A Pseudoautosomal Random Amplified Polymorphic DNA Marker for the Sex Chromosomes of *Silene dioica*

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ABSTRACT

The segregation pattern of an 810-bp random amplified polymorphic DNA (RAPD) band in the F_1 and backcross generations of a *Silene dioica* (L.) Clairv. family provides evidence that this molecular marker is located in the pseudoautosomal region (PAR) of the *X* and *Y* chromosomes. The marker was found through a combination of bulked segregant analysis (BSA) and RAPD techniques. Recombination rates between this pseudoautosomal marker and the differentiating portion of the *Y* chromosome are 15% in both generations. Alternative explanations involving nondisjunction or autosomal inheritance are presented and discussed. Chromosome counts provide evidence against the nondisjunction hypothesis, and probability calculations argue against the possibility of autosomal inheritance. This constitutes the first report of a pseudoautosomal DNA marker for plant sex chromosomes.

SILENE dioica is a dioecious angiosperm with an *XX*/*XY* system of sex determination similar to that found in mammals. Pistillate plants are homogametic (*XX*), and staminate plants are heterogametic (*XY*). The diploid number of chromosomes is 24. The *X* chromosome has a relative length of more than 14% of the haploid genome and the *Y* of more than 21%, being the largest of the complement (Ciupercescu *et al.* 1990). The *Y*:*X* size ratio has been estimated to be between 1.5 (Van Nigtevecht 1966) and 1.4 (Ciupercescu *et al.* 1990).

On the basis of the study of mutants that lacked different regions of the *Y* chromosome, Westergaard (1953) described the *Y* chromosome of the closely related dioecious species *Silene latifolia* as having a pairing, or pseudoautosomal region (PAR), and a nonpairing, or differentiating region. Crossing over between the *X* and the *Y* chromosomes can only occur in the PAR. According to this model, the differentiating region of the *Y* chromosome contains female suppressing, male promoting, and male fertility regions.

In screening primers to search for *Y* chromosome random amplified polymorphic DNA (RAPD) markers, we found that primer OPA-09 provided one each of "male" (*Y* chromosome) and "female" (paternal *X* chromosome) markers. When we analyzed a larger array of F_1 progeny, the female marker was absent from a few females and present, along with the male marker, in a few males. We hypothesized that this marker is undergoing crossing over and therefore should belong to the pseudoautosomal region. A backcross analysis supported this hypothesis.

Several reports have been made on markers and functional genes in the pseudoautosomal region of sex chromosomes of mammals (reviewed in Ellis and Goodfellow 1989) and, in particular, of humans (reviewed in Rappold 1993). To our knowledge, this is the first report of a pseudoautosomal molecular marker for plant sex chromosomes.

MATERIALS AND METHODS

Sex-linked molecular markers were sought through a combination of bulked segregant analysis (BSA) (Michelmore *et al.* 1991) and RAPDs (Mulcahy *et al.* 1992; Zhang *et al.* 1997). In BSA, individuals segregating for a trait of interest, here gender, are pooled (or bulked) together. Within each pool, individuals are identical for the selected trait and arbitrary at all unlinked loci (Michelmore *et al.* 1991). Because mothers contribute their *X* chromosomes to both female and male offspring, BSA reveals only markers derived from the male parent, that is, paternal-*X* markers (in the daughters) and *Y* chromosome markers (in the sons). In a previous study, we screened each of several Silene families from different populations with 80, 10-base random primers from Operon Technologies (Alameda, CA), obtaining several *Y* and paternal *X* markers. In this study we are concerned with two of these sex-linked markers.

To produce the bulks, a female and a male individual from a *S. dioica* population from Sherringham (England) were crossed under controlled greenhouse conditions. Female and male bulks each contained 15 individuals. We took an equal weight of leaf tissue from each of the 15 individuals and performed pooled DNA extractions following Bernatzky and Tanksley (1986). Extractions were made from leaves in a flowering shoot to ensure that DNA belonging to the desired sex was being used (this was meant to avoid the possibility of extraneous plant material growing in the same pot). PCR was

