The Development of Two Flanking SCAR Markers Linked to a Sex Determination Locus in Salix viminalis L.

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Most studies of sex determination systems in plants involve dioecious annuals that have known sex chromosomes. Despite the absence of such structures in the majority of dioecious plants, gender seems to be under relatively strict genetic control in some species. Genetic markers linked to a female sex-determination locus in Salix viminalis L. have been discovered through bulked segregant analysis of three full-sib families using approximately 1,000 arbitrary primers. Two RAPD markers that were present in the common female parent as well as in predominantly female progeny of these families were subsequently sequenced and converted to sequence characterized amplified region (SCAR) markers. The two SCAR markers are correlated with gender in the three full-sib families and are present in 96.4% of the female progeny and 2.2% of the males, providing evidence of linkage to a putative female-specific locus associated with gender determination in S. viminalis. Estimates of recombination suggest that the two markers are flanking a putative sex determination locus, SDL-II, in certain families of S. viminalis.

Mechanisms of sexual differentiation in plants are almost overwhelmingly diverse. Most models of sex determination in dioecious plants are based on annuals such as Silene or Rumex that possess heteromorphic sex chromosomes (Parker 1989). Although these species represent useful models for understanding the control of gender in a broad sense, the lability of sex determination mechanisms in plants without sex chromosomes suggests that such models may prove to be unsuitable for the majority of dioecious plants. In the last decade, the application of molecular techniques to plant developmental biology has advanced the isolation and characterization of many floral meristem identity genes (Ainsworth 2000; Theissen 2001; Weigel and Meyerowitz 1994). However, it is unclear what direct effect such genes may have on sex determination in monoecious and dioecious species. One model proposed for the evolution of dioecy from hermaphroditic ancestors suggests that two simultaneous but independent mutations, which cause both male and female sterility and also suppress recombination between these mutations to prevent the formation of hermaphrodites, could be responsible for such a state (Lewis 1942; Ross 1978). A more reasonable hypothesis is that gynodioecy or androdioecy resulting from a single mutation may have provided a likely intermediary step between hermaphroditism and dioecy (Ainsworth 2000; Charlesworth and Gutman 1999). Furthermore, despite evidence that sex determination is under genetic control in dioecious species with heteromorphic sex chromosomes (e.g., Rumex and Silene), it is doubtful that the same mechanism would control sex in individual flowers in monoecious plants or in dioecious plants without sex chromosomes (Ainsworth et al. 1998). How, then, is sex determined in these species? Do one or a few primary sex determination loci activate sex differen-
tion genes, or does a series of organ suppression genes activated by environmental clues control this process? Are sex determination mechanisms so diverse that no one mechanistic model can account for sexual differentiation among all plant species? Given the multiple, independent instances of evolution of dioecy, the latter is virtually certain.

Interactions between genetic loci and plant growth hormones have been proposed for sexual differentiation in monoecious maize (Dellaporta and Caldon-Urrea 1994, reviewed in Lebel-Hardenack and Grant 1997) and dioecious annual mercury (Durand and Durand 1991; Louis 1989; Louis et al. 1990). However, willows and poplars seem to be somewhat different from other described dioecious species in this regard. There is no robust evidence of sex chromosomes in Salicaceae, yet both species are represented by fairly stable sexually dimorphic systems not readily influenced by plant growth substances (Ainsworth et al. 1998), although some level of hermaphroditic and female-biased sex ratios has been reported for both Salix and Populus spp. (Alström-Rapaport et al. 1997; Lester 1963; Mosseler and Zsuffa 1989). It has been hypothesized that female-biased sex ratios in Salix viminalis are a result of familial relationships rather than abiotic agents (Ahman 1997), and observed sex ratios in certain genetic backgrounds suggest a multilocus epistatic model of sex determination in S. viminalis (Alström-Rapaport et al. 1998). These results could have strong implications for breeding strategies for the development of S. viminalis as a short-rotation energy crop (Ahman 1997; Alström-Rapaport et al. 1998).

