Mapping of Sex Determination Loci on the White Campion (*Silene latifolia*) Y Chromosome Using Amplified Fragment Length Polymorphism

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ABSTRACT

S. latifolia is a dioecious plant with morphologically distinct sex chromosomes. To genetically map the sex determination loci on the male-specific Y chromosome, we identified X-ray-induced sex determination mutants that had lost male traits. We used male-specific AFLP markers to characterize the extent of deletions in the Y chromosomes of the mutants. We then compared overlapping deletions to predict the order of the AFLP markers and to locate the mutated sex-determining genes. We found three regions on the Y chromosome where frequent deletions were significantly associated with loss of male traits. One was associated with hermaphroditic mutants. A second was associated with asexual mutants that lack genes needed for early stamen development and a third was associated with asexual mutants that lack genes for late stages of stamen development. Our observations confirmed a classical genetic prediction that *S. latifolia* has three dispersed male-determining loci on the Y chromosome, one for carpel suppression, one for early stamen development, and another for late stamen development. This AFLP map provides a framework for locating genes on the Y chromosome and for characterizing deletions on the Y chromosomes of potentially interesting mutants.

ALTHOUGH the majority of flowering plants are hermaphroditic, ~5% of flowering plants are dioecious (reviewed in Lebel-Hardenack and Grant 1997); *i.e.*, they are either male or female. Dioecy has evolved recently in independent lineages of flowering plants and they are often closely related to hermaphroditic species. By comparing the developmental programs of related hermaphroditic and dioecious species, we can follow the evolutionary steps to dioecy. In some dioecious species, sex determination is controlled by genes on sex chromosomes (Parker 1990). Silene species of the subgenus Elisanthe (Fenzl) have either two identical X chromosomes if they are female or one X and a larger Y chromosome if they are male (reviewed in Scutt *et al.* 1999). During meiosis, the X and Y chromosomes only pair over the pseudoautosomal region on their q arms (Figure 1; Westergaard 1958; Buzek *et al.* 1997). This sex determination mechanism must have evolved relatively recently in the Silene genus since the majority of species have hermaphroditic flowers. In fact, most species are gynodioecious (*i.e.*, populations consist of hermaphroditic and female plants; Desfeux *et al.* 1996), which is considered to be a common first step toward the evolution of dioecy (Charlesworth and Charlesworth 1978). Because the dioecious Silene species have the same number of chromosomes as hermaphroditic Silene ($2N = 24$), the sex chromosomes must have evolved from an autosome pair. The availability of close relatives in which the ancestral autosome pair has either remained autosomal or differentiated into sex chromosomes makes the Silene genus an attractive model to study the molecular changes that led to sex chromosome evolution (reviewed in Moneger *et al.* 2000).

The dioecious *Silene latifolia* is the most extensively characterized species of the Elisanthe. In females, stamen development is arrested before microspore mother cells differentiate. Carpel development is arrested at a slightly earlier stage in male flowers, before vascular cells differentiate in the rudimentary gynoecium (Grant *et al.* 1994). Femaleness is recessive to maleness and could be caused by either loss of nuclear-encoded gene functions or cytoplasmically inherited male sterility (Desfeux *et al.* 1996). Regardless of the cause of repressed stamen development, the *S. latifolia* Y chromosome carries stamen-promoting genes that overcome the female deficiency. In addition, genes on the Y chromosome cause arrest of carpel development. Autosomal mutants that allow limited carpel development in XY individuals have been described (Lardon *et al.* 1999) but the strongest carpel-suppressing locus is located on the Y chromosome. Deletion of the Y-linked locus leads to full restoration of carpel development (Westergaard 1946).

Classical cytogenetic studies by Westergaard identified three regions on the *S. latifolia* Y chromosome that carried sex-determining genes (Westergaard 1946).

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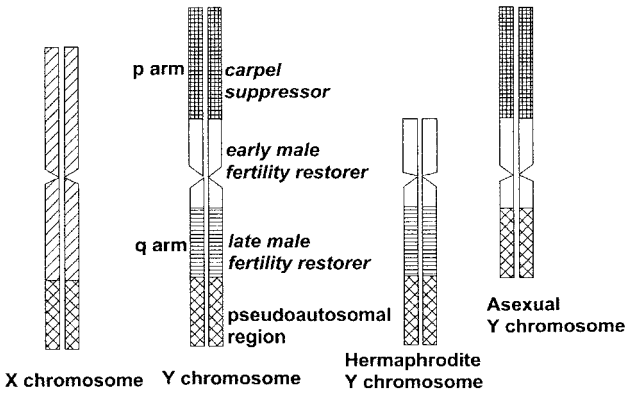

FIGURE 1.—*S. latifolia* sex chromosomes as described by WESTERGAARD (1946, 1958). Regions deleted in the hermaphroditic and asexual mutant classes studied by Westergaard are indicated with the type of genes predicted to be located in each region.

