

Early Events in the Evolution of the *Silene latifolia* Y Chromosome: Male Specialization and Recombination Arrest

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ABSTRACT

Understanding the origin and evolution of sex chromosomes requires studying recently evolved X–Y chromosome systems such as those in some flowering plants. We describe Y chromosome deletion mutants of *Silene latifolia*, a dioecious plant with heteromorphic sex chromosomes. The combination of results from new and previously described deletions with histological descriptions of their stamen development defects indicates the presence of two distinct Y regions containing loci with indispensable roles in male reproduction. We determined their positions relative to the two main sex determination functions (female suppressing and the other male promoting). A region proximal to the centromere on the Y *p* arm containing the putative stamen promoting sex determination locus includes additional early stamen developmental factors. A medial region of the Y *q* arm carries late pollen fertility factors. Cytological analysis of meiotic X–Y pairing in one of the male-sterile mutants indicates that the Y carries sequences or functions specifically affecting sex chromosome pairing.

IT is widely accepted that X–Y sex chromosome systems evolved from a pair of autosomes, which initially acquired the mutation(s) responsible for sexual dimorphism, the primary sex determination genes (reviewed in CHARLESWORTH 1991). Two main processes are generally considered to occur during this earliest stage of X–Y evolution. One is restriction of crossing over in the region(s) containing these loci, which might be called a proto-X–Y system. The second is the accumulation of two kinds of genes advantageous for male sex functions: sexually antagonistic male-benefit alleles and alleles enhancing male gamete success.

Sexually antagonistic male-benefit alleles are predicted to accumulate close to the sex-determining genes, because expression of their disadvantageous effects in females will be reduced by tight linkage with the sex-determining region (FISHER 1931; RICE 1987; reviewed in VICOSO and CHARLESWORTH 2006). Recombination between the primary sex-determining region and loci at which male-benefit alleles have accumulated on the Y will generate poorly adapted phenotypes, selecting for tighter linkage in the region. The process of selection and recruitment of male-benefit alleles may be followed by further selection favoring restriction of recombination, which may eventually be completely suppressed,

and the entire proto-Y-chromosome region may become nonrecombining. This can explain why, in many sex chromosome systems, recombination is suppressed across most of the Y, not just in the primary sex-determining region (reviewed in CHARLESWORTH *et al.* 2005). In mammals, for example, only small parts of the Y (the “pseudo-autosomal” regions, PAR1 and PAR2; see PERRY and ASHWORTH 1999; CHARCHAR *et al.* 2003) still recombine with the X.

After crossing over ceases, the sequences of the initially homologous regions of the proto-sex chromosomes diverge, both due to further mutations in genes linked to the sex-determining region, as just outlined, and to substitutions of neutral and weakly deleterious mutations under genetic drift. The low effective population size of the evolving Y chromosome also leads to genetic degeneration within the nonrecombining sex-specific region, including reduction of genes’ functions, loss of genes, and accumulation of transposable elements (reviewed in CHARLESWORTH and CHARLESWORTH 2000).

Empirical evidence for the involvement of sexually antagonistic male-benefit alleles and for their presence on the Y driving the evolution of low crossing over is limited. The classical evidence for accumulation of male-benefit alleles closely linked to sex-determining genes is the presence, in the fish *Poecilia reticulata*, of male sexual attractiveness alleles in both the nonrecombining and pseudo-autosomal regions of the Y

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chromosome (WINGE 1927; BULL 1983; LINDHOLM and BREDEEN 2002). These alleles reduce males' survival to maturity, even under laboratory conditions (BROOKS 2000), so they are clearly disadvantageous, except for their effects on male sexual attractiveness. They are probably not sex limited in expression (BULL 1983), but the benefits of attractiveness in females are less important than for males, and there are fitness costs of ornamentation in terms of conspicuousness to predators; thus, these are probably sexually antagonistic genes of the kind hypothesized. There is also some quantitative genetic support for sexually antagonistic alleles in *Drosophila melanogaster* (GIBSON *et al.* 2002), and there is indirect evidence in the neo-Y system of *D. americana* (MCALLISTER and EVANS 2006). Recently, a QTL mapping study inferred the presence of sexually antagonistic genes for sexually dimorphic characters in *Silene latifolia*, including flower size (SCOTTI and DELPH 2006). However, these conclusions need to be verified by other kinds of markers, as the genes were inferred to be located in two PAR regions (which has not previously been suspected). Moreover, the situation is not the one hypothesized above: females apparently do not express the phenotypes (expression is sex limited), and the genes were hypothesized to be maintained polymorphic by antagonistic pleiotropy in males.

An indirect line of evidence is based on the human Y, the best-studied Y chromosome so far. As the theory above predicts, the Y sequence has revealed numerous genes involved in spermatogenesis. Some of these are genes that were once autosomal or X-linked and have transposed onto the Y (LAHN and PAGE 1999b; SKALETSKY *et al.* 2003), while others are multi-copy genes, present in ampliconic regions, mostly with no homology with either X-linked or autosomal genes (SKALETSKY *et al.* 2003). The human Y is thus enriched for male function genes, many of which are indispensable for male reproduction (their loss leads to oligospermia or azoospermia, disorders that disallow reproduction under natural conditions (these data are reviewed in KENT-FIRST 2000; ELLIS and AFFARA 2006).

There is evidence that recombination arrest between the mammalian sex chromosomes evolved in successive stages, as the above model predicts. This is deduced from the observation of X chromosome evolutionary strata. The strata are genomic regions with different divergence between homologous X and Y gene pairs, with the least diverged region (indicating the most recent recombination arrest) located closest to the PAR1 on the genetic map of the X, and the most diverged (in which recombination between the X and Y ceased longest ago) furthest from the PAR1 (LAHN and PAGE 1999a; SANDSTEDT and TUCKER 2004). The gene order of mammalian X chromosomes is highly conserved, whereas the Y chromosomes are very rearranged, which suggests that inversions have occurred on the Y during these chromosomes' history, estimated to be a period of

~300 million years (LAHN and PAGE 1999a). The divergence values are not discontinuous enough to define boundaries definitively indicating inversions, so loss of crossing over may have involved rearrangements and/or gradual recombination arrest (IWASE *et al.* 2003; MARAIS and GALTIER 2003; SKALETSKY *et al.* 2003; ROSS *et al.* 2005).

