Theor Appl Genet (2000) 100:432–438

S.M. Reamon-Büttner · C. Jung

AFLP-derived STS markers for the identification of sex in *Asparagus officinalis* L.

Received: 31 May 1999 / Accepted: 22 June 1999

Abstract For a simple, rapid and PCR-based screening of sex in the cultivated asparagus (*Asparagus officinalis* L.), we developed five STS markers from previously mapped, low-copy, sex-linked AFLP markers. A male/female PCR assay was feasible with these STS markers either by direct amplification or by digestion with restriction enzymes. Similar to the AFLP markers from which they were derived, STS4150.1, STS4150.2, STS4150.3 and STS3156 did not give recombinants in five different populations. STS3660 could be scored codominantly, enabling the differentiation of XY from YY males in the screened F_2 mapping population. The use of the sex-linked STS markers should allow early identification of sex, thus accelerating the breeding process for new asparagus varieties. Further, 10 additional AFLP markers obtained with *Pst*I/*Mse*I primer combinations have been mapped on the L5 chromosome, bringing the total number of known AFLP and STS markers flanking the sex locus to 24. These markers can be utilized for fine mapping of the sex gene in asparagus, which will pave the way for a map-based cloning approach.

Key words AFLP · STS · Sex-linked DNA markers · PCR markers · *Asparagus officinalis* L.

Introduction

Polymerase chain reaction (PCR)-based markers such as sequence-tagged-site (STS) markers are important to plant breeding programmes and genome analysis because they are simple, fast, cheap and easy to handle. Primers are usually designed from sequences of previously mapped molecular markers including random amplified polymorphic DNA (RAPD) (Talbert et al. 1996; Deng et al. 1997; Kawchuk et al. 1998), restriction fragment length polymorphisms (RFLPs) (Blake et al. 1996; Erpelding et al. 1996; Williams et al. 1996; Tsumura et al. 1997; Blair and McCouch 1997; Harry et al. 1998) and amplified fragment length polymorphisms (AFLPs) (de Jong et al. 1997; Qu et al. 1998; Bradeen and Simon 1998). Polymorphisms are detected either from direct amplification with the primer pairs or after digestion of the PCR products with restriction enzymes.

The cultivated asparagus (*Asparagus officinalis* L.) is a dioecious plant. Sexual dimorphism is apparently controlled by gene(s) located on a pair of homomorphic L5 chromosomes (Löptien 1979). In asparagus cultivation, male plants are desired due to their high yield and longevity. For the breeding of all male asparagus varieties, YY males or 'supermales' are key plant material because when a YY male is crossed to a female (XX) plant, a homogeneous population of male (XY) plants will be obtained in the progeny. The YY genotype can only be verified through laborious and time-consuming testcrosses. Therefore, if codominant sex-linked molecular markers are available, this should lead to a fast identification of YY males and, in turn, speed-up the breeding process. Moreover, the application of sex-linked molecular markers to asparagus breeding will allow screening of the sex of the plants at the seedling stage, as opposed to the present waiting time of about 2 years when asparagus plants begin to flower.

Sex-linked molecular markers in asparagus are not only important to breeding but also to the understanding of sex determination. For these purposes, several molecular marker techniques have been employed to find sex-linked markers in this species (Restivo et al. 1995; Lewis and Sink 1996; Jiang and Sink 1997; Jiang et al. 1997). A RAPD marker that mapped 1.6 cM to the sex locus, *M*, has been converted into a sequence-characterized amplified region (SCAR) marker (Jiang and Sink 1997). The RAPD and the SCAR markers, however, were not applicable to other asparagus germplasm studied. Re-

Communicated by G. Wenzel

S.M. Reamon-Büttner · C. Jung
Institute of Crop Science and Plant Breeding,
Christian-Albrechts-University of Kiel, Olshausenstrasse 40,
D-24118 Kiel, Germany
Fax: +49 (431) 880 2566

cently, we have identified and mapped 9 sex-linked AFLP markers on the L5 chromosome in asparagus (Reamon-Büttner et al. 1998). These markers were generated by the primer combinations *Eco*RI/*Mse*I. One of our aims was to convert them into sequence-tagged-site (STS) markers that could be utilized for a simple, fast, PCR-based, early screening of the sex of the plants as well as for the identification of YY males.