He examined three sex determination mutants. Two were hermaphroditic with full carpel development and stamen maturation. Both mutations were linked to large overlapping deletions of the nonrecombining p arm of the Y chromosome (Figure 1). Westergaard proposed that at least one carpel-suppressing locus was located on this arm. The third mutant was asexual. It had repressed carpel development as in normal males but, although stamens formed, they were arrested in growth before the pollen matured. It was partially female fertile, allowing Westergaard to follow the genetic transmission of the asexual mutation and prove that it was linked to a deletion on the q arm. Since the asexual mutants supported stamen development beyond the stage of arrest in normal females, Westergaard concluded that other stamen-promoting genes affecting early stages of stamen development must exist in the center of the chromosome between the regions deleted in the other two mutant classes (WESTERGAARD 1958). Westergaard's predictions have been supported by subsequent mutant studies. Hermaphroditic mutants generated by X- or γ-irradiation of pollen were shown to have deletions in the distal p arm as in Westergaard's mutants (LARDON *et al.* 1999). In addition, FARBOS *et al.* (1999) have identified asexual mutants that arrest stamen development at earlier stages than the mutants of Westergaard. Importantly, these also have deletions in the p arm of the Y chromosome, validating Westergaard's predictions of an early stamen development locus between the carpel suppressor and the late stamen development locus.

To locate sex-determining genes, we have characterized X-ray-induced Y chromosome deletions in a collection of sex determination mutants. We used amplified fragment length polymorphism (AFLP) markers that cosegregated with wild-type Y chromosomes to determine the extent of deletions in the Y chromosome of each mutant. Overlapping deletions in the Y chromosomes of mutants with common phenotypes revealed three dispersed regions where sex determination genes with distinguishable functions are predicted to be located. The number of sex determination loci and their predicted location resemble the chromosome organization suggested by Westergaard on the basis of his cytogenetic analysis (WESTERGAARD 1958).

MATERIALS AND METHODS

Plant material for mutagenesis: U9 plants used in this study are from an inbred line derived from eight generations of brother × sister matings (a gift of J. van Brederode, University of Utrecht, The Netherlands). Plants for mutagenesis were the progeny of one pair of plants from this inbred population. Pollen from several sibling male plants was irradiated and used to fertilize several sibling female plants. MR4X64 plants were generated through mating two clonally propagated plants. MR4 was a female plant and MR64 was a male derived from an earlier mating of a wild-type male with MR4 (gift of D. Ye, Max-Planck-Institut fuer Zuechtungsforschung, Cologne, Germany). MR4 and MR64 were propagated vegetatively. Pollen of MR64 plants was irradiated and used to fertilize MR4 females to generate plants of the MR4X64 genetic background. In addition, untreated MR64 pollen was used to pollinate MR4 plants to make a wild-type population.

X-irradiation: Pollen was collected and X-irradiated in a Siefert (Ahrensburg, Germany) X-ray machine to dosages of 4500, 5000, 6000, or 7600 R. Immediately after X-irradiation, the pollen was dusted on the styles of female plants.

Selection of mutants: Young plants were grown in an unheated greenhouse with natural light over the winter for vernalization, and the flower phenotype of each plant was visually examined in the spring and summer. Two flowers per plant were checked for deviation from strict male or female development. Hermaphroditic and asexual mutants were selected and grown with 15 hr per day of light to maintain flowering. As much as possible, mutants were vegetatively propagated to prevent loss of each genotype.

Heritability tests: All hermaphroditic mutants were crossed as male parents to normal females from the same original line. Flowers from at least 30 progeny from each mutant were examined for sex.

DNA isolation: DNA was isolated from young leaves as described in DONNISON *et al.* (1996).

AFLP: AFLP was performed by the standard method (VOS *et al.* 1995). DNAs were cut with *Eco*RI and *Mse*I restriction enzymes and appropriate adaptor sequences were ligated to the ends. Two selective primers, one for the *Eco*RI cut end and one for the *Mse*I cut end, were used in combination to amplify DNA from each genotype. Each primer had three randomly chosen bases at the 3′ end to ensure specific amplification of sequences with three complementary bases adjacent to the restriction site. Primer combinations that led to amplification of male-specific polymorphic bands in DNA from both U9 and MR4X64 plants are listed in Table 1. Primers that amplified male-specific genotypes in only one genetic background can be seen in Table 1 on our web page (http://www.bio.unc.edu/research/silene). Selective amplification of DNA was carried out with fluorescent dye-labeled primers from Perkin-Elmer (Boston, MA). The DNA fragments were electrophoretically separated and visualized on an ABI 377 machine. We used GeneScan Analysis software from Perkin-Elmer to process the data. AFLP reactions from eight males, eight females and 44 sex determination mutants were scored on the same gels. Bands were scored as present or missing only if the observation was repeated at least twice.

