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Localization of Male-Specifically Expressed *MROS* Genes of *Silene latifolia* by PCR on Flow-Sorted Sex Chromosomes and Autosomes

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ABSTRACT

The dioecious white campion *Silene latifolia* (syn. *Melandrium album*) has heteromorphic sex chromosomes, XX in females and XY in males, that are larger than the autosomes and enable their separation by flow sorting. The group of *MROS* genes, the first male-specifically expressed genes in dioecious plants, was recently identified in *S. latifolia*. To localize the *MROS* genes, we used the flow-sorted X chromosomes and autosomes as a template for PCR with internal primers. Our results indicate that the *MROS3* gene is located in at least two copies tandemly arranged on the X chromosome with additional copy(ies) on the autosome(s), while *MROS1*, *MROS2*, and *MROS4* are exclusively autosomal. The specificity of PCR products was checked by digestion with a restriction enzyme or reamplification using nested primers. Homology search of databases has shown the presence of five *MROS3* homologues in *A. thaliana*, four of them arranged in two tandems, each consisting of two copies. We conclude that *MROS3* is a low-copy gene family, connected with the proper pollen development, which is present not only in dioecious but also in other dicot plant species.

THE majority of flowering plants are hermaphrodites, forming flowers with both female and male reproductive organs. Approximately 11% of plant species have unisexual flowers and 4% are dioecious with separate female and male individuals (for review, see Grant *et al.* 1994). Heteromorphic sex chromosomes, which in plants are usually larger than autosomes, have evolved in only a few species like *Silene latifolia* or *Rumex acetosa*. Little is currently known about a molecular mechanism of sex determination in dioecious plants.

S. latifolia has recently become a popular model to study dioecy and evolution of plant sex chromosomes (for review, see Charlesworth and Guttman 1999; Monéger *et al.* 2000). It possesses a pair of heteromorphic sex chromosomes; females are homogametic ($2n = 24$, XX) and males heterogametic ($2n = 24$, XY). The pair of sex chromosomes, X and Y, represents ~16% of the total size of the male diploid genome (Matsunaga *et al.* 1994). The Y chromosome is 1.4 times larger than the X, which in turn is 1.6 times larger than the longest pair of autosomes and 1.9 times bigger than the average size of autosomes (calculated according to Ciupercescu *et al.* 1990). The intrinsic differences between autosomes and X and Y chromosomes make it possible to discriminate and sort individual chromosome types by flow cytometry (Veuskens *et al.* 1992). It is assumed that *S. latifolia* sex chromosomes evolved recently in contrast with the ancient origin of mammalian or insect sex chromosomes, thus offering the opportunity to study the early stages of sex chromosome evolution.

A number of research groups have recently isolated sex-specifically expressed or sex chromosome-linked genes and other DNA sequences in an attempt to find sex-determining genes in *S. latifolia*. They used (i) cDNA or genomic library subtraction methods (Donnison *et al.* 1996; Matsunaga *et al.* 1996; Barbacar *et al.* 1997; Robertson *et al.* 1997; Scutt *et al.* 1997; Scutt and Gilmartin 1998); (ii) microdissected and degenerate oligonucleotide-primed (DOP)-PCR amplified sex chromosomes as probes for screening of the cDNA library (Delichére *et al.* 1999) or for FISH (Bužek *et al.* 1997; Scutt *et al.* 1997); and (iii) PCR-based methods such as randomly amplified polymorphic DNA (Mulcahy *et al.* 1992). Although these attempts often resulted in isolation of repetitive sequences (Donnison *et al.* 1996; Bužek *et al.* 1997; Scutt *et al.* 1997), a group of four male-specifically expressed genes, *MROS* genes, was discovered (Matsunaga *et al.* 1996, 1997). In addition, although not involved in sex determination, an active gene located on the Y chromosome, *SlY1*, with a functional homologue on the X, has been isolated (Delichére *et al.* 1999).

MROS (*m*ale *r*eproductive *o*rgan-*s*pecific) genes are

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specifically expressed in male reproductive organs (Matsunaga *et al.* 1996, 1997): pollen grains (*MROS1*), late stages of anther maturation (*MROS2*), mature anther tapetum (*MROS3*), and early male flower buds (*MROS4*). The *MROS* genes were isolated by differential screening of the cDNA library prepared from male flower buds with cDNA from male and female flower buds separately. The *MROS* genes are present both in female and male genomes, so they cannot be located exclusively on the Y chromosome (Matsunaga *et al.* 1996, 1997). Other research groups isolated these genes or their alleles independently; *Men-1* (Scutt *et al.* 1997) is homologous to *MROS1*, *Men-9* (Robertson *et al.* 1997) and *CCLS-4* (Hinnisdaels *et al.* 1997) are homologous to *MROS3*. Genetic analysis using a single-strand conformation polymorphism (SSCP) technique has indicated that only the *MROS3* gene is located on the X chromosome, with a degenerate homologue (pseudogene) on the Y chromosome, whereas *MROS1* and *MROS2* are autosomal (Guttman and Charlesworth 1998). Moreover, two genomic clones of *MROS3* were isolated recently and a single pollen PCR-based typing analysis suggests their localization either on the autosomes or in the homologous region of the X and Y chromosomes (Matsunaga *et al.* 1999).