To develop sex-linked STS markers in asparagus, we isolated the 9 AFLP markers from the gel, cloned them into plasmid vectors, determined and analysed their nucleotide sequence and designed STS primers. In this paper, we report the development of five STS markers from low-copy AFLP sequences consisting of four dominant and one codominant markers. Of these, three STS markers can be readily detected through direct amplification with the primer sets, while two, by digestion with restriction enzymes. We also report the mapping on the L5 chromosome of 10 additional AFLP markers obtained with *Pst*I/*Mse*I primer combinations. This brings the total of known AFLP and STS markers flanking the sex locus to 24.

Materials and methods

An F_2 population (F_2-950545) consisting of 69 plants with 15 females and 54 males was used for the mapping of the AFLP and STS markers. This population was obtained from the selfing of an andromonoecious plant. Selfing of an andromonoecious will give the segregation 1 XX: 2 XY:1 YY in the progeny. To verify the universality of the STS markers, we screened an additional 122 plants from four populations with varying genetic backgrounds. For the bulked segregant analysis with *Pst*I/*Mse*I primer combinations, 10 plants per bulk (XX, XY and YY) from F_2-950545 were used. Linkage analysis and mapping of the AFLP and STS markers were carried out with MAPMAKER/EXP 3.0 (Lander et al. 1987).

The bulked segregant analysis, non-radioactive AFLP protocol and the cloning of AFLP markers were described in detail previously (Reamon-Büttner et al. 1998). Briefly, asparagus genomic DNA was digested with *Eco*RI or *Pst*I and *Mse*I. The digested DNA was ligated to respective linker-adaptors, and the preamplification step was performed using primers with one selective nucleotide. After preamplification, the PCR product was diluted tenfold in 1 × TE buffer. Subsequently, amplification involving primers with three selective nucleotides was conducted. After tracking dye was added to the PCR reaction, electrophoresis followed on 7% LongRanger (Biozym) sequencing gels with a Li-Cor DNA sequencer (MWG-Biotech).

To clone the AFLP markers, we carried out a radioactive AFLP protocol as described previously (Schondelmaier et al. 1996). The fragments were isolated from the dried gel, reamplified using the primers that gave the polymorphism, then cloned into plasmid vectors [pCR-Script Amp SK (+), Stratagene]. To identify the correct clones, inserts were amplified with *Eco*RI+3 and *Mse*I+3 primers, electrophoresed on an 8% polyacrylamide gel on the Li-Cor DNA sequencer and the mobility of amplified fragments compared with the original AFLP marker. Clones having inserts migrating the same distance as the AFLP marker were considered to most likely be correct ones. These clones were [^{32}P]-labeled and used as probes to female and male asparagus genomic DNA digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I for further tests. Southern hybridization was done according to a standard procedure at 60°C (Sambrook et al. 1989). Washing was carried out twice (30 min each) with 0.5 × SSC/0.2% SDS at 60°C. The nucleotide sequence of cloned sex-linked AFLP markers was determined using a SequiTherm EXCEL^{Tm} cycle sequencing kit (Biozym).

Primer sets were designed using OLIGO 5.0. In general, the primers include the complete restriction enzyme sequence and the three selective bases. PCR was carried out with a Perkin Elmer 9600 thermocycler programmed as follows: 30 s, 94°C; 30 s, 94°C/ 30 s, 55–61°C/ 40 s, 72°C (32 cycles); 5 min, 72°C. The annealing temperature depended on the primer set used. If no polymorphism between female and male plants with a primer set was detected, the putative restriction sites of the cloned AFLP marker were analysed using DNASIS or Webcutter (http://www.ccsi.com/firstmarket/cutter/cut2.html). PCR products were digested with enzymes cutting within the male-specific AFLP sequence and electrophoresed on 3% Metaphor gels (Biozym).