TABLE 1

Primer combinations that generate male-specific AFLP markers

Name of polymorphic bands found in both U9 and MR4X64	Primer combination to identify male-specific PCR product[a] *Eco* + 3/*Mse* + 3
L1	AAG/CCT3
L2	ACA/CCT2
L3	ACT/CTA2
L4	ACT/CAG3
L5	ACA/CCT7
L6	AAG/CGA2
L7	ACT/CCA4
L8	AAG/CGA3
L9	AAG/CGA6
L10	ACA/CCT1
L11	AAC/CAT1
L12	AGC/CAG2
L13	ACA/CGT4
L14	AAG/CCT2
L15	ACT/CAT2
L16	ACT/CAG1
L17	ACA/CTC1
L18	AGC/CAG1
L19	ACA/CCG1
L20	ACT/CTA1
L21	ACT/CCA1
L22	AAG/CGC1
L23	ACA/CCG3
L24	ACA/CCT5
L25	AAG/CGC3
L26	ACG/CCA1
L27	ACT/CAG2
L28	ACA/CGA1
L29	ACA/CGC2

[a] Specific bases (replacing NNN) added to 3′ ends of the AFLP primers designed to amplify *Eco*RI/*Mse*I restriction fragments. (*Eco*RI primer: 5′ GACTGCGTACCAATTCA + NNN; *Mse*I primer: 5′ GATGAGTCCTGAGTAAC + NNN). Since several male-specific bands were often amplified using a single primer pair, the number following the specific bases distinguishes individual male-specific bands.

Statistical analysis: Chi-square and Fisher's exact test analyses were performed using the SAS statistics package. The AFLP marker order was predicted using the RHMINBRK version 3.0 minimal breaks program and the RH2PT version 3.0 mapping program of the RHMAP Radiation Hybrid analysis package (Boehnke *et al.* 1991). Radiation hybrid mapping orders markers under the assumption that the probability of radiation-induced breakage between two markers is proportional to the distance between the markers. When two markers are deleted together more often than expected by chance, we say that the markers are linked; *i.e.*, the breakage probability is <1. The conditional probabilities of breakage and the logarithm of the odds of linkage (LOD) for every possible marker pair were calculated using the RH2PT program.

The markers were put in order using a minimum breaks criterion and implementing a simulated annealing algorithm to examine the possible orders consistent with the data (Boehnke *et al.* 1991). This approach predicts marker orders that lead to the minimal number of chromosome breaks needed to account for all deletions in the data set. More than one deletion per chromosome (which is often true for radiation-induced breakage) is allowed in the analysis.

RESULTS

Collection of mutants with loss of male traits: In each inbred population tested, all males shared a common Y chromosome from the original male parent. Mutants from the U9 population were given a name starting with the letter U and mutants from the MR4X64 population were given a name starting with M. The second letter in the mutant name refers to the mutant phenotype, H for hermaphroditic and S for asexual (sterile) mutants. Irradiated pollen was used to fertilize females of the same population. Flowers of the next generation were screened for deviations from male or female flower development. This strategy favored identification of Y-linked genes because the female parents were not expected to have a homologous allele to complement loss-of-function mutations. Hermaphroditic and asexual mutants were selected for further study. It was not possible to identify mutants that reversed sex because they would be indistinguishable from wild-type males or females. In a screen of 10,000 MR4X64 plants, we selected >80 mutants. An additional 38 mutants were selected from 5000 mutagenized plants of the U9 population as described earlier (Donnison *et al.* 1996). Most hermaphroditic mutants were phenotypically similar with a mature gynoecium composed of five carpels and 10 stamens (Figure 2, B and C). Asexual mutants all had a rudimentary gynoecium as in normal males but stamen development was prematurely arrested. Stamen primordia formed in all cases but the timing of developmental arrest was variable. In the class of mutants we called early stamen development mutants (Figure 2, D and E), locules stopped growing when they were <1 mm long and filaments did not elongate. In the intermediate stamen development class (Figure 2F), stamen development progressed slightly further (filaments elongated to up to 1 mm). These intermediate class mutants were morphologically similar to asexual mutants with deletions in the p arm described by Farbos *et al.* (1999). The asexual mutants US11 and US9 (Figure 2, G and H) are representative examples of the late stamen development mutant class. Their stamens are arrested after morphologically distinct locules develop and the filaments elongate to a few millimeters in length. The asexual mutant described by Westergaard was morphologically similar to this class (Westergaard 1946). Mature pollen was not produced from any of the asexual mutants we identified.