Here we describe physical localization of *MROS1* to *MROS4* genes of *S. latifolia* by PCR on the flow-sorted X chromosomes and autosomes. We show that all these *MROS* genes are localized on the autosomes. In addition, at least two copies of *MROS3* are X-linked, organized in the head-to-tail tandem array. Our data from PCR experiments and database homology searching indicate that *MROS3* gene homologues are present also in other Silene species as well as in *A. thaliana*.

MATERIALS AND METHODS

Plant material: *S. latifolia* Poiret, *S. diclinis* (Lag.) Lainz, and *S. vulgaris* (Moench) Garcke plant material comes from the seed collection of the Institute of Biophysics, Brno, Czech Republic. As a routine source of *S. latifolia* mitotic chromosomes for flow sorting, hairy root cultures were established from a tetraploid female line after infection with *Agrobacterium rhizogenes*, strain A4RS (Široký *et al.* 1999). To synchronize the cell cycle in root tip meristems, aphidicolin (30 μM, Sigma, St. Louis) was added for 12 hr. Mitoses were then accumulated with 15 μM oryzalin (4 hr, Elanco).

Chromosome isolation and sorting: Synchronized root tips were cut 1 cm from the root tip, rinsed in distilled water, and fixed for 20 min at 5° in 2% (v/v) formaldehyde made in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, pH 7.5) supplemented with 0.1% Triton X-100 (Doležel *et al.* 1992). After three 5-min washes in Tris buffer, meristem tips (1 mm) of 200 roots were cut and transferred to a 5-ml polystyrene tube containing 1 ml LB01 lysis buffer (Doležel *et al.* 1989). The chromosomes were released by mechanical homogenization with a Polytron PT1200 homogenizer (Kinematica AG, Littau, Switzerland) at 15,000 rpm for 10 sec. The suspension was passed through a 50-μm pore size nylon mesh, stained with 4′6-diamidino-2-phenylindole (DAPI) at a final concentration of 2 μg/ml and analyzed at rates of 200–400 particles per sec using a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA). The cytometer was equipped with an argonion laser tuned to multiline UV and run with a 300-mW output power. The system threshold was set on the fluorescence pulse height (FL1-H) and the gate window was set on a dot plot of FL1-H *vs.* forward light scatter to eliminate debris with extremely high or low fluorescence intensity. To achieve the highest purity in sorted fractions, two-step sorting was employed (Lucretti *et al.* 1993). Sorting gates were set on a dot plot of fluorescence pulse area *vs.* fluorescence pulse width and at least 25,000 chromosomes were sorted at rates of 5–10 per sec into a polystyrene tube containing 400 μl of 1.5× LB01. Before the second sorting run, DAPI was added to the suspension, enriched for given chromosome type, and chromosomes were sorted at rates of 20 per sec. For the analysis of purity in sorted fractions using fluorescence *in situ* hybridization (FISH), 1000 chromosomes were sorted into a 15-μl drop of buffer containing 5% sucrose on microscope slides (Kubaláková *et al.* 2000). The slides were air dried after sorting and maintained at room temperature until use. For PCR, chromosomes were sorted into 33 μl of sterile deionized water in 0.5-ml PCR reaction tubes and stored at −70°.

Fluorescence *in situ* hybridization: As a FISH probe for 25S rDNA, an internal biotinylated 2.5-kb *Eco*RI fragment of 25S rRNA gene was used. The hybridization mix (20 μl per slide) consisted of 200 ng of the labeled probe, 6 μg autoclaved salmon sperm DNA (Serva), 4 μl of 50% solution of dextran sulfate (Sigma), 10 μl formamide (Sigma), and 2 μl of 20× SSC. Denatured probe was added to the denatured slides with sorted chromosomes and hybridized for 12 hr. After a stringent washing, biotin was detected by FITC-conjugated avidin (Vector, Burlingame, CA). FISH signals were observed under Olympus AX 70 fluorescent microscope.

PCR on sorted chromosomes: Before PCR, chromosomes were spun down and then 16 μl of master mix containing PCR buffer (Promega, Madison, WI), dNTP, $MgCl_2$, and primers were added. The final concentrations of the reagents were 0.2 mM dNTP, 0.2 μM primers, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.0. After initial denaturation at 94° for 10 min, temperature was decreased to 85° and 1 μl (5 units) of *Taq* polymerase (Promega) was added. DNA was amplified using 40 cycles (94°/40 sec–1 min, 50–60°/40 sec–1.5 min, 72°/1–2 min) with the final primer extension step at 72°/ 5 min. Temperatures and incubation times in PCR profile were varied depending on primers used. In all PCR experiments a PTC-200 thermal cycler (MJ Research) was used. Identity of PCR products was verified using restrictase digestion performed according to manufacturer's instructions. For reamplification with nested primers, 0.5 μl of original PCR product was used as a template for 20 cycles of PCR at conditions described above.

