Isolation of *Y* Chromosome-Specific Sequences From *Silene latifolia* and Mapping of Male Sex-Determining Genes Using Representational Difference Analysis

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Manuscript received May 16, 1996
Accepted for publication September 3, 1996

ABSTRACT

The genomic subtraction method representational difference analysis (RDA) was used to identify male-specific restriction fragments in the dioecious plant *Silene latifolia*. Male-specific restriction fragments are linked to the male sex chromosome (the *Y* chromosome). Four RDA-derived male-specific restriction fragments were used to identify polymorphisms in a collection of X-ray-generated mutant plants with either hermaphroditic or asexual flowers. Some of the mutants have cytologically detectable deletions in the *Y* chromosome that were correlated with loss of male-specific restriction fragments. One RDA-derived probe detected a restriction fragment present in all mutants, indicating that each has retained *Y* chromosomal DNA. The other three probes detected genomic fragments that were linked in a region deleted in some hermaphroditic and some asexual mutants. Based on the mutant phenotypes and the correlation of cytologically visible deletions with loss of male-specific restriction fragments, these markers were assigned to positions on the *Y* chromosome close to the carpel suppression locus. This RDA mapping also revealed a *Y*-linked locus, not previously described, which is responsible for early stamen development.

THE majority of flowering plants are hermaphroditic and capable of self-fertilization. There has, however, been a tendency in plants for evolution of species that are sexually dimorphic. The dioecious plants bear flowers of only one sex (either male or female) on each plant (reviewed in DELLAPORTA and CALDERON-URREA 1993; GRANT *et al.* 1994a). Dioecious species are scattered among many genera and families such that most of their closest relatives are hermaphroditic (YAMPOLSKY and YAMPOLSKY 1922). For example, the genus *Silene* includes ~30 species indigenous to central Europe. Four species are dioecious, and the others are either true hermaphrodites or gynodioecious (plants produce either female or hermaphroditic flowers) (CHATER and WALTERS 1964; HEGI 1979). This genus is therefore an excellent model for studying the genetic changes leading to evolution of a dioecious species from a hermaphroditic progenitor. Comparison of the function of genes that regulate the decision to become male or female (sex determining genes) from dioecious plants to homologous genes in related hermaphroditic species would reveal how the change from hermaphrodite to sexually dimorphic has occurred. We are presently concerned with the first step toward this comparison, identification of the genes of sex determination in a sexually dimorphic species and investigation of their mode of action.

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The sex of the dioecious species *Silene latifolia* (also known as *S. alba* or *Melandrium album*) is determined by loci located on morphologically distinct chromosomes. Male plants have 22 autosomes and a pair of dimorphic sex chromosomes, *X* and *Y*. Females have 22 autosomes and two *X* chromosomes (WARMKE and BLAKESLEE 1939; WARMKE 1946; WESTERGAARD 1940, 1948). The genetics of sex determination in *S. latifolia* has been well characterized by classical cytogeneticists (WARMKE and BLAKESLEE 1939; WARMKE 1946; WESTERGAARD 1946, 1958). Studies based on a collection of sterile and hermaphroditic mutant plants led WESTERGAARD to propose a model in which the *Y* chromosome comprises three distinct regions and two sex determining functions: a region responsible for anther maturation (WESTERGAARD 1958), a region which acts as a gynoecium suppressor, and a region with homology to the *X* chromosome where pairing occurs between *X* and *Y* chromosomes in meiosis. Similar mutant analysis has more recently confirmed WESTERGAARD's identification of male determining genes on the *Y* chromosome (VAN NIGTEVECHT 1966).

The mutants on which WESTERGAARD and VAN NIGTEVECHT based their conclusions are no longer available. We therefore generated a new collection of phenotypically similar mutants by X-irradiation of pollen. The selection scheme used favored the isolation of mutations in male-determining genes on the *Y* chromosome. In order to physically locate the mutations, we identified male-specific DNA sequences to use as markers for physical mapping. Male-specific markers for mapping have been obtained previously by screening of male and fe-

male DNA for the ability to produce specific DNA fragments on PCR amplification with short oligonucleotides using a technique called random amplified polymorphic DNA or RAPD (MULCAHY *et al.* 1992; VEUSKENS 1993). Although they were male-specific, we found that RAPD polymorphisms were often difficult to reproduce due to the large genome of *S. latifolia.* In addition, they tended to amplify members of highly repetitive multigene families that were not useful for isolation of flanking male-specific DNA sequences (T. HULLAR and S. R. GRANT, unpublished data). Here, we have applied a recently described genomic subtraction protocol, representational difference analysis (RDA) to identify male-specific markers. These reveal restriction enzyme polymorphisms between male and female genomes. We found them to be highly reproducible. We used the identified markers to analyze the DNA of plants with *Y*-linked mutations affecting sex determination in order to establish the relative positions of the markers and genes for male sex determination.

MATERIALS AND METHODS

Plant materials: Wild-type *S. latifolia* plants were derived from an inbred line propagated by seven generations of brother-sister mating (from a gift of J. VAN BREDERODE, State University, Utrecht, The Netherlands). Hermaphroditic and sterile mutant plants were obtained by pollinating wild-type female plants with X-ray mutagenized pollen from the same genotype (GRANT *et al.* 1994a). Mutants were propagated vegetatively by cuttings.

Isolation of genomic DNA for subtraction: Genomic DNA was isolated from leaf material collected from male and female plants. Leaves from ≥10 plants were pooled from each sex. DNA was extracted in a solution containing 1% 2-mercapthoethanol, 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl at 50° according to the protocol of (SHURE *et al.* 1983).