The heritability and sex linkage of hermaphroditic mutations were tested by backcrossing to females of the same genetic background (the heritability of U9 hermaphroditic mutations was described previously in Donnison *et al.* 1996). In most cases, all the progeny were female, indicating that Y chromosomes were too damaged to

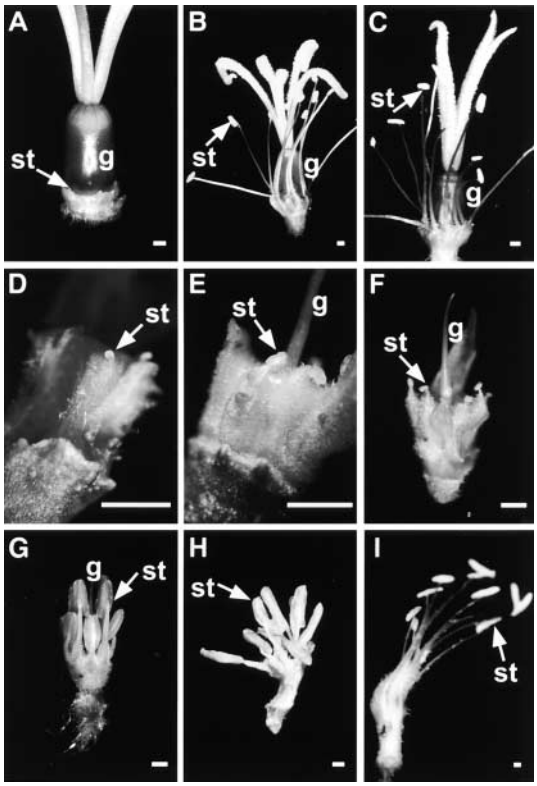

FIGURE 2.—Representative mutant flowers. Nonreproductive organs have been removed from mature flowers to reveal stamens and gynoecium of each mutant type. (A) Normal female. (B) Hermaphroditic mutant MH12. (C) Hermaphroditic mutant MH115. In B and C, both stamens and carpels are mature and as numerous as if male and female were added together. (D–H) Asexual mutants US15 (D), which has the earliest stamen arrest seen, US16 (E) and US10 (F) with intermediate stages of stamen arrest, and US11 (G) and US9 (H) with late stamen arrest. (I) Normal male. Arrows with "st" indicate stamen primordium. The gynoecium or gynoecium primordium is marked by "g" where visible. Bars, 1 mm (photographs not taken at same magnification).

support viable pollen. Only two hermaphroditic mutants, UH1 and MH78, passed the trait on to the next generation. In both cases, all progeny were hermaphroditic or female, indicating that the mutations were linked to the Y chromosome. It was not possible to test the heritability of the asexual mutations as they caused complete sterility. The lack of heritability of male traits limited the methods we could use to map the mutations. Segregation mapping was impossible because we were characterizing the nonrecombining portion of the Y chromosome. We therefore analyzed X-ray-induced deletions to determine if the presence of deletions on the Y chromosome was correlated with mutant phenotypes and to make a genetic map of the Y chromosome on the basis of the extent of overlapping deletions in the mutants.

Generation of male-specific AFLP markers: Our mutants could have DNA lesions that are not on the Y chromosome as well as Y-linked mutations. To focus on the Y chromosome of the mutants, we initially selected AFLP markers that were male specific (*i.e.*, that mapped genetically to the nonrecombining region of the Y chromosome). AFLP (Vos *et al.* 1995) allowed us to identify male-specific DNA markers easily. A total of 59 primer combinations were used to amplify genomic DNA from eight males and eight females from each of our two independent populations. Bands that were found only in males were scored as Y linked. The probability that these bands are not Y linked is represented by the equation $P = 2(1 - (1/4)^n)(1/4)^n$, where $n = 8$ (Michelmore *et al.* 1991) or $P = 3.1 \times 10^{-5}$. Twenty-one primer combinations revealed a total of 48 male-specific AFLP bands in the U9 population. The MR4X64 population had 56 male-specific bands from 22 primer combinations. All primer combinations that amplified male-specific bands are listed at our website (http://www.bio.unc.edu/research/silene). However, the two populations did not share all the same male-specific bands. Only 29 male-specific bands (from 19 different primer combinations) were shared by both populations (Table 1). These were used for mapping.

Mapping deletions in sex determination mutants establishes a marker order and locates sex determination loci: We chose 44 representative mutants, some from each population, to test for the loss of male-specific markers that were common to both populations. The mutants included 18 hermaphroditic mutants and 26 asexual mutants chosen to represent the three stages of stamen development arrest that we observed in the mutant collection (Figure 2). (Photographs of the mutants used can be seen at our website: http://www.bio.unc.edu/research/silene.) We analyzed each marker individually to determine if marker loss could be associated with loss of any sex determination loci. (An Excel file with presence or absence of each marker in each mutant can be found at our website. The data are illustrated in Figure 3.)