Mammalian X–Y systems are ancient and thus not suited to gaining a detailed understanding of the early stages of this evolution or for testing the sexual antagonism hypothesis. The recent evolutionary history of flowering plants (LEEBENS-MACK *et al.* 2005), and the scattered distribution of dioecy (individuals with separate sexes) among them, suggest recent evolution of dioecy in angiosperms, sometimes even within plant genera, such as the genus *Silene* (DESFEUX *et al.* 1996), yet some dioecious plants have X–Y sex chromosome systems (WESTERGAARD 1958; AINSWORTH *et al.* 1998; CHARLESWORTH 2002). Early events in X–Y evolution can thus be studied in plants (reviewed in NEGRUTIU *et al.* 2001; VYSKOT and HOBZA 2004). Dioecious species of the genus *Silene* have started to be studied in detail. Their sex chromosomes are estimated to have evolved recently, only ~10–20 MYA (NICOLAS *et al.* 2005; BERGERO *et al.* 2007). The available evidence suggests that these Y chromosomes are not highly degenerated; the DNA is not hypermethylated (SIROKY *et al.* 1998), nor is there histone H4 hypoacetylation compared with the X (VYSKOT *et al.* 1999). The nonrecombining Y region includes most of the length of the Y, which is longer than the X, and a PAR region is present on the *q* arm (LENGEROVA *et al.* 2003). Partial deletions of the *S. latifolia* Y chromosome have revealed three regions controlling different sex functions. One region suppresses female sex organ development (deletion strains have hermaphrodite flowers; WESTERGAARD 1946; LARDON *et al.* 1999), while deletions of two other regions lead to anther arrest (deletion strains bear asexual flowers in which male structures closely resemble those in females; DONNISON *et al.* 1996; FARBOS *et al.* 1999) or late male sterility (WESTERGAARD 1946), showing that these regions carry genes involved in male sex organ development and male fertility, respectively. The generally accepted cytogenetic model (Figure 1) is that the regions corresponding to hermaphroditic and asexual phenotypes contain the two sex determination functions that are responsible for the sexual dimorphism in this species.

More detailed dissection and localization of different functional regions of the Y chromosome is now possible with the help of molecular markers. Sex-linked markers in *S. latifolia* (supplemental Table S1 at <http://www.genetics.org/supplemental/>; Figure 1) have been used to estimate the recombination map of the X and relate this to estimated X–Y sequence divergence (FILATOV 2005a; NICOLAS *et al.* 2005; BERGERO *et al.* 2007) and to compare the X and Y gene orders using deletion maps

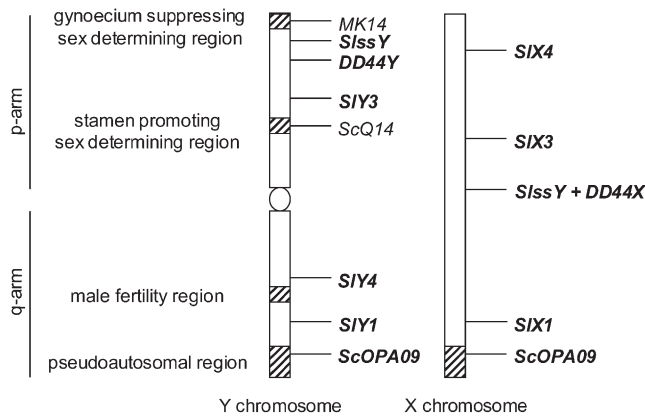


FIGURE 1.—The *S. latifolia* sex chromosomes and the current sex determination model according to NEGRUTIU *et al.* (2001) and CHARLESWORTH (2002). Y-chromosome deletion mutants (WESTERGAARD 1958; DONNISON *et al.* 1996; FARBOS *et al.* 1999; LARDON *et al.* 1999; LEBEL-HARDENACK *et al.* 2002; ZLUVOVA *et al.* 2005a) define at least two critical sex-determining regions on the differential arm of the Y-chromosome: the regions responsible for suppressing female organ development and for early stamen development (see text), while the arm homologous with the X (*i.e.*, containing the PAR) carries a region responsible for late stamen development (plants lacking this region are male sterile). When compared to the known gene order on the X chromosome, the presence of an inversion can be deduced. Molecular markers are listed on the right, those present both on the X and Y chromosomes are in bold. The *p* and *q* arms are indicated.

of the Y (LEBEL-HARDENACK *et al.* 2002; ZLUVOVA *et al.* 2005a). They have also been used to understand the origins of the sex chromosome pair and whether translocations from other chromosomes have been involved (FILATOV 2005a; MARKOVA *et al.* 2006). These results suggest that, similarly to the mammalian sex chromosomes, restriction of recombination between the *S. latifolia* X and Y evolved gradually (FILATOV 2005a; NICOLAS *et al.* 2005; BERGERO *et al.* 2007). They further suggested that a large inversion inferred on the Y chromosome may have caused the observed rearranged order of genes but probably did not cause X–Y recombination arrest (ZLUVOVA *et al.* 2005a), since the genes within the inverted region include pairs with a wide range of X–Y silent site divergence values.

Here, we study a set of 16 *S. latifolia* Y chromosome deletions described previously (FARBOS *et al.* 1999; LARDON *et al.* 1999) plus 12 new deletions. For each deletion that affects male functions, we describe the anther and pollen phenotypic defects in detail to estimate the number of genes carried on the Y chromosome whose loss abolishes male functions. These could be genes in two categories: (i) genes with copies on the X whose X alleles cannot replace the Y gene functions (suggesting that the Y allele has become more male-promoting, or that the X carries an allele that has weakened in male function; among the latter will be the gene in which a mutation caused the appearance of females in the

species); and (ii) genes with no X copies, such as the Y-linked genes in *Drosophila* (CARVALHO 2002).

We also tested each mutant for the presence or absence of molecular markers to find out which of them are due to deletion of Y chromosomal regions. The mapping work revealed no inconsistencies with previous maps of the Y and largely confirmed previous conclusions. This allowed us to base our conclusions on the best ordering of the deletions so far possible, as well as to demonstrate Y linkage of the new mutants studied. Finally, we examined X–Y chromosome meiotic pairing in all the mutants and identified three Y deletion mutants exhibiting altered X–Y pairing during meiosis.

MATERIALS AND METHODS

Rationale and methodology of the screen: The results in this article are based on screening deletion mutants obtained by irradiating pollen grains, pollinating wild-type females with irradiated pollen, and screening for abnormalities in the M1 generation. Recessive mutations on the autosomes or the X chromosome should be masked by their wild-type alleles in the maternal genome, whereas dominant or dosage-sensitive mutations in autosomal and X-linked genes will be detected in the screen. Plants with deletions of Y-linked genes that do not have functional homologs within the genome (type i in the list above) will also show phenotypic defects, as will plants with mutations in genes of type ii listed above, but recessive mutations in Y-linked genes with functional homologs elsewhere in the genome will show no phenotypic defects. Y chromosome deletions can be distinguished from autosomal and X-linked mutations by using Y-linked markers. Thus, although a large number of genes may exist on the *S. latifolia* Y chromosome, our screen should detect a specific category of interest: those with no functional homologs within the genome.