Subtraction: The procedure was a modification of RDA (LISITSYN *et al.* 1993). Four subtraction experiments were made, each with a different restriction endonuclease (either *Bam*HI, *Bgl*II, *Eco*RI, or *Hin*dIII). Male and female DNAs were cut with the chosen restriction enzyme and adapters composed of unphosphorylated complementary oligonucleotides were ligated to the fragments. Oligonucleotide sequences were based on those described in LISITSYN *et al.* (1993), designed so that they would recreate the original restriction site at the border of the genomic DNA. After amplification of the restriction-digested male and female DNAs, adapters were removed by restriction enzyme digestion and new unphosphorylated oligonucleotide adapters were ligated to the tester (male) DNA only. Because the adapter oligonucleotides did not have 5′ phosphates, the oligonucleotide was only able to be ligated to the 5′ end of the genomic DNA fragment. Thus, each male sequence had one oligonucleotide attached to the 5′ end as illustrated in LISITSYN *et al.* (1993). The male fragments were denatured and annealed to an excess of denatured amplified female DNA. After hybridization, male-male hybrids had single stranded identical oligonucleotide sequences extending from the 5′ ends of both strands. Male-female hybrids had an extension on only one end and female-female hybrids had none. The 5′ extensions were duplicated using TAQ polymerase. This generated a sequence complementary to the original oligonucleotide adapter on the 3′ end of each male-male hybrid. The resulting fragments were PCR amplified using a primer identical in sequence to the original oligonucleotide. PCR amplification was carried out using a buffer of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 5% DMSO. Cycling conditions were as follows: 1 min at 95°, 1 min at 60°, and 2 min at 72° in all experiments. Only male-male hybrids could be exponentially amplified leading to a significant enrichment of male-specific sequences. In each experiment, three rounds of hybridization of tester to an increasing excess of driver were made: round 1, 20–100×; round 2, 800×; round 3, 400,000× (or 100,000× for *Hin*dIII).

Cloning of RDA products: The final subtraction product was digested with the appropriate restriction enzyme to remove the adaptor sequences. The products were ligated to pBluescriptKS+ (Stratagene) linearized at the multiple cloning site by digestion with *Bam*HI (for *Bam*HI or *Bgl*II digested genomic DNAs), *Eco*RI or *Hin*dIII as appropriate. *Escherichia coli* strain XL1Blue (Stratagene) was transformed with the ligations. DNA was prepared from 40 randomly picked isolated colonies by alkaline lysis mini-prep (SAMBROOK *et al.* 1989) and digested to release the cloned insert from the Bluescript vector. DNA fragments were separated by gel electrophoresis and capillary blotted to nylon membranes. The blots were hybridized to a probe derived from the control subtraction labeled by replication with klenow polymerase in the presence of nucleotides conjugated to digoxygen (Boehringer Mannheim). Colonies to which the inserts hybridized were discarded and the remaining colonies were characterized for size of cloned inserts and cross-hybridization.

Preparation of Southern blots: Genomic DNA was isolated from leaf material collected from individual male, female and mutant plants in addition to pooled male and female samples (over 10 plants for each sex). DNA was extracted using a modification of the protocol of with an extraction buffer containing 42% urea, 313 mM NaCl, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 1% N laryl sarcosine. Genomic DNA was digested for 4 hr at 37° with 2–3 units of enzyme per microgram of DNA. Digests were extracted with phenol/chloroform, ethanol precipitated and redigested with the same enzyme at the same concentration for an additional 4–6 hr. A sample of each digest was then checked on an ethidium stained agarose gel for completion of digestion. After digestion, DNA was phenol/chloroform extracted and ethanol precipitated before loading 15 μg DNA/lane on 0.9% agarose gels. DNA was blotted onto Hybond N+ nitrocellulose filters (Amersham), alkali fixed and hybridized in 0.1% SDS, 5× Denhardts and 5× SSPE with ^{32}P labeled probe at 67°. Probes were prepared using random six nucleotide primers (Pharmacia) and Klenow DNA polymerase. Exposure to X-ray film was for 6–14 days.

Northern blots: Ten micrograms of polyA+ RNA from either isolated anthers, male flower buds <5 mm long, male leaves, isolated carpels, female flowers <5 mm long, female leaves, roots of mixed male and female plants or entire seedlings of both sexes was separated by electrophoresis on 1% agarose gels. Nucleic acids were transferred to nylon membranes (Hybond N, Amersham) by capillary blotting. Hybridization of probes and washing of filters was performed as for Southern blots.

DNA sequencing: Both strands of each clone were sequenced by the dideoxy chain termination method using a T7 polymerase kit from Amersham. One clone of every representative insert class was sequenced using T3 and T7 primers.

Karyotype analysis: Root tip metaphase cells were prepared and stained for photography of chromosomes as described in (GRANT *et al.* 1994a).

RESULTS

Experimental rationale: RDA uses PCR to amplify DNA sequences that are polymorphic between two ge-

nomes. The technique is ideal for the study of complex genomes such as human or *S. latifolia*. Only a subset of fragments derived from each genome by restriction endonuclease digestion, those which are small enough to be amplified by PCR, are compared. The diploid *S. latifolia* genome is $\sim 9 \times 10^9$ bp making it essential to reduce the complexity of sequences involved in hybridization in order to achieve complete annealing in a reasonable period of time. Using different restriction enzymes for the initial DNA fragment preparation allowed us to sample different parts of the genome in separate experiments.

Selection of subtracted clones: The genomes of a pool of 10 male and 10 female plants were cut with *Bam*HI, *Bgl*II, *Hin*dIII or *Eco*RI. After ligation to sex-specific adapters, male and female DNAs were amplified. The resulting amplicons were hybridized and amplified according to the RDA protocol as described in Methods. Sequences that were unique to the male amplicon were enriched. After three or four rounds of enrichment, a small number of discrete PCR bands were visible on ethidium stained agarose gels (data not shown). This material was cloned into plasmid replicons and 40 antibiotic resistant bacterial transformants from each treatment were randomly chosen for DNA isolation. An important control to identify nonspecific artifacts of RDA was performed by a second "reverse" hybridization. Female DNA cut with the appropriate restriction enzyme and amplified with the male-specific adapters was hybridized to an excess of male DNA and subjected to RDA. Reverse hybridization PCR products were used to probe the RDA-derived cloned DNAs, blotted onto nylon membranes. Clones that hybridized to the probe were considered unlikely to be male-specific restriction fragments and were discarded. One clone from the *Bam*HI experiment and 36 from the *Bgl*II experiment were identified as potentially male-specific. All of the clones generated after digestion of genomic DNA with *Hin*dIII or *Eco*RI were homologous to the reverse hybridization probe. The discarded clones from the *Bam*HI digested DNA were shown to be highly repetitive sequences by hybridizing to radiolabeled total male or female DNAs in separate experiments (data not shown). Their repetitive nature may make it difficult to remove these clones by hybridization.