Results

The different AFLP and the AFLP-derived STS markers linked to the sex locus were mapped on the L5 chromosome in the population F_2-950945 (Fig. 1). The genetic map consisted of 19 AFLP markers, five STS markers and the sex locus, *M*. AFLP markers were designated EM (*Eco*RI and *Mse*I) or PM (*Pst*I and *Mse*I). The AFLP markers were all linked to maleness in coupling phase, i.e. a fragment was present in male plants but absent in

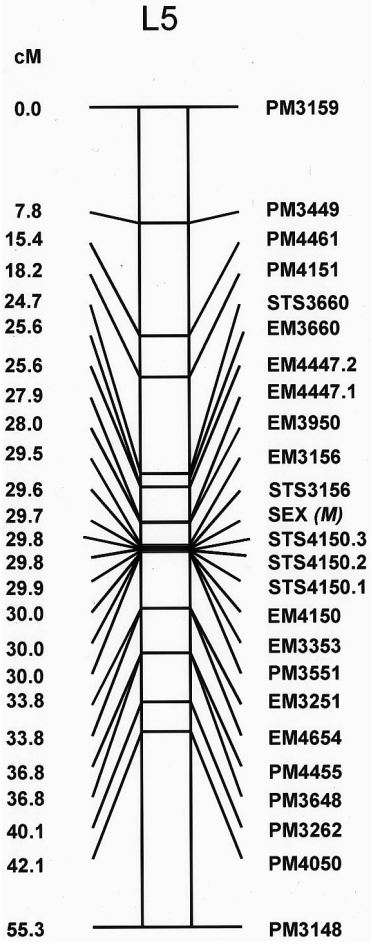

Fig. 1 Genetic map of the L5 chromosome showing 19 AFLP markers, five STS markers and the sex locus, *M*

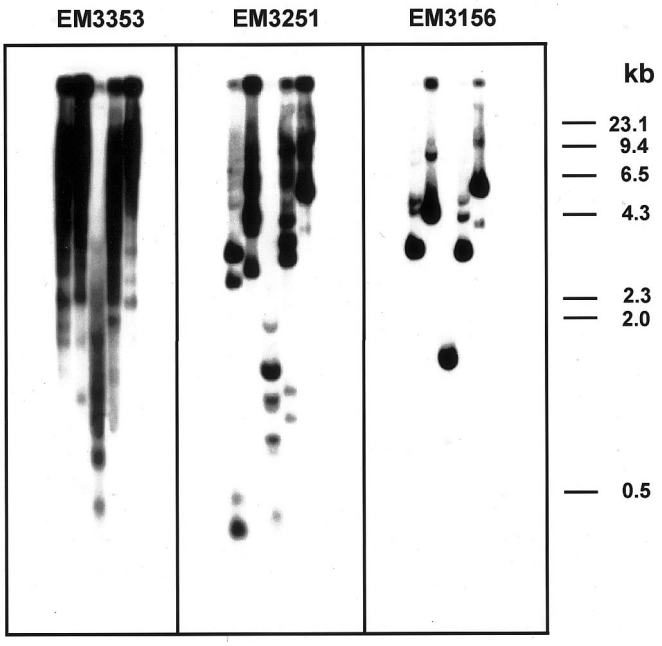

Fig. 2 Southern hybridization with cloned sex-linked AFLP markers – as examples, EM3353, EM3251, EM3156– showing high- to low-copy sequences. For each filter, asparagus genomic DNA was digested with (*left to right*): *Eco*RI, *Eco*RV, *Sau*3AI, *Hin*dIII and *Bam*HI. Each slot contained approximately 10 µg DNA. Exposure time:1 day

