Genetic and Functional Analysis of *DD44*, a Sex-Linked Gene From the Dioecious Plant *Silene latifolia*, Provides Clues to Early Events in Sex Chromosome Evolution

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ABSTRACT

Silene latifolia is a dioecious plant with heteromorphic sex chromosomes. The sex chromosomes of *S. latifolia* provide an opportunity to study the early events in sex chromosome evolution because of their relatively recent emergence. In this article, we present the genetic and physical mapping, expression analysis, and molecular evolutionary analysis of a sex-linked gene from *S. latifolia*, *DD44* (*D*ifferential *D*isplay 44). *DD44* is homologous to the oligomycin sensitivity-conferring protein, an essential component of the mitochondrial ATP synthase, and is ubiquitously expressed in both sexes. We have been able to genetically map *DD44* to a region of the Y chromosome that is genetically linked to the carpel-suppressing locus. Although we have physically mapped *DD44* to the distal end of the long arm of the X chromosome using fluorescence *in situ* hybridization (FISH), *DD44* maps to the opposite arm of the Y chromosome as determined by our genetic map. These data suggest that chromosomal rearrangements have occurred on the Y chromosome, which may have contributed to the genetic isolation of the Y chromosome. We discuss the implications of these results with respect to the structural and functional evolution of the *S. latifolia* Y chromosome.

THE presence of a heteromorphic Y chromosome defines males in the dioecious plant, *Silene latifolia* (Grant *et al.* 1994a; Monéger *et al.* 2000; Matsunaga and Kawano 2001; Negrutiu *et al.* 2001; Charlesworth 2002). An active-Y sex determination system also occurs in humans; however, while mammalian sex chromosomes diverged almost 300 mya (Lahn and Page 1999a), *S. latifolia* sex chromosomes appeared between 8 and 24 mya (Desfeux *et al.* 1996). Therefore, the X/Y chromosome pair in *S. latifolia* offers a unique opportunity to analyze the structure and function of sex chromosomes at an early stage of evolution (Negrutiu *et al.* 2001). In particular, we want to answer two questions: (1) Are the same evolutionary events that helped shape human sex chromosomes occurring in *S. latifolia* and (2) to what extent have these events occurred in *S. latifolia*?

Preliminary evidence suggests that the sex chromosomes of *S. latifolia* are following a similar pattern of evolution to that of the human sex chromosomes. The inhibition of recombination between the proto-X and proto-Y chromosomes has already occurred in *S. latifolia* sex chromosomes (Charlesworth and Guttman 1999). The *S. latifolia* Y chromosome can be divided into a nonpairing region (NPR) and a smaller pseudo-autosomal region (PAR) localized at the tip of the q arm (Westergaard, 1958; Lardon *et al.* 1999a). Second, the observation that one of the X chromosomes in females is hypermethylated, a common hallmark of inactivated genes, suggests that an X-inactivation system may have evolved in *S. latifolia* to offset the decreased fitness caused by overexpression of X-linked genes (Vyskot *et al.* 1993; Siroky *et al.* 1998; Vyskot 1999). Finally, functional degradation of the Y is supported by the observation that YY progeny and androgenic haploid plants with only the Y chromosome are not viable (Westergaard 1958; Ye *et al.* 1991). Furthermore, decreased sequence variability of the Y-linked gene, *SlY1*, compared to its X-linked homolog, is a hallmark characteristic of degenerating Y chromosomes (Filatov *et al.* 2000, 2001).

However, there are some differences between *S. latifolia* and human sex chromosomes. The *S. latifolia* Y chromosome is clearly at an earlier stage of evolution and has not degenerated to the extent of the human Y chromosome. First of all, C-banding experiments and methylation analyses show that the *S. latifolia* Y chromosome is largely euchromatic with the exception of centromeric and subtelomeric DNA (Grant *et al.* 1994a; Siroky *et al.* 1998; Grabowska-Joachimiak and Joachimiak 2002). This, and the fact that the Y chromosome only recently diverged from the X, suggests that

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many actively transcribed genes may still be on the Y chromosome. Also, the Y chromosome has not degenerated in size relative to the X. In fact, the Y chromosome of *S. latifolia* is 1.4 times larger than the X (Ciupercescu *et al.* 1990; Grabowska-Joachimiak and Joachimiak 2002). Preliminary evidence suggests that the increase in the size of the Y is at least partially due to the accumulation of repetitive DNA sequences (Donnison and Grant 1999).

We are interested in knowing whether the *S. latifolia* Y chromosome is in the process of evolving structural and functional coherence, much like the Y chromosome of humans (Lahn and Page 1997; Delbridge and Graves 1999), even though the X and Y chromosomes of *S. latifolia* only recently diverged. Clustering of genes with similar functions on the *S. latifolia* Y chromosome may indicate that the organization of the Y chromosome occurs rapidly after the divergence of the sex chromosomes from their autosomal ancestors. However, if the *S. latifolia* Y chromosome is not well ordered, it may mean more time is required to fine-tune this organization. Evidence derived from genetic and deletion mutant analysis indicates that at least the rudiments of functional units exist on the *S. latifolia* Y chromosome (Westergaard 1958; Donnison *et al.* 1996; Farbos *et al.* 1999; Lardon *et al.* 1999b). However, the genes located within these units have yet to be discovered and mapped. Thus, the structural and functional organization of genes on the *S. latifolia* Y chromosome remains enigmatic.

We began our study with a screen designed to identify sex-linked genes expressed specifically in male premeiotic floral meristems, because we are interested in addressing the hypothesis that the *S. latifolia* Y chromosome is functionally coherent. In this article, we present the genetic and molecular analysis of one of the genes identified from this screen, *DD44* (*D*ifferential *D*isplay 44). *DD44* has a simple sex-linkage pattern, with an X- and Y-linked allele, although it is ubiquitously expressed in males and females. This expression profile is not unexpected, as *DD44* is homologous to the oligomycin sensitivity-conferring protein (OSCP), an essential component of the mitochondrial ATP synthase. Interestingly, genetic and physical mapping of *DD44* suggests that the position of the Y-linked allele of *DD44* is the result of a chromosomal rearrangement. We compare our findings with those for other sex-linked genes in *S. latifolia* and discuss the implications of these data for the functional coherence of the *S. latifolia* Y chromosome.

MATERIALS AND METHODS

Plant materials: Male and female *S. latifolia* plants of the U9 and MR4X64 ecotypes (described in Lebel-Hardenack *et al.* 2002) as well as the 15X10 ecotype (gift of J. Antonovics, University of Virginia) were used for wild-type populations. Sterile and hermaphrodite plants were selected from M1 populations derived from X-ray-irradiated pollen as described in Lebel-Hardenack *et al.* (2002). Families of 10 male and 10 female F_1 progeny used for segregation analysis were obtained by cross-pollination of a female of each ecotype with a male from each of the other two ecotypes. Hairy root (HR) cultures were obtained by infecting plant leaf explants with an oncogenic *Agrobacterium rhizogenes*, strain A4RS, and culturing the explants on B5 medium without hormones. During prolonged cultivation, subclones with various deletions were isolated. HR cultures of a tetraploid female (Siroky *et al.* 1999), a female with a deletion of the distal region of the q arm, and a male with a deletion of one arm of the Y chromosome were used as sources of mitotic chromosomes.

Differential display: Differential display (Liang and Pardee 1992; Martin and Pardee 1999) was performed between mRNA populations isolated from male and female dissected floral meristems from developmental stages prior to or at the stage of sex determination (up to stage 6; Grant *et al.* 1994b). Total RNA was prepared using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). Poly(A)$^+$ RNA was isolated using Dynabeads (Dynal, Oslo). The differential display was carried out using the RNAimage kit 2 (Genhunter, Nashville, TN). Differential display fragments were cloned into the TOPO pCR2.1 cloning vector (Invitrogen Life Technologies) and sequenced with the M13 reverse primer.

Extraction of nucleic acids: DNA was isolated from young leaves by CsCl banding. Briefly, 5–10 g of ground frozen tissue was incubated in extraction buffer [0.1 M EDTA pH 8.0, 3× SSC, 0.1 M sodium diethyldithiocarbamic acid (Sigma, St. Louis)] at 37° for 15 min followed by two rounds of phenol:chloroform extraction and ethanol precipitation. Precipitated DNA was spooled and resuspended in 7.5 ml of TE buffer, pH 8.0, at 65° for 15 min with periodic agitation. CsCl (8.73 g) and 20 μl of ethidium bromide (20 mg/ml) were added to resuspended DNA and the sample was spun overnight at 55,000 rpm in an L7-55 Ultracentrifuge (Beckman, Palo Alto, CA). The genomic DNA band was removed with a pipette, the ethidium bromide was extracted multiple times with 7:1 (v/v) isopropanol:water, and the DNA was ethanol precipitated. Precipitated DNA was air dried, resuspended in TE, and stored at 4°.