The development of molecular markers linked to sex has been attempted in a number of dioecious species through genetic mapping or breeding work (reviewed in Ainsworth 2000). We previously reported the discovery of a single Random Amplified Polymorphic DNA (RAPD) marker, UBC354560, linked to a putative sex determination locus, SDL-II, in S. viminalis through bulked segregant analysis (BSA) (Alström-Rapaport et al. 1998). Genomic DNA from progeny of three full-sib families derived from a single maternal parent was used to identify UBC354560. The objectives of this study were (1) to increase the number of RAPD primers screened in an effort to find a marker flanking the putative sex-determination locus and (2) to convert RAPD markers to sequence characterized amplified region (SCAR) markers (Paran and Michelmore 1993) in order to generate sequence-specific markers that could potentially be used to screen other willow families or species.

Materials and Methods

Plant materials and protocols used in this study were as described in Alström-Rapaport et al. (1998) for identification of RAPD markers associated with gender in three full-sib families (families 960, 961, and 962) of S. viminalis, including PCR amplification of progeny DNA with RAPD primers, and confirmation of band segregation through nonradioactive digoxigenin labeling and hybridization. Briefly, families 960 and 962 were initially chosen for BSA, based on expected phenotypic sex ratios and model predictions that they should segregate at a single locus for an allele that determined gender. Family 961 was subsequently chosen because it was produced from the same maternal parent as families 960 and 962. Each family was divided into female and male progeny bulks consisting of 1 ng genomic DNA from each of 10 individual genotypes for each gender. In the previous study, 380 random decamer primers were evaluated to find a single marker. For this study, additional primers from Operon Technologies (Alameda, CA) and the University of British Columbia (Vancouver, BC) were screened on gender-based bulked DNA from families 960, 961 and 962.

The initial marker, RAPD UBC354560, present in the mother (S. viminalis clone 78-0-90) of families 960, 961 and 962 was amplified, electrophoresed in a 1.5% agarose/0.5X TBE gel, excised, and purified with GeneClean II (Qiogene, La Jolla, CA). A custom primer was synthesized (Life Technologies, Rockville, MD) to append a Hind III site to the 5′ end of the UBC decanucleotide sequence used to amplify the gender-associated marker RAPD UBC354560 described in Alström-Rapaport et al. (1998). The sequence of the custom primer was the following: 5′-TCTGTTTTCAGGGCCG-3′. Amplification of maternal genomic DNA with the custom primer was accomplished through 35 cycles of 94°C 1 min, 42°C 1 min, and 72°C 2 min, initiated with a 5 min 94°C denaturation step, and terminated with a 5 min 72°C polymerization step. Each reaction contained 50 mM KCl, 10 mM Tris–HCl pH 8.0, 1.5 mM MgCl2, 200 μM dNTPs, 1 μg BSA, 10 ng primer, and 5 μl of a 1:100 dilution of the GeneClean purified product from the initial RAPD amplification. The reamplified product was digested with Hind III, ligated to pBluescript KS+ (Stratagene, La Jolla, CA), transformed into E.coli DH5α (Life Technologies/Invitrogen, Carlsbad, CA) cells by electroporation, plated on selective media, and incubated at 37°C for 16 h. Twenty-four transformed bacterial colonies were selected, representing one clone species that carried the willow UBC354560 insert. Plasmid DNA from two clones was purified (Qiagen, Valencia, CA) and sequenced using an ABI 373 Sequencer, following the Applied Biosystems (Foster City, CA) Dye terminator protocol. The resulting consensus sequence was used to design SCAR primers (MACVector 4.1, International Biotechnology, Inc., New Haven, CT; primer size = 18–25 nt; Tm = 45–70°C; % G + C = 45–55) that amplified a unique 520-bp product in S. viminalis clone 78-0-90.