Initial DNA digestion with rarely cutting restriction enzymes appears to be more useful for RDA using *S. latifolia*. Digestion of genomic DNA with *Bam*HI, *Hin*dIII and *Eco*RI lead to products of a broad size range. In contrast, *Bgl*II-digested DNA was composed mostly of large restriction fragments, too large to be in the amplicon for subtraction. This leads to lower sequence complexity in the amplicon and more of the RDA clones derived from *Bgl*II-digested DNA did not hybridize to the reverse hybridization probe. The single *Bam*HI clone that did not hybridize to the reverse hybridization probe and 18 of the 36 nonhybridizing *Bgl*II clones were analyzed further and yielded 10 independent sequences. Four of these were further characterized as described below.

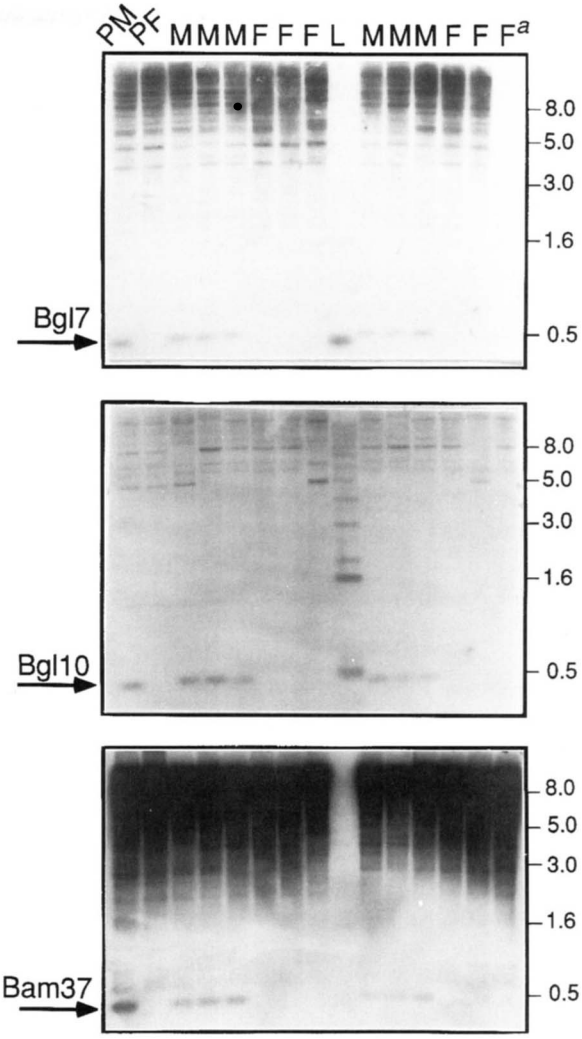

FIGURE 1.—Male-specific DNA fragments identified using RDA. Male and female genomic DNAs cut with *Bgl*II (top two panels) or *Bam*HI (bottom panel) probed with RDA-derived sequences Bgl7, Bgl10 or Bam37 as indicated. Arrows indicate male-specific polymorphisms. Size markers are indicated in kilobases on the right. Genomic DNA pooled from over 10 male siblings (PM), genomic DNA pooled from over 10 female siblings (PF), DNA from individual male siblings of the inbred population (M), DNA from individual female siblings (F), 1-kb ladder (GIBCO-BRL) that cross hybridizes to some probes (L) were probed in each panel. [a]In the top panel probed with Bgl7, lane F has no DNA.

Southern blots of male and female individuals confirms that RDA-derived restriction fragments are uniquely found in males: A male-specific restriction fragment of the same size as the probe was detected when the male-specific RDA-derived clones were labeled and used to probe DNA gel blots of genomic DNA digested with the same restriction enzyme used to produce the original amplicon. DNA was tested from at least six individual sibling plants of each sex. Gel blots probed with the 450-bp *Bam*HI clone, Bam37, the 471-bp *Bgl*II clone, Bgl7, or the 331-bp *Bgl*II clone, Bgl10 are shown in Figure 1. Notably, a fragment identical in size to the male-specific

DNA probe (indicated by an arrow at the left of the gel in Figure 1) was detected in the genomic DNA of all male plants tested and was lacking in all female plants tested. The probability that these male-specific polymorphisms are not linked to the sex of the plant is 4.8×10^{-4} according to the formula used for bulk segregant analysis of cosegregation of dominant markers, $2(1 - [1/4]n)(1/4)n$ where $n = 6$, the number of plants of each sex (MICHELMORE *et al.* 1991). None of the probes detecting male-specific fragments were single copy sequences. Instead they were all members of repetitive sequence families. In all cases, the additional homologous sequences were common between both male and female plants although some polymorphisms, not related to the sex of the plant, could be seen in some individuals. The number of hybridizing bands varied from <10 in clone Bgl10 (and Bgl16, data not shown) to many in clones Bam37 and Bgl7 (Figure 1). The latter two clones appear to contain moderately repetitive DNA sequences. The majority of hybridization with all tested RDA probes is to large fragments. In *Bgl*II-digested DNA, the majority of restriction fragments visualized by ethidium staining sites are in this size range. However *Bam*HI cut DNA leads to a broad range of restriction fragment lengths. We attempted to ensure that DNA digests were complete (MATERIALS AND METHODS), but we cannot rule out that some hybridization bands are to undigested DNAs. Only the fragments identical in size to the RDA-derived probes are unique to male genomes and likely to be linked to the *Y* chromosome.