Total RNA was isolated from open flowers, flower buds (<1 mm), young leaves of male and female *S. latifolia*, and isolated meristems of mixed male and female *S. latifolia* seedlings using TRIZOL reagent (Invitrogen Life Technologies). Poly (A)$^+$ RNA was procured from total RNA using the Oligotex mRNA midi kit (QIAGEN, Valencia, CA) and treated with DNase I using the DNA-free kit (Ambion, Austin, TX).

Southern and Northern blot analyses: Genomic DNA and RNA gel blots were made using standard techniques (Sambrook *et al.* 1989). Probes used for Southern or Northern blots were labeled with [α-^{32}P]ATP using the Prime-It II random primer labeling kit (Stratagene, La Jolla, CA). Standard conditions for Southern blot hybridization and washing were 2-hr preincubation in hybridization solution [1× SSPE, 1% (w/v) SDS, 10% (w/v) polyethylene glycol (PEG), 125 mg heparin, 50 mg herring sperm DNA] at 65°; overnight incubation in 10 ml hybridization solution with labeled probe; and 15-min wash with 2× SSC, 0.1% SDS at 65° followed by three 15-min washes with 0.5× SSC, 0.1% SDS at 65°. Standard conditions for Northern blot hybridization and washing were 2-hr pre-incubation in ULTRAhyb (Ambion) at 42°; overnight incubation with labeled probe in 10 ml of ULTRAhyb (Ambion) at 42°; and two 5-min washes with 2× SSC, 0.1% SDS followed by three 15-min washes with 0.1× SSC, 0.1% SDS at 42°.

Isolation of *DD44* cDNA and genomic clones: PCR was used to amplify a 214-bp *DD44* partial cDNA clone isolated from differential display using primers DD44F1 and DD44R1 (see below). The resulting PCR product was gel purified using a

QIAquick gel extraction kit (QIAGEN) and used to probe a male *S. latifolia* floral Lambda Zap II cDNA library (Stratagene, La Jolla CA) and a male *S. latifolia* Lambda FIX II genomic library (Stratagene) for *DD44* cDNA and genomic clones, respectively. Plasmid containing the *DD44* cDNA was rescued from positive cDNA clones using the Rapid Excision kit (Stratagene). A *DD44* genomic clone was digested with *Not*I, and the resulting 18-kb insert was subcloned into linearized (*Eag*I) pBR322 for ease of manipulation and labeling.

Primers and PCR reactions: Unless otherwise noted, all products were amplified from 150 ng of template using Taq polymerase (Invitrogen Life Technologies), and all PCR reactions used the following conditions: 94° for 2 min; 30 cycles of 94° for 30 sec, *X*° for 45 sec, 72° for *Y* min (where *X* and *Y* are defined below for each reaction); followed by 72° for 5 min.

The 214-bp *DD44* probe was amplified using primers DD44F1 (5′ GTGTTCGACATGTCCATCAGAACC 3′) and DD44R1 (5′ CCATCACTTCTTATTTTATGCAGG 3′) with a 54° annealing temperature and 45-sec extension. The same primers and conditions were used to amplify the male-specific doublet from genomic DNA, and products were run on a 4% agarose gel to visualize the doublet.

To obtain the complete genomic sequence and structure of the *DD44* X- and Y-linked alleles (*DD44X* and *DD44Y*), we performed three sequential PCR amplifications of three regions of *DD44.* All PCR amplifications of *DD44Y* were performed from a U9 male, while all PCR amplifications of *DD44X* were performed from a U9 female. The first amplification was from exon 3 to the 3′ flanking region using a *DD44X*- or *DD44Y*-specific reverse primer, DD44XR2 (5′ GCCAACA AAATTAGCGTAGCG 3′) or DD44YR2 (5′ TCCGACGAAAAC GAGGGAAG 3′), and a shared forward primer in exon 3, DD44F2 (5′ CTGTCAGTGCCCTCGGATATAAGA 3′). *DD44X* and *DD44Y* products were amplified using a 56° annealing temperature and 2-min extension time. From these sequences, a hypervariable region was found in the middle of intron 3, and reverse primers specific to *DD44X* and *DD44Y* were designed: DD44XR3 (5′ CCTTTGGTCTGGTATGGAGGGA 3′) and DD44YR3 (5′ GACAGAGAAATGAGAACTTCCACAA TAAA 3′). For the second PCR amplification, these sequence-specific primers were used in conjunction with a common forward primer found in exon 2, DD44F3 (5′ CGAAGAGCTT TGCTACCAAGGC 3′). This genomic segment of *DD44X* was amplified using a 57° annealing temperature and 3-min extension time. For amplification of this segment of *DD44Y*, long-range PCR was performed with the TaKaRa DNA polymerase (PanVera, Madison, WI) using the following conditions: 94° for 2 min; 30 cycles of 98° for 10 sec, 63° for 7 min; followed by 72° for 5 min. For the final PCR amplification, a hypervariable region in intron 2 was used to design one last set of reverse primers specific to *DD44X* and *DD44Y*: DD44XR4 (5′ CCCAAA CCACGGCATACATGTAG 3′) and DD44YR4 (5′ GGAGCTGA GGAGGCTTGGGA 3′). These primers were used in conjunction with a forward primer starting at the start codon, DD44F4 (5′ ATGTCAATGGCGAACCGCAT 3′), to amplify the remaining genomic sequence. *DD44X* and *DD44Y* products were amplified using a 60° annealing temperature and 45-sec extension time. Primer pairs DD44F3/DD44XR3 and DD44F3/DD44XR4 amplified products from the U9 female and not the U9 male (data not shown), whereas primer pairs DD44F2/DD44YR2, DD44F3/DD44YR3, and DD44F3/DD44YR4 amplified products from the U9 male and not the U9 female (data not shown).

Amplification of *DD44* Y-specific markers from genomic DNA isolated from Y-deletion mutants was performed using either DD44F1 or DD44R1 as described above, which amplifies a doublet in males, or DD44F1 and DD44YR2, which amplifies a band only in males, with a 54° annealing temperature and 1-min extension. Amplification of *SlY1* from genomic DNA isolated from Y-deletion mutants was performed using SlY1 + 11 (Filatov *et al.* 2000) and SlY1_4551R (5′ GTAGAATGGC AATATAGTACTCCATAACTA 3′), which produce a band only in males, with a 56° annealing temperature and a 2-min extension time. A second set of *SlY1*-specific primers, SLY1_4505F (5′ GGTTGAAACATGTTCATTAGTTATGGA 3′) and SLY1_5813R (5′ CATACTCCACCCAAGTATTCTTTCC 3′), was also used to map *SlY1* in Y deletion mutants using a 56° annealing temperature and 2-min extension time. Amplification of *SlY4* from genomic DNA isolated from Y-deletion mutants was performed using *SlY4*-specific primers with the previously published step-down PCR protocol (Atanassov *et al.* 2001). Amplification of these same products from hairy root genomic DNA was performed as above, except 15 ng of template was used. The Y-specific marker, *Bgl10*, was amplified from genomic DNA of these hairy root lines using primers and conditions used in Lebel-Hardenack *et al.* (2002).

Thermal asymmetric interlaced PCR: Thermal asymmetric interlaced (TAIL) PCR was used to identify variable genomic sequences flanking the *DD44* 214-bp differential display sequence. TAIL PCR conditions were as described in Liu *et al.* (1995) and Liu and Whittier (1995); however, we used a series of five nested primers instead of three, similar to an approach used by Terauchi and Kahl (2000). The last round of PCR was divided into three reactions, each with a different nested primer. This aided in the identification of gene-specific bands, as these bands gave a characteristic "step ladder" of products of decreasing size in each of these reactions. Walking was performed in the 3′ direction using the following gene-specific primers in combination with previously published degenerate primers (Liu and Whittier 1995; Liu *et al.* 1995):

DD44TAILF1 (5′ aagtgttcgacatgtccatcagaaccag 3′);
DD44TAILF2 (5′ accagggcaagacagatggagaggtt 3′);
DD44TAILF3 (5′ tcaacatatgatggcactggtgctaca 3′);
DD44TAILF4 (5′ tcccgtcattacagtttcccct 3′);
DD44TAILF5 (5′ ctaagcgccctttttgtatctctca 3′).

RT-PCR: Poly(A)$^+$ RNA was prepared from total RNA as described above. cDNA was prepared from poly(A)$^+$ RNA using the M-MLV reverse transcriptase in conjunction with an oligo(dT) primer using the RETROscript kit (Ambion). PCR reactions were performed using DD44F1 and DD44R1 primers using conditions described above.

DNA sequencing and analysis: Before sequencing, PCR products were cloned into the TOPO pCR2.1 cloning vector (Invitrogen Life Technologies). Sequencing of cDNA, genomic, and PCR-amplified clones of *DD44* was performed by the University of North Carolina Automated DNA Sequencing Facility (Chapel Hill, NC). BLAST searches were performed with default settings using the National Center for Biotechnology (NCBI) search engine (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analyses and contig assemblies were performed using the programs Sequencher version 4.0.5 (Gene Codes Corporation, Ann Arbor, MI) and Vector NTI version 6.0 (InforMax, Bethesda, MD). Sequence alignments were performed by using the CLUSTALW algorithm of the Vector NTI program, AlignX.