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run with 10-base primer OPA-09 from Operon Technologies (5′ GGGTAACGCC 3′). Each PCR contained the following: 1.25 μl 10× buffer (100 mm Tris-HCl, pH 8.3, 500 mm KCl, 15 mm $MgCl_2$, 0.01% gelatin), 1 μl dNTP (1.25 mm each of dATP, dCTP, dGTP, dTTP), 0.55 μl $MgCl_2$ (20 mm), 0.5 μl primer (10 μm), 0.1 μl Taq polymerase (5 U/μl), 2.5 μl DNA (5 ng/μl), and 6.75 μl nanopure water. Cycling was carried out in Perkin-Elmer (Norwalk, CT) and Barnstead Thermolyne (Dubuque, IA) Amplitron II thermocyclers: three cycles of 94° for 1 min, 35° for 1 min, 72° for 2 min; 42 cycles of 94° for 5 sec, 35° for 1 min, 72° for 2 min. Reactions were run in duplicate to ensure repeatability. Electrophoresis was performed in 1.5% agarose gels, and the banding pattern in male and female bulks was compared. Any band unique to the male bulk was considered a putative *Y* chromosome marker, and any band unique to the female bulk was considered a putative paternal-*X* marker. Band size was estimated by comparison to a 100-bp DNA standard (Pharmacia, Piscataway, NJ). To further test a given marker, we ran both parents and four to six female and male individuals of the F_1 progeny and followed the segregation pattern of the sex markers.

The backcross was done under controlled greenhouse conditions between one of the "unusual" males (possessing both the male and the female markers) and the original mother. The resulting backcross progeny was bulked according to sex, DNA was extracted, and PCR performed by the same methods as described for the F_1 generation.

To observe mitotic figures and count numbers of chromosomes, rosettes from adult plants were induced to produce roots for root tip chromosome squashes. We applied rooting hormone (Hormo-Root "B," Rockland, Gilbertsville, PA) to a rosette's base and then placed it in presoaked peat plugs. When roots emerged from the mesh surrounding the peat plugs, they were collected into a 0.05% solution of colchicine and soaked for 4–6 hr at room temperature with aeration. Root tips were then fixed in 3:1 absolute alcohol:glacial acetic acid at room temperature overnight. After acid hydrolysis in 1 N HCl for 10 min at 65°, the material was stained in Feulgen reagent for 3 hr in the dark. Root tip squashes were performed in 45% acetic acid. Slides were permanized by freeze-popping the cover slip, dehydrating in 95%, 100% ethanol and Histosol, and mounting in acrylic medium (Polyscience, Niles, IL). Observations were made using bright-field, fluorescent, and confocal microscopes from the Central Microscopy Facility at the University of Massachusetts. Linkage analysis was done using the software LINKAGE 1 (Suiter *et al.* 1983).

RESULTS

Primer OPA-09 produced a unique 590-bp band in the male bulk and a unique 810-bp band in the female bulk. The parents, six of the male and six of the female progeny that composed the bulk, were examined individually. The 810-bp band was present in the father and in six individual daughters, following the segregation pattern expected for a paternal-*X* marker. The 590-bp band, present in the father and in six sons, is a typical *Y* chromosome marker (data not shown).

In the process of assembling a new bulk, including the individuals tested above and other F_1 individuals from the same original cross, we were surprised to find that the band for locus *OPA-09* ^{810} was faintly present in the male bulk as well as in the female bulk. We then screened each individual in the new bulk and found that 2 of 16 females were lacking and 3 out of 17 males possessed the band for locus *OPA-09* ^{810}. The band for locus *OPA-09* ^{590} remains consistently present in males only (in Figure 1, one of the females with the band for *OPA-09* ^{810} was scored in a separate gel and is therefore not included in this figure; Table 1).

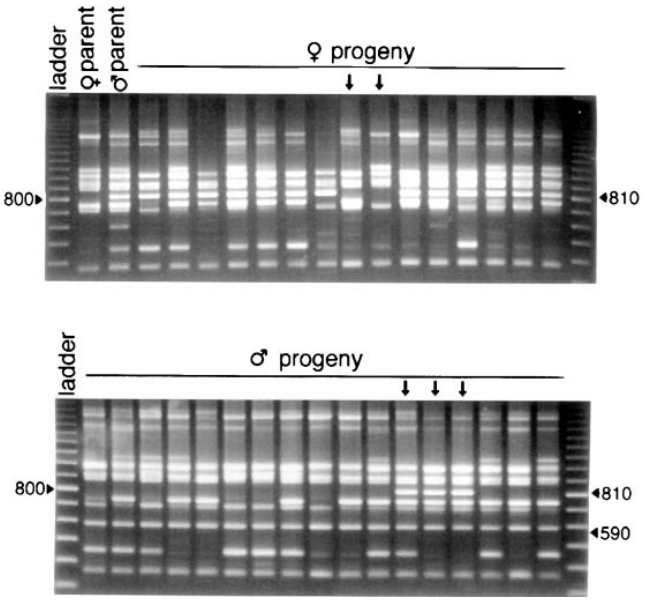

Figure 1.—Segregation of a 590-bp and an 810-bp marker in the F_1. Arrows on lanes indicate females lacking the 810-bp marker and males having it. Sizes are in bp, ladder is 100 bp (Pharmacia).

