. The C-function gene was expressed in the female-type, developing gynoecia in female-like flowers and male-type suppressed gynoecia in asexual flowers of K034. Suppressed and rod-like elongating gynoecia were also observed in intersexual flowers of a triploid intersexual individual in *Rumex acetosa* (Ainsworth et al. 2005). In *R. acetosa* intersexual flowers, the C-function gene was expressed in elongating and suppressed gynoecia. These results indicate the possibility that the C-function gene is involved in the elongation of the gynoecium. Moreover, the data suggest that female organ suppression is not controlled by the upstream gene of the C-function gene, but rather by the downstream gene of the C-function gene.

In wild-type males with the normal Y chromosome, B- and C-function genes continue to be expressed in developing stamens and petals. Despite the fact that K034 possessed the Y chromosome, the expression of B- and C-function genes was no longer detectable after stage 8 in the stamen primordia in asexual and female-like flowers of K034 (Fig. 4s, t, w, x). At this stage, the stamen primordia of K034 were arrested. These results suggest that B- and C-function genes are involved in the development of stamens. The deletion of the stamen-promoting factor would affect the upstream gene of B- and C-function genes.

**Materials and Methods**

**Plant materials**

An inbred *S. latifolia* line, the K line, was produced by 11 generations of sibling mating; this K line provided healthy plants. K034 was spontaneously identified from the K line. Clones of K034 were obtained from vegetatively propagated cuttings and from progeny of backcrosses of K034 with wild-type males. The phenotype and karyotype in the original K034, cutting clones of K034 and progeny obtained in the first backcross generation were checked. All experiments indicated that the plants were from the original K034. Hairy root cultures were obtained by infecting plant leaf explants with the oncogenic *Agrobacterium rhizogenes* strain 15834 (kindly provided by Professor H. Kamata, University of Tsukuba), and culturing the explants on B5 medium without hormones. Total RNA was extracted from young flower buds (<1 mm) using the RNasy Mini Kit (Qiagen, Valencia, CA, USA).

**Light microscope observations**

Plant materials >1 mm in length were observed under a stereoScope (MZ16; Leica Imaging Systems, Cambridge, UK). Fluorescence and cytological microscopy were performed using a fluorescence microscope (BX 52; Olympus Optical Co., Tokyo, Japan) equipped with a color charge-coupled device (CCD) camera (DP50 and DP70 Olympus).
Scanning electron microscopy

The protocols of Uchida et al. (2003) were used as a reference for SEM, with some modifications. The flower buds were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C, washed three times with distilled water, dehydrated in an ethanol series (50, 70, 80, 90, and 95%), each step for 20 min at room temperature) and stored in 100% ethanol overnight. The ethanol was replaced with isopentyl acetate, and the buds were dried using a critical-point dryer (HCP-2; Hitachi, Japan), and sputter-coated with platinum using an ion sputter (D-1010; Hitachi). The buds were then examined in the S-3000 N, operated at 5 kV in high-vacuum mode.

In situ hybridization

Total RNA (100 ng) was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (GE Healthcare Biosciences, Buckinghamshire, UK). The probes used were the 3' ends of the MADS box genes SLM1 and SLM2, with SLM1-specific primers (SLM1F2, 5'-CCAGATCGTACGTAACAGAGATAA-3') and SLM1R1, 5'-CACACACGCTCACAAGTACA-3') and SLM2-specific primers (SLM2F2, 5'-GATTGAGGAGGATCTACCC-3') and SLM2R2, 5'-GATTGAGGAGGATCTACCC-3') without the conserved MADS domains. The amplified insert was used to produce digoxigenin (DIG)-labeled sense and antisense RNA probes using a DIG RNA Labeling Kit SP6/T7 (Roche Applied Science, Indianapolis, IN, 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). The 8-μm sections were cut with a microtome and mounted on slides at 37 °C overnight. In situ hybridization was performed as described by Kazama et al. (2005).

Fluorescence in situ hybridization

To check the long arm of the Y chromosome, the 8 kb KpnI subfamily genomic clone in the bacterial artificial chromosome (BAC) clone #13B12 was labeled with Cy3-dUTP (GE Healthcare Biosciences) in a standard nick translation reaction using DIG nick translation mix (Roche). The labeled probe was purified using a DIG RNA Labeling Kit SP6/T7 (Roche). Root tips from hairy root cultures of S. latifolia were enzymatically converted into protoplasts with dextran sulfate and 2 M HCl treatment, and metaphases were accumulated with oryzalin (15 μM, 9 h). Cells with acetone for 30 s. The hybridization mixture (for one slide, 8 μl) contained 15 ng μl⁻¹ of each labeled DNA, 50% formamide, 10% dextran sulfate and 2× SSC; this was denatured at 80 °C for 10 min and applied to the slides. Slides were covered with coverslips and washed in 2× SSC at 37 °C. Slides were treated with avidin coupled with Alexa Fluor 488 (1:750, Invitrogen) at 37 °C for 30 min and washed as before. Slides were mounted in DAPI II (Vysis, Downers Grove, IL, USA) containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) as a counterstain. Images were collected with a Leica Q550CW digital image analyzer and captured by a CCD camera using Leica QFISH software Version 3.0 (Leica).

Mapping of MS7 using Y deletion mutants

To determine the physical position of MS7 on the Y chromosome, PCR with primers MS7f (5'-GATGACGGACCTATA-3') and MS7r (5'-CGCTGACTCCCTTTACA-3') was performed on a panel of S. latifolia Y chromosome deletion mutants, which were described by Lebel-Hardenack et al. (2002). The panel was kindly provided by Dr. S. Grant, University of North Carolina. The position of MK17 on the Y chromosome was based on the map constructed by Zlouva et al. (2005).

Mapping deletions in K034

Based on the Y chromosome map reported by Lebel-Hardenack et al. (2002), Moore et al. (2003), Zlouva et al. (2005) and Hobza et al. (2006), we examined deletions on the Y chromosome of K034 by PCR analysis using the primers for 27 AFLP markers reported by Lebel-Hardenack et al. (2002), Y-linked genes (SIY1, Delichere et al. 1999; SIY4, Atanassov et al. 2001; DD44Y, Moore et al. 2003; SlAP3Y, Matsunaga et al. 2003; SIY3, Nicolas et al. 2005; SmY, Filatov 2005) and Y-linked PCR markers (Bgl6 and Bgl10, Donnison et al. 1996; ScD05, ScQ14 and ScX11, Zhang et al. 1998; MS4, Obara et al. 2002; MK17, Hobza et al. 2006).

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References

An asexual and female-like mutant with Y deletions