Predictions of the order of the AFLP markers on the Y chromosome: We used the presence or absence of markers to determine the order of the AFLP markers using methods developed for radiation hybrid mapping (Boehnke *et al.* 1991). In radiation hybrid mapping, irradiated cells are fused to healthy cells from a different species and chromosomal fragments from the irradiated cells become introgressed into the chromosomes of the healthy cell. Markers specific to the irradiated genome are tested for presence or absence in each cell line derived from the fusions. The closer two markers are on the chromosome, the more likely it is that they will be on the same chromosomal fragment. Conversely, the farther apart two markers are on the chromosome the more likely it is that they will be on different fragments. The frequency at which markers are coretained determines the marker order and relative distance between markers (Cox *et al.* 1990; Boehnke *et al.* 1991; Falk 1991). We applied the converse of this model to our data by analyzing the frequency of codeletions to establish gene order. The parameters for the probability of breakage and the probability of retention are depen-

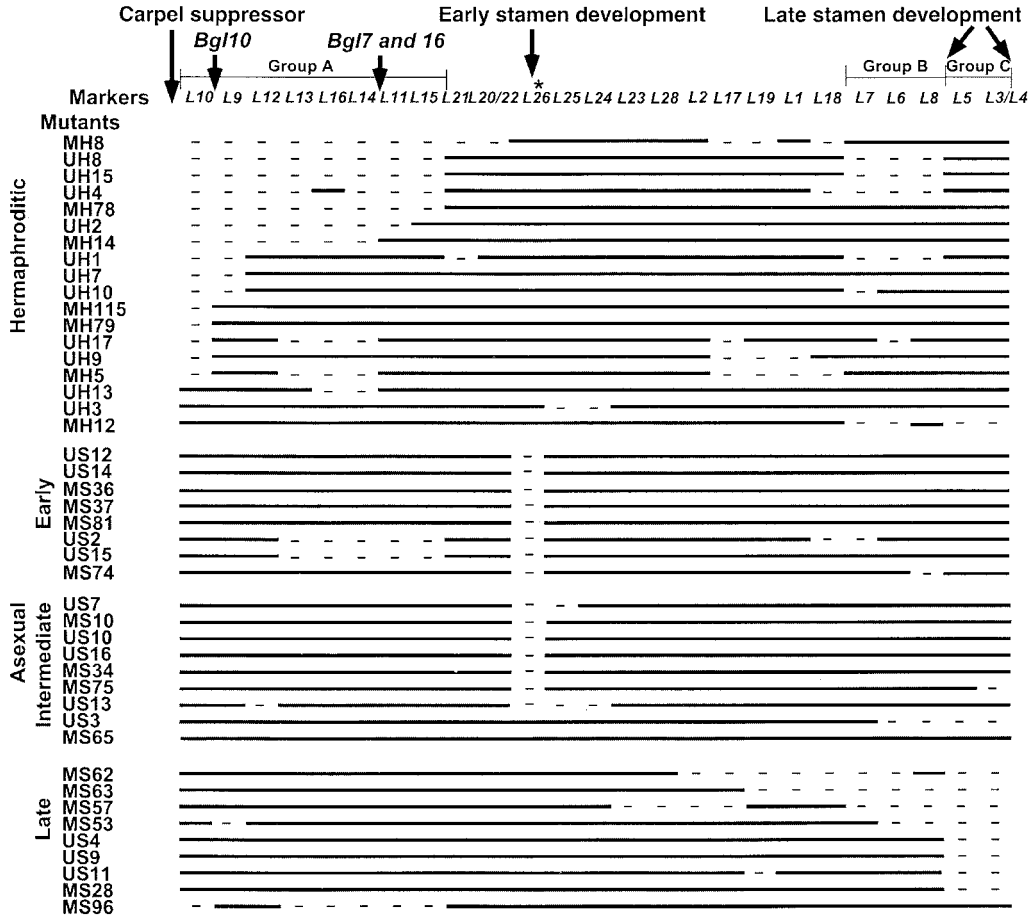

FIGURE 3.—AFLP marker order based on minimal breaks analysis. The markers are represented in order along the top. Each mutant is named on the left. The extent of the bar beside each mutant name represents the markers present in each mutant. Dashes indicate the marker was missing. Presence or absence of markers was confirmed by repeating the AFLP analysis at least twice. Lines over the marker names represent linkage groups with >1000:1 odds of linkage. Male-specific RDA markers *Bgl7*, *Bgl10*, and *Bgl16* were positioned on the map using data from Table 3.

dent on a given radiation dose. Since multiple radiation levels were used to generate mutations in this experiment, we did not estimate the genetic distances between the markers.

We first predicted the LOD for each marker pair. This information allowed us to establish a preliminary marker order and identify groups of markers with a high probability of linkage. Three groups had a LOD of >3, *i.e.*, a chance of linkage better than 1000:1. A LOD score of at least 3 is generally accepted as an indicator that two sequences are linked (HARTWELL *et al.* 2000). The largest group is illustrated in Figure 3 as Group A. The markers were associated with a LOD score >3 but <4. Markers *L6*, *L7*, and *L8* formed a second group (Group B; LOD > 4) and markers *L3*, *L4*, and *L5* formed a third group (Group C; LOD > 4). Since all the markers were initially mapped to the nonrecombining part of the Y chromosome, it should be possible to link all of the markers into one group. However, since the Y chromosome of *S. latifolia* is extremely large, with an estimated 860 Mbp of DNA (MATSUGANA *et al.* 1994), we expect that analysis of significantly more deletion mutants will be required to generate a single high probability linkage group defining the Y chromosome.