One mutant (*pf5*, see below) arose spontaneously in a family grown from a cross between plants from two laboratory strains provided by M. Ruddat (University of Chicago). All of the other mutants were generated by gamma-irradiation of pollen from a single male plant, which was used to pollinate a single female parent (LARDON *et al.* 1999). The mutant plants were vegetatively propagated, and flower buds of several replicate individuals were analyzed for each deletion.

Y-specific markers were used to confirm the presence of Y deletions and to localize them on the previously published *S. latifolia* Y deletion map (ZLUVOVA *et al.* 2005a; HOBZA *et al.* 2006). Details of the markers are shown in supplemental Table S1 (at <http://www.genetics.org/supplemental/>), which includes references to the publications in which the conditions for the PCR amplifications are described. All the markers that we genotyped in the mutants were completely Y-linked.

Cytological and histological analysis: Mitotic and meiotic preparations were performed as described in LARDON *et al.* (1999). Flower bud fixation, paraplast embedding, histological preparations, and staining were performed as described previously (ZLUVOVA *et al.* 2005b). Preparations were observed using an Olympus Provis AX70 microscope, and pictures were captured using ISIS (Metasystems, Sandhausen, Germany).

RESULTS

Generation of Y deletion mutants with sex phenotypes: We expected our screen to recover mainly dominant or

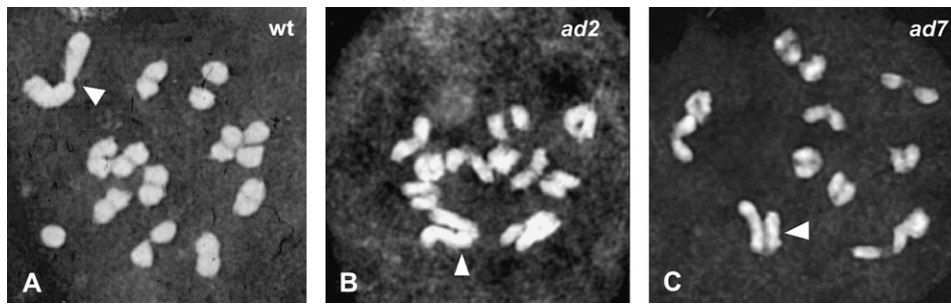


FIGURE 2.—Meiotic preparations of wild type and two Y-deletion mutants showing altered X–Y behavior in meiosis. Wild-type diakinesis. Note the terminal pairing of X and Y (A). Diakinesis in *ad2* mutant in which X and Y do not pair. Note that the distal regions of the X are not aligned with any of the Y distal regions (B). Diakinesis in *ad7* showing distal pairing of X and Y. One distal region of each chromosome is aligned (C). White arrowheads indicate X–Y chromosome pairs.

partially dominant mutations due to deletions or mutations of Y-linked genes that have no functional homologs elsewhere in the genome (see MATERIALS AND METHODS), *i.e.*, either null mutations or deletions (which are expected to behave as nulls). The screen of 2262 M1 plants identified a total of 36 plants with sex function mutations. Sixteen were described previously (14 hermaphroditic *bsx* mutants, 1 of them autosomal, and 2 asexual *asx* mutants; FARBOS *et al.* 1999; LARDON *et al.* 1999). Here, we describe for the first time 12 stamen mutants (out of the remaining 20 mutants with stamen defects), for which we also demonstrate Y linkage below. No other morphological defects in floral traits, other than one floral homeotic and meristic mutation (SCUTT *et al.* 1999) or the occasional albino, dwarf, or narrow-leaved plantlets, were observed (LARDON *et al.* 1999). Thus, male defects were found disproportionately compared with other phenotypes. This is consistent with these mutations being due to Y chromosome partial deletions.

Here, we characterize the male defects of the 12 new deletions and add more details to the descriptions of those previously studied. Our new histological analyses disclosed defects in a broad range of anther developmental processes. They include mutants with alterations in the early formation of the anthers, referred to as *anther development* mutations (*ad1–7*), two of which—denoted here by *ad6* and *ad7*—were preliminarily described as “*misparieta*” mutants (*msp1* and 2 in HINNISDAELS *et al.* 1997), and in microspore maturation (*pollen fertility* mutations, denoted by *pf1–5*). The *bsx* mutants are almost all male and female fertile (LARDON *et al.* 1999), but all mutants in the other categories above (*asx*, *ad*, and *pf*) are male sterile. The mutants have maintained their distinctive phenotypes when repeatedly propagated through cuttings (at least 10 vegetative clones per mutant in the last 10 years for all mutant subgroups in the *asx*, *ad*, and *pf* classes).

Meiotic behavior of mutants with stamen and pollen defects: In most mutants, meiotic X–Y pairing was unchanged (Figure 2A), but abnormalities were seen in three independent lines. Two mutants, *ad2* and *ad3*, showed variable rates of interstitial X–Y pairing, suggesting an inversion including the PAR region (Figure 2B),

while *ad7* exhibited a high frequency of X–Y pairing (84%), including extensive pairing in X–Y regions that never normally pair (Figure 2C).

Deletions of Y chromosome markers: The mutations are inferred to be due to partial deletions of the Y chromosome (see below), and, in some mutants, deletions are cytologically visible (Table 1). The largest deletion causing loss of male functions, *asx1*, lacks ~40% of the *p* arm of the Y (Table 1) (FARBOS *et al.* 1999). Most of the *p* arm (the arm that does not pair with the X) is covered by deletions detected in our screen. Deletions leading to hermaphrodite mutants have previously been estimated to extend to as much as 50% of the wild-type Y chromosome *p* arm (the largest is the *bsx1* mutant reported by LARDON *et al.* 1999). Since these plants are largely male fertile, genes controlling stamen development and pollen fertility functions cannot be located in the part of the Y covered by these deletions.

We tested each mutant strain for the presence of 13 previously described Y-linked markers, 6 genic and 7 anonymous (supplemental Table S1 at <http://www.genetics.org/supplemental/>). The 2 *asx* mutants, and the 12 *pf* and *ad* mutants (6 of which have no cytologically detectable deletions), were found to lack at least 1 of these markers, confirming that these involve deletions of parts of the Y chromosome. Figure 3 outlines the deletions found in the mutants.