Detection of larger male-specific DNA restriction fragments with RDA generated probes: When the male-specific probes were hybridized to genomic DNA digested with other restriction enzymes, male-specific polymorphic bands were detected with each digest. In Figure 2A, a 4.1-kb DNA fragment homologous to the 292-bp *Bgl*II generated probe Bgl16 is found only in pooled male DNA (indicated by longest arrow on left) and not in female DNA. A 6-kb hybridizing fragment is only found in male DNA cut with *Eco*RI (indicated by medium length arrow on left) and a fragment of ~11 kb (indicated by the shortest arrow) is found only in male DNA digested with *Bam*HI. The 450-bp Bam37 probe also detects DNA fragments only present in male DNA cut with the same three restriction enzymes (indicated by arrows in Figure 2B). The 450-bp male-specific *Bam*HI fragment seen in Figure 1 is indicated by the shortest arrow on the left of the blot. The ability of the probes to detect male-specific polymorphisms with a variety of restriction enzymes proves that the DNA fragments digested with the same enzymes used to produce the RDA probes as in Figure 1, are not artifacts of differential methylation of *Bam*HI or *Bgl*II restriction sites in male and female genomes. The higher molecular weight polymorphic DNA fragments potentially contain some less abundant sequences flanking the Bgl16 or Bam37 cloned RDA sequences that could be used to isolate *Y*-linked sequences from a male genomic DNA bank.

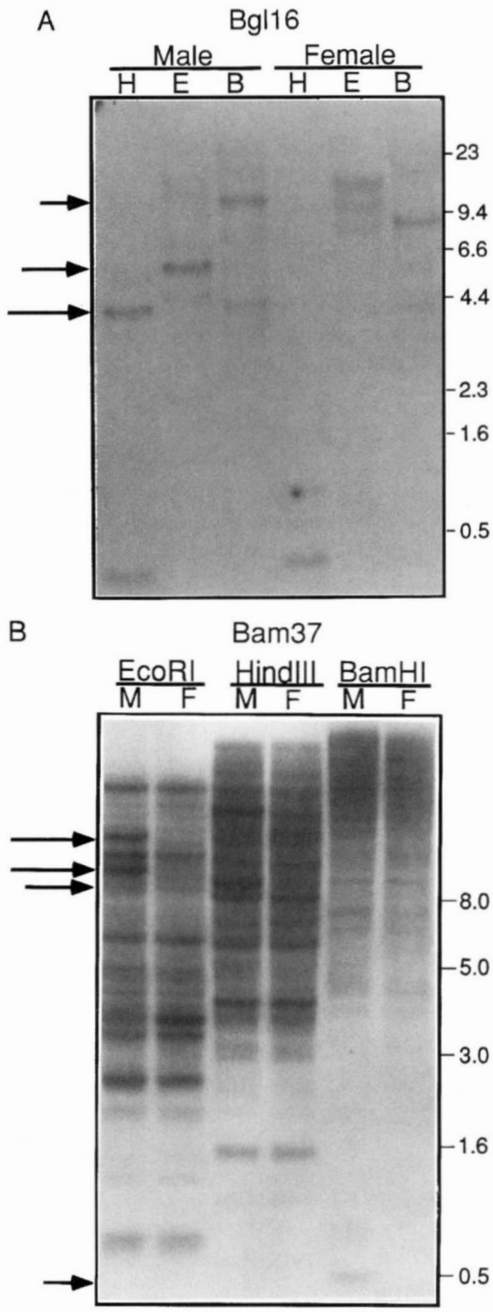

FIGURE 2.—RDA probes hybridize to male-specific fragments in DNA digested with various enzymes. Enzyme-specific male polymorphisms homologous to A, Bgl16; B, Bam37 seen with pooled male (M) or pooled female (F) genomic DNA cut with *Hin*dIII (H), *Eco*RI (E) and *Bam*HI (B) restriction enzymes. Arrows indicate male-specific hybridization signals. Arrow lengths correlate to male-specific bands in lanes from left to right. Longest arrows refer to left lanes and shortest arrows refer to right lanes. DNA sizes in kilobases are indicated on the right.

The RDA sequences are unlikely to represent expressed genes: Sequencing of the four clones revealed that none of them (including Bgl7 and Bgl16) shared significant sequence homology (GenBank database accession Nos.: Bgl7, X99865; Bgl10, X99866; Bgl16, X99867; and Bam37, X99864). No significant homology to known genes in the National Center for Biotechnol-

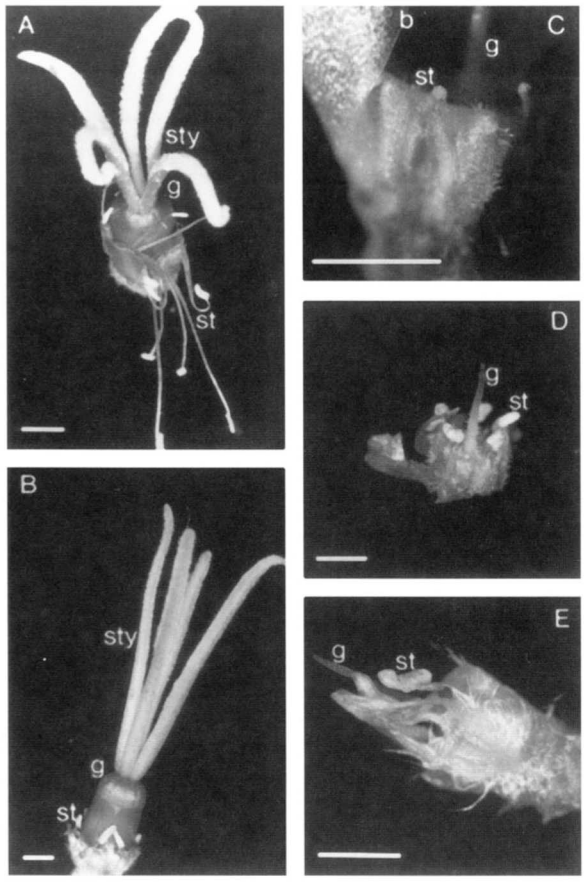

FIGURE 3.—Flowers of hermaphroditic and asexual mutants with sepals and petals removed to reveal reproductive organs. (A and B) Two different hermaphroditic mutants. Gynoecium (g) including styles (sty) and stamens (st) are indicated. (C–E) Three different asexual mutants. An outer bract (b) is showing in C at the left. Rudimentary gynoecium and stamens are shown. Size bar is equal to 2 mm.