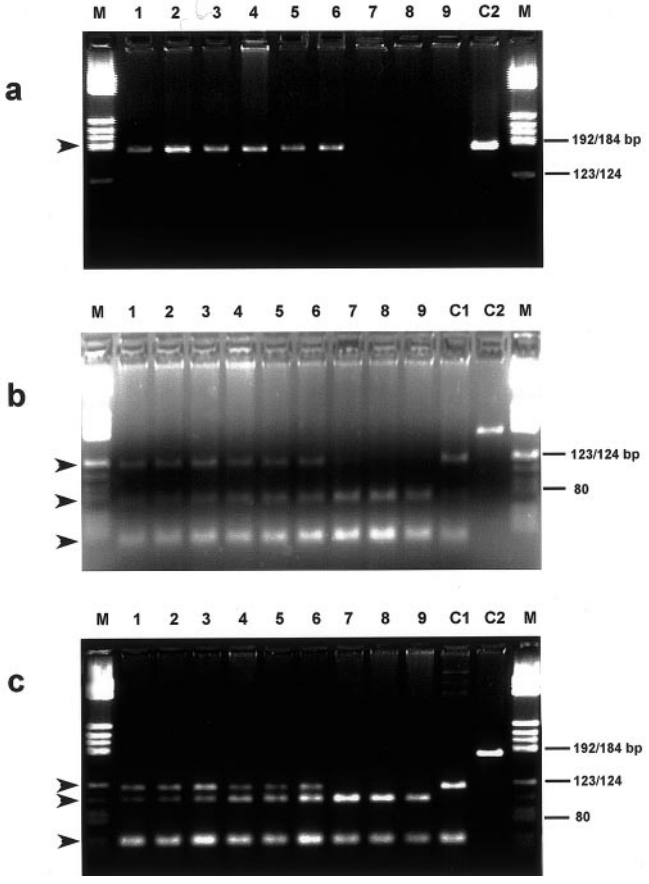

Fig. 3a–c STS markers: **a** STS4150.1, **b** STS4150.2, **c** STS4150.3. Polymorphism was obtained with STS4150.1 by direct amplification, while polymorphism with STS4150.2 and STS4150.3 was obtained after digestion with *Sau*3AI and *Taq*I, respectively. **Lanes: 1–3** YY males, **4–6** XY males, **7–9** female plants, **C1** digested PCR product with the cloned AFLP marker EM4150 as PCR template, **C2** the PCR product with the cloned AFLP marker EM4150 as PCR template, **M** molecular weight marker. The PCR products were electrophoresed on a 3% Metaphor gel

female plants. Moreover, stronger banding intensities were observed in YY males than in XY males, thus allowing codominant scoring in the F_2 population. In general, the AFLP markers obtained with *Pst*I and *Mse*I primers mapped farther from the sex locus. The STS markers were found in the vicinity of the AFLP markers from which they were derived.

Southern hybridization with cloned AFLP markers revealed banding patterns corresponding to low-copy to repetitive sequences. Shown as examples in Fig. 2 are the banding patterns exhibited by EM3353, EM3251 and EM3156. For the conversion of AFLP into STS markers, three low-copy AFLP fragments, namely, EM4150, EM3156 and EM3660, were chosen for the primer design. Several primer sets were also designed from the high copy sequences, but the corresponding PCR products exhibited complex banding patterns on the agarose gel (data not shown). Therefore, further experiments with highly repetitive sequences were not pursued. A total of five STS were developed from 3 low-copy AFLP sequences consisting of four dominant and one codominant markers. Polymorphisms were obtained by direct amplification with the primer sets or by the digestion of PCR products with restriction enzymes. In succeeding paragraphs, the five AFLP-derived STS markers are presented in detail.

Three STS markers could be derived from the AFLP marker EM4150. As determined by sequence analysis, EM4150 is 183 bp in length. It detected RFLPs between male and female asparagus genomic DNA restricted with *Hin*dIII and *Xba*I. The first STS marker (STS4150.1) could be directly amplified with the primer set asp4150–8/asp4150–12. When this primer set was used, a PCR product of 178 bp was expected. As predicted, a single fragment of this size was amplified in male plants (YY and XY) but not in female plants (Fig. 3a). This polymorphism resulted after incorporating a single male-specific nucleotide at the 3' end of primer asp4150–12. It would also be later confirmed by a comparison of male and female sequences obtained with related primers (i.e. asp4150–10/asp4150–11).