Histological characterization of the mutants: The histological results for the *asx*, *ad*, and *pf* mutants are summarized in Table 2, and a more detailed description of mutants lacking one or more markers is given below and in supplemental Figures S1 and S2 (at <http://www.genetics.org/supplemental/>). The histological analyses of anthers define a variety of clear-cut male sex defects. The first two major categories, asexuals (*asx*) and anther developmental mutants (*ad*), have early anther developmental defects, while the third category includes pollen fertility mutants (*pf*). With detailed phenotypic data, these categories subdivide into a total of nine groups.

The asexual class: Anthers of both *asx1* and *asx2* mutants are similarly arrested before differentiation of the parietal layers and the mutants are thus completely

TABLE 1
Cytological characterization of male-defective mutants of *S. latifolia*

Mutant names	Size of deletion (%)	Meiosis ^a			Other changes
		Diakinesis	Metaphase I	X–Y pairing	
<i>ad1</i>	9	12	12	Wild type	
<i>ad2</i>	12	12	12	No or interstitial (36%)	
<i>ad3</i>	Not measurable	12	12	Interstitial (11%)	10% abnormal segregation in meiosis II
<i>ad4</i>	13	12	12	Wild type	
<i>ad5</i>	Not measurable	12	12	Wild type	
<i>pf1</i>	4	12	12	Wild type	
<i>pf2</i>	Not measurable	Not studied	Not studied	Wild type	
<i>pf3</i>	Not measurable	12	12	Wild type	
<i>pf4</i>	Not measurable	12 and 13 (10%)	Mitotic divisions of one bivalent	Wild type	
<i>pf5</i>	Not measurable	14		Wild type	
<i>ad6 (msp1)</i>	8	12	12	Wild type	
<i>ad7 (msp2)</i>	7	12	12	Extensive X–Y pairing (84%)	
<i>asx1</i>	20	No meiosis		Not testable	
<i>asx2</i>	12	No meiosis		Not testable	

The second column of the table shows the sizes of the Y deletions measured in mitotic cell preparations, as a percentage of the entire Y chromosome. An extensive meiotic analysis was performed to estimate bivalent formation during diakinesis and metaphase I (columns 3 and 4), other meiotic aberrations (column 5), and X–Y pairing behavior (column 6).

^aSee LARDON *et al.* (1999): number of bivalents are known to vary from $2n = 12$ to $2n = 14$ in *S. latifolia* genotypes, with no consequences for the sex phenotype.

sterile. These flowers have suppressed gynoecia, as in wild-type male flowers, and rudimentary anthers similar to those in developing flowers of wild-type female (see FARBOS *et al.* 1999) (Figure 4A shows the wild-type female anther rudiments, and Figure 4B shows *asx1*).

The anther developmental class: The seven mutants in this class (*ad1–ad7*) have anthers arrested at early stages during the formation of parietal and/or sporogenous layers and are therefore categorized as anther developmental mutants. The *ad* class includes deletions affecting different stamen whorls and distinct stamen parts, and different anther tissues are affected, and the timing of the defects also differs (Table 2). These mutants are again completely sterile either because they do not form pollen grains (*ad2, ad4, ad6, and ad7*) or because the few pollen grains produced are sterile (*ad1, ad3, and ad5*). These mutants can be further subcategorized into four distinct and stable phenotypic groups with specific defects, as follows:

- i. The *ad1* mutant has two types of defects. The inner, antipetalous subwhorl of anthers reaches only the four-lobed stage (*i.e.*, the parietal layers are not yet formed, similarly to the *asx* mutants), such anthers accumulating deposits around the connective region (Figure 4C and supplemental Figure S2L at <http://www.genetics.org/supplemental/>). The outer, antisepalous anther subwhorl forms all anther layers found in wild-type plants (*i.e.*, epidermis, endothecium, middle layers, and tape-

tum), but the pollen grains are abnormally small and nonviable (Figure 4Q and supplemental Figure S2K; for the wild type, see Figure 4P).

- ii. In the *ad6* mutant, developmental arrest occurs at the stage corresponding to the formation of parietal layers in the wild type (for the wild type, see the Figure 4D). Although only the primary parietal layer is formed, the microspore mother cells can undergo meiosis (Figure 4E and supplemental Figure S1E). The *ad2* phenotype is very similar (supplemental Figure S1D).
- iii. The *ad3* mutant forms asymmetrically developed anthers; the adaxial locules are well developed (*i.e.*, all anther layers, and microspores, form correctly), but the formation of abaxial locules is highly reduced (Figure 4F and supplemental Figure S1G). The *ad5* mutant displays a similar asymmetry and phenotype (supplemental Figure S1H).
- iv. In the *ad7* mutant, the secondary parietal layer often appears to differentiate directly, without formation of all anther layers (Figure 4H and supplemental Figure S1K). The stage of developmental arrest corresponds to the formation of the secondary parietal layer in the wild type (Figure 4G). Mutant *ad4* has a similar phenotype, except that anthers are highly variable while degenerating (supplemental Figure S1J).

The pollen fertility class: In the “late” mutants with impaired pollen fertility (*pf1–pf5*), all anther parts are

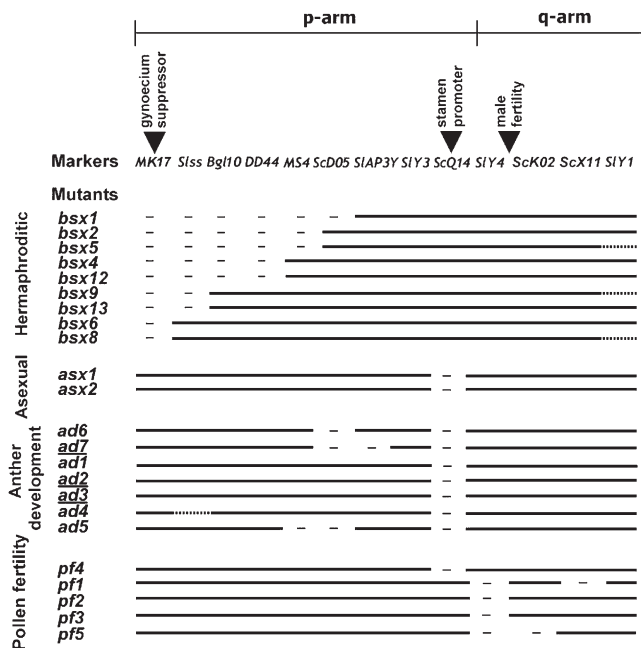


FIGURE 3.—Outline of the deletions observed in the mutants analyzed. The markers are shown at the top, ordered according to the Y map (HOBZA *et al.* 2006), with inverted triangles indicating the sex determination functions located on the *p* arm and a pollen fertility region on the *q* arm. The pseudo-autosomal region is on the right. The mutant names are given on the left. The underlined mutant names state for mutants with pairing phenotypes. Full lines indicate markers present in each deletion mutant, dashes indicate markers that were determined to be missing, and dotted lines indicate that the presence of the marker was not tested in the strain. Deletions are not to scale. Note that in several mutants more than one marker is deleted.