Examination of flowers confirmed that the two females without the 810-bp marker are phenotypically normal females and the three males with it are normal males. We elaborated two hypotheses to explain these results. In one scenario, the father has a nondisjunction during meiosis, producing *XY* pollen (*OPA-09* ^{810} marker present in the *X* chromosome and *OPA-09* ^{590} marker in the *Y*) and *O* pollen. When crossed to a female parent lacking any markers, it gives *XXY* males [both markers present and still phenotypically male due to the *Y* chromosome being strongly male determinant (Westergaard 1953)] and *XO* females (810-bp marker absent, phenotypically female).

Figure 2 shows the expected segregation of the mark-

TABLE 1

Genotypic frequencies for sex-linked RAPD loci *OP-A09*^{810} and *OP-A09*^{590} in the F_1 of a *S. dioica* cross

Genotype			
OPA-09 ^{810}	*OPA-09* ^{590}	Phenotype	Frequency
+	−	Female	14
−	+	Male	14
−	−	Female	2
+	+	Male	3
			$N = 33$

+, presence of RAPD band; −, absence of RAPD band.

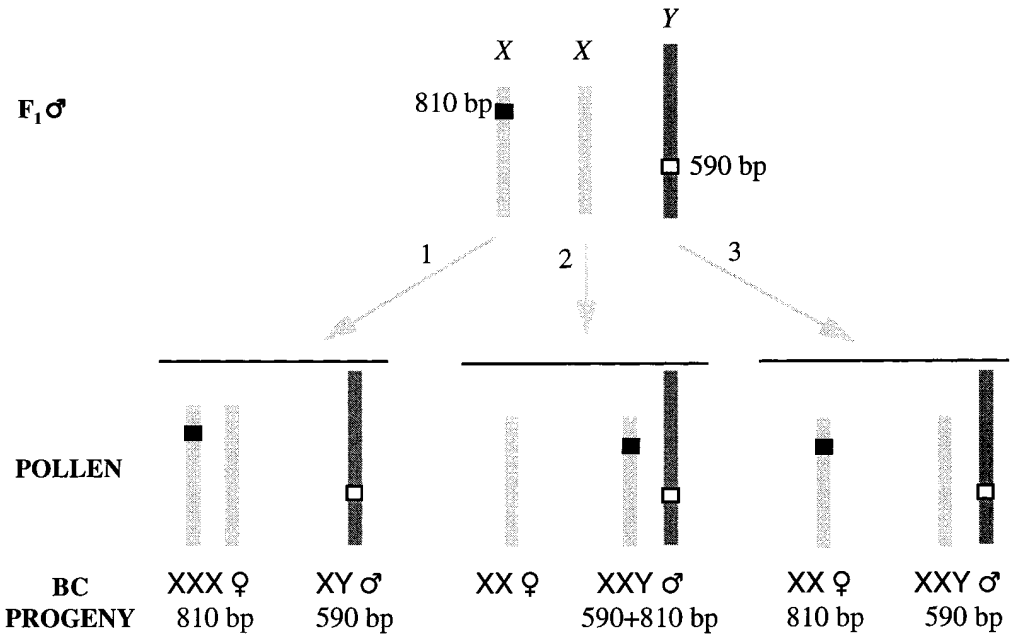

Figure 2.—Model for the segregation of *X* and *Y* chromosome markers during pollen production of an *XXY* male and the respective progeny types sired in the backcross.

ers when an *XXY* male is backcrossed to his mother, who lacks any markers. If we suppose the probability of all three outcomes (arrows 1–3 in Figure 2) to be equal, we would expect one-sixth of female and one-third of male progeny to lack the 810-bp marker, and one-third of female and one-sixth of male progeny to have it.