Assuming that the markers were all linked to the Y chromosome, we predicted the order of the markers using the minimum breaks analysis program RHMINBRK version 3.00 (BOEHNKE *et al.* 1991). This program calculates the minimal number of chromosome breaks required to account for the observed deletions in the mutant chromosomes. Not all 29 markers were used. Markers *L20* and *L22* were present or absent in the same set of mutants. Since it would be impossible to distinguish the marker order between them, only L22 was included in the analysis. *L4* and *L3* were also similar with the exception that two L4 data points were ambiguously scored (from mutants UH2 and US15). Since ambiguous data are ignored by the program, we also removed *L4*. Three other markers, *L26*, *L27*, and *L29*, were also left out of the analysis because their nearest neighbors could not be predicted using the two-point analysis program RH2PT (BOEHNKE *et al.* 1991). RH2PT calculates a predicted distance between each marker pair, which is used to establish an initial marker order on the basis of closest neighbors. The minimal breaks program refines this marker order to get the best map. *L26* was predicted to be an infinite distance from all markers except *L25*, but the predicted distance from *L25* was too great to support a predicted linkage. Because *L27* was deleted only in one mutant and *L29* was not deleted in any mutants, they could fit equally well into several different positions in predicted maps. Consequently, all three markers were disruptive to predicting a reliable marker order. If they

were included in the analysis, several quite different marker orders were predicted, using either the simulated annealing or the maximum-likelihood algorithm. However, each predicted map was composed of three similar blocks of markers: Group A, the markers numbered *L21–L29* in Figure 3, and a block including both Groups B and C. Although markers within each block were assigned consistent orders, the blocks could be arranged in all possible combinations. Therefore, we removed the disruptive markers.

Consistent marker orders were predicted after removing *L26*, *L27*, and *L29* from the analysis. Ten closely related marker orders were predicted, each of which could account for the data with the lowest minimal number of 89 chromosome breaks. The only inconsistencies between the 10 predicted orders affected the relative order of markers within three small groups. The order of *L16*, *L14*, and *L13* could be rearranged in several different combinations. In addition, *L21* and *L22* could be reversed, as could *L19* and *L1*. The first of the 10 predicted orders listed in the output from the computer analysis is shown along the top of Figure 3.

Identification of sex determination loci: X-irradiated mutants can have more than one deletion or other types of rearrangements that would lead to loss of AFLP markers and not all rearrangements are necessarily responsible for the mutant phenotypes we selected. However, we assumed that it would be possible to identify trends in which a significant proportion of mutants with a common phenotype would have lost markers close to the affected gene. Therefore, we looked for strong correlation between loss of markers and mutant phenotypes to indicate the position of the sex-determining genes. The pattern of marker distribution among the mutant classes allowed us to predict the location of three sex determination loci as described below. In all cases, we do not know if only one or several linked genes that affect a common phenotype are lost in each mutant. We refer to a single locus when discussing mutations associated with a common sex determination phenotype even though we do not know how many genes are involved.

A locus for carpel development is located at the left end of the map: L10 showed the strongest evidence for an association with hermaphroditic mutations, suggesting that the genes for suppressing carpel development were located to the left of *L10* or between *L10* and *L9* (Figure 3). We performed a chi-square analysis and Fisher's exact test for each marker to determine the probability that it had a significant association with any sex determination phenotype (Table 2). The Fisher's exact test indicated that the markers at the left end of the map—*L9*, *L10*, and *L14*—were highly unlikely to be associated with hermaphroditic mutations by chance alone (Table 2). Three hermaphroditic mutants (UH13, UH3, and MH12) retained *L9* and *L10*. Therefore, they could be useful for finer mapping of the carpel-suppressing locus.

A locus for late stamen development is located at the right end of the map: Markers on the right end of the map (Figure 3) were frequently deleted in mutants with late stage arrest of stamen development. The pattern of deleted markers is consistent with the prediction that a locus needed for late stages of stamen development is located within Group C. However, we cannot determine the position of the locus between *L3*, *L4*, and *L5*. The Fisher's exact tests also indicated that late arrest of stamen development was associated with the Group C markers with low Pr values (Table 2). One late stamen development mutant, MS96, retained the markers in linkage group C and could be useful for closer identification of the late-acting male fertility locus.