RESULTS

Flow sorting of chromosomes and purity of fractions: Cultures from a tetraploid female (Figure 1a) were used to obtain a higher number of metaphase X chromosomes and autosomes per cell without a risk of contamination with Y chromosomes. Flow cytometric analysis of chromosome suspensions clearly discriminated a dominant peak corresponding to the population of autosomes and a smaller peak corresponding to the X chromosomes (Figure 1b). While the fractions of sorted autosomes obviously did not contain the (larger) X

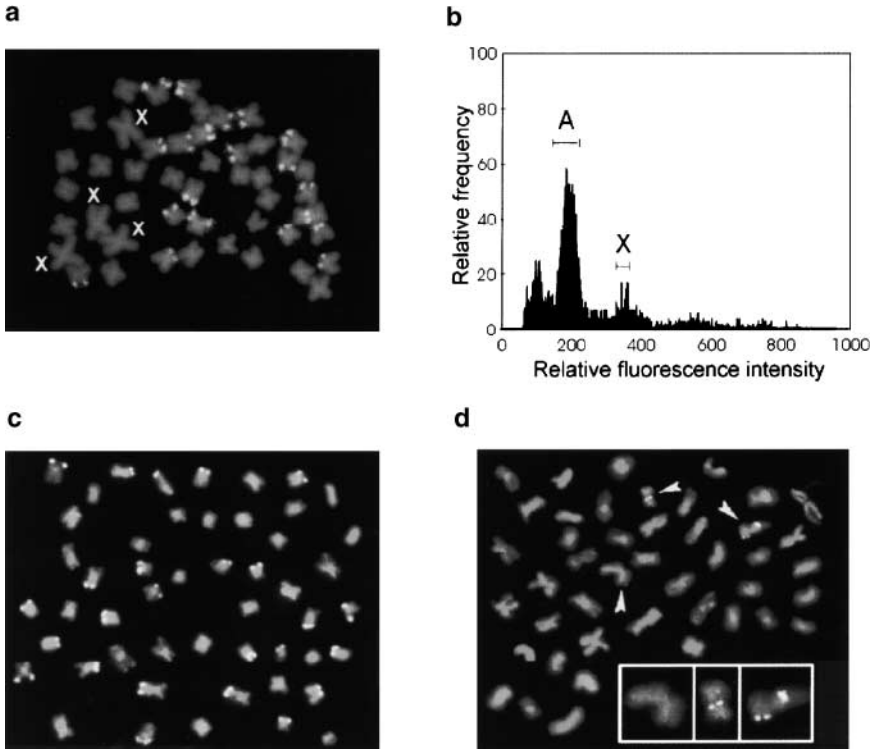

FIGURE 1.—Sorting of chromosomes and their purity. (a) Fluorescence *in situ* hybridization on tetraploid metaphase plate ($4n = 48$, 4X) used for the sorting. "X" indicates the X chromosomes; bright spots at the end of 10 pairs of autosomes show 25S rDNA locations. (b) Flow karyotype obtained after the analysis of DAPI-stained chromosome suspension. Fluorescence *in situ* hybridization on flow-sorted autosomes (c) and the X chromosomes (d) with biotin-labeled 25S rDNA probe (bright spots represent the hybridization signals). Inserts in d show three possible variants of contaminating autosomal doublets found—both autosomes without any 25S rDNA label (left), only one autosome with the 25S rDNA cluster (middle), and two autosomes with the 25S rDNA signals (right).

chromosomes or their separated chromatids (Figure 1c), sorted fractions of X chromosomes were contaminated to some extent with doublets of shorter autosomes (Figure 1d). To improve the purity of the X chromosome sorting, DNA content and chromosome length were analyzed simultaneously (see MATERIALS AND METHODS). Furthermore, a two-step sorting procedure was always used to ensure maximum purity. Chromosomal fractions were sorted in parallel into Eppendorf tubes for PCR analysis and onto microscopic slides to check their purity after DAPI staining and FISH.

Because of the high sensitivity of PCR, contamination of a chromosomal fraction lacking the gene in question with a few different chromosomes harboring this DNA sequence may result in a weak amplification fragment. To check the extent of cross-contamination by missorted chromosomes, we applied both chromosomal fractions (X chromosomes and autosomes) onto microscopic slides in parallel with flow sorting of chromosomes into Eppendorf tubes for PCR. The chromosomal fractions on slides were then hybridized with labeled 25S rDNA. While the 25S rDNA clusters are distally located on 5 of 11 autosome pairs (45%) they are absent from the sex chromosomes (MATSUNAGA *et al.* 1994). The presence of 10 25S rDNA clusters on the tetraploid material used is shown using the FISH technique (Figure 1a). Microscopic observations of 500 chromosomes of each fraction after the fluorescence *in situ* hybridization with 25S rDNA probe did not indicate a detectable "statistical" contamination of autosomes by X chromosomes because 45% of chromosomes in the autosomal fraction were 25S rDNA positive (Figure 1c). However, in the X chromosome fraction, all autosomes were present in the form of doublets (Figure 1d). We observed ~12% contamination of the X chromosomes by autosomes (44 X chromosomes and 6 autosomes in Figure 1d), *i.e.*, 1% contamination by each type of 11 different autosomes.

A hairy root culture prepared from a male plant was used as a source of the Y chromosomes. Unfortunately, microscopic analysis of slides with sorted chromosomes showed an abundant presence of chromosomal clumps (consisting mainly of two to four autosomes) in the Y interest sorting zone, which prevented the sorting of the Y chromosomes to a purity acceptable for PCR (not shown).