ogy Information (NCBI) database was detected using BLAST and FASTA searches. However, some homologies to repetitive DNAs were seen in short stretches of Bgl16 (72% identity to a retrotransposon from *Arabidopsis thaliana* over 67 bases) and Bam37 (89% identity to a human Alu repeat sequence over 27 bases). All four sequences have multiple stop codons in all reading frames and no long open reading frames suggesting none encode expressed genes. All four cloned sequences were radiolabeled and used together to probe an RNA gel blot of 10 μg of polyA+ RNA from various *Silene latifolia* tissues including flowers, leaves roots and seedlings. No transcripts were detected (data not shown).

Generation of mutants with altered sex: We used X-ray mutagenesis of pollen to generate lesions in the male *Y* chromosome leading to production of flowers expressing altered gender. In two rounds of mutagenesis, we irradiated pollen from males of an inbred line (7 generations brother × sister mated) and crossed it to sibling females to generate seed. X-ray dosages varied from 600 to 7000 r as described previously (GRANT *et al.* 1994a). Plants were selected in the first generation

TABLE 1

Sex of progeny of hermaphroditic mutants backcrossed to normal females

Mutant used as male parent	No. female	No. male	No. hermaphroditic	Total No. of plants scored
H1	23	0	1	24
H7	11	0	0	11
H8	14	0	0	14
H9	8	0	0	8
H10	27	0	0	27
H15	15	0	0	15
H16	9	0	0	9
H17	53	0	0	53
H20	20	0	0	20

when recessive mutations would only be visible if they were *Y* linked. Five asexual and 22 hermaphroditic mutants were isolated from 472 plants generated from pollen irradiated at 7600 r and eight hermaphroditic and 30 asexual mutants were found from ~5000 plants generated form pollen irradiated at 4500 or 5000 r. We also found some mutations affecting leaf shape but most of the mutations affected flower development.

Examples of hermaphroditic and asexual mutant flowers are shown in Figure 3. The mutants varied considerably in flower morphology suggesting that several different loci were affected in various mutants in the collection. Four of the 22 hermaphrodites derived from the 7600-r treated pollen were andromonoecious, having a mixture of male and hermaphroditic flowers (not shown). Andromonoecious mutants had variable numbers of carpels in hermaphroditic flowers (two to five). Nineteen of the 30 hermaphroditic mutants had five carpels as in a mature wild-type female and 10 stamens as in a normal male flower. Seventeen of these were self-fertile. A typical flower of such a mutant, H8, is shown in Figure 3A with its sepals and petals removed to reveal the reproductive organs. Five hermaphroditic mutants had unusually short stamen filaments with pollen bearing anthers like mutant H20 shown in Figure 3B. Other hermaphroditic mutants had a variable number of carpels ranging from two to five carpels per flower. The number of carpels even differed between flowers on the same plant (not shown).

Flowers of asexual mutants have a small rod of cells in the center which is the rudimentary gynoecium typical of wild-type male flowers (Figure 3, C–E) (GRANT *et al.* 1994b), and their stamens are arrested in development before maturity. The stamen filament length and the degree of development of anthers before premature arrest occurred varied between different mutants. However, the stage of stamen arrest was fairly uniform on different flowers of each asexual mutant. Six asexual mutants resembled mutant S12 shown in Figure 3C, which expresses early arrest of stamen development at a stage similar to stamen arrest in wild-type females

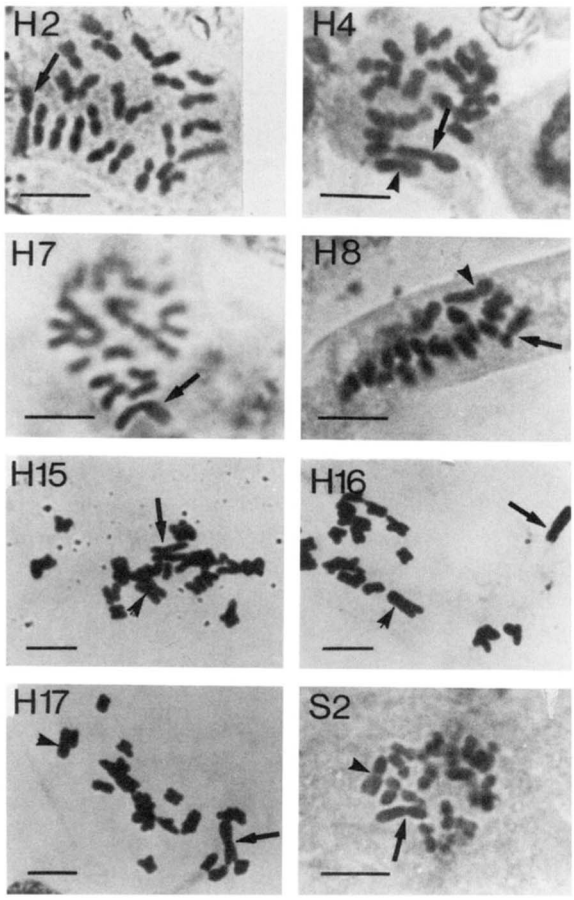

FIGURE 4.—Karyotypic analysis reveals *Y* chromosome deletions in some hermaphroditic and asexual mutants. Root tip metaphase chromosomes from the mutants indicated were stained with acetocarmine. *Y* chromosomes (long arrow) are indicated along with the *X* chromosome (arrow head) where visible. The size bar is equal to 5 μm.

(GRANT *et al.* 1994b). The majority of asexual mutants have stamens that develop further before their arrest, as shown in Figure 3, D and E.