Genomic sequences for *DD44X* (GenBank accession no. AF543833) and *DD44Y* (GenBank accession no. AF543834) were aligned manually and unalignable regions removed. The divergence between the *DD44X* and *DD44Y* was analyzed using DnaSP version 3.0 software (Rozas and Rozas 1999). The coding regions were analyzed to estimate the synonymous and nonsynonymous divergence values per synonymous and nonsynonymous site (K_s and K_a; see Li 1997). A low K_a/K_s ratio indicates that the sequence encodes a functional protein

(see Li 1997). Divergence between the sequences of the introns of *DD44X* and *DD44Y* was also estimated, to compare the extent of divergence in the *DD44* gene with values for other X- and Y-linked loci in *S. latifolia*.

Statistical analysis: Chi-square and Fisher's exact test analyses were performed using the SAS statistics package. Order of Y-linked genes with respect to previously described amplified fragment length polymorphism (AFLP) markers was predicted as described previously (Lebel-Hardenack *et al.* 2002), using the RH2PT version 3.0 program of the RHMAP Radiation Hybrid analysis package (Boehnke *et al.* 1991).

Probe preparation and labeling for fluorescence *in situ* hybridization: The 18-kb *DD44* genomic clone in pBR322 was cut with *Sal*I and the insert was separated by gel electrophoresis (0.5% agarose). DNA was extracted from the gel using the QIAquick gel extraction kit (QIAGEN). The probe was labeled by Cy3-dUTP (Amersham Pharmacia Biotech, Little Chalfont, England) in a standard nick translation reaction using Nick Translation Mix (Roche, Nutley, NJ). Labeled probe was purified by the QIAquick nucleotide removal kit (QIAGEN).

Preparation of metaphase chromosomes for fluorescence *in situ* hybridization: Root tips from germinating seeds or hairy root cultures of *S. latifolia* (see above) were used as a source of chromosomes for mitotic spreads and prepared according to Siroky *et al.* (1999, 2001).

Fluorescence *in situ* hybridization: Slides were treated with RNAse (100 μg/ml in 2× SSC; QIAGEN), for 1 hr at 37° and washed three times with 2× SSC. Remnants of the cytoplasm were removed by 10 μg/ml Pepsin (Sigma) in 0.01 N HCl for 10 min at 37°, washed as before, dehydrated in ascending ethanol series, and air dried. The hybridization mixture (for one slide, 30 μl) consisted of 200 μg of labeled probe, 15 μl formamide, 6 μl of 50% dextran sulfate solution, and 3 μl of 20× SSC. The volume was brought to 30 μl by adding TE, pH 8. Heat-denatured (76°) hybridization mix was applied to slides, covered by a cover slip, and placed on a cycler equipped with a flat plate (cycler model PHC-3; Techne, Cambridge, England). Slides with probe mixes in them were denatured at 75° for 5 min and brought to 37° using stepwise cooling. The slides were hybridized for 40 hr. Posthybridization wash consisted of three washes in 2× SSC at 42°, two stringent washes (5 min each) in 0.1× SSC at 42°, and 4× SSC supplemented by 0.1% Tween 20 (0.1%; Sigma). Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) as a counterstain and viewed under an Olympus AX 70 epifluorescent microscope equipped by filter sets for DAPI and Cy3. Images were captured by a CCD camera using ISIS software (Meta Systems GmBH, Altlussheim, Germany) as black and white figures and merged.

RESULTS

Identification of candidate sex-linked, male-specific genes in *S. latifolia*: We used a twofold approach to identify sex-linked genes in *S. latifolia*. First, we performed differential display on mRNA isolated from male and female premeiotic floral buds (stages 1–6) to identify transcripts that were potentially expressed only during the initial stages of male floral differentiation and development. It is during these early stages of floral development, before there are any gross morphological differences in male and female floral development, that we expected to find genes involved in sex determination to be expressed. Second, we used the partial transcripts identified from differential display to probe a Southern blot of total genomic DNA pooled from either five males or five females and digested with *Eco*RI, *Bam*HI, or *Hin*dIII. Transcripts that gave at least one male-specific restriction fragment length polymorphism (RFLP) were selected for a more detailed segregation analysis to confirm their chromosomal linkage and expression analyses to confirm whether they were expressed specifically in male tissues.

We identified 13 putative male-specific transcripts from our initial differential display analysis. Six of these showed at least one male-specific RFLP in our initial screen with Southerns of pooled male *vs.* female genomic DNA (data not shown), and two of these subsequently showed evidence of sex linkage in further segregation analyses (below and data not shown). Here, we present the genetic and physical mapping, expression analysis, and molecular evolutionary analysis of the gene that showed the simplest pattern of sex linkage, *DD44*.

***DD44* is linked to the X and Y chromosomes:** We performed a segregation analysis of *DD44* to verify the chromosomal linkage of male-specific RFLPs. We mated parents from two different *S. latifolia* populations that are polymorphic for *DD44*. The fate of male-specific RFLPs was followed in 10 male and 10 female F_1 progeny (Figure 1). There were two male-specific RFLPs of *DD44*: one (>12 kb) was inherited by all female progeny, the X-linked allele; another (8 kb) was inherited by all male progeny, the Y-linked allele. Using the formula from Delichère *et al.* (1999), the probability that these RFLPs are not linked to the sex chromosomes is 9.5×10^{-7} [$P = (\frac{1}{2})^n$, where $n = 20$]. Thus, based on this RFLP segregation analysis, *DD44* is sex linked.

***DD44* encodes the OSCP of the mitochondrial ATP synthase:** We screened a cDNA library derived from male floral mRNA with the 214-bp *DD44* differential display product to identify a full-length cDNA for *DD44*. We obtained a 976-bp cDNA clone containing 639 bp of the 693-bp *DD44* open reading frame (ORF) and 337 bp of the 3′ flanking region. The initial 54 bp of the ORF was deduced from the genomic sequence of *DD44* (detailed below). Our probe sequence was found in the region spanning the last 71 bp of the *DD44* ORF into the 3′ flanking sequence.

Blast alignment of the translated *DD44* ORF against the GenBank nonredundant protein database yielded greatest similarity to the δ-subunit of the mitochondrial F_1-ATP synthase of the sweet potato, *Ipomoea batatas* (*E* value = 8×10^{-69}; accession no. BAA77508), which is homologous to the OSCP from other eukaryotes (Kimura *et al.* 1990) and to the OSCP of *Arabidopsis thaliana* (*E* value = 3×10^{-54}; accession no. NP_196849). *DD44* shares 65.6% nucleotide identity with the F_1-ATPase δ-subunit of *I. batatas* and 63% nucleotide identity with the *A. thaliana* sequence. At the protein level, *DD44* is 62.9% identical (72.7% similar) to the *I. batatas* sequence and 53.1% identical (68% similar) to the *A.*

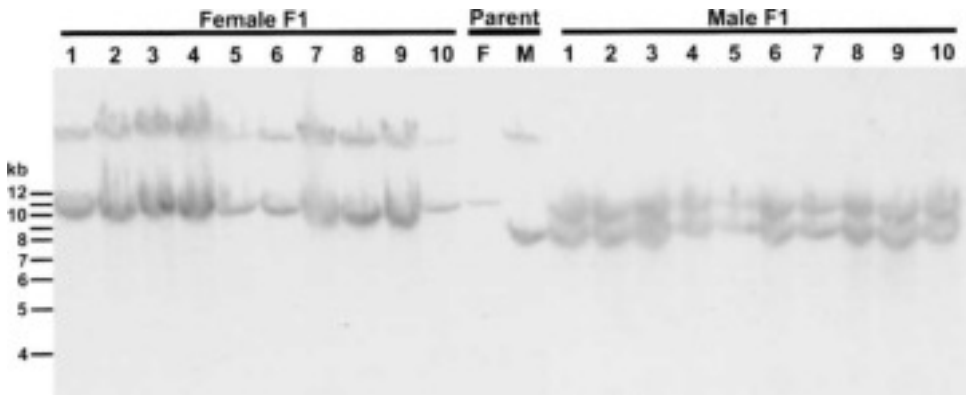


FIGURE 1.—Segregation analysis of male-specific RFLPs in a family of 10 female F_1 and 10 male F_1 progeny obtained by crossing a 15X10 female parent (F) to a U9 male parent (M). Genomic DNAs (20 μg) from each individual were digested with *Eco*RI, separated on a 0.7% gel, and blotted. The Southern blot was probed with the 214-bp partial *DD44* differential display product. The Y-linked RFLP of *DD44* is ~8 kb and is inherited by all 10 male F_1 progeny. The X-linked RFLP of *DD44* is >12 kb and is inherited by all females.

thaliana sequence. *DD44* contains the conserved functional domain of the OSCP family between aa55 and aa224 (34.5% identity, 46% similarity to the pfam00213 consensus domain). The presence of this domain, and the high degree of similarity with other plant OSCPs, places *DD44* in this category of conserved proteins.