A locus for early stamen development is linked to L26: L26 was the only marker associated with asexual mutants with early or intermediate stamen arrest. The hypothesis that these markers are unrelated to early stamen devel-

TABLE 2

Probability of association of each marker with a sex determination phenotype based on chi square and Fisher's exact test

Locus[a]	Pr value[b]	Significant association with locus[c]
L10	Pr = 2.235×10^{-7}	Carpel suppressor
L9	Pr = 0.0014	Carpel suppressor
L12	Pr = 0.595	
L13	Pr = 0.0796	
L16	Pr = 0.0910	
L14	Pr = 0.0124	Carpel suppressor
L11	Pr = 0.1890	
L15	Pr = 0.4333	
L20	Pr = 1.000	
L21	Pr = 0.6575	
L22	Pr = 1.000	
L26	Pr = 1.901×10^{-9}	Early and intermediate stamen development
L25	Pr = 0.1854	
L24	Pr = 0.4669	
L23	Pr = 0.6410	
L28	Pr = 0.5952	
L2	Pr = 0.1057	
L17	Pr = 0.3098	
L19	Pr = 0.1089	
L1	Pr = 0.4183	
L18	Pr = 0.7615	
L7	Pr = 0.2188	
L6	Pr = 0.0947	
L8	Pr = 0.6894	
L5	Pr = 2.855×10^{-6}	Late stamen development
L4	Pr = 1.277×10^{-5}	Late stamen development
L3	Pr = 7.404×10^{-6}	Late stamen development

Null hypothesis: Deletion of markers is random over all phenotypic classes.

[a] Loci are in predicted order shown in Figure 3.

[b] Pr = value for Fisher's exact test.

[c] Significant association = Pr value < 0.05. Class of mutants is determined from the chi-square table (data not shown).

opment was rejected ($Pr < 1.9 \times 10^{-9}$) according to the Fisher's exact test. Mutants US3 and MS65 also had intermediate arrest of stamen development but retained marker *L26*. Therefore, they may be useful for further mapping studies. We could not predict the position of *L26* in the AFLP map but our data strongly suggest that it is in a different section of the Y chromosome from either of the other two sex determination loci positioned at the ends of the map. Because *L26* is statistically associated with a sex determination locus, we attempted to find its position on the predicted AFLP map as illustrated in Figure 3. We chose this position because the two-point analysis indicated that *L26* had a weak association with *L25* and to minimize the number of chromosome breaks predicted in mutants UH3, US7, and US13.

The AFLP map can be used to locate additional sequences on the Y chromosome: Once we had a predicted marker order, we were able to locate other Y-linked sequences by analysis of their presence or absence in the collection of mutants used to make the map. Even a subset of mutants could be used. Previously (Donnison *et al.* 1996; Donnison and Grant 1999), we identified male-specific DNA sequences using representational difference analysis (RDA; Lisitsyn *et al.* 1993) and without the help of statistical analysis, we predicted map positions for three of them (*Bgl7*, *Bgl10*, and *Bgl16*). We have now designed PCR primers that would amplify a male-specific band from the *Bgl10* sequence. This allowed us to score the mutants used for AFLP mapping for the presence or absence of *Bgl10*. The data presented in Table 3 allowed us to locate *Bgl10* between *L10* and *L9*, which confirmed our earlier prediction that it was the closest of the three RDA markers to the carpel suppression locus. This method of gene location requires a small amount of mutant DNA for the PCR analysis and can be applied to locate any gene for which a male-specific PCR polymorphism can be identified.

We used restriction fragment length polymorphism data collected earlier on some of the mutants used in this study to add *Bgl7* and *Bgl16* to the AFLP map. Both mapped between *L14* and *L11* (Figure 3). In Donnison *et al.* (1996), we predicted that *Bgl7* and *Bgl16* were close to a locus for early stamen development because they were deleted in two asexual mutants with early stamen abortion, US2 and US15 (Figure 2C). The AFLP analysis does not support this conclusion. Instead, our data reveal two deletions in these mutants: one includes the location of *Bgl7* and *Bgl16* and the other includes *L26* (Figure 3). Since other mutants with early stamen arrest share the loss of *L26*, we conclude that the affected sex determination locus is in the region of *L26* and the second deletion has no effect on stamen development.

TABLE 3

Locating polymorphic Y-linked sequences using the mutant collection and the AFLP map

Plants	*Bgl10*[a]	*Bgl7*	*Bgl16*
Female MR4X64 1-4	—[b]	—	—
Male MR4X64 1-4	X[c]	X	X
Female U9 1 and 2	—	—	—
Male U9 1 and 2	X	X	X
MH8	—	ND[d]	ND
UH8	—	—	—
UH15	—	—	—
UH4	—	—	—
MH78	—	ND	ND
UH2	—	—	—
MH14	—	ND	ND
UH1	—	X	X
UH7	—	X	X
UH10	—	X	X
MH115	—	ND	ND
MH79	—	ND	ND
UH17	X	X	X
UH9	X	X	X
MH5	X	ND	ND
UH13	X	—	—
UH3	X	X	X
MH12	X	ND	ND
US14	X	X	X
US13	X	X	X
MS10	X	X	X
US16	X	X	X
US15	—	—	—
MS34	X	ND	ND
MS36	X	ND	ND
MS75	X	ND	ND
MS74	X	ND	ND
MS81	X	ND	ND

Mutants were scored at least three times for loss of male-specific PCR fragments.
[a] RDA marker name × band present.
[b] AFLP band missing.
[c] AFLP band present.
[d] Mutant not tested.