formed, but the microspores either degenerate after meiosis or else the pollen grains are sterile. These five independent pollen fertility mutants belong to four distinct phenotypic groups, each mutant having a defined tapetal defect, as follows (see also Table 2):

- i. Mutant *pf1* forms all anther layers (similarly to the wild type; Figure 4I), but the tapetum does not differentiate (Figure 4J and supplemental Figure S2B).
- ii. Mutant *pf2* forms all layers properly, like the wild type (for the wild type, see Figure 4K), but the tapetum is released from the degenerating middle layer (Figure 4L and supplemental Figure S2D).
- iii. Unlike the wild type (Figure 4M), mutant *pf3* microspores are irregular in shape (Figure 4N and supplemental Figure S2F). Mutant *pf5* has similar defects (supplemental Figure S2G).
- iv. In mutant *pf4*, all parts of the anther are properly formed, but later on, the middle layer and tapetum do not degenerate (Figure 4O and supplemental Figure S2I).

Location of Y regions with stamen indispensable functions: To determine the number of male indispensable genes responsible for the mutant phenotypes, we

ascertained their locations by arranging the Y chromosome deletions of each phenotypic group according to the presence or absence of molecular markers. The deletion results showed that male indispensable genes were located in only two or three Y regions. On the *p* arm, there were one or two regions containing the loci causing the *asx* and *ad* mutations, *i.e.*, genes with mainly early stamen functions. All the *pf* loci (affecting late male functions) located to one region on the *q* arm.

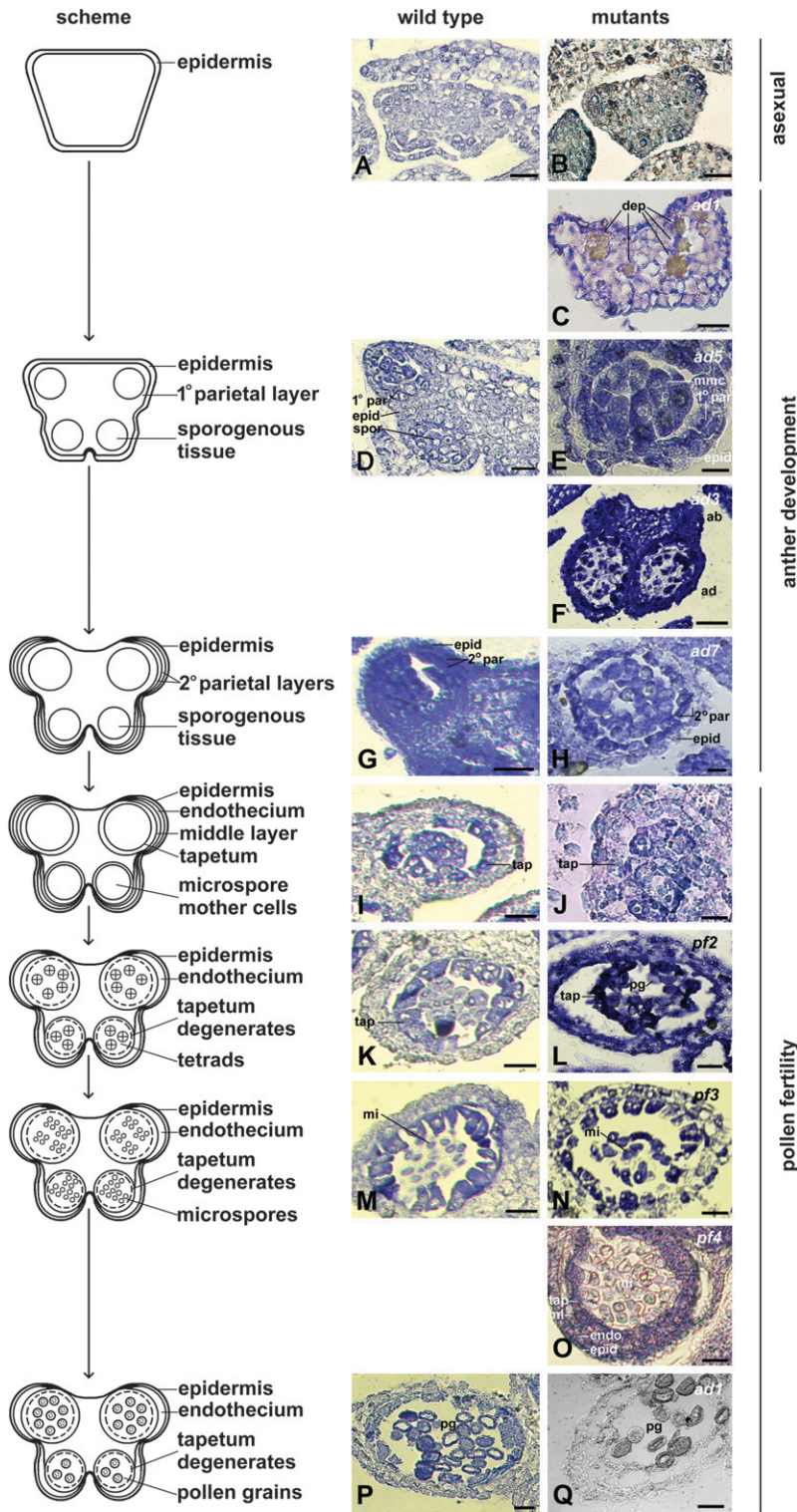
The overlapping series of deletions at the distal end of the Y chromosome *p* arm (Figure 3) produced hermaphroditic (*bsx*) mutants. These deletions (up to 50% of the chromosome arm) indicate that the gynoceium suppressing factor, or female suppression sex determination gene of males (often denoted by *GSF* or by *Su^f*), is located at this end of the chromosome (LARDON *et al.* 1999; also see FARBOS *et al.* 1999), consistent with previous results (WESTERGAARD 1958). The loci that, according to the current deletion map (Figure 3), are closest to the *GSF* region, correspond to mutants *ad5–7* (which are codeleted with the *ScD05* marker), but the physical distance between the two classes of genes is unknown. *ad*-type defects are never seen in *bsx* mutants. The size range of the *bsx* deletions suggests that the distance between *GSF* and the closest *ad* loci must be >50% of the *p* arm. As explained above, the cytogenetic results indicate that the *GSF*-containing region of the Y does not contain genes controlling stamen development or pollen fertility functions. Consistent with the cytology, several markers that are never deleted in hermaphrodites have been shown to be codeleted in stamen function mutants (Figure 3). *ScQ14* (ZHANG *et al.* 1998) has thus been inferred to be close to a stamen-promoting factor or factors (ZLUVOVA *et al.* 2005a) and *SIY4* (ATANASSOV *et al.* 2001) to a male fertility factor or factors (MOORE *et al.* 2003). Interestingly, plants with a range of male defects lack the *ScQ14* marker, including all the asexual and anther-developmental mutants (*asx* and *ad1–ad7*) and also one of the pollen fertility mutants (*pf4*; see Figure 3 and further details below). In contrast, the deletions in the four mutants with only pollen fertility defects (*pf1*, *pf2*, *pf3*, and *pf5*), defining a locus or loci involved in late anther functions, lack the *SIY4* gene, which is currently mapped on the *q*-arm. As all these deletions are cytologically small (Table 1 and Figure 3), these loci must be located close to *SIY4* in a different region of the Y chromosome from the *ScQ14* marker.