We tried to characterize the function of the OSCP in higher plants by screening for homozygous T-DNA insertion lines in the OSCP homolog of *A. thaliana* (At5g13450). BLAST analysis of At5g13450 against left border sequences of T-DNA insertion lines from the Salk Institute Genomic Analysis Library (SIGnAL; La Jolla, CA) identified a T-DNA insertion line (SALK_010674) with an insertion ~68 bp upstream of the start codon of At5g13450. T3 seed was obtained from the Arabidopsis Biological Resource Center (ARBC; Columbus, OH). No T-DNA insertion lines were found in the coding region of the gene. We tested 17 T3 progeny harvested after kanamycin selection for insertions in the At5g13450 promoter and identified 14 individuals heterozygous for the insertion and no individuals homozygous for the insertion (data not shown). The lack of homozygous insertions suggests that the OSCP of *A. thaliana*, and by homology *DD44*, is essential for proper growth and development in plants. To determine whether the T-DNA insertion in the At5g13450 promoter was embryo lethal or gametophytic lethal, we germinated 375 seeds from one of the T3 heterozygous insertion lines on Gamborg B-5 media with 50 μg/ml kanamycin. There were 191 kanamycin-resistant plants and 184 kanamycin-sensitive plants. The observed ratio significantly varies ($P < 0.001$) from the expected ratio of 2:1 resistant-to-sensitive plants expected for an embryo-lethal insertion; however, this ratio does not significantly vary from the expected ratio of 1:1 resistant-to-sensitive plants expected if the insertion is lethal to one gametophyte (HOWDEN *et al.* 1998). Therefore, the T-DNA insertion in the *A. thaliana* OSCP is gametophyte lethal, although this experiment does not differentiate between male and female lethality.

***DD44* is expressed in male and female tissues:** We tested whether *DD44* was expressed specifically in male tissues, as differential display is prone to false positives. We performed Northern analysis on total RNA isolated from either male or female leaves, mature flowers, and unopened floral buds, using the 214-bp *DD44* differential display product as a probe (Figure 2A). The *DD44* probe hybridized to a single 1.15-kb band that was present in all tissue types in both males and females. We also performed reverse transcription (RT)-PCR using primers designed to amplify the 214-bp *DD44* differential display sequence on cDNA synthesized from poly(A)$^+$ mRNA isolated from male and female leaves, mature flowers, and unopened floral buds, and also from apical meristems isolated from seedlings of mixed sex. *DD44* was amplified from all tissue types and in both sexes (Figure 2B). No products were observed in controls processed without reverse transcriptase (data not shown). Thus, *DD44* is expressed in males and females and expression is not tissue specific. This expression profile also suggests that *DD44* is performing a general "housekeeping" function, necessary throughout male and female plant development.

The PCR primers used to amplify the *DD44* differential display sequence also amplify a male-specific polymorphism from male genomic DNA. PCR from genomic DNA using these primers amplifies a doublet in males and only a single band in females (Figure 2B). The larger PCR product was determined to be linked to the Y chromosome by a segregation analysis of F_1 progeny from a cross of polymorphic parents (Figure 3A). This same doublet was specifically amplified from cDNA from all male tissues in our RT-PCR analysis, while only the lower, shared band was amplified in females (Figure 2B). The size difference between the male-specific upper band and the shared lower band is small enough (<20 bp) that it is not resolved using Northern analysis. These data indicate that males express both the Y-linked allele and the shared, X-linked allele of *DD44* in all tissues examined.

Genomic structure of *DD44X* and *DD44Y*: Initially, we deduced the genomic structure of *DD44* by sequencing

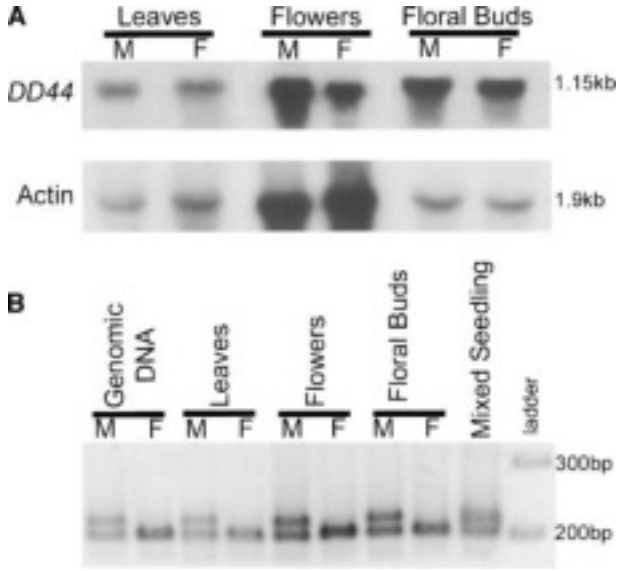


Figure 2.—Northern and RT-PCR expression analysis of *DD44*. (A) A Northern blot of total RNA isolated from male (M) and female (F) leaves, open flowers, and floral buds was probed with the 214-bp partial *DD44* differential display product. A 1.15-kb band was recognized in all tissues examined, regardless of sex, indicating *DD44* is not differentially expressed. (B) RT-PCR of the *DD44* differential display sequence from cDNA prepared from poly(A)$^+$ RNA from male and female leaves, mature flowers, floral buds, and mixed-sex seedling apical meristems amplifies a 214-bp band in all tissues regardless of sex. Furthermore, a doublet, the top band of which is male specific, is amplified in all samples from male tissue. Thus, both the X- and the Y-linked copies of *DD44* are expressed in males, and only the X-linked allele is expressed in females.

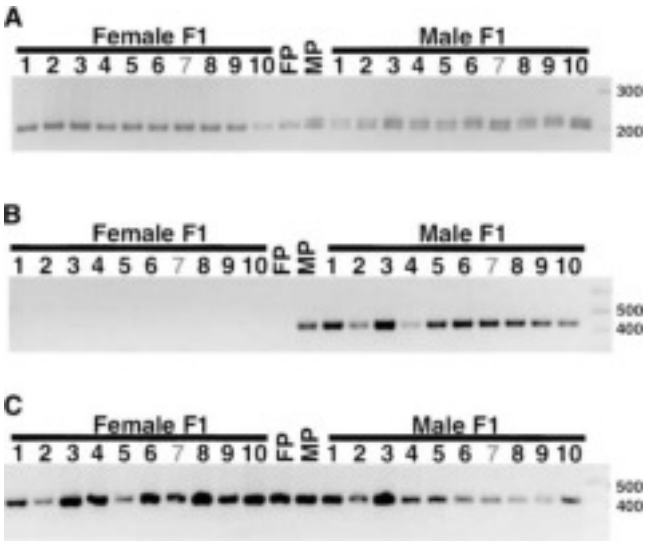


Figure 3.—Segregation analysis of Y-linked and X-linked PCR polymorphisms of *DD44*. Products were amplified from genomic DNA of a family of 10 female F_1 and 10 male F_1 progeny obtained by crossing a 15X10 female parent (FP) to a MR4X64 male parent (MP). (A) PCR using the primers DD44F1 and DD44R1 amplifies a doublet in males, the upper band of which is Y-linked and segregates only to male progeny. (B) PCR using primers DD44F1 and DD44YR2 amplifies a male-specific band that is Y-linked and segregates only to male progeny. The DD44YR2 primer was used to amplify the 3′ region of *DD44Y* from U9 male genomic DNA for sequencing. (C) PCR using primers DD44F1 and DD44XR2 amplifies a band in both the male and the female parent that is X-linked and segregates to all progeny. The DD44XR2 primer was used to amplify the 3′ region of *DD44X* from U9 female genomic DNA for sequencing.

an 8-kb region of an 18-kb genomic clone identified by screening a *S. latifolia* male genomic DNA phage library with the 214-bp *DD44* differential display sequence. The *DD44* sequence from the genomic clone contained six exons (totaling 693 bp) and five introns (totaling 3436 bp). However, we were interested in obtaining genomic sequence and structure for the *DD44* X- and Y-linked alleles. Therefore, we used TAIL PCR from total male genomic DNA to walk both 5′ and 3′ from the 214-bp *DD44* differential display sequence in hopes of identifying variable flanking sequences corresponding to the X and Y alleles of *DD44*.

We identified two alleles in the 3′ flanking region of *DD44* that were characterized by an ~40-bp indel. Reverse primers specific to each allele were designed and tested in conjunction with the forward primer found in the shared *DD44* partial cDNA sequence (DD44F1). One primer (DD44YR2) amplified a male-specific, 420-bp fragment that segregated only to F_1 male progeny; therefore, it amplified the Y-linked allele (Figure 3B). The other primer (DD44XR2) amplified a 400-bp fragment from both males and females and segregated to all progeny regardless of sex; therefore, it amplified the X-linked allele (Figure 3C).