The *MROS1*, *MROS2*, and *MROS4* genes are autosomal: Flow-sorted autosomes and X chromosomes were used as templates for PCR with primers specific for the individual *MROS* genes (Table 1). Primers used for amplification of *MROS1*, *MROS2*, and *MROS4* were designed on the basis of the known cDNA nucleotide sequences (MATSUNAGA *et al.* 1996, 1997); in all cases the primer sites were located in coding regions of the *MROS* genes. The only exception was the MROS1-F5 primer, located in the *MROS1* promoter region. While the *MROS1* and *MROS4* genes possess introns, there are no introns in *MROS2*. In all PCR experiments, the autosomal fractions contained 11 times more chromosomes (1100) than the fraction of the X chromosomes (100) to ensure the same numbers of each specific chromosome (since $n = 11A + X$). Using the *MROS1* gene-specific

TABLE 1

List of genes, primers, and primer sequences used to amplify the *MROS* genes in this study

Gene	Primer	Primer sequence
MROS1	MROS1-F1	5′-CAA ATG GCT CTC TCA TTC GC-3′
	MROS1-R1	5′-GTA CAA CAC ACA CAC CCA TA-3′
	MROS1-F5	5′-CCG CTT GTG ACC AAC ATA TC-3′
MROS2	MROS2-F1	5′-ACT AGA AAT AAT GGG GTC AC-3′
	MROS2-R1	5′-GCA TGC ATT AAT CTC CCT AG-3′
MROS3	INF2[a]	5′-GGA ACC CAA TCA CGC TTG CA-3′
	R3X2[a]	5′-TCA AGC ACG ACG AAC AAA-3′
	INF1	5′-GAA TGA GAA CCA AGG TGA TAA TCG-3′
	INR1	5′-GAC TTT CTT TGG TGA CAT TT-3′
	BF1	5′-GAC CCA GTT GAA ATG ATC AC-3′
MROS4	MROS4-F1	5′-TAG TTG TGC AAA TGG CTC CCT-3′
	MROS4-R1	5′-TCC GAA ACA CAA TGG CCT TC-3′
	MROS4-F2	5′-TCA CCT ATA TAC CCC TTC AG-3′
	MROS4-R2	5′-CTT ACA TTG GCT GAA CTC GA-3′

[a] The primers INF2 and R3X2 according to Guttman and Charlesworth (1998).

primers MROS1-F5 and MROS1-R1, a single band of the expected size (1375 bp) was obtained with the sorted autosomes as a template, but not with the X chromosomes (Figure 2a). PCR with the *MROS2* gene-specific primers MROS2-F1 and MROS2-R1 also resulted in a specific band of 785 bp only in the autosomal fraction (Figure 2b), while PCR on the X chromosomes did not yield any product. Similarly, the primers designed for the *MROS4* gene led to amplification of a specific band of ~600 bp only in the autosomes (Figure 2c). Female and male genomic DNA representing positive controls as well as negative controls without any DNA template were always included. For all three pairs of primers, female and male templates yielded the same products as in the autosomal fractions, while no product was detected in blank controls.

Due to the cross-contamination of the X chromosomal fraction with autosomes (as demonstrated in Figure 1d), it was necessary to perform control PCR experiments with increasing numbers of sorted chromosomes as a template. PCR with primers MROS4-F1 and MROS4-R1, specific for the autosomal *MROS4* gene, resulted in a weak band of the expected size when only 55 autosomes (statistically representing 5 of each from 11 different autosomes) were used, while giving a strong band starting at 110 autosomes (Figure 3a). On the other hand, PCR with the same primer pair using an increasing number of X chromosomes resulted in a very weak band only when 500 X chromosomes were applied (Figure 3b). We believe that these data reflect contamination of 500 X chromosomes by <55 autosomes, thus representing 11% contamination at maximum.

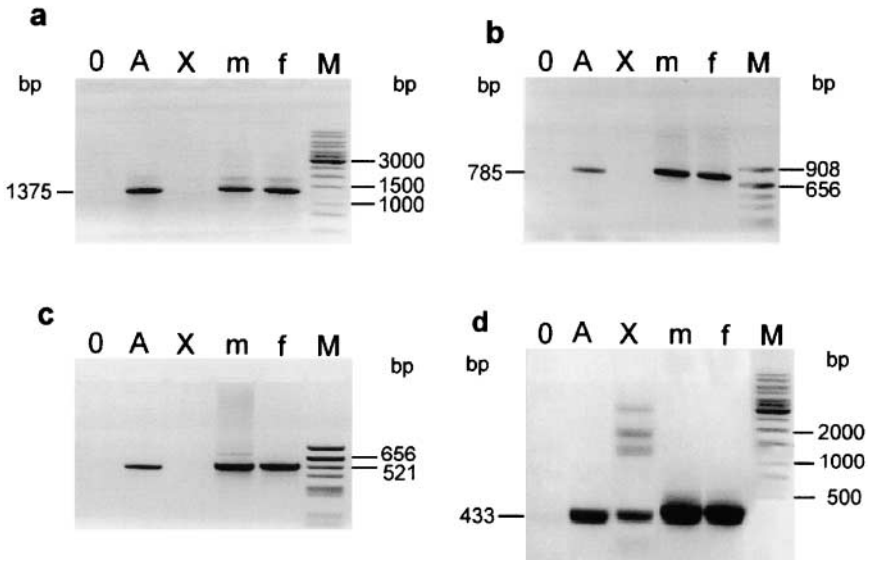

Figure 2.—Agarose gel electrophoresis of PCR products obtained with (a) *MROS1* gene-specific primers MROS1-F5 and MROS1-R1, (b) *MROS2* gene-specific primers MROS2-F1 and MROS2-R1, (c) *MROS4* gene-specific primers MROS4-F1 and MROS4-R1, and (d) *MROS3* gene-specific primers INF2 and R3X2. No template (0), 1100 autosomes (A), 100 X chromosomes (X), male genomic DNA (m), and female genomic DNA (f). M, DNA length markers: 1-kb ladder (a and d) and pBR322/*Alu*I (b and c).