The second STS marker (STS4150.2) was generated with the primer set asp4150–10/asp4150–11, in which the amplification of a 178-bp fragment was likewise expected. No direct polymorphism was observed. Male and female plants exhibited a single amplicon of 178 bp, as visualized on the gel. After *Sau*3AI digestion of the PCR product, however, three fragments (115, 72 and 42 bp) were observed in YY and XY male plants, while two

fragments were visible (72 and 42 bp) in female plants (Fig. 3b). In the EM4150-plasmid clone which was used as a control, a 178-bp fragment was also amplified (Fig. 3b, C2) and after *Sau*3AI digestion, the 115-bp and 42-bp fragments were revealed (Fig. 3b, C1). Subsequent analysis would reveal that there are two *Sau*3AI restriction sites on the male-specific sequence. A 21-bp digestion product could no longer be detected on the gel. Assuming that YY males are homozygous for this STS marker locus (STS4150.2), then we can only expect the 115-bp, 42-bp and 21-bp fragments, which sum up to 178 bp, after *Sau*3AI digestion. However, we noted the additional presence of a 72-bp fragment in the YY males, a fragment which was present in both XY (male) and XX (female) plants. An explanation for this discrepancy would come later when the STS primers were determined to be amplifying not just the male specific sequence but also a population of related sequences of similar size when genomic DNA was used as template for PCR.

The third STS marker (STS4150.3) was also generated with the primer set asp4150–10/asp4150–11. Digestion of the 178-bp PCR product with *Taq*I produced three fragments of sizes 119, 104 and 59 bp. All three fragments were observed in YY and XY male plants, while the 104-bp and 59-bp fragments were only observed in female plants (Fig. 3c). In the EM4150-plasmid clone, only the 119-bp and 59-bp fragments after *Taq*I digestion were detected (Fig. 3c, C1). The presence of a single *Taq*I restriction site on the 178-bp sequence would confirm the presence of fragments of sizes 119 and 59 bp. A similar situation was encountered with STS4150.3 as regards the discrepancy of fragments detected in the YY males and in the EM4150-plasmid clone. Like the AFLP marker EM4150 from which they were derived, STS4150.1, STS4150.2 and STS4150.3 mapped closely to the sex locus and did not give any recombinants in five different populations.

To gain more insights into the PCR product being amplified by the STS primer set asp4150–10/asp4150–11, we isolated from the gel the 178-bp PCR product from a YY male plant and used it as a probe to male and female asparagus genomic DNA restricted with *Xba*I. Hybridization with this PCR product gave a smear on the autoradiograph (data not shown), in contrast to the cloned AFLP marker EM4150 where RFLPs between male and female plants with asparagus genomic DNA digested with *Xba*I could be detected. This indicates that with the STS primers, in addition to the male-specific fragment, a population of fragments of similar sizes are being amplified. Furthermore, we cloned the 178-bp fragment obtained with the primer set asp4150–10/asp4150–11 from a YY male and a female plant. Four clones from the YY male and two clones from the female plant were randomly selected and their nucleotide sequence determined. Of these, three different sequences were found, subsequently designated as seq-1011/5, seq-1011/21 and seq-1011/22 (Fig. 4). Pairwise comparison using BLASTN algorithms revealed sequence homologies of between 88% and 90%, suggesting an amplification of related sequences. Two clones from the YY male exhibited seq-1011/5, corresponding to the cloned AFLP marker EM4150, while the other two clones displayed seq-1011/21. The clones from the female plant possessed seq-1011/21 and seq-1011/22. Analysis of the *Sau*3AI or *Taq*I restriction sites on the three sequences could account for all the fragment sizes obtained with the STS markers using male and female asparagus genomic DNA (Fig. 4).