DISCUSSION

Three sex determination loci have been identified on the Y chromosome: We have identified a set of AFLP markers that are genetically linked to maleness and therefore are expected to be located on the nonrecombining region of the Y chromosome. By the association of loss of male-specific AFLP markers with loss of male-specific traits, we located sex determination genes on three dispersed regions on the Y chromosome. Although we could not generate a map that clearly identified the order of all the AFLP markers along the nonrecombining region of the chromosome, the predicted order with the most statistical support indicates that a locus for carpel development is at one end of the map and a locus for late stages of stamen development is at the opposite end. A third set of mutants was strongly associated with deletion of a third part of the chromosome, which contains the marker *L26*. This marker is clearly on a distant segment of the nonrecombining region of the Y chromosome from the other two sex determination loci.

We used weak association of loss of markers in a small number of mutants to assign *L26* to a position between the carpel-suppressing locus and the late stamen development locus. Several of the mutants with intermediate stamen development that lost *L26* resemble mutants described by Farbos *et al.* (1999). The Farbos asexual mutants have a deletion in the same arm of the Y chromosome as hermaphroditic mutations characterized by Lardon *et al.* (1999), which supports our assigned location. This is also the approximate location for early stamen development genes predicted by Westergaard (1958).

Mutations leading to early or intermediate arrest of stamen development shared a strong association with *L26*, as expected if loss of a single locus controls early and intermediate stages of stamen development. However, it is hard to explain the differences we see between these mutants by environmental variation alone because we have observed these plants for several flowering seasons and we find that the stages of stamen arrest are consistent for each mutant. Since we cannot predict the distance between *L26* and its nearest markers, a very large segment of the Y chromosome may be represented by this one marker on our predicted map. Several genes that affect different early or intermediate stages of stamen development could be located in this area. In this case, mutants that have lost only *L26* may have very different overlapping deletions that result in loss of different sets of stamen development genes. Much finer genetic analysis is needed to explore this possibility.

The *S. latifolia* Y chromosome requires recombination suppression to maintain dioecy: Our AFLP map predicts that the locus needed to suppress carpel development is at one end of the nonrecombining region of the Y chromosome and the locus for late stamen development is at the opposite end, supporting the predictions of Westergaard (Figure 1). Several models have been put forward to suggest that sex chromosomes have evolved to maintain the linkage of male fertility factors in species as diverse as mammals and angiosperms (Lahn and Page 1997; Charlesworth and Guttman 1999; Delbridge and Marshall Graves 1999; Charlesworth 2001). Westergaard himself proposed that recombination suppression was essential to maintain the dioecious breeding system in Silene (Westergaard 1958). In *S. latifolia,* maleness depends on simultaneously inheriting both the Y-linked carpel-suppressing locus and the stamen development loci. Since these genes are on distant segments of the Y chromosome, they could segregate randomly unless recombination between them was suppressed. Random segregation would result in some of the offspring of every male-by-female mating being sterile. As a result, new mutations that disrupt the carpel-suppressing locus of males or restore male fertility to females would be more fertile than the dioecious parents. Selection would favor such mutations. Therefore, recombination suppression is essential to maintain the dioecious species (Charlesworth 1991; Charlesworth and Guttman 1999). The location of the carpel-suppressing locus and the late stamen development locus is sufficient to account for the extent of the nonrecombining region. At present, we do not know what physical features of the X and Y chromosomes suppress recombination in this region. However, the AFLP map we generated in this study will allow us to roughly identify the physical location of genes that are on the Y chromosome. This is a first step toward identifying gross deviations in gene order between the Y chromosome and its former partner, the X chromosome, that could account for loss of recombination and morphological differentiation of the sex chromosomes in a dioecious plant.

Practical uses of the map: The AFLP map we generated identified the mutants that were most likely to have small deletions close to sex determination loci that are useful for more fine-scale genetic and physical mapping. For example, hermaphrodites UH3, MH12, and UH13 all retain marker *L10*. As a next step in identifying sex determination genes, it will be possible to estimate the size of deletions in these mutants by physical mapping using the most closely linked AFLP sequences as probes. The AFLP map can also be used to locate the relative positions of Y-linked sequences such as cDNA clones. For example, sequences that are specifically found in males could be mapped using the same method we used in Table 3 to determine if they are close enough to the sex determination loci to merit further study. The male-specific AFLP sequences we have characterized will be useful for physical mapping of the Y chromosome using either pulsed-field gel electrophoresis or *in situ* hybridization (Buzek *et al.* 1997; Ekong and Wolfe 1998). This will facilitate comparison of the overall organization of the *S. latifolia* Y chromosomes with the structure of chromosomes in hermaphroditic Silene species to elucidate the evolutionary steps that led to a sex-chromosome-based sex determination system.

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