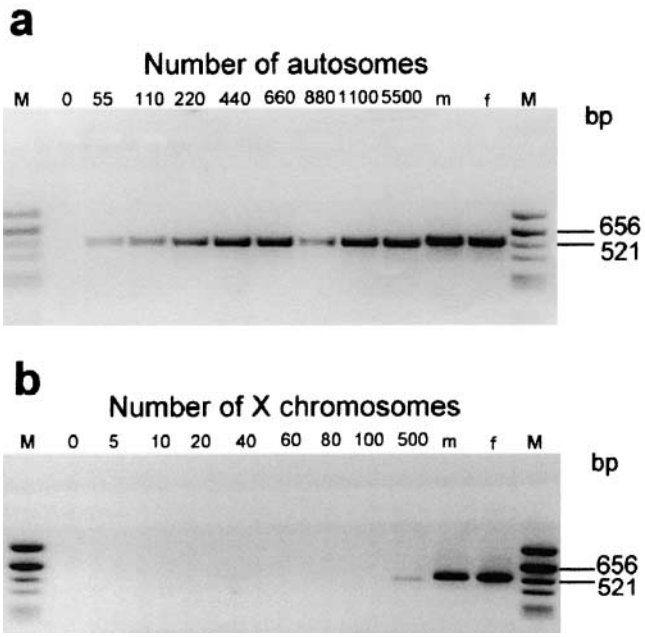

FIGURE 3.—Agarose gel electrophoresis of PCR products obtained using the *MROS4* gene-specific primers MROS4-F1 and MROS4-R1. Numbers of the flow-sorted autosomes (a) or X chromosomes (b) are indicated. No DNA template (0), male genomic DNA (m), and female genomic DNA (f) were used as controls. M, DNA length marker (pBR322/*Alu*I).

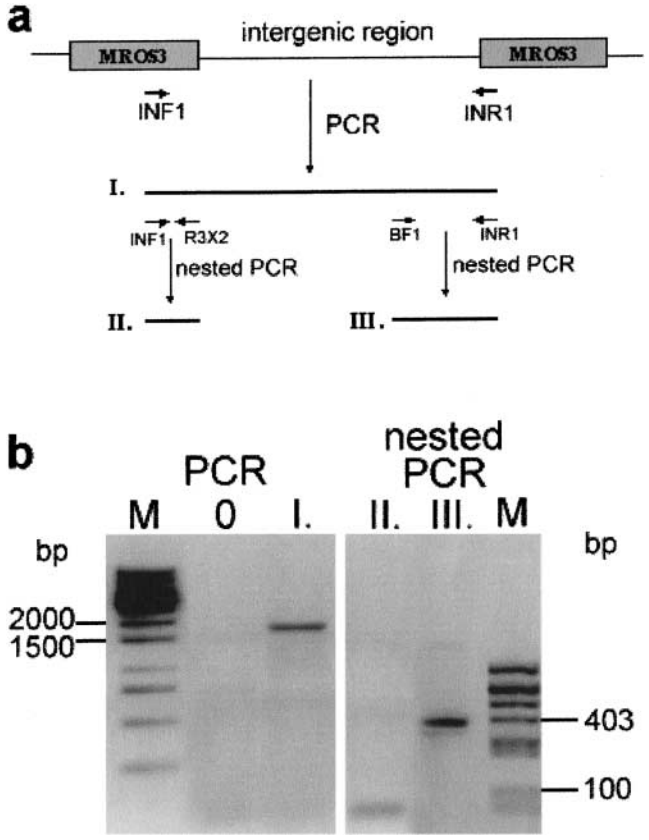

FIGURE 4.—A diagram of the tandem arrangement of two copies of the *MROS3* gene separated with an intergenic spacer, PCR product, and two reamplification products expected on the basis of this hypothetical scheme (a). Agarose gel electrophoresis of PCR products (b) obtained with inverse and nested primers, respectively, as shown in a. As a template for PCR, 1100 sorted X chromosomes were used. M, DNA length markers: 1-kb ladder (left) and pBR322/*Alu*I (right).