To obtain the complete genomic sequence and structure of the *DD44* X- and Y-linked alleles (*DD44X* and *DD44Y*), we performed three sequential PCR amplifications of three regions of *DD44* using *DD44X*- or *DD44Y*-specific primers as described in materials and methods. Most of the genomic structure of *DD44X* and *DD44Y* is similar (Figure 4). However, there was surprising difference in the length of the intron 2 between *DD44Y* and *DD44X*. While intron 2 of *DD44X* was only 1.6 kb, it was 7.5 kb in *DD44Y*. Only the 5′ and 3′ flanking sequences of *DD44Y* intron 2 could be aligned with intron 2 sequence of *DD44X*. Blastx analysis of the 5′ region of the internal region of intron 2 specific to *DD44Y* showed similarity (E value = 1×10^{-16}) to a retrotransposon polyprotein from the flatworm, *Schistosoma japonicum* (accession no. AAK14815).

Deletion mapping of *DD44*, *SlY1*, and *SlY4* on the Y chromosome: We used the Y chromosome AFLP-deletion map developed by Lebel-Hardenack *et al.* (2002) to map the location of *DD44* and the previously published Y-linked genes *SlY1* and *SlY4* (Delichère *et al.* 1999; Atanassov *et al.* 2001) on the Y chromosome with respect to the sex-determining loci. We performed PCR from genomic DNA isolated from the Y-deletion

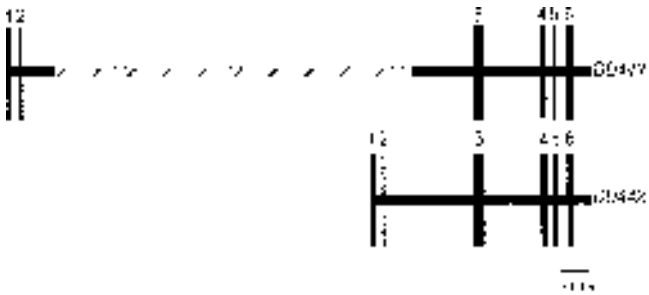


FIGURE 4.—Genomic structure of *DD44X* and *DD44Y*. *DD44X* and *DD44Y* each have six exons (numbered) and five introns. Interestingly, *DD44Y* has a 7.5-kb intron 2, whereas the corresponding intron is 1.6 kb in *DD44X*. The crosshatched area of intron 2 of *DD44Y* cannot be aligned with intron 2 of *DD44X*.

mutants using primers designed to amplify the Y-linked allele of each gene. We then scored for the presence or absence of the Y-specific PCR product in each mutant. This information was used to place the Y-specific allele onto the Y-deletion map with respect to the known AFLP markers (Figure 5).

For mapping *DD44Y*, we scored for the presence or absence of the two *DD44* Y-linked PCR polymorphisms (Figure 3, A and B) in the Y-deletion mutants. Both Y-linked PCR polymorphisms were absent in 10 of the hermaphrodite mutants, but present in all sterile mutants. *DD44* was significantly associated with the carpel-suppressing locus on the basis of chi-square and Fisher's exact test ($Pr = 9.76 \times 10^{-4}$), and *DD44* has a LOD > 3 of being linked to the group A linkage group of markers, which contains the carpel-suppressing locus. Specifically, the *DD44* Y-linked polymorphisms were missing in hermaphrodite H10, but present in mutant H115, thus placing *DD44* between markers L10 and L9 on the AFLP deletion map (Figure 5). The carpel-suppressing locus is deleted from the Y chromosome of hermaphrodite mutants and dominantly inhibits carpel formation in males (LARDON *et al.* 1999a). Classical cytological studies on Y-deletion mutants by WESTERGAARD (1958), as well as more recent molecular investigations (DONNISON *et al.* 1996; FARBOS *et al.* 1999; LARDON *et al.* 1999b), have placed the carpel-suppressing locus on the p arm of the Y chromosome. Therefore, the association of *DD44Y* with the carpel-suppressing locus would place *DD44Y* on the p arm of the Y chromosome.

For mapping *SlY1*, we first designed primers that amplified either a 1.4-kb band (primers SlY1 + 11 and SLY1_4551R) or a 1-kb band (primers SLY1_4505F and SLY1_5813R) only from males in our three *S. latifolia* ecotypes (data not shown). Both primer sets show the same amplification pattern from our population of Y-deletion mutants (Figure 5). *SlY1* does not amplify from the hermaphrodite mutants UH15, UH4, and UH1, all of which are missing loci L7, L6, and L8 in the group B linkage group. Furthermore, *SlY1* does not amplify from the early sterile mutant US2, which is missing L7, and the late steriles MS63 and MS57, which are missing group B markers, as well as the group C markers. *SlY1* was not significantly associated with any sex-determination loci, although *SlY1* has a LOD > 3 of being linked to the group B linkage group of markers (Figure 5). Group B markers are not linked to sex-determining loci, although they are missing from many of the large-deletion late-sterile mutants.

To map *SlY4*, we used previously published Y-specific primers (ATANASSOV *et al.* 2001) that amplify a 600-bp male-specific band in our three *S. latifolia* ecotypes (data not shown). Amplification of *SlY4* from Y-deletion mutants was lacking in a number of late-sterile mutants (all but US9 and US11), which are missing group B and group C markers, as well as a group of early-to-intermediate mutants (US2, MS74, and MS10), two of which are missing group B markers. *SlY4* was significantly associated with the late stamen development locus on the basis of chi-square and Fisher's exact test ($Pr = 1.8 \times 10^{-4}$), and its nearest neighbors are the group C linkage group markers (pairwise LOD is between 1.5 and 1.8). Therefore, *SlY4* resides between markers L8 and L5 at the boundary of groups A and B (Figure 5).

Both *SlY1* and *SlY4* map to the opposite end of the Y-deletion map from *DD44*; however, neither gene corresponds to a sex-determining locus, although *SlY4* is deleted primarily in late-sterile mutants, the locus of which is thought to reside on the q arm of the Y chromosome (WESTERGAARD 1958; LARDON *et al.* 1999a). Both *SlY1* and *SlY4* were determined to be on the q arm of the Y chromosome on the basis of deletion mapping in an independent set of mutants (DELICHÈRE *et al.* 1999; ATANASSOV *et al.* 2001). This would physically place *SlY1* and *SlY4* on the opposite arm of the Y chromosome as *DD44*.

Physical mapping of *DD44* on the sex chromosomes using fluorescence *in situ* hybridization: We were able to physically map the location of *DD44* on the *S. latifolia* sex chromosomes using the technique of fluorescence *in situ* hybridization (FISH), using the 18-kb *DD44* genomic clone as a probe. A Southern blot of the genomic clone digested with *Eco*RI, *Hin*dIII, or *Bam*HI probed with radioactively labeled total male genomic DNA gave only a weak hybridization signal (data not shown), indicating our clone did not contain highly repetitive DNA sequences. We subsequently subcloned the 18-kb genomic fragment into pBR322 for ease of manipulation and probe labeling. The 18-kb *DD44* genomic clone was used as a probe for FISH in metaphase chromosome spreads prepared from protoplasts derived from mitotically synchronized root tips of either *S. latifolia* seedlings or *S. latifolia* HR cultures. The *DD44X*-specific PCR primer pair (DD44F1/DD44XR2) amplified a band from our *DD44* genomic clone; thus our genomic clone represents an X-linked allele (data not shown).