A second marker associated with gender, RAPD OPAE81300, discovered through bulked segregant analysis of additional RAPD primers, was excised, gel-purified, random prime-labeled using digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN), directly reamplified with Operon primer AE08, and hybridized to nylon filters containing DNA from RAPD amplifications of parental and selected progeny as described in Alström-Rapaport et al. (1998) to confirm homology of the sequences. An unlabeled second amplification product was cloned into the TA cloning vector (Invitrogen), following the manufacturer’s protocol, and plated on selective media. Two of 24 transformed
Table 1. Observed frequency of SCAR markers among full-sib progeny of three families of S. viminalis that share a common maternal parent

<table>
<thead>
<tr>
<th>Family</th>
<th>960</th>
<th>961</th>
<th>962</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCAR</td>
<td>+:− (♀)</td>
<td>+:− (♀)</td>
<td>+:− (♀)</td>
</tr>
<tr>
<td>UBC 54420</td>
<td>20:0 0:16</td>
<td>20:0 0:17</td>
<td>33:2 0:17</td>
</tr>
<tr>
<td>OPAE08780</td>
<td>19:1 0:14</td>
<td>20:0 0:17</td>
<td>10:1 1:9</td>
</tr>
</tbody>
</table>

Ratios within progeny sets indicate the number and gender (♀ = female; ♂ = male) of offspring exhibiting the marker (+ = marker present) versus the number not exhibiting the marker (− = marker absent). Discrepancy in observed number of progeny within families between markers was due to insufficiency of some genotype templates during final testing of the SCAR markers.

Results

We screened 380 random amplified polymorphic DNA (RAPD) primers to find the first marker associated with gender in willow, RAPD UBC 354420. For this study, 573 additional primers were examined before a second, flanking, marker was found. Out of 953 total primers screened, only 773 amplified any product. One hundred sixty-one primers exhibited marker differences associated with male or female bulked genotypes in at least one family. Eight markers were present in bulks of all three families, and only two suggested 1:1 segregation in a 10 female genotype: 10 male genotype population. Reaction components were as described above to design primers to amplify a unique 780-bp DNA fragment in S. viminalis clone 78-0-90.

PCR amplification using the newly synthesized SCAR primers was tested on parental and progeny templates for families 960, 961 and 962. Reaction components were as described above, with the following exceptions: 25 ng of each primer and 10 ng of parental and progeny template DNA from the three families were used, and the annealing temperature was increased to 55°C. DNA template from family 962 was limited for testing of SCAR OPAE08780, and this is reflected in the number of genotypes available for use in later analyses (Table 1 and Figure 1). Amplified products were resolved as described in Alström-Rapaport et al. (1998) using standard agarose gel electrophoresis separation. Genetic distances were calculated from the ratio between recombinant and nonrecombinant phenotypes (Hartl 1998) for the putative sex-determination locus (Table 1 and Figure 1).

Figure 1. Putative sex determination locus II in S. viminalis female clone 78-0-90. Genetic distance estimates between each marker and the putative locus are based on crossing over frequency in 110 progeny for SCAR UBC 354420 and 92 progeny for SCAR OPAE08780 from three full-sib families with a common mother. Genetic distance between markers was calculated using the 92 progeny common to both data sets.

\[ 2.7 ± 0.5 \text{ cM} \]
\[ 3.3 ± 1 \text{ cM} \]

(5'-GAGAGGAGGGAGATTTAAG-3') and a 22-nt reverse primer (5'-CGCGTATGGTTAATCATCAC-3') designed from this sequence amplified a 520-bp marker designated SCAR UBC 354420. The DNA consensus sequence derived from RAPD OPAE081300 was actually 1271 bp in length and contained 27.9% G + C (A = 478; C = 163; G = 191; T = 439). A 20-nt forward primer (5'-TGTTATGTTGTGATGGA-3') and a 20-nt reverse primer (5'-CAATCCAATGTTTTGGA-3') designed from the sequenced RAPD OPAE081300 fragment amplified a 780-bp dominant SCAR marker designated SCAR OPAE08780. Both markers displayed 1:1 genotypic segregation between male and female progeny when PCR-amplified in families 960, 961, and 962 (Table 1). Unlike SCAR UBC 354420, which was present in the maternal parent and one paternal parent (family 961), SCAR OPAE08780 was present only in the maternal parent of the families tested.