The *MROS3* gene is both autosomal and X-linked with tandemly arranged copies on the X chromosome: *MROS3* genes were amplified using the primers described previously by GUTTMAN and CHARLESWORTH (1998). The *MROS3* gene lacks introns. PCR with *MROS3* primers INF2 and R3X2 yielded a strong band of 433 bp in the autosomes. In the X chromosomes, a specific 433-bp band plus several additional bands corresponding to fragments >1000 bp were present (Figure 2d). This result indicates that the *MROS3* gene is present on the X chromosome in multiple copies, probably tandemly arranged. To confirm the tandem arrangement of *MROS3* copies, we designed a pair of "inverse" primers INF1 and INR1 (Table 1) in the terminal regions of the *MROS3* gene and directed outward as shown in Figure 4a. PCR with these primers resulted in amplification of a fragment of ~1700 bp from X chromosomes (Figure 4b) but not from autosomes (not shown). This PCR product represents the intergenic region (spacer) between two copies of *MROS3* genes flanked by the terminal parts of *MROS3* genes. The specificity of this PCR product was confirmed by reamplification using two pairs of nested primers (Figure 4a). One pair of nested primers (INF1 + R3X2) reamplified a specific 73-bp-long region from the 3′ end of the first (left) *MROS3* gene; the second pair of nested primers (BF1 + INR1) reamplified the promoter region of the second (right) *MROS3* gene (Figure 4b). The lengths of both nested PCR products were 73 and 400 bp, respectively, as expected from the known nucleotide sequence. In addition, we performed PCR using only one primer, INF1 or R3X2, to reveal potential tandemly arranged *MROS*3 genes in inverted orientation (not shown). The absence of any PCR product suggested that there are no tandemly arranged *MROS3* genes in inverted orientation that are close enough to be amplified by PCR.

Evidence for specificity of PCR products: On the basis of the knowledge of nucleotide sequences of *MROS* genes (MATSUNAGA *et al.* 1996, 1997), the specificity of PCR-amplified products was checked by restriction enzyme digestion into fragments of the expected sizes or by reamplification of PCR products using nested primers (Figure 5). The PCR fragment corresponding to the *MROS1* gene (1375 bp) was reamplified with one original primer MROS1-R1 in combination with the nested primer MROS1-F1 located 247 bp from the 5′ end, resulting in the PCR reamplification product of 1128 bp. Digestion of the *MROS2* PCR product (785 bp) with *Msp*I resulted in two fragments of 597 and 188 bp,

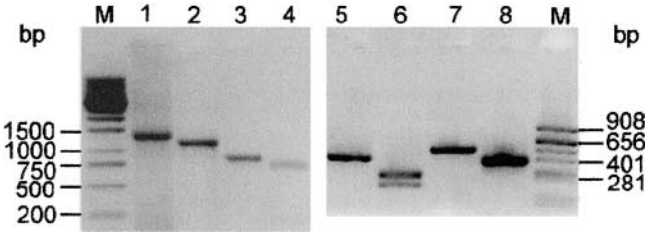

FIGURE 5.—Verification of the *MROS* PCR products by agarose gel electrophoresis of restriction fragments or reamplified products driven by nested primers. *MROS1* identification, the basic PCR product (lane 1), and its product derived from the nested primers (lane 2). The *MROS2* PCR product (lane 3) and the same after *Msp*I digestion (lane 4; the smaller fragment is hardly visible). The PCR product with *MROS3* primers (lane 5) and cut with *Msp*I (lane 6). The PCR product with *MROS4* primers (lane 7) and the same after reamplification with nested primers (lane 8). DNA length markers: 1-kb ladder (left) and pBR322/*Alu*I (right).

as expected according to the one interprimer *Msp*I restriction site. Similarly, the fragment obtained by PCR with *MROS3* gene-specific primers (433 bp) was digested with *Msp*I and yielded, as expected, two fragments of 257 and 176 bp in length. The PCR fragment corresponding to the *MROS4* gene was reamplified using nested primers MROS4-F2 and MROS4-R2 located 60 and 71 bp apart from the 5′ and 3′ ends, respectively. As expected, the reamplification resulted in a PCR product 131 bp shorter than the original PCR fragment.

The *MROS3* gene is also present in other plant species: To check whether the *MROS3* gene is also present in other closely related plant species, we used MROS3 gene-specific primers INF1 and R3X2 for PCR with genomic DNA template prepared from other Silene species, the dioecious *S. diclinis* and the gynodioecious species *S. vulgaris*. In both species, we obtained a *MROS3* band of the same size as in *S. latifolia* (not shown). We also used two copies of the *S. latifolia MROS3* gene, *MROS3a* and *MROS3b*, published recently by MATSUNAGA *et al.* (1999) to search the entire *Arabidopsis thaliana* genome. This database search revealed that *A. thaliana* has five genes homologous to *MROS3*. They were named *AtMROS3a* (accession no. AAC19282), *AtMROS3b* (AAC 19269), *AtMROS3c* (AAF02885), *AtMROS3d* (AAF 02163), and *AtMROS3e* (AAF02153). None of the *AtMROS3* genes have an intron. A database search showed that *AtMROS3a* and *AtMROS3b* as well as *AtMROS3d* and *AtMROS3e* are arranged as tandem repeats on chromosomes IV and III, respectively. Figure 6a shows a multiple alignment of the five *A. thaliana* and two *S. latifolia MROS3* homologues and an evolutionary tree derived from this alignment (Figure 6b).