To test this nondisjunction hypothesis, we performed chromosome counts in the males in which locus *OPA-09* ^{810} was present and in the females that lacked it. All counts resulted in 24 chromosomes, thus providing no evidence of aneuploidy. Figure 3 shows a mitotic chromosome spread of one of these females. Twenty-two autosomes are present, and the two *X* chromosomes are indicated with arrows.

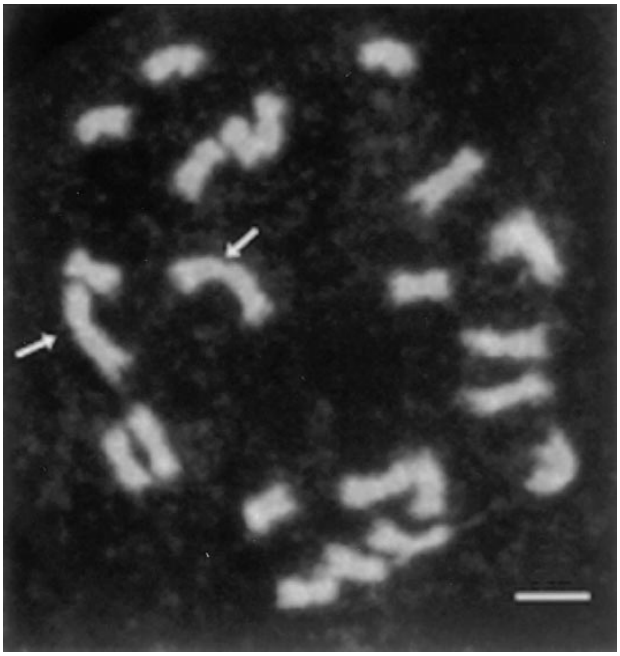

Figure 3.—Mitotic chromosome spread of an F_1 female (22, *XX*) that lacks the band for locus OPA-09^{810}. Arrows indicate the two *X*-chromosomes. Bar, 5 μm.

An alternative hypothesis is that locus *OPA-09* ^{810} could be located in the PAR. This would make it show incomplete sex linkage. Figure 4 describes the expected F_1 progeny under the crossing-over hypothesis. A male containing the 810-bp marker in its *X* chromosome and the 590-bp marker in its *Y* chromosome is crossed to a female lacking these markers. In this case, both markers become linked in the *Y* chromosome through crossing over, fitting the results presented above. Under this hypothesis the frequency of genotypes can be divided into "parental" (3 and 4) and "recombinant" (1 and 2) types. Recombinants comprise 15% of the F_1 progeny (Table 1). Locus *OPA-09* ^{590} is present in all males.

The segregation of markers in the backcross of one of the males containing the two markers is shown in Figure 5. Table 2 shows the frequency of genotypes in that backcross. Of the 17 backcross generation daughters analyzed, 13 lack the band for *OPA-09* ^{810}, like their mother, and 4 have the marker (recombinant females). A total of 15 of the backcross generation sons contain both markers, like their father, and one of them lacks the band for *OPA9*^{810} (recombinant male). The percentage of recombinant phenotypes in the backcross is 15%, as in the F_1. Locus *OPA-09* ^{590} is present in all males.

DISCUSSION

Because it is not clear whether the segregation pattern of an *XXY* male is predictable, we would like to evaluate two likely outcomes and contrast the expectations derived from them with our results. The predictions outlined in the Results section (equal probability of all possible gametes; Figure 2) do not fit the results because