The *asx* and *ad* mutants thus probably have overlapping deletions around the *ScQ14* marker, which may extend to different distances on either side (the physical sizes of the regions between *ScQ14* and the adjacent markers on either side are unknown and could be large). The *ad5* and *ad6* mutants apparently also have a second, more distal, deleted region partially shared with the *ad7* mutant (defined by the three consecutive markers *MS4*, *ScD05*, and *SIY3*; see Figure 3). Mutant

TABLE 2
Outline description of the phenotypes of the deletion mutants and suggested function(s) of the genes involved

Mutant class and Y deletion sizes	No. of deletion mutants	Mutant group names and possible gene functions involved in each subgroup (Roman numerals)	Phenotypic defects	Figures
Asexual; interstitial, up to 40% of proximal <i>Yp</i>	2	<i>asx1</i> , <i>asx2</i> specifying primary parietal cell fate	Anther arrest before differentiation of the parietal layers, as in wild-type females. The two mutants have an identical phenotype.	4B, S1B
Anther development; interstitial, up to 26% of proximal <i>Yp</i>	7	<i>ad1-ad7</i>	Anthers arrested early during formation of parietal and sporogenous layers. Completely sterile.	
		i. <i>ad1</i> stamen sub-whorl coordination	Inner, anipetalous, anther subwhorl is arrested at the four-lobed stage, deposits accumulate around the connective region. Outer, antisepalous anthers form abnormally small and unviable pollen grains.	4C, 4Q, S2K, S2L
		ii. <i>ad2</i> , <i>ad6</i> maintaining parietal layer	Anther arrest at the stage of parietal layer formation in the wild type, but microspore mother cells can undergo meiosis. The two mutants have very similar phenotype.	4E, S1E, S1D
		iii. <i>ad3</i> , <i>ad5</i> locule synchronization	Asymmetrically developed anthers (abaxial locules form only primary parietal layers). The two mutants have an identical phenotype.	4F, S1G, S1H
		iv. <i>ad4</i> , <i>ad7</i> specification of parietal layers	Secondary parietal layer often differentiated directly into tapetum-like cells without formation of all anther layers. The two mutants have very similar phenotype. <i>Ad7</i> is altered in X-Y pairing.	4H, S1J, S1K
Pollen fertility; interstitial, up to 8% of proximal or medial <i>Yq</i>	5	<i>pf1-pf5</i>	Microspores either degenerate after meiosis, or the pollen grains are sterile.	
		i. <i>pf1</i> tapetum differentiation	Tapetum does not differentiate.	4J, S2B
		ii. <i>pf2</i> tapetum/middle layer signaling	Tapetum is released from the degenerating middle layer.	4L, S2D
		iii. <i>pf3</i> , <i>pf5</i> microspore shape	Microspores are irregular in shape. The two mutants have very similar phenotype.	4N, S2F, S2G
		iv. <i>pf4</i> middle layer and tapetum degeneration function.	Tapetum and middle layer do not degenerate.	4O, S2I

The left-hand column lists the three major male-defective phenotypic classes and shows the total numbers of deletions found in each of them. The third column describes the general phenotype of each group (Roman numerals indicate subgroups).



asexual

anther development

pollen fertility

FIGURE 4.—Histological analyses of *S. latifolia* wild type and Y chromosome deletion mutants. An outline of anther development is given on the left. The middle column shows sections of wild-type anthers, and the right-hand column displays sections of anther developmental mutants. Detailed descriptions of the wild-type anther development and Y-chromosome deletion mutant histology are given in supplemental Figures S1 and S2 at <http://www.genetics.org/supplemental/>. The anther rudiment in female plants stops developing before the formation of the parietal layers (A). The anthers of the Y chromosome deletion mutant *asexua 1* (*asx1*) strongly resemble female anther rudiments (B). The inner subwhorl anthers of the anther development mutant *ad1* also do not develop beyond this stage, probably because of the accumulation of deposits around the connective (C). Wild-type male anthers develop the primary parietal layer and the sporogenous tissue (D). In the anther development mutant *ad6*, the primary parietal layer is altered, but the sporogenous tissue is able to form microspore mother cells (E). In the *ad3* mutant, abaxial locule development is arrested prematurely (F). Unlike the wild-type anthers, where no difference in the staining ability of the secondary parietal layers is observed (G), the inner secondary parietal layer of the *ad7* mutant differentiates into a densely stained tapetum-like layer (H). In the wild-type anthers, the tapetum becomes densely stained after the formation of all anther layers, and this process is connected with its differentiation (I). However, the tapetum is not stained in the pollen fertility mutant *pf1* (J). During meiosis and tetrad formation, the tapetum starts to degenerate and its cells are released from each other but remain attached to the other anther layers (K). In the *pf2* mutant, the tapetum is released from the anther layers and forms an agglutinate inside the anther (L). The wild-type microspores, when released from the tetrads, are round shaped (M), but in mutant *pf3*, the microspores are irregular in shape (N). Mutant *pf4* forms round microspores, and the endothelial and tapetal cells do not degenerate, as deduced from the fact that the endothecium is present and the tapetal cells remain attached to each other (O). When compared to the wild type (P), both the anther locules and the pollen grains of the outer subwhorl of the mutant *ad1* are abnormally small (Q). dep, Deposits; epid, epidermis; 1° par, primary parietal layer; spor, sporogenous cells; mmc, microspore mother cells; ab, abaxial locule; ad, adaxial locule; 2° par, secondary parietal layer; tap, tapetum; mi, microspores; pg, pollen grains. Bars, 10 μ m (A–C, E, H, J, O), 25 μ m (D, G, I, K–N, P, Q), and 50 μ m (F).

ad5 lacks the first two markers and *ad7* lacks the second two, while *ad6* lacks only the marker believed to be in the middle, *ScD05*. Thus, the abnormal X–Y pairing, which is seen only in *ad7*, could be due to loss of a gene (or sequences) located in this distal region, since the deletion in this mutant appears to be more extensive on the side where *SlY3* was mapped by ZLUVOVA et al.