DISCUSSION

Very little is known about molecular mechanisms of sex determination in dioecious plants and the structure and evolution of plant sex chromosomes. *S. latifolia* has heteromorphic sex chromosomes that are much bigger than autosomes and it has been suggested that they represent an early stage in the evolution of sex chromosomes. Until now not many genes have been isolated in *S. latifolia* (MATSUNAGA *et al.* 1996, 1997; HINNISDAELS *et al.* 1997; ROBERTSON *et al.* 1997; SCUTT *et al.* 1997; DELICHÉRE *et al.* 1999) and only a few of them—*MROS3* and *SlX/SlY* genes—have been localized on the sex chromosomes (GUTTMAN and CHARLESWORTH 1998; DELICHÉRE *et al.* 1999). In these studies genetic mapping methods were used, which cannot distinguish between localization of genes on autosomes and in the pseudoautosomal region of sex chromosomes. For example, the X-linkage of the *MROS3* gene was derived from SSCP analysis, but the same pattern could also occur with some probability for an autosomal gene (GUTTMAN and CHARLESWORTH 1998). To supplement genetic analyses, direct physical techniques to localize genes on individual chromosomes should be used. These include fluorescence *in situ* hybridization and PCR on microdissected (MACAS *et al.* 1993a) or flow-sorted chromosomes (MACAS *et al.* 1993b). *In situ* hybridization of plant chromosomes is not yet a reliable technique for mapping short (<10 kb) single copy DNA sequences, while microdissection is a rather laborious method yielding only a small number of chromosomes of interest. PCR on flow-sorted chromosomes is a more straightforward and reliable approach allowing localization of single or low-copy genes or other DNA sequences.

Here we used this technique to localize the *MROS* genes on *S. latifolia* chromosomes. Chromosome sorting by flow cytometry requires chromosome suspensions with sufficient concentrations of intact chromosomes. The first protocol for chromosome isolation in *S. latifolia* involved the use of hairy root cultures (VEUSKENS *et al.* 1992). The procedure achieved a high degree of mitotic synchrony and clonal maintenance of large numbers of roots of either sex. Here we have used the same protocol to grow and synchronize hairy roots. However, unlike the earlier procedure, intact chromosomes were released from root meristems mechanically after mild formaldehyde fixation according to DOLEŽEL *et al.* (1992). The fixation made chromosomes resistant to mechanical shearing forces, and thus two-step sorting could be employed. Furthermore, fixed chromosomes were suitable for FISH after sorting onto microscope slides (*cf.* DOLEŽEL *et al.* 1999, 2001). While the purity of the X chromosome fraction was very good, we had problems sorting the Y chromosomes in sufficient purity, due to the presence of autosomal clumps. Similarly, VEUSKENS *et al.* (1992, 1995) reported chromosome clusters to be the sole contamination in the Y fractions. Nevertheless, the authors reported 60–80% (VEUSKENS *et al.* 1992) and even 90% (VEUSKENS *et al.* 1995) purity in the Y chromosome fractions. Although we have used a different procedure for the chromosome release, the