Heritability of hermaphroditic mutations: To assess the heritability of mutations, hermaphrodites were crossed as male parents to normal females. We have examined the offspring of nine hermaphrodites derived from the 7600 r irradiated pollen (Table 1). The hermaphrodite trait was transmitted to the next generation in only one family. All offspring of this mutant were female except for one hermaphrodite. Upon self fertilization of this sole backcross-derived hermaphrodite plant, a ratio of 2:1 female to hermaphrodite plants were observed in the next generation (50 flowering plants examined). This non-Mendelian segregation ratio reflects two things. First, the *Y* chromosome is potentially not inherited through the egg of a hermaphroditic plant. Studies of embryo regeneration from pollen cells demonstrated that only *X* bearing pollen grains could go through embryogenesis (YE *et al.* 1990), so it is possible that *Y* bearing ovules are not viable. This would lead to a 1:1 ratio of hermaphrodite to female in selfed progeny of a *Y*-linked hermaphrodite. The second problem in segregation is due to the tendency of this line to produce more female than male offspring. Unbalanced sex ratios are commonly observed in *S. latifolia* populations and are thought to be caused by a difference in transmission of *X* and *Y* chromosomes through pollen (TAYLOR 1994). In the other eight families, all offspring were female. This indicates that the *Y* chromosomes of these plants were not transmitted through the pollen, a common feature of damaged chromosomes (STADLER and ROMAN 1948). No male progeny were found as expected if the lesion is linked to the *Y* chromosome.

TABLE 2

Presence of RDA markers in hermaphroditic mutant *Silence latifolia* plants

Mutant	Bgl7	Bgl10	Bgl16	Bam37	Karyotype
H1	+	−	+	+	ND
H2	−	−	−	+	LVD
H3	+	+	+	+	ND
H4	−	−	−	+	NVD
H5	+	+	+	+	ND
H6	+	+	+	+	ND
H7	+	−	+	+	NVD
H8	−	−	−	+	LVD
H9	+	+	+	+	ND
H10	+	−	+	+	ND
H11	−	+	−	+	ND
H12	+	+	+	+	ND
H13	−	+	−	+	ND
H14	+	+	+	+	ND
H15	−	−	−	+	LVD
H16	+	−	+	+	LVD
H17	+	+	+	+	NVD
H18	+	+	+	+	ND

−, Male-specific restriction fragment is absent; +, male-specific restriction fragment is present; LVD, large visible deletion in *Y* chromosome removing most of one arm; NVD, no visible deletion in *Y* chromosome; SVD, small visible deletion of part of one arm of *Y* chromosome; ND, karyotype not analyzed.

Some sex mutants have cytologically detectable *Y* chromosome deletions: We have examined the chromosomes in root tip metaphase cells of seven hermaphroditic and one asexual mutant. An example of metaphase chromosomes from a root tip cell of each mutant is shown in Figure 4 and the results are summarized as part of Table 2. The wild-type *Y* chromosome is the largest and it is completely metacentric (MATSUNAGA *et al.* 1994). The hermaphrodite mutants, H4, H7 and H17 had *Y* chromosomes that resembled wild type in their morphology and size. In contrast, the asexual mutant, S2 and four of the hermaphrodites, H2, H8, H15, and H16 had visible deletions in the *Y* chromosome (Figure 4).

Presence of male-specific RDA-derived restriction fragments in sex mutants: In order to determine if any of the mutant phenotypes could be correlated with loss of male-specific restriction fragments, we probed Southern blots of total genomic DNA isolated from 18 fully

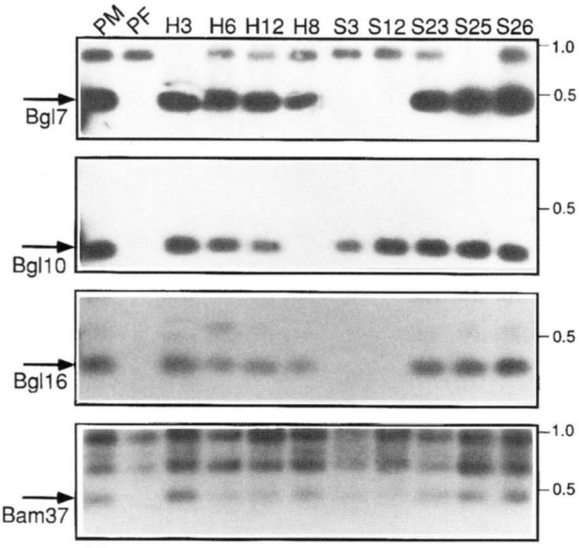

FIGURE 5.—Genomic DNAs from individual mutants probed with RDA markers reveal DNA rearrangements. *Bgl*II (top three panels) or *Bam*HI (bottom panel) digested DNA pooled from over 10 normal male siblings (PM). DNA pooled from over 10 normal female siblings (PF) or DNA from individual hermaphroditic mutants (H3, H6, H12, H8) or asexual mutants (S3, S12, S23, S25, S26) probed with RDA-derived clone Bgl7, Bgl10, Bgl16 or Bam37 as indicated. Male-specific fragments are indicated by an arrow. Size markers in kilobases are indicated on the right.

hermaphroditic and 25 asexual mutant plants with the four RDA-derived male-specific fragments described above. In each case, the genomic DNA was cut with the restriction enzyme that would produce a homologous fragment of the same size as the probe. Loss of the band corresponding to the cloned fragment could occur due to mutation of one of the flanking *Bam*HI or *Bgl*II restriction sites, deletion of part or all of the sequence or a DNA rearrangement involving that sequence. An example of Southern blots with DNA from pooled male and female plants and four hermaphrodite (H3, H6, H8 and H12) and five asexual mutants (S3, S12, S23, S25 and S26) probed with each clone is shown in Figure 5. Male-specific bands are indicated by an arrow to the left of each panel. As expected, all four sequences are present in pooled male DNA and missing in pooled female DNA. The presence or absence of the male-specific restriction fragments in each mutant is summarized in Tables 2 and 3 and conclusions regarding the position of the markers is discussed below.