The *S. latifolia* diploid genome consists of two pairs

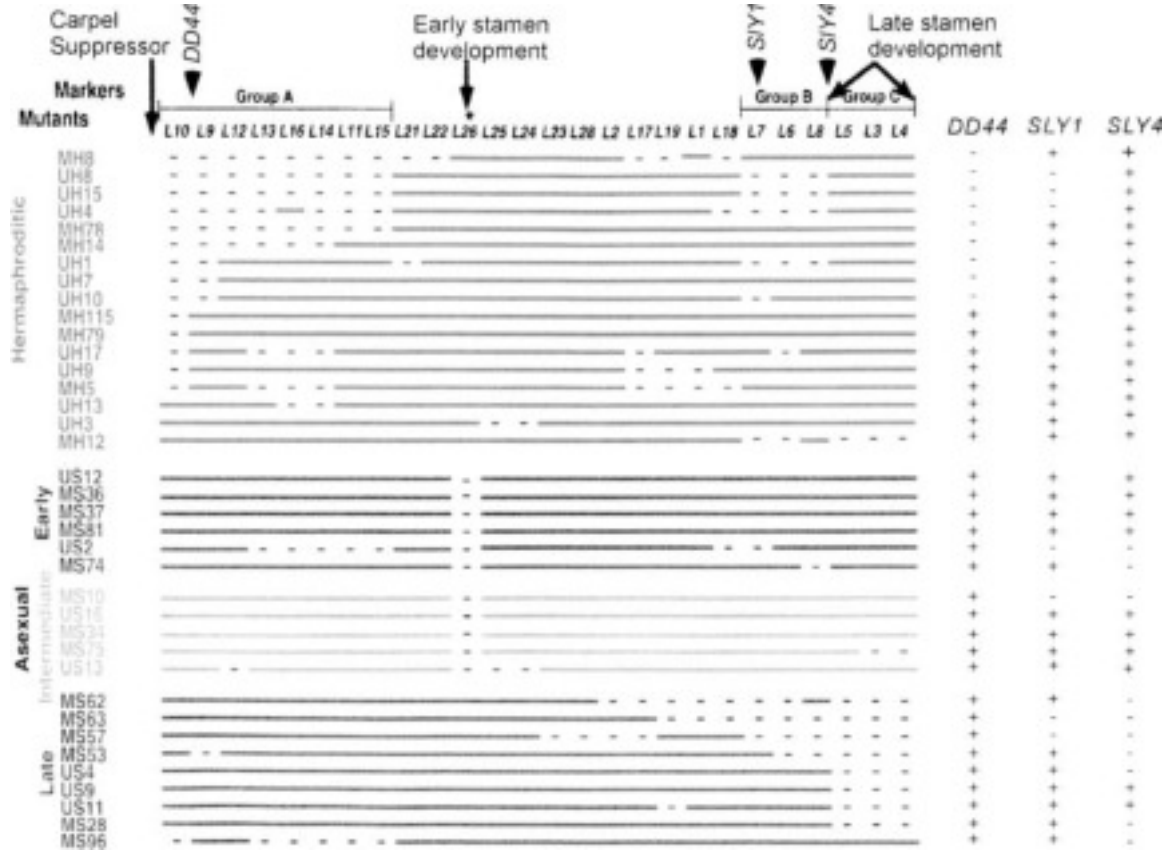


FIGURE 5.—AFLP deletion map of the *S. latifolia* Y chromosome showing relative positions of *DD44Y*, *SlY1*, and *SlY4* as determined by PCR analysis from genomic DNA of Y-deletion mutants. The map is arranged according to LEBEL-HARDENACK *et al.* (2002), with mutant identifiers on the left vertical axis (M, MR4X64 ecotype; U, U9 ecotype; H, hermaphrodite; S, sterile), and AFLP loci (L nos.) on the top horizontal axis. To the right of the map are the PCR results for each Y-linked gene; Y deletion mutants were scored for the presence (+) or absence (−) of Y-linked PCR products. The most parsimonious positions of each gene with respect to AFLP loci are indicated at the top, as well as loci associated with sex determination. *DD44* is missing from the majority of hermaphrodite mutants, but from none of the asexual mutants, and maps in linkage group A between markers L10 and L9. *SlY1* is missing from 7 out of 10 mutants lacking the L7 locus, and therefore we placed it at this locus in linkage group B. *SlY4* is predominantly absent in late-sterile mutants and maps between markers L8 and L5 of linkage groups B and C.

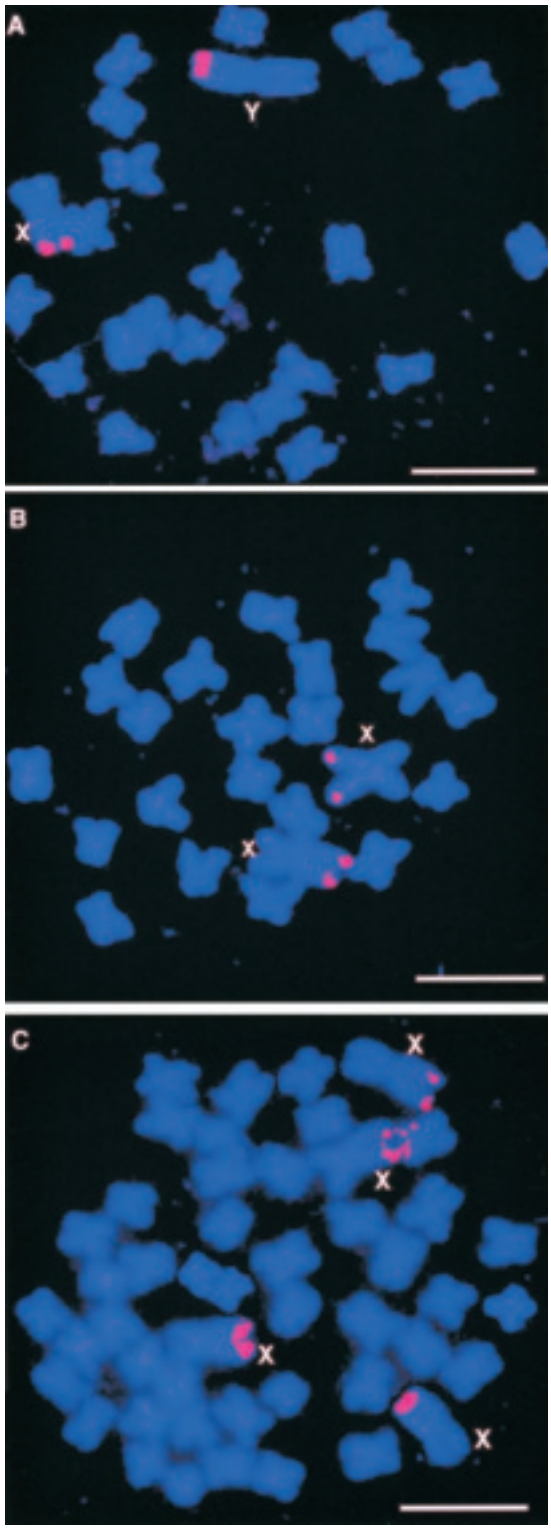
of 11 autosomal chromosomes and two larger sex chromosomes: Females have two submetacentric X chromosomes and males have one X and one larger, metacentric Y chromosome (CIUPERCESCU *et al.* 1990). FISH, using the 18-kb *DD44* genomic clone as a probe, shows fluorescent hybridization signals in the distal region of one arm of both the X and the Y chromosomes of males (Figure 6A), to both X chromosomes in a diploid female (Figure 6B), and to all four X chromosomes of a tetraploid female (Figure 6C). These results support the conclusion drawn from our segregation analysis that *DD44* is sex linked. Furthermore, these FISH data physically map *DD44* to the distal region of one arm of the sex chromosomes.

In metaphase X chromosomes, the identification of the p and q arms is relatively straightforward, as the X chromosome is submetacentric (arm ratio = 1.44 ± 0.15; CIUPERCESCU *et al.* 1990). It is clear from Figure 6, B and C, that *DD44* hybridizes to the longer arm, or q arm, of the X chromosome. This conclusion is further supported by FISH analysis of *DD44* in the female diploid HR line that has a deletion of the distal end of the q arm, as determined by DAPI banding patterns (Figure 7A). In this line, *DD44* does not hybridize to the X chromosome carrying the distal q deletion (Figure 7A).

Cytological identification of which arm the Y chromosome *DD44* binds to is more difficult as it is metacentric (arm ratio = 1.09 ± 0.04; CIUPERCESCU *et al.* 1990), and it is not possible upon visual inspection to differentiate the p and the q arms. FISH was performed in the male HR line where one arm of the Y is missing to address this issue. Our *DD44* genomic probe hybridizes to the distal end of the remaining Y chromosome arm, suggesting that *DD44Y* is not deleted in this line (Figure 7B).

To identify which arm remained in this Y-deletion HR line, we performed PCR from DNA isolated from the HR Y-deletion line, as well as the male line with no deletion, using four Y-specific markers: *DD44Y*, *Bgl10*, *SlY1*, and *SlY4*. PCR analysis using *DD44Y*-specific prim-

ers amplifies a Y-specific band from the Y-deletion HR line (Figure 8, A and B). Additionally, the Y-specific marker *Bgl10*, which is found in the same linkage group as the carpel-suppressing factor (Lebel-Hardenack *et al.* 2002), also amplifies from this Y-deletion mutant (Figure 8C). However, both *SlY1* and *SlY4* Y-specific primers do not amplify any bands from this Y-deletion HR line (Figure 8, D and E). We suggest that it is the p arm that remains in this Y-deletion HR line, as both *SlY1* and *SlY4* have previously been mapped to the q arm of the Y chromosome (Delichère *et al.* 1999; Atanassov *et al.* 2001). Thus, we predict that *DD44Y* hybridizes to the distal p arm of the Y, whereas *DD44X* hybridizes to the distal q arm of the X.



Divergence of *DD44X* and *DD44Y*: We were interested in assessing the nucleotide divergence between *DD44X* and *DD44Y* to see whether or not both of these alleles showed signs of selective constraint. We performed an alignment of all coding regions, introns (parts of intron 2 and intron 3 do not align and were removed from the analysis), and 311 bp of the 3′ flanking region, to compare the *DD44X* and *DD44Y* sequences. Table 1 summarizes the total number of sites examined, the numbers of synonymous and nonsynonymous substitutions for each exon, the numbers of substitutions for each intron, and the nucleotide divergence for each region. The noncoding regions show a divergence of ~7%. The results for the coding sequences can be summarized in terms of synonymous and nonsynonymous substitutions per site, K_s and K_a, respectively (Table 1). The exons show a silent site divergence of ~9.6%, slightly higher than that for intron sites, and a nonsynonymous divergence of 2.5%. Exon 1 shows very high nucleotide divergence ($K_{Total} = 0.15$, $K_s = 0.27$) compared to the other exons.