Among the three families, 96.6% of female progeny and 20% of male progeny exhibited amplification of SCAR UBC 354420 \( (\chi^2 = 49.6, df = 1, P < .001) \), and 96.1% and 2.4% of males and females, respectively, exhibited amplification of SCAR OPAE08780 \( (\chi^2 = 40.2, df = 1, P < .001) \). Estimates of recombination frequency were used to calculate mapping distance between each marker and SDL-II, the putative sex determination locus, as well as between markers within the three families. Our calculations indicate that SCAR UBC 354420 is 2.7 ± 0.5 cM away from SDL-II, that SCAR OPAE08780 is 3.3 ± 1 cM from the locus, and that the two markers are separated from each other by a distance of 4.4 ± 2.1 cM (Figure 1).
for either marker. Both SCAR UBC 354520 and SCAR OPAF08780 sequences successfully amplified in other species of willow (see Discussion, below).

Discussion

The discovery of two sex-linked molecular markers associated with femaleness in certain genetic backgrounds of *Salix viminalis* strengthens the hypothesis that sex determination is associated with one or more autosomal loci, in accord with the model proposed by Alström-Rapaport et al. (1998) for sex determination in the species. Markers associated specifically with female progeny suggest either (1) the inheritance of a sequence on a sex chromosome from a male parent or (2) close linkage with a female sex-determination gene (Ainsworth 2000). The fact that more than 2,000 primer sequences have been screened in both *Populus* and *Salix* (Alström-Rapaport 1997; Alström-Rapaport et al. 1998; McLetchie and Tuskan 1994; this study), and only two have exhibited an association with gender, provides evidence for the lack of sex chromosome structure in Salicaceae. Furthermore, since both markers are essentially biparentally inherited, the former hypothesis seems improbable. According to the quantitative genetics model of sex determination in *S. viminalis*, the markers we discovered are most likely associated with one allele of a hypostatic locus, since, in some families, the presence or absence of the markers is not directly associated with phenotypic gender ratios (see discussion, Alström-Rapaport et al. 1998). Within the three *S. viminalis* families, we anticipated that markers would be located close enough to a putative sex determination locus to be useful in identifying a candidate sex-determination gene via chromosome walking or landing within a BAC library of the maternal *S. viminalis* clone 78-0-90. Although the base chromosome number in *Salix* is identical to *Populus*, the DNA content of diploid *Salix* was estimated to be 0.76–0.98 pg (Thibault 1998), which is somewhat smaller than that of *Populus* (1.2–1.3 pg; Bradshaw and Stettler 1993). Based on an average mapping distance of 180 kb/cm in *Populus trichocarpa* (Bradshaw et al. 1994), we estimate that a span of approximately 414–1170 kb separates these flanking markers. This is far too large a distance to attempt chromosome walking, and, considering both the lack of suitable markers found in our initial attempts and the small number of genotypes available from pedigrees of the three families, it is unlikely that we would readily be able to find more tightly linked markers without detailed sequence information from this species.

Despite the current limitations in locating a sex-determination gene in *S. viminalis*, these markers may be useful in characterizing female progeny from intraspecific *S. viminalis* crosses within certain genetic backgrounds. Moreover, because it is probable that a multilocus sex determination system is the prevailing mode of sexual differentiation within Salicaceae, these markers may be associated with gender in other *Salix* spp. We observed an association of SCAR UBC 354520 with gender ($\chi^2 = 4.77$, df = 1, $P < .05$; data not shown), based on screening both markers in a total of 18 female and 26 male clones in five different species of *Salix* (1–14 clones per sex per species), although in that case the marker association was with males, not females. We speculate that key loci involved in sex determination may be conserved among species of the genus *Salix*, and, if so, we should find evidence that they are present and associated with gender in species other than *S. viminalis*. As a result, we have used the SCAR markers developed here to survey available pedigrees of *Salix eriocephala* Michx. in order to test whether one or both loci serve as indicators of sexual differentiation in an alternate genetic background (Gunter et al., in review).

References


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