The fourth STS marker (STS3156) was developed from the AFLP marker EM3156, which is 60 bp in size. EM3156 detected RFLPs between male and female asparagus genomic DNA digested with *Hin*dIII and *Xba*I. With the primer set asp3156–1/asp3156–2, a single fragment of 62 bp could be amplified in YY and XY males, but only a faint band or no band at all could be found in

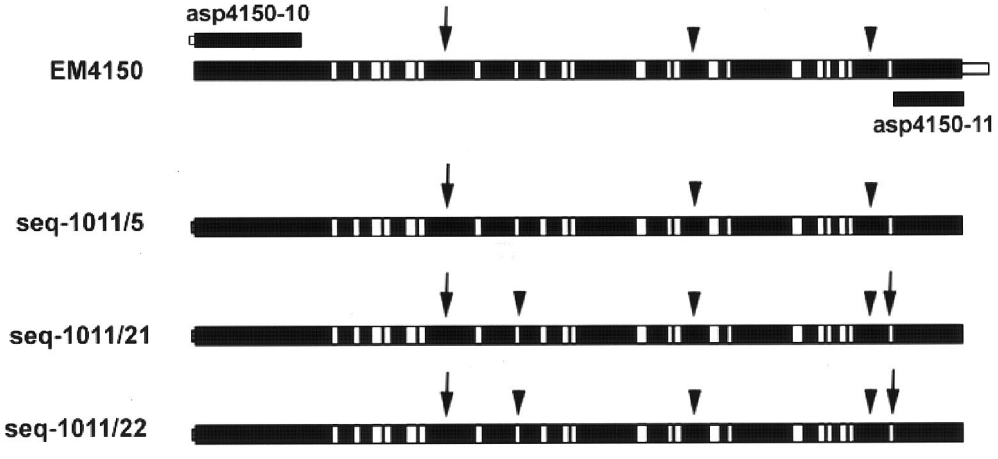

Fig. 4 Schematic alignment of the three sequences obtained with the primer pair asp4150–10/asp4150–11, namely, seq-1011/5, seq-1011/21 and seq-1011/22, all of which are 178 bp in length. EM4150 is the cloned AFLP marker (183 bp). When EM4150 is used as the basis, the sequences extend by one nucleotide on the left side. EM4150 is longer by six nucleotides on the right side. Seq-1011/5 is the male-specific sequence corresponding to EM4150. Polymorphic sites are indicated by *unshaded areas*. *Taq*I restriction sites are designated by arrows while *Sau*3AI restriction sites are indicated by *arrowheads*

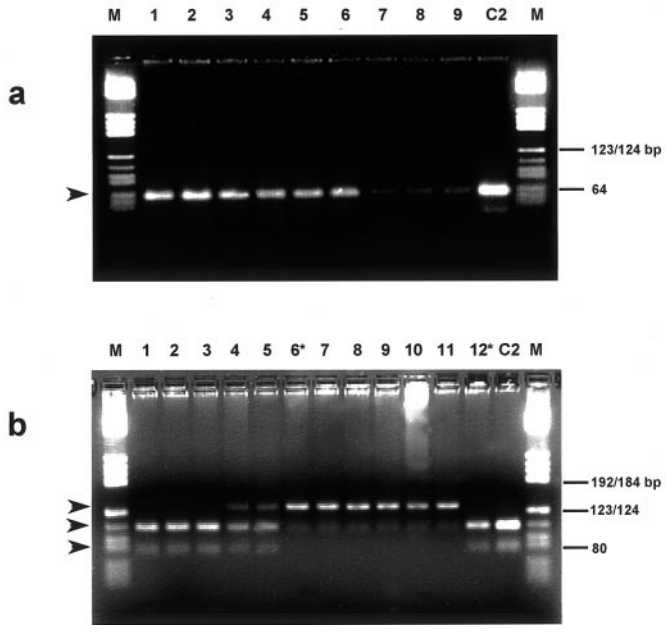

Fig. 5a, b STS markers: **a** STS3156 and **b** STS3660. Polymorphism was obtained in both markers by direct amplification. **Lanes: 1–3** YY males, **4–6** XY males, **7–12** female plants. In Fig. **5b**, **6*** and **12*** are recombinant plants. **C2** The PCR product with the cloned AFLP marker EM3156 or EM3660 as template, **M** molecular weight marker. The PCR products were electrophoresed on a 3% Metaphor gel

female plants (Fig. 5a). The two additional nucleotides, i.e. 60–62 bp in the PCR product, resulted by including the complete restriction sequence of *Eco*RI or *Mse*I in the primers at the 5' end. The first 5' nucleotide in the restriction sequence of *Eco*RI or *Mse*I is lost through digestion of DNA and subsequent ligation to adaptors during AFLP analysis. No recombinant was found with EM3156 and STS3156 in screened populations.