(2005a). Alternatively, this gene or sequences could be located near the *ScQ14* region, since *ad7* may have a deletion that extends beyond the region deleted in the other *ad* mutants but not as far as *SlY4* (since the *pf* mutants have normal chromosome pairing).

The mutants *asx1* and *asx2*, in which, as just explained, *ScQ14* is absent, have large deletions of the Y

chromosome *p* arm (Table 1). These mutants lack the stamen-promoting, sex-determining function, often denoted *SPF* (FARBOS *et al.* 1999). Since *asx2* has a cytologically smaller deletion than *asx1*, the stamen-promoting, sex-determining gene must lie within the region corresponding to the *asx2* deletion (24% of the *p* arm of the Y), and the distance between *ScQ14* and the *SPF* locus (and a gene or genes essential for early stamen development, defined by the *ad* mutants) must thus be $\leq 24\%$ of the *p* arm.

The probability of all the deletions with the *ad* phenotypes occurring in one region of the Y and all the *pf* deletions in another single region is very low, if each of the eight *ad* and *pf* groups (Table 2) is deleted for a different gene, assuming that the genes are randomly distributed on the Y chromosome. We discuss below possible explanations for the finding that there are apparently two distinct gene regions on the Y chromosome with indispensable roles in anther formation, one containing the *asx* and *ad* loci on the *p* arm (early stamen developmental factors) and one bearing the *pf* loci on the *q* arm (pollen fertility factors).

DISCUSSION

Our screen identified eight different phenotypic groups of Y-linked mutants with a broad range of stamen development and pollen fertility traits (Table 2) in addition to the sex determination functions corresponding to the *asx* and *bsx* mutations previously described. Defects in late stamen functions, notably tapetum malfunction, like those in our *pf* mutants have been reported repeatedly in various plant species (VIZIR *et al.* 1994; GORMAN and McCORMICK 1997; SANDERS *et al.* 1999).

The *asx* and *ad* mutants are, however, unusual. In *Arabidopsis thaliana*, only a few mutants have been identified with defects in the specification, organization, or development of anther cell types, similar to those in our *ad* class (*e.g.*, *ad2* and *ad4*; see VIZIR *et al.* 1994; SANDERS *et al.* 1999). Mutants with such defects were also not found in large-scale screens for fertility defects using EMS or irradiation mutagenesis in other model species such as maize and tomato (GORMAN and McCORMICK 1997; NEUFFER *et al.* 1997). This suggests that the *S. latifolia* Y chromosome carries an unusual kind of locus with early stamen functions (or an unexpectedly large number of such loci) in addition to a locus that generates the more common type of mutations affecting late stamen functions.

Types of male-indispensable genes identified: Since several types of mutations appeared more than once, the Y has probably been saturated with respect to mutations affecting indispensable male functions, *i.e.*, all regions whose deletion can affect such functions have been identified. Our mapping of *asx*, *ad*, and *pf* classes

shows that the corresponding deletions span fairly large Y chromosome regions (Table 2). The observed *asx-ad* phenotypes produced by overlapping deletions can thus be explained in two ways (similar reasoning can be applied to the *pf* deletions). One possibility is that the different phenotypes observed with deletions in a single region resulted from deletion of different loci, indicating that several different genes with male indispensable and closely related functions lie in this Y region. Alternatively, the region contains a major gene involved in early stamen development, plus other (modifier) genes that interact with this gene, so that the phenotype is different in each distinct deletion.

We cannot distinguish between these two possibilities. A variant of the second possibility above is that effects such as position effect variegation (PEV) could influence the expression of nondeleted genes located near the breakpoints. The fact that independent mutants within a group (*e.g.*, the *ad3* and *ad5* mutants) were always phenotypically identical or very similar suggests that PEV is unlikely, since it generally leads to variable phenotypes with the same rearrangement (WEILER and WAKIMOTO 1998 and references therein).

Male specialization of the Y: The screen identified almost exclusively male function genes, and the mutant phenotypes are dominant or at least semidominant. Dominant mutations are generally much less frequent than recessive ones (ORR 1991; for an estimate in *S. latifolia*, see LARDON *et al.* 1999), so that it is not expected that one will find many such mutations in a screen. For male reproductive functions, however, the theory outlined in the Introduction predicts that Y chromosomes will be enriched for such genes, due either to (i) evolution of Y-linked alleles that actively increase male functions relative to their X-linked homologs (so that loss of the Y copy impairs male function), or (ii) transposition of genes onto the Y but not to the X; in addition, loss of the SPF should lead to complete sterility, since the X chromosome has a nonfunctional allele at this locus (defined as the locus that is mutated in females and causes their male sterility). Y chromosome genes of either type i or ii might have deleterious effects in females and fit into the category of sexually antagonistic genes proposed by RICE (1987) that are expected to accumulate on the Y once dioecy evolves.

In *S. latifolia*, the *SLAP3Y* gene is already known to be an example of recruitment to the Y of an autosomal gene, whose function has subsequently become more male specialized (MATSUNAGA *et al.* 2003). This gene appears to map in the *asx-ad* region (Figure 3). Although it is not possible to tell which of the two categories of changes mentioned above has been involved in cases other than this, the results described above suggest that the *S. latifolia* Y chromosome has started to undergo the predicted changes and carries several genes promoting male reproductive functions that cannot be replaced by X-linked alleles or autosomal genes, *i.e.*, has evolved

some male specialization. The process may be accentuated by degeneration and subsequent deletion of Y-linked genes not involved in male function (such as the *MROS3* gene; reviewed in CHARLESWORTH and CHARLESWORTH 2000), but there is so far little evidence for this in *S. latifolia*.