The silent site divergence between *DD44X* and *DD44Y* is similar to that for the three other *S. latifolia* X/Y gene pairs compared (whose K_s values range from 4% for *SlX/Y1* to 18% for *SlX/Y4* and 20% for a short region of sequence that can be aligned between *MROS3-X* and *-Y*). This shows that *DD44X* and *DD44Y* have been separated by a similar evolutionary time to the other sex-linked gene pairs. The analysis also shows that *DD44X* and *DD44Y* are under selective constraint. K_a/K_s ratios for divergence between expressed genes in plants, either between homologous genes in different species or between similar paralogous genes in the same species, are commonly between 10 and 20% (*e.g.*, Li 1997; Lagercrantz and Axelsson 2000). Values considerably <1 are expected if both genes were actively expressed, whereas if the Y-linked copy were not expressed as a functional protein, a value close to 1 could be found,

Figure 6.—FISH using the 18-kb *DD44* genomic probe on *S. latifolia* mitotic chromosomes. *DD44* hybridization signals are in red, and chromosomes are counterstained with DAPI. Mitotic spreads from (A) a standard male, (B) a standard female, and (C) a tetraploid female with sex chromosomes indicated (X and Y) are shown. The *DD44* genomic probe hybridizes to the distal end of one arm of either the X or the Y chromosome. A and B are mitotic spreads from root tips of germinating seedlings, and C is from the tetraploid female hairy root culture. Bar, 10 μm.

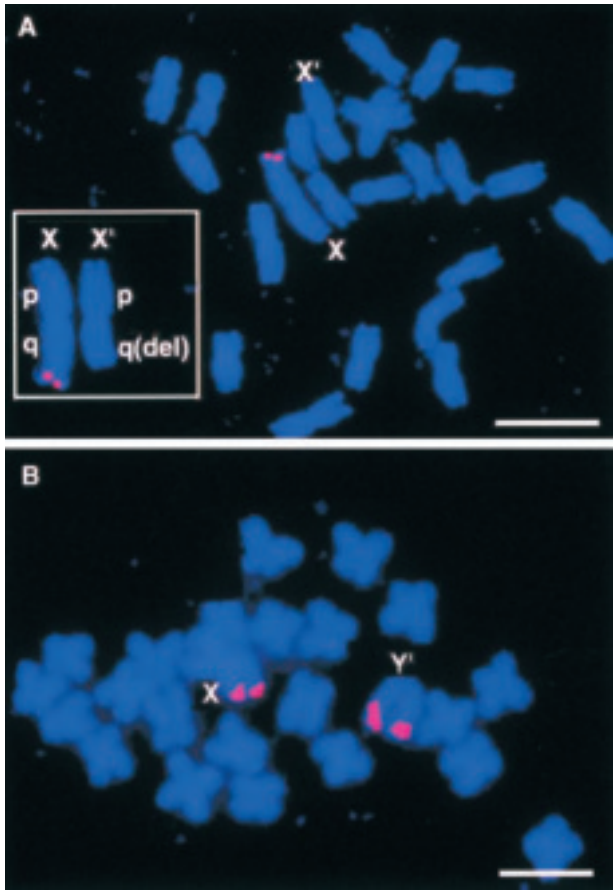


Figure 7.—FISH using the 18-kb *DD44* genomic probe on *S. latifolia* mitotic chromosomes with deletions in parts of the X or Y chromosome (indicated by X′ or Y′). *DD44* hybridization signals are in red, and chromosomes are counterstained with DAPI. (A) Mitotic spread from the female hairy root culture with a deletion of the distal end of the q arm on one X chromosome (as determined by DAPI banding). The *DD44* genomic probe hybridizes to the distal end of the q arm of one X chromosome, but not to the distal end of the arm of the X chromosome containing the deletion (see inset). (B) Mitotic spread from the male hairy root culture with a deletion of one arm of the Y chromosome and an intact X chromosome. The *DD44* genomic probe hybridizes to the distal end of the intact X chromosome and to the distal end of the remaining arm of the Y chromosome. Bar, 10 μm.

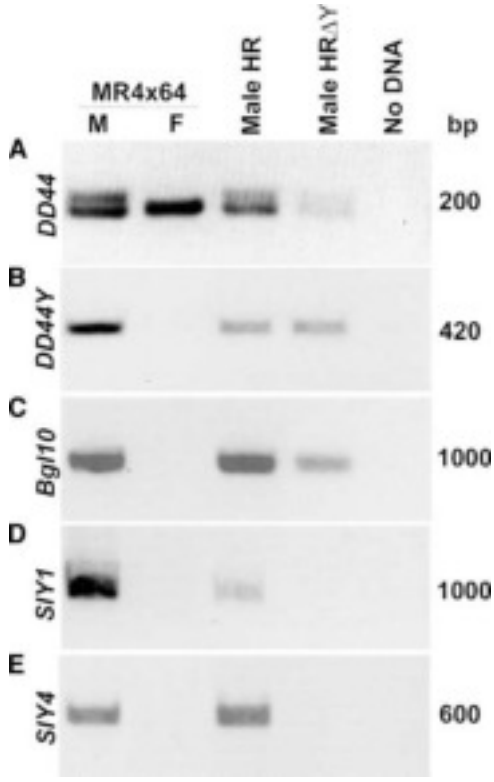


Figure 8.—PCR amplification of Y-specific sequences for *DD44*, *Bgl10*, *SlY1*, and *SlY4* from genomic DNAs isolated from an MR4X64 ecotype male (M) and female (F), the male HR line with a normal Y chromosome, and the male HR line that has a deletion of one arm of the Y chromosome. (A and B) PCR amplification with (A) the primers DD44F1/DD44R1 (*DD44*) or (B) DD44F1/DD44R2 (*DD44Y*) produces the characteristic male-specific band in all lines tested. (C) Similar results were obtained with the Y-specific PCR marker *Bgl10*. In contrast, both (D) *SlY1* and (E) *SlY4* amplified only from the male genomic DNA and from genomic DNA of the male HR line with a normal Y chromosome; neither amplified from genomic DNA of the male HR line with a deletion of one arm of the Y chromosome.

as in the case of *MROS3-Y vs. -X* (Guttman and Charlesworth 1998). For *DD44X/Y*, the K_a/K_s ratio is 0.26. Thus, the sequence analysis supports our results described above in suggesting that *DD44Y* is functional. The K_a/K_s for *DD44X/Y* is higher than that published for two other sex-linked genes, *SlX/Y1* ($K_a/K_s = 0.045$; Atanassov *et al.* 2001) and *SlX/Y4* ($K_a/K_s = 0.166$; Atanassov *et al.* 2001).

DISCUSSION

***DD44* is a class 1, Y-linked gene:** The simple linkage pattern and universal expression patterns of *DD44* are analogous to the class 1 group of genes of the nonrecombining region of human Y chromosome. These Y-linked genes are single-copy genes with functional X homologs, the expression of which is not developmentally restricted (Lahn *et al.* 2001). There are two other classes of Y-linked genes: class 2, which does not have functional X homologs, is typically multicopy, and is expressed specifically in developing male testis, and class 3, which does not fit either profile for the first two classes and includes the *SRY* sex-determining gene (Lahn *et al.* 2001).

Class 1 Y-linked genes are considered to be housekeeping genes and are thought to be involved in fundamental cellular processes (Lahn *et al.* 2001). We propose that *DD44* encodes a presumptive housekeeping gene. *DD44* is highly similar on the protein level to the δ-subunit of the mitochondrial ATP synthase from the sweet potato,

TABLE 1

Total number of sites examined, the number of silent and nonsynonymous substitutions, and sequence divergence between *DD44X* and *DD44Y* for each exon and intron region

				No. of mutations			Sequence divergence		
Coding sequence	Sites	Silent sites	Nonsynonymous sites	Total mutations	Silent mutations	Nonsynonymous mutations	All sites (K_{Total})	Silent sites (K_s)	Nonsynonymous sites (K_a)
Exon 1	84	21.83	62.17	13	7	6	0.155	0.275	0.113
Exon 2	45	9.33	35.67	0	0	0	0	0	0
Exon 3	244	56.5	187.5	11	6	5	0.045	0.097	0.027
Exon 4	113	28.33	84.67	3	2	1	0.027	0.071	0.012
Exon 5	87	20.33	66.67	1	0	1	0.012	0	0.015
Exon 6	117	25.83	91.17	2	2	0	0.017	0.077	0
Total coding	690	162.15	527.85	30	17	13	0.043	0.096	0.025
Noncoding sequence	Sites	Silent sites	Nonsynonymous sites	Mutations	Silent mutations	Nonsynonymous mutations	All sites (K_T)		
Intron 1	112	112	0	12	12	0	0.107		
Intron 2	1572	1572	0	115	115	0	0.073		
Intron 3	643	643	0	43	43	0	0.067		
Intron 4	91	91	0	7	7	0	0.077		
Intron 5	158	158	0	6	6	0	0.038		
3′ flanking region	311	311	0	22	22	0	0.071		
Total noncoding	2887	2887	0	205	205	0	0.071		

I. batatas, which is homologous to the OSCP in other eukaryotes (Kimura *et al.* 1990). The OSCP is a component of the stalk of the ATP synthase complex and functions as a link between the F_1 catalytic domain and the F_0 proton channel (Walker and Collinson 1994). The *Escherichia coli* ATP synthase δ-subunit, which is homologous to the eukaryotic OSCP, is characterized by a six-α-helix bundle in the N terminus, which interacts with the F_1 core, and a less-defined C-terminal domain, which is required for binding to the F_0 structure (Wilkens *et al.* 1997). Yeast with a null mutation in the OSCP cannot grow on media with a nonfermentable carbon source, suggesting the OSCP is necessary for oxidative phosphorylation and essential for proper growth and development (Uh *et al.* 1990; Prescott *et al.* 1994).