The primers for the fifth STS marker (STS3660) were derived from the AFLP marker EM3660, which is 101 bp, and likewise revealed RFLPs in male and female asparagus genomic DNA digested with *Eco*RI and *Eco*RV. Using the primer set asp3660–1/asp3660–2, we expected a fragment of 103 bp, at least in male plants. Here again, the primers start with the complete restriction sequence of *Eco*RI or *Mse*I, but instead of a single fragment, three were observed. A major band (103 bp) and a faint band (78 bp) were amplified in YY males (Fig. 5b). Female plants also exhibited a major band (130bp) and a faint band (103 bp). In XY males, the bands of 130, 103 and 78 bp were all present. Therefore, STS3660 could be scored codominantly. Among 69 plants we found five recombinants in the population F_2-950545. Three of the recombinant plants corresponded to those in EM3660; the two other recombinants were male plants, and both exhibited the marker genotype *Mm* in EM3660 but had the genotypes *MM* and *mm* in STS3660. This shows that the polymorphism seen in EM3660 may not be the same as that in STS3660. The linkage of markers EM3660 and STS3660 to the sex locus was only observed in the population F_2-950545.

Discussion

In our experience, AFLP is a very efficient marker system. In combination with bulked segregant analysis and a non-radioactive detection system, it proved to be the fastest way of finding tightly linked molecular markers to the sex locus in asparagus. It was possible to map 19 sex-linked AFLP markers in the population F_2-950545 using bulked segregant analysis with 509 *Eco*RI or *Pst*I and *Mse*I primer combinations. Three of the sex-specific markers remained very tightly linked in five screened populations. Other marker systems such as isozyme, RAPD and RFLP were not very successful in detecting very tightly linked markers in asparagus (Restivo et al. 1995; Lewis and Sink 1996; Jiang et al. 1997). With RAPD markers, only two specific markers amplified from one primer mapping 1.6 cM to the sex locus were found after screening more than 760 arbitrary decamer primers (Jiang and Sink 1997).

Although the present sex-linked AFLP markers can be readily used for the early screening of sex in asparagus as well as in the identification of YY males in F_2 populations, their usefulness can be increased by converting them into STS markers. This should allow a simple and rapid PCR-based, large-scale screening of plants. For the conversion of the AFLP to STS markers, we found it important to first determine the nature of the cloned AFLP markers, for instance copy number and distribution in the genome. Primer design from low- to single-copy sequences is surely not as complicated as with highly repetitive sequences.

Southern hybridization with the cloned sex-linked AFLP markers has shown that single-locus AFLP markers could consist of low-copy to highly repetitive sequences. As demonstrated by fluorescence *in situ* hybridization (FISH), sex-linked AFLP sequences, for example AFLP markers EM4447.1 and EM4150, could be found on all the chromosomes of asparagus (Reamon-Büttner et al. 1999). These results show that AFLP marker polymorphisms are basically defined by nine nucleotides at the *Eco*RI end and seven nucleotides at the *Mse*I end, and the fragment size. We did not obtain single-copy AFLP sequences as has been found in potato (Meksem et al. 1995) or in rice (Cho et al. 1996). We also were not able to derive STS markers from the highly repetitive AFLP sequences. However, developing STS markers from low-copy AFLP sequences with sizes ranging from 60–183 bp was feasible.