Early and late stamen functions define distinct Y regions: According to the hypothesis proposed by CHARLESWORTH and CHARLESWORTH (1978), male-benefit genes should start to accumulate on the proto-Y chromosome while it still recombines with the proto-X, forming clusters around the initial sex-determining genes. Once a nonrecombining region has evolved, male-benefit genes will continue to evolve throughout the Y-specific region (RICE 1987).

Each of the phenotypic categories of Y-linked mutants described above (*bsx*, *asx-ad*, and *pf*) is associated with deletions of specific parts of the Y chromosome, and the three major categories of mutants are each found only in deletions of a single Y region. These results thus suggest only two distinct regions with Y-specific genes having roles in anther formation. One region, located on the *Yp* arm, contains the gene(s) responsible for all nine early stamen developmental mutants, including the *asx* mutants and the four *ad* groups defined in Table 2 (all nine of these mutants are deleted for the *ScQ14* marker; see Figure 3). In contrast, four of the five late stamen mutants (*pf*) have deletions in a different region, on the *q* arm, defined by loss of the *SIY4* marker. This distinct pattern would be highly unlikely if these mutants represent 14 genes scattered throughout the Y chromosome.

The female suppression region (containing the *GSF* gene defined by the *bsx* mutants) is large (about one-half of the distal Y chromosome *p* arm) but contains no genes indispensable for stamen functions. A stamen-promoting region is defined by the *asx* mutants. This region, including the genes indispensable for early stamen functions that are deleted in all 7 *ad* mutants and 1 of the 5 *pf* mutants, defines the proximal ~20% of the *p* arm. It may include the gene that is mutated or absent in females that causes their male deficiency, which may correspond to the locus defined by our *asx* mutations and thus represents the Y chromosomal *SPF* gene. If the *asx-ad* region contains several different genes with male-indispensable functions (see above), any of them could play the role of a male sex determination function and could thus be the *SPF* gene.

Finally, very late functions involved in pollen fertility (defined by the *pf* mutants) are located distant from the sex determination regions in the current *S. latifolia* Y, close to the breakpoint of the large Y inversion inferred to lie between *SIY1* and *SIY4* (Figure 1; see also below). The inversion must have occurred recently, as genes with high and low X–Y sequence divergence (FILATOV 2005b; NICOLAS *et al.* 2005) are found in adjacent locations in the Y map (ZLUVOVA *et al.* 2005a). If the

recent ancestral state was the same gene order as the current X chromosome, the *pf* gene(s) must thus have been located either near the *SIY1* gene, *i.e.*, close to the PAR on the *q* arm or else at the end of the arm distant from the PAR (*i.e.*, near the *GSF* locus on the *p* arm). On the second hypothesis, the inversion (Figures 1 and 3) may be reinforcing reduced X–Y recombination either directly through the well-known effect of inversions in causing lower survival of recombinant gametes or zygotes due to chromosome breaks, or as a secondary consequence of allowing sequence divergence of this region of the Y.

Meiotic X–Y pairing: Three of our mutants have altered X–Y pairing in meiosis. Among them, the *ad7* deletion clearly indicates that the Y chromosome carries elements or functions essential for X–Y pairing arrest. *S. latifolia* mutants with pairing along the sex chromosomes may have been observed by WESTERGAARD (1946; type Y², with the differential or *p* arm only about one-fourth as long as the pairing arm), but he interpreted his cytological observations in terms of a translocation of a part of the X chromosome to the Y, with loss of almost all of the *Yp* arm. This interpretation cannot explain the *ad7* deletion, because this mutant's Y chromosome is only slightly smaller than the wild-type Y (Table 1) and still possesses most of the Y-specific markers we tested, including markers from most Y regions (Figure 3 and supplemental Table S2 at <http://www.genetics.org/supplemental/>). The cause of the lack of pairing throughout most of the X and Y chromosomes is unknown. A reinversion in the mutant of the putative inverted region of the Y chromosome (Figure 1) (ZLUVOVA *et al.* 2005a) seems implausible. If this is correct, the *ad7* mutation indicates that the Y carries gene(s) or sequences restricting X–Y pairing. It is not possible to test whether pairing in the *ad7* deletion affects X–Y recombination, because the mutant is male sterile (but restoration of recombination seems unlikely given the large sequence divergence that is likely in this region of the Y).

The region deleted when the pairing function is mutated is not close to the pseudo-autosomal region (which might suggest that it was involved in loss of recombination in the most recently evolved stratum of the sex chromosomes). Interestingly, however, it is close to the region carrying the *SIY3* gene (Figure 1), a gene pair with the highest level of X–Y sequence divergence yet observed (NICOLAS *et al.* 2005; BERGERO *et al.* 2007). As explained above, this region also contains a male-promoting gene, which could be one of the primary sex determination loci. In this region, the *S. latifolia* X and Y chromosomes are as highly diverged as homeologous chromosome pairs in many allopolyploid plant species. This lack of close homology may not necessarily prevent chromosome pairing. A classical example of homeologous chromosome pairing occurs in wheat, and it is suppressed by an allelic variant of the *Ph1* locus (RILEY

and CHAPMAN 1958; MARTINEZ-PEREZ *et al.* 2001; GRIFFITHS *et al.* 2006). It is possible that the *S. latifolia* genome has evolved a similar mechanism to restrict sex chromosome pairing.

Whatever the mechanism, our results are consistent with restricted X–Y recombination in *S. latifolia* having evolved in steps, as suggested by previous results on differences in sequence divergence of X–Y gene pairs spanning most X–Y regions (FILATOV 2005a; NICOLAS *et al.* 2005; BERGERO *et al.* 2007). The meiotic behavior of the *ad7* mutant supports this by indicating that pairing can be stopped through sex chromosome pair-specific processes. In *S. latifolia*, more genes (for estimating X–Y divergence, which can be used to infer when recombination ceased) and FISH experiments with BAC clones will in the future allow better inferences of the extent of X–Y rearrangements, including detecting other, smaller inversions that may have occurred at different times in the evolution of the Y chromosome (some of which might have been involved in suppressing its recombination with the X).

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Note added in proof: The rearrangements of the genomic material on the Y chromosome are not confined to a single pericentric inversion, as shown by HOBZA *et al.* (R. HOBZA, E. KEJNOVSKY, B. VYSKOT and A. WIDMER, 2007, The role of chromosomal rearrangements in the evolution of *Silene latifolia* sex chromosomes. *Mol. Genet. Genomics*, in press). This does not affect any of our conclusions, except that it makes the hypothesis of a reinversion, which we reject in the DISCUSSION, even less plausible.

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