In plants, characterization of the structure and function of the mitochondrial OSCP has been examined in *I. batatas*, where the OSCP is represented by many isoforms encoded by multiple genes in the hexaploid genome (Kimura *et al.* 1990; Morikami *et al.* 1992). Expression studies by Maeo *et al.* (1999) show that the promoters of two OSCP isoforms from sweet potato, F_1δ-1 and F_1δ-2, drive expression of the β-glucuronidase (GUS) gene in transgenic tobacco in all tissues examined and that expression is strongest in the vascular tissue of leaves, stems, roots, and in the meristematic region of roots. These expression patterns correlate with cells with large numbers of mitochondria, such as the companion cells of phloem tissue (Maeo *et al.* 1999).

There are two other published Y-linked genes of *S. latifolia* with similar properties to *DD44*: *SlY1* and *SlY4* (Delichère *et al.* 1999; Atanassov *et al.* 2001). Both genes are single copy with functional X homologs (*SlX1* and *SlX4*), and both are similar to genes with cellular housekeeping functions: *SlY/X1* are members of the large WD-repeat family of proteins (Delichère *et al.* 1999), and *SlY/X4* are putative fructose-2,6-bisphosphatases (Atanassov *et al.* 2001). Similarly to *DD44*, both the X and the Y alleles of *SlX/Y1* and *SlX/Y4* are expressed in males, and the X alleles are actively expressed in females (Delichère *et al.* 1999; Atanassov *et al.* 2001). However, *SlX/Y1*, unlike *DD44* and *SlX/Y4* (Atanassov *et al.* 2001), show some tissue-specific expression patterns, being primarily expressed in the floral tissues, with only weak expression in vegetative tissues (Delichère *et al.* 1999). Thus, both *SlY1* and *SlY4* fall into the class 1 class of Y-linked genes, similar to *DD44*. Interestingly, the K_a/K_s value of *DD44X/Y* (0.235) is greater than that of *SlX/Y4* ($K_a/K_s = 0.166$; Atanassov *et al.* 2001) and *SlX/Y1* ($K_a/K_s = 0.045$; Atanassov *et al.* 2001), although all values are relatively low. Low K_a/K_s values are indicative of functional constraint. Such low

K_a/K_s values are characteristics of class I genes that have escaped genetic deterioration (Lahn and Page 1999a).

Genetic and physical mapping of *DD44* provides evidence for rearrangements on the *S. latifolia* Y chromosome: We used two approaches to determine the position of *DD44* on the Y chromosome. Physical and genetic mapping data support the localization of *DD44Y* to the distal region of the p arm of the Y and *DD44X* to the distal region of the q arm on the X. We hypothesize that this difference is due to a chromosomal rearrangement on the Y chromosome.

Chromosomal rearrangements are thought to have played a central role in the evolution of human sex chromosomes (Graves *et al.* 1998; Lahn and Page 1999a). In particular, a series of at least four chromosomal inversions are thought to have occurred over the 310 million years of human sex chromosome evolution (Lahn and Page 1999a). These inversion events have contributed to the genetic isolation and subsequent deterioration of the human Y chromosome (Lahn *et al.* 2001). We do not think a large inversion event is responsible for the rearrangement of *DD44Y* relative to *DD44X* for two reasons. First, the X-linked copy of *DD44* has been genetically mapped to a large interval between the X-linked markers, *SlX1* and *SlX4* (V. Hykelova and V. Laporte, personal communication). Our Y-mapping data separate *DD44Y* from *SlY1* and *SlY4* onto separate arms. Thus, it is possible that a small-scale chromosomal rearrangement, such as a transposition, may be responsible for the separation of *DD44Y* from *SlY1* and *SlY4*.

Second, our analysis of the sequence divergence between *DD44X* and *DD44Y* does not support a large chromosomal rearrangement event leading to the isolation of *DD44Y* from *SlY1* and *SlY4*. Specifically, the K_s value of *DD44X/Y* (0.095), a rough measure of evolutionary distance, is intermediate between that of *SlX/Y1* (K_s = 0.04; Atanassov *et al.* 2001) and *SlX/Y4* (K_s = 0.181; Atanassov *et al.* 2001). These data do not resolve clear evolutionary strata on the *S. latifolia* sex chromosomes, like those observed in human sex chromosomes (Lahn and Page 1999a), because the K_s values of these *S. latifolia* sex-linked genes are relatively low. These values fall within the range of K_s values of sex-linked genes of the most recently isolated stratum on the human X chromosomes ($K_s < 0.23$, estimated by a molecular clock to correspond to 30–50 mya). The *S. latifolia* sex chromosomes are either slightly or considerably (*SlX/Y1*) less diverged than this and probably younger (estimated age between ~8 and 24 mya). Therefore, the *S. latifolia* sex-linked genes may have diverged at the same time due to a single isolation event, with the differences in K_a values being due to differences in functional constraint and with perhaps a second, more recent, event accounting for the low *SlX/Y1* silent divergence. If these genes are part of the same evolutionary stratum, then the localization of *DD44Y* on the opposite arm of the Y from *SlY1* and *SlY4* may be due to a small-scale rearrangement event after the initial, large-scale chromosomal rearrangement.

Is the *S. latifolia* Y chromosome functionally coherent? The human Y is considered by some to be functionally coherent (Lahn and Page 1997; Lahn *et al.* 2001); it has accumulated and maintained genes involved in male sex differentiation and development, and these genes are arranged in discrete functional units along the Y. This hypothesis is largely supported by the identification and mapping of testis-specific genes on the Y chromosome that have no X homolog, also known as class 2 genes (Lahn and Page 1997; Lahn *et al.* 2001). Some of these genes are thought to have resided on an autosomal ancestor of the Y, while others, such as *CDY* and *DAZ*, translocated to the Y chromosome (Saxena *et al.* 1996; Lahn and Page 1999b; Lahn *et al.* 2001). Has the evolutionarily young *S. latifolia* Y chromosome developed functional coherency, or is functional coherence a trait that is acquired only late in the evolution of the Y chromosome?

To date, only class 1 Y-linked genes have been characterized on the Y (*DD44Y*, *SlY1*, and *SlY4*). Importantly, no class 2 genes, those expressed specifically in males and that have no X homologs, have been identified for the *S. latifolia* Y chromosome. Other Y-linked genes reported for *S. latifolia* include *MROS3*, which has a Y-linked pseudogene (Guttman and Charlesworth 1998); ORF285, which is not actively transcribed (Nakao *et al.* 2002) and is similar to a putative non-LTR reverse transcriptase in *A. thaliana* (*E* value = 1×10^{-12}; accession no. NP_178768); and a number of sequences that share similarity to retrotransposons and repetitive elements from plants and animals (Donnison and Grant 1999). Cytogenetic and molecular analyses of *S. latifolia* hermaphrodite and asexual mutants with Y deletions suggest at least three genes exist on the Y that are involved in the differentiation and development of male sex organs (Westergaard 1958; Donnison *et al.* 1996; Farbos *et al.* 1999; Lardon *et al.* 1999b); however, of the >50 male-specific genes that have been reported for *S. latifolia*, (Matsunaga *et al.* 1996, 1997; Barbacar *et al.* 1997; Lebel-Hardenack *et al.* 1997; Robertson *et al.* 1997; Scutt *et al.* 1997, 2002), only two are potentially Y linked (Scutt *et al.* 2002). Also, there is evidence that a functional homolog of the carpel-suppressing locus exists on the autosomes (Farbos *et al.* 1999). Thus, current evidence suggests that the gene composition of the *S. latifolia* Y chromosome is not heavily weighted with genes involved in male development and does not fit the criteria for functional coherency. This is possibly because *S. latifolia* is evolutionarily young and that functional coherency is a trait acquired only late in the evolution of the Y chromosome. However, the final word on this issue can be made only after the identification and mapping of more sex-linked genes in *S. latifolia*.

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