For the conversion of the AFLP to STS markers, it was necessary to establish a test system to identify the correct clones. We found a heterologous population of fragments among our cloned AFLPs, from which only one "true'' sequence corresponds to the original AFLP marker. Since the cloned AFLP markers are not single-

copy sequences, it was likewise necessary during primer design that the STS primers should have the following make-up: 5'-*Eco*RI or *Mse*I complete restriction sequence + three selective bases + short sequence from the cloned AFLP marker- 3'. As the primer sequences moved farther from the restriction sites, varying amplification products were detected. It was only from the primers which contained the complete restriction sites and selective bases that it was possible to retrieve the sequences identical to the cloned AFLP marker. In the design of primers from AFLP sequences for a PCR-based assay for the *ph1b* deletion in wheat (Qu et al. 1998), the primers also contained the restriction sites, the selective bases and an additional short sequence 3' from the selective nucleotides.

In this paper we present five sex-specific STS markers that can be utilized for marker-assisted screening of sex in asparagus. These STS markers exhibited, in general, the same polymorphism as the AFLP markers from which they were derived. Three STS markers were generated from the AFLP marker EM4150, which did not give recombinants in five screened populations. For a fast assay, a direct plus/minus screening with STS4150.1 is possible. Assays with STS4150.1 may be further optimized for large-scale screening; for instance, through the use of molecular beacons (Tyagi and Kramer 1995; Tyagi et al. 1998), thereby eliminating gel electrophoresis. Screenings with STS4150.2 and STS4150.3 both offer no difficulty except that the PCR products require digestion with *Sau*3AI and *Taq*I, respectively. However, these enzymes can be directly added into the reaction tube after PCR, and the resulting fragments can be highly resolved on a 3% Metaphor gel. Using these STS markers should basically give the same results, although the scorings with STS4150.2 and STS4150.3 as compared to STS4150.1 would be more accurate. This is because the polymorphism detected with STS4150.1 is a result of one male-specific nucleotide at the 3' end of one of the primers and, therefore, may be affected by changes in the annealing temperature during PCR. However, the PCR conditions we used gave consistent results. If it is necessary to choose between STS4150.2 and STS4150.3, the fragments obtained with STS4150.3 are larger, thereby allowing better resolution on the gel. Besides, *Taq*I is much cheaper to use than *Sau*3AI. Therefore, of the three STS markers from EM4150, STS4150.3 meets the criteria needed for a reliable and a cheap PCR-based marker.

STS3156 is another marker that can be used for the direct plus/minus screening of sex in asparagus. Although the fragment generated by this STS marker is small (62 bp), it can still be detected on a 3% Metaphor gel. In a few cases in female plants, at an annealing temperature of 61°C, a faint band was observed. We are trying to optimize this STS marker because the AFLP marker (EM3156) from which it was derived is a low-copy sequence and did not give recombinants in the screened populations. From our present data, we cannot determine whether EM3156 or EM4150 maps closest to the sex locus. The closest marker should give a more accurate result as regards identification of sex in asparagus. In contrast to the other STS markers, STS3660 could be scored codominantly in the screened F_2 population. This STS marker can, therefore, be utilized in a PCR assay to differentiate the XY and YY males. However, STS3660 and EM3660 appear to be population-specific markers. Linkage with these markers was observed only in the population F_2-950545 and with recombination.

In conclusion, we have demonstrated that it was possible to derive STS markers from short, low-copy, AFLP sequences. The sex-linked STS markers will allow a simple, rapid, PCR-based assay of male and female plants in asparagus. Since screening can be undertaken as early as the seedling stage, and it takes about 2 years for asparagus to start flowering, their use can tremendously reduce the breeding time of this crop species. Being PCR-based markers, they are amenable to automation for the large-scale screening of plants. The AFLP and STS markers that we have mapped on the L5 will also be useful for fine-scale mapping of the sex gene, thereby paving the way for a map-based cloning approach.

Acknowledgements We wish to thank Anke Schmidt for excellent technical support and Felix Dreyer for helpful discussion and assistance, particularly during the construction of the genetic map. This project has been funded by the Deutsche Forschungsgemeinschaft (DFG grant Ju 205/2–2).

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