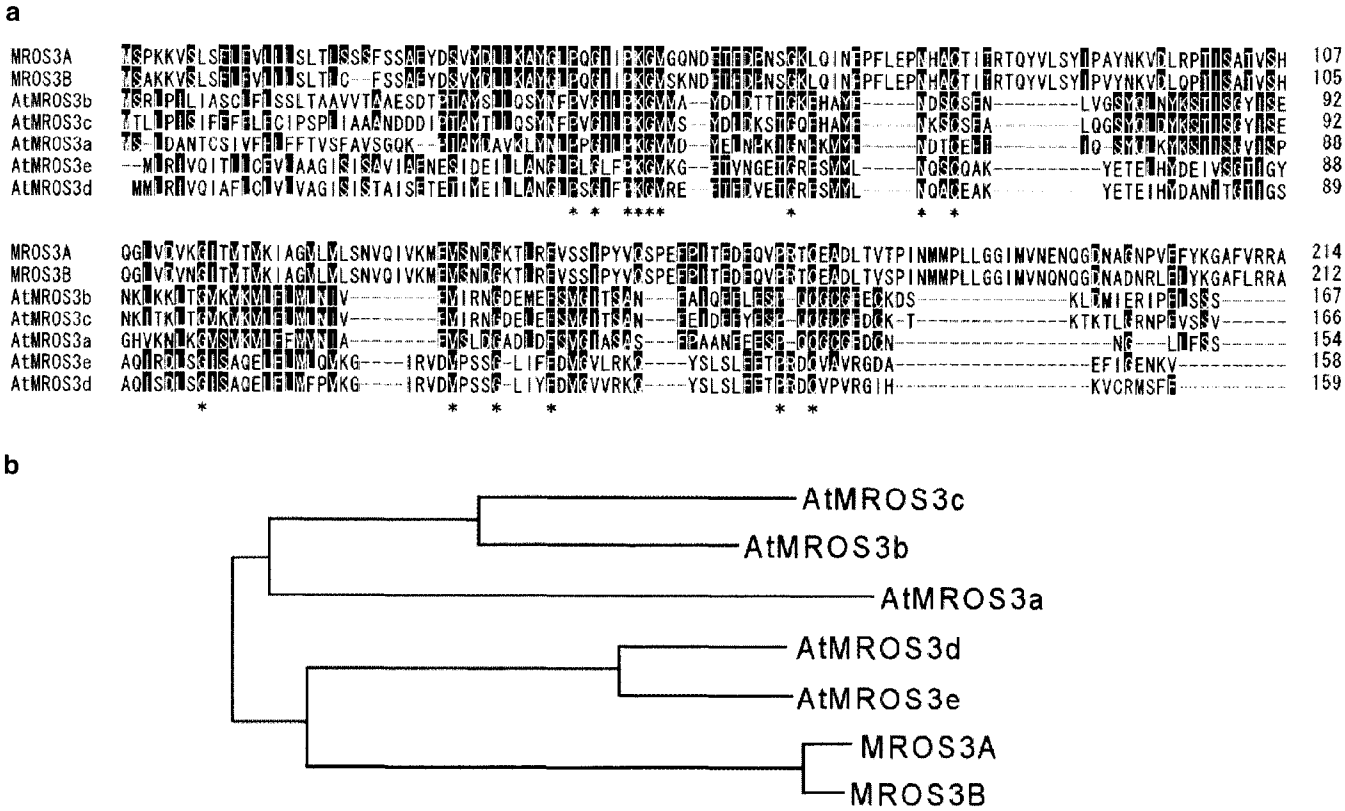

FIGURE 6.—(a) Alignment of deduced amino acid sequences of five *A. thaliana AtMROS3* genes including *AtMROS3a* (accession no. AAC19282), *AtMROS3b* (AAC19269), *AtMROS3c* (AAF02885), *AtMROS3d* (AAF02163), and *AtMROS3e* (AAF02153) and two *S. latifolia MROS3* genes including *MROS3A* (AB029398) and *MROS3B* (AB029399). The numbering on the right denotes the position of amino acid residues from the putative translational initiation. Dashes indicate gaps introduced to maximize the extent of homology among sequences. Solid boxes indicate conserved amino acids residues. Asterisks indicate complete consensus sequences. (b) Phylogenic analysis of deduced amino acid sequences of *AtMROS3* genes and *MROS3* genes. The clustering was performed using the program CLUSTAL X (THOMPSON *et al.* 1997). The branch lengths are proportional to the genetic distances by the neighbor-joining method (SAITOU and NEI 1987).

resolution of flow karyotypes was comparable and thus the reason for lower purity in our Y fractions is not clear. KUBALÁKOVÁ *et al.* (2000) noted that the chromosome morphology may be changed during chromosome isolation, sorting, and drying on a flat surface and recommended identification of sorted chromosomes after specific labeling. As VEUSKENS *et al.* (1992, 1995) evaluated the purity in the sorted Y chromosome fractions on the basis of chromosome morphology alone, one may speculate that the fractions were contaminated by the X chromosomes and/or autosomes, which were not detected.

PCR on flow-sorted plant chromosomes has proved to be a powerful and direct method for physical localization of genes and other DNA sequences. Here, for the first time, physical localization of genes using PCR and sequence-specific primers on the flow-sorted plant sex chromosomes is demonstrated. The sensitivity of PCR on sorted chromosomes is a critical factor because only a few copies of a gene of interest are sufficient to serve as a template. Moreover, the efficiency of PCR on chromosomes consisting of condensed DNA complexes with proteins could be lower in comparison to PCR using pure DNA. In our experiments, a relatively very high sensitivity was achieved and we were able to amplify the single copy *MROS4* gene using 55 autosomes, representing an average of only 5 of each chromosome. This sensitivity is comparable to the data described by other authors (MACAS *et al.* 1993a,b).

We showed that all four *MROS* genes are located on autosomes with at least two additional copies of *MROS3* on the X chromosome. Our results support the previously published data indicating the X-linkage of *MROS3* and autosomal localization of *MROS1* and *MROS2* (GUTTMAN and CHARLESWORTH 1998), as well as results describing two autosomal copies of the *MROS3* gene (MATSUNAGA *et al.* 1999). In addition, GUTTMAN and CHARLESWORTH (1998) isolated an *MROS3* pseudogene located on the Y chromosome. We can conclude that the *MROS3* gene is not a single copy gene but rather a low-copy gene forming a gene family with a few members spread on the autosomes as well as the X and Y chromosomes.

MROS3 genes are also present in other Silene species.

In addition, *MROS3* homologues also were found in a nonrelated *A. thaliana* genome, suggesting an ancient origin of this gene. We showed that at least two *MROS3* genes are arranged in tandem on the X chromosome of *S. latifolia*. Surprisingly, a database search revealed that in *A. thaliana* the *MROS3* homologues also are tandemly arranged. However, on the basis of our results we cannot conclude whether this duplication event(s) took place in an ancestral genome or independently in *S. latifolia* and *A. thaliana*, giving rise to orthologous or paralogous genes, respectively.

The five *A. thaliana MROS3* homologues have the same characteristics as the *S. latifolia MROS3* genes: (i) The N-terminal regions in their encoded proteins are hydrophobic putative signal peptides (Figure 6a), (ii) the consensus sequence (P-G–PKGV) is found in homologous regions of the proteins (Figure 6a), and (iii) they lack introns. The proportion of proteins belonging to families of more than five members, such as *AtMROS3*, is relatively higher in the *A. thaliana* genome than in other eukaryotic genomes (The Arabidopsis Genome Initiative 2000). Moreover, most gene families are organized in tandem arrays. The tandem pairs *AtMROS3a* and *AtMROS3b* on chromosome IV and *AtMROS3e* and *At MROS3d* on chromosome III are examples of this. *At MROS3c* is localized on chromosome I. *AtMROS3* genes fall into two groups in accordance with their chromosomal localization (Figure 6b). This suggests that the tandem arrays were generated by duplication of an ancestral duplicate gene. The duplication is conserved on the X chromosome of *S. latifolia* (Figure 4). It is possible that the X chromosome has some regions homologous to chromosome III or IV of *A. thaliana* and a study of chromosomal synteny may be interesting.

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