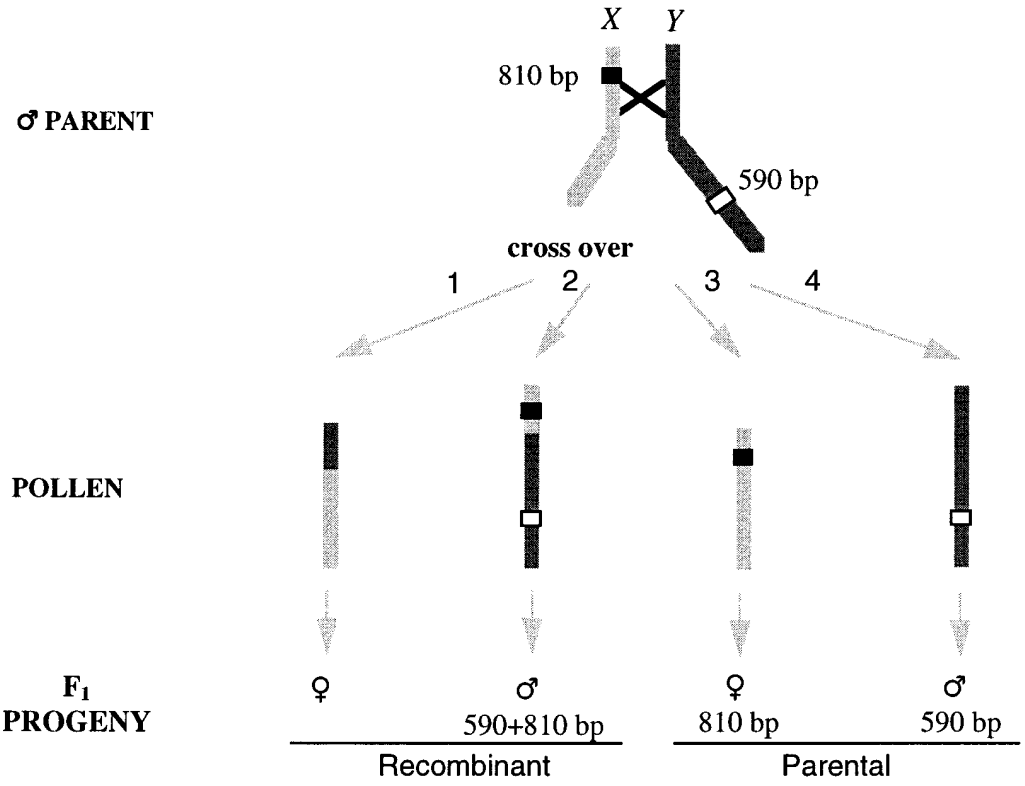

Figure 4.—Model for the location of sex-linked markers in the *X* and *Y* chromosomes and their segregation under a crossing-over hypothesis.

the least-represented class is females without and males with the marker, rather than the opposite (Table 2).

Westergaard (1948) notes that in *S. latifolia*, *XXY* males produce an excess of *X* and *XY* gametes and few *XX* and *Y* gametes. Therefore, the majority of the offspring in the backcross will have the sex chromosome constitutions *XX* or *XXY* (Figure 2, arrows 2 and 3), but a small proportion of *XXX* or *XY* may also appear (Figure 2, arrow 1). Based on this evidence, we would expect outcomes 2 and 3 in Figure 2 to be equally prevalent. In terms of the molecular phenotypes, this translates into having similar numbers of males and females with and without the 810-bp marker. If we add some of the progeny which, according to Westergaard (1948), may occur at low frequency (outcome 1, Figure 2), this means we would expect more than half of the females to have the 810-bp marker and more than half of the males to lack it. Clearly, none of the above predictions fits the data (Table 2). Nevertheless, the strongest evidence against the nondisjunction hypothesis is provided by the lack of sex-chromosome aneuploidy in the individuals with the "unusual" genotypes.

Another alternative to the PAR hypothesis is that of

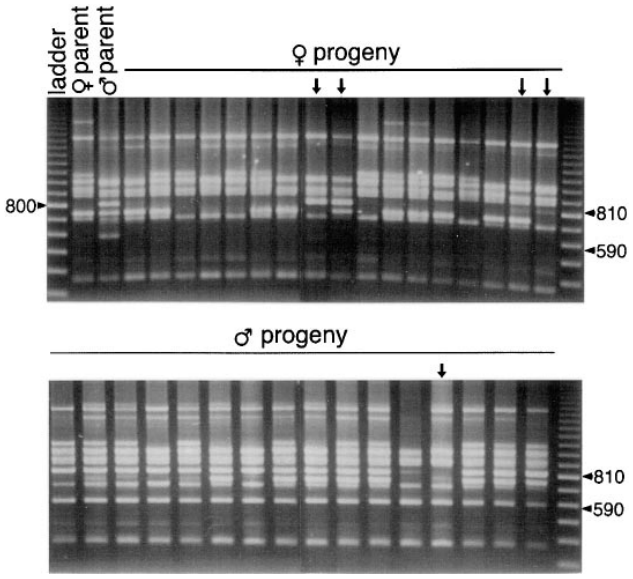

Figure 5.—Segregation of two sex-linked RAPD markers (indicated by arrows on the sides) in the backcross. Arrows on lanes indicate females with the 810-bp marker and a male without it. Sizes are in bp, ladder is 100 bp (Pharmacia).