DISCUSSION

Our results demonstrate the utility of RDA for the identification of DNA markers polymorphic between male and female *S. latifolia* plants. We isolated sequences that were male specific in siblings from an inbred population. Since autosomal genes are randomly segregating with respect to loci on the sex chromosomes, our RDA-derived clones are most likely to be

TABLE 3

Presence of RDA markers in asexual mutant *Silene latifolia* plants

Mutant	Bgl7	Bgl10	Bgl16	Bam37	Karyotype
S1	−	+	−	+	ND
S2	−	+	−	+	SVD
S3	+	+	+	+	ND
S4	+	+	+	+	ND
S5	+	+	+	+	ND
S6	+	+	+	+	ND
S7	+	+	+	+	ND
S9	+	+	+	+	ND
S10	+	+	+	+	ND
S11	+	+	+	+	ND
S12	+	+	+	+	ND
S13	+	+	+	+	ND
S14	+	+	+	+	ND
S15	−	+	−	+	ND
S16	+	+	+	+	ND
S17	+	+	+	+	ND
S18	+	+	+	+	ND
S19	+	+	+	+	ND
S20	+	+	+	+	ND
S21	+	+	+	+	ND
S22	+	+	+	+	ND
S23	+	+	+	+	ND
S24	−	+	−	+	ND
S25	+	+	+	+	ND
S26	+	+	+	+	ND

Symbols as in Table 2.

Y chromosome-specific sequences. Using this technique, we have identified four independent male-specific restriction fragments. However, all probes hybridized to additional DNA in common between males and females. Thus, at least a part of each sequence must have homology to moderately repetitive sequences that are not sex chromosome linked. They will, however, make useful probes for mapping *Y*-linked sex determining genes and for the isolation of larger genomic DNA fragments that may carry transcribed sequences or single copy sequences that are unique to males.

Loss of male-specific RDA markers in sex mutants identifies sex determining regions of *Y* chromosome: Of the four RDA clones tested, one detected a *Y* chromosome-specific sequence present in all tested mutants, indicating that all mutants carry *Y* chromosome DNA. The other three sequences were lost in some hermaphroditic and some asexual mutants (Tables 2 and 3). Two of the three clones, Bgl7 and Bgl16, shared exactly the same pattern of presence or absence in mutant DNAs, and the third (Bgl10) shared the same pattern as a cDNA clone isolated independently from a male-specific cDNA subtraction experiment (D. YE, H. KOUTNIKOVA, H. SAEDLER and S. GRANT, unpublished results). Thus, it would appear that there may be islands of difference on the *Y* chromosome detected with three of our RDA-derived clones. The ability to use RDA to quickly identify limited regions of the *Y* chromosome carrying sex-linked