TABLE 2

Genotypic frequencies for sex-linked RAPD loci *OP-A09*^{810} and *OP-A09*^{590} in a backcross of *S. dioica*

Genotype			
OPA-09^{810}	*OPA-09*^{590}	Phenotype	Frequency
−	−	Female	13
+	+	Male	15
+	−	Female	4
−	+	Male	1
			$N = 33$

+, presence of RAPD band; −, absence of RAPD band.

autosomal inheritance. For both the F_1 and backcross generations, the segregation for marker *OPA-09* ^{810} does not fit the inheritance pattern of a non-sex-linked autosomal gene, that is, of being equally distributed among males and females ($\chi^2 = 8.06$ and $\chi^2 = 8.55$, respectively; $P < 0.005$). The strong distortion for the presence of the allele in females of the F_1 (Table 1) and males of the backcross (Table 2) suggests incomplete sex linkage. Locus *OPA-09* ^{810} shows linkage to maleness and locus *OPA-09* ^{590} in the backcross. The χ^2 for independent assortment in the two segregating populations is 16.1 and 16.6, respectively ($P < 0.001$), and the recombination rate is $r = 0.15 \pm 0.06$ for each population.

Because *OPA-09* ^{810} segregation indicates recombination between the *X* and *Y* chromosomes, the partial sex linkage fits the description of a pseudoautosomal locus (Rouyer *et al.* 1986). No recombinants are detected for *OPA-09* ^{590}, suggesting that it lies in the differentiating region of the *Y*. The recombination rates obtained do not give us information about the location of one marker with respect to the other, but rather of the distance of the *OPA-09* ^{810} marker from the pseudoautosomal boundary. Thus, the evidence suggests that we have found a DNA sequence belonging to the pseudoautosomal region of the sex chromosomes of *S. dioica*, located 15 cM from the pseudoautosomal boundary.

To facilitate finding PAR markers with BSA, we suggest that bulks should consist of few (five) individuals. These will generate some false positives that will be identified as such during further study. Small bulks, however, will allow detection of partial sex-linked markers because, among five individuals, recombination frequencies of <20% are unlikely to succeed in breaking the sex linkage. Subsequent examination of putative sex-linked markers should be extended to individuals not included in the original five, and this larger population may reveal, as happened here, a breakdown in the apparent sex linkage. Presumably, these PAR markers will tend to be fairly close to the differential region. Otherwise, recombination would have obscured the partial sex linkage.

Studies on the human PAR have found loci with as low as 2.5% recombination, located close to the pseudoautosomal boundary, and as high as 50%, mapping close to the telomere (Ellis and Goodfellow 1989; Rappold 1993). Human PAR sequences have relatively high recombination rates, possibly a consequence of the lack of recombination along the differential arms of the sex chromosomes (Rouyer *et al.* 1986). To date, there is no information indicating this might also be the case with plants. Future comparisons to recombination rates for other pseudoautosomal loci in Silene will provide insights as to the relative length of the recombining region and the location of this locus with respect to the telomeres of the sex chromosomes in this species.

Differences in the genetic behavior of the PAR between species is thought to reflect the degree of sex-chromosome differentiation from an hypothesized ancestral pair of homologs (Rouyer *et al.* 1986). Silene has been placed at an intermediate stage in the evolution of sex chromosomes, between the incipient system in the fish Lebistes and many plants and the more evolved one in Drosophila (Darlington 1958). In this context, it will be interesting to be able to study the genetic behavior of this angiosperm's PAR.

As to the function of the PAR, it is thought to maintain pairing and correct segregation of the sex chromosomes during meiosis in animals (Mohandas *et al.* 1992). However, neither *X-Y* pairing nor chiasmata are found in eutherian mammals (Pathak *et al.* 1980) or in marsupials (Sharp 1982).

Our finding constitutes a first step in the search for PAR genes in angiosperms. A prediction for PAR genes is that they should escape *X* inactivation (Burgoyne 1982). The closely related species *S. latifolia* has been found to have hypermethylation of one of the *X* chromosomes in the female, implying that it is transcriptionally inactive as described for mammals (Vyskot *et al.* 1993). In that study no evidence of hypomethylation of the PAR could be found. However, PAR markers will allow us to test more specifically for hypomethylation of this region in an angiosperm.

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