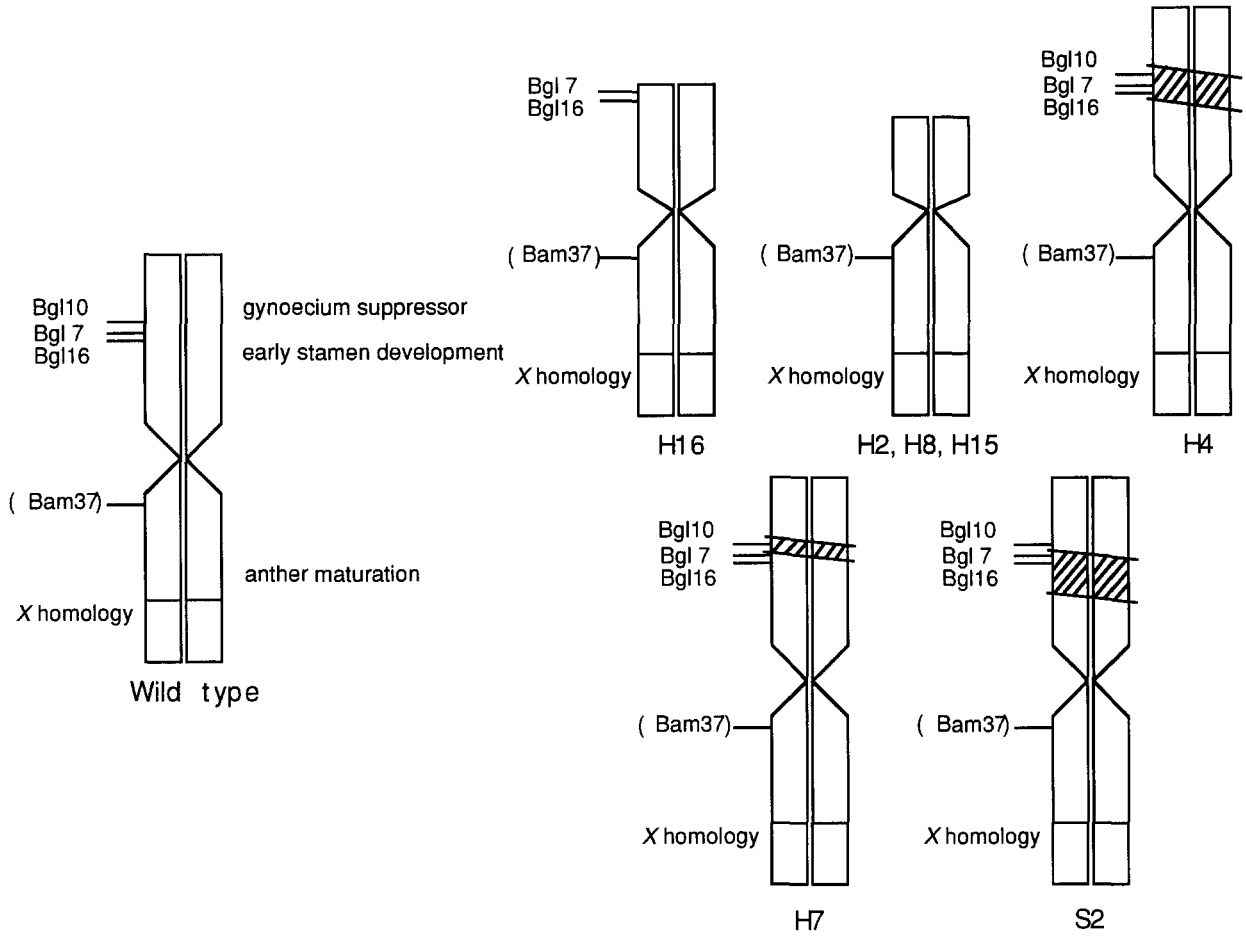

FIGURE 6.—Hypothetical location of RDA markers on the *Y* chromosome of wild-type males and chosen mutants. Each image represents the *Y* chromsome of the mutant designated below it. The relative predicted position of the RDA-derived markers and sex determining genes (gynoecium suppressor, stamen initiation, anther maturation) are based on WESTERGAARD (1958) and the markers missing in the mutants described in this paper. Bam37 is marked in brackets because we cannot predict its map position based on our data. Indent in chromosome arms indicates the centromere. Hatched bars across chromosome arms indicate location of internal deletions.

deletions will be very important to overcome the disadvantages for mapping of the large size and repetitive nature of the *Y* chromosome.

Assignment of marker positions on the *Y* chromosome: Comparison of male-specific restriction fragments missing in mutants and karyotypic evidence of deletions in some *Y* chromosomes allowed us to make a first assignment of marker position in relation to genes for sex determination as illustrated in Figure 6. The markers Bgl7, Bgl10 and Bgl16 are missing in mutants H2, H8 and H15 which have large deletions in one *Y* chromosome arm. However, all three are also missing from hermaphroditic mutant H4 with no visible deletion of the *Y* chromosome. This indicates that the three markers are linked in a relatively small *Y* chromosome region. Bgl10 alone is missing in mutant H16 with a large *Y*-linked deletion. Therefore we assigned it to the position closest to the end of the chromosome, assuming that this large deletion is not internal. The Bgl10 marker appears to be close to a female suppressor locus because it is the marker most frequently absent in hermaphroditic mutant plants. It is also the only marker lost in hermaphroditic mutant H7 which has no visible alterations of the *Y* chromosome (Figure 6). Finally, Bgl10 is lost only in hermaphroditic mutants and retained in all asexual mutants. The cytogenetic work of Westergaard (WESTERGAARD 1946, 1958) defined a region of the *Y* chromosome bearing genes involved in suppression of carpel development on the opposite arm from the region of homology to the *X* chromosome. Following WESTERGAARD's observations, we assigned the Bgl10 probe to the arm distal to the *X* homology. Bgl7 and Bgl16 must also be linked on that arm.

Identification of a locus involved in early stamen development: Since two clones, Bgl7 and Bgl16, can be deleted in both hermaphroditic and sterile mutants, we predict that a carpel suppressor gene and a stamen promoter gene must be linked on one arm of the *Y* chromosome. Therefore, we assigned an early stamen development locus to same arm of the chromosome as the gynoecium suppressor close to Bgl7 and Bgl16. This locus was not identified in earlier genetic studies. WESTERGAARD originally proposed that genes to promote the

early stages of stamen development could be located on the same arm as the carpel suppressor (WESTERGAARD 1958), but he did not analyze asexual mutants with rearrangements leading to very early arrest of stamen development (WESTERGAARD 1946). The asexuals, which lost Bgl7 and Bgl16 marker sequences (mutants S1, S2, S15 and S24), all arrest anther development at a stage comparable with mutant S12 shown in Figure 3C. (If S12 has a mutation in the same gene, it has occurred without affecting the DNA homologous to Bgl 7 and Bgl16). WESTERGAARD analyzed asexual mutants in which stamen development was aborted after anther differentiation and some filament elongation had occurred, similar to the mutant in Figure 3E. These had a deletion in the *Y* arm with the *X* chromosome homology which he proposed contained a region for anther maturation as illustrated in Figure 6 (WESTERGAARD 1946, 1958).

Since Bam37 is present in all mutants, we assume it is on another part of the *Y* chromosome not included in any of the deletions. Therefore we have assigned it to the opposite chromosome arm from the other markers. However, it may be on the same arm closer to the centromere.

Using RDA markers and deletion mutants of *S. latifolia* to locate sex determining genes of the *Y* chromosome: RDA offers us the potential to efficiently identify a large number of male-specific markers to characterize the nature of DNA rearrangements in our collection of X-ray generated mutants. Ionizing radiation such as X-rays generates deletion mutations and translocations at a higher rate than chemical mutagens. Our karyotypic analysis of our mutant collection indicates that deletions in the male sex chromosome are common. The markers we have already identified by RDA allowed us to begin to characterize the rearrangements of the *Y* chromosome in some mutants and to deduce the order of the markers and their relation to sex determining genes. We can now use these markers to identify mutations linked to small deletions of the *Y* chromosome. Mutant H1 with its heritable mutation and H7, which has an apparently full-sized *Y* chromosome, may have suitable mutations since both have lost the Bgl10 marker. Mutants with small deletions can be used to generate further linked markers by using RDA to identify restriction fragments present in related wild-type males and missing in the mutants. As more mutants are examined with linked markers, it will be possible to identify regions of the *Y* chromosome containing sex determining genes and ultimately to clone those genes based on their proximity to DNA markers.

We thank N. LISITSYN for providing us with a detailed protocol and advice for the RDA procedure. We gratefully acknowledge the technical assistance of B. HUNKIRCHEN, R. SCHMIDT and T. GUERTLER. We also thank S. WHITFIELD for assistance with the illustrations and J. L. DANGL, S. HARDENACK and Anonymous Reviewer 2 for helpful suggestions on the manuscript. I.S.D. was supported by a British Royal Society Fellowship and a Max Planck Society Fellowship. S.R.G. was supported by a grant from the Bundesministerium für Forschung und Technologie no. 0316101A.

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Communicating editor: D. CHARLESWORTH