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A. Jamsari · I. Nitz · S. M. Reamon-Büttner · C. Jung

BAC-derived diagnostic markers for sex determination in asparagus

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Abstract A *Hin*dIII BAC (bacterial artificial chromosome) library of asparagus (*Asparagus officinalis* L.) was established from a single male plant homozygous for the male flowering gene (*MM*). The library represents approximately 5.5 haploid genome equivalents with an average insert size of 82 kb. A subset of the library (2.6 haploid genome equivalents) was arranged into DNA pools. Using nine sex-linked amplified fragment length polymorphism (AFLP) and two sequence-tagged site (STS) markers, 13 different BAC clones were identified from this part of the library. The BACs were arranged into a first-generation physical map around the sex locus. Four PCR-derived markers were developed from the BAC ends, one of which could be scored in a co-dominant way. Using a mapping population of 802 plants we mapped the BAC-derived markers to the same position close to the *M* gene as the corresponding AFLP and STS markers. The markers are useful for further chromosome walking studies and as diagnostic markers for selecting male plants homozygous for the *M* gene.

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A. Jamsari · I. Nitz · S. M. Reamon-Büttner · C. Jung (✉)
Plant Breeding Institute,
Christian-Albrechts-University of Kiel,
Olshausenstrasse 40, 24098 Kiel, Germany
e-mail: cjung@plantbreeding.uni-kiel.de
Tel.: +49-431-8807364
Fax: +49-431-8802566

Present address:
I. Nitz, Federal Dairy Research Centre Kiel,
Institute of Physiology and Biochemistry of Nutrition,
Hermann-Weigmann-Strasse 1, 24103 Kiel, Germany

Present address:
S. M. Reamon-Büttner, Drug Research and Medical Biotechnology,
Fraunhofer Institute for Aerosol Research and Toxicology,
Nikolai-Fuchs-Strasse 1, 30621 Hanover, Germany

Introduction

Asparagus (*Asparagus officinalis* L.) is an important perennial, dioecious crop plant with $2n=2x=20$ chromosomes and a haploid genome size of 1,323 Mb (Bennett and Leitch 2003). As an edible vegetable, it is cultivated worldwide for its succulent stems. Previous studies have shown that sex dimorphism is genetically determined by a dominant gene, *M* (Flory 1932, reviewed by Uno 2002) that is located on the L5 chromosome (Löptien 1979). Female plants are homozygous for the recessive alleles (*mm*); male plants are either homozygous (*MM*) or heterozygous (*Mm*) at the sex locus (Löptien 1979). Andromonoecious plants, which are sometimes found among asparagus populations, are heterozygous for the *M* gene and are able to set seeds after selfing. Male plants are desired in asparagus breeding and cultivation because they have a higher yielding capacity, are more tolerant to diseases and have a greater longevity than female plants. For breeding, *MM* males, sometimes referred as 'supermales', are preferred as pollinators because they produce 'all male' hybrid populations following crossing with females. However, *MM* males cannot be differentiated phenotypically from the heterozygous males, even at the flowering stage. Hence, time-consuming testcrosses have to be carried out for distinguishing homozygous males from heterozygous ones.

Efforts have been made to identify genetic markers linked to the *M* gene. These have included the use of morphologic, isoenzyme (Maestri et al. 1991) and DNA-based markers (Jiang and Sink 1997; Reamon-Büttner et al. 1998; Reamon-Büttner and Jung 2000) linked to the *M* locus. Several sex chromosome-derived sequences with known and unknown functions in flower development have been isolated in different dioecious plant species, for example *Silene latifolia* (Okada et al. 2001; Scutt et al. 2002), *Atriplex garrettii* (Ruas et al. 1998) and *Rumex acetosa* (Shibata et al. 2000). In other dioecious plant species, including *S. latifolia*, morphologic, biochemical and genetic events leading to sexual dimorphism have been defined (Negrutiu et al. 2001; Wolf et al. 2001), but

the corresponding mechanisms at the molecular level are still unclear. No sex-determining plant gene has been isolated to date. Thus, map-based cloning of the sex gene of asparagus would contribute to the understanding of the molecular aspects of sex determination in dioecious plants and would also have an important impact on plant breeding.

We describe here the construction of an asparagus BAC (bacterial artificial chromosome) library for map-based cloning of the sex gene, selection of sex-specific BAC clones and development of diagnostic markers derived thereof. The markers were used to construct a high-density map around the *M* gene.

Materials and methods

Plant material

High-molecular weight (HMW) DNA was extracted from a homozygous (*MM*) male asparagus (*Asparagus officinalis* L.) plant, no. 950545/8. This plant originated from an F_2 mapping population produced by selfing of an andromonoecious plant (Reamon-Büttner and Jung 2000). We used 802 plants to construct the high-resolution genetic map. These plants resulted from the selfing of two andromonoecious F_1 and three andromonoecious F_2 plants initially derived from crossing a male (*MM*) and a female (*mm*) plant (Reamon-Büttner et al. 1998).

Construction of the BAC library

HMW DNA was isolated from 4-week-old ferns (modified stems or cladophylls) using the nuclear-DNA-based TAMU protocol (http://HBZ.TAMU.EDU/bacindex2.html) adapted to asparagus. Since asparagus contains several phenolic compounds, megabase-sized DNA can be isolated in sufficient quantities by adding 2% polyvinylpyrrolidone (PVP) to the isolation and washing buffer. Agarose plugs were equilibrated with 100 µl *Hin*dIII reaction buffer, and the diffusion of *Hin*dIII into the agarose plugs was enabled by incubation for 1 h on ice before digestion. For the first size selection, the agarose gel was run at 5.8 V/cm, angle 120°, with a pulse ramping from 1 2 to 45 s for 16 h at 14°C. DNA fragments corresponding to 100–350 kb were subjected to a second size selection (14°C, 16 h, current 5.8 V/cm, angle 120°, switch time 5 s). DNA in the range of 100 kb was excised from the CHEF gel.

For cloning, the BAC vectors pBeloBAC11 (Shizuya et al. 1992) and pBeloBACkan (Mozo et al. 1998) were purified with a QIAGEN Maxi kit (QIAGEN, Valencia, Calif.) according to the manufacturer's protocol. Vector DNA (10 µg) was completely digested with *Hin*dIII (pBeloBAC11) or *Bam*HI (pBeloBACkan). Vector preparation, ligation and transformation were carried out according to the TAMU protocol (http://HBZ.TAMU.EDU/bacindex.html).

The clones were picked by hand and by a QPix machine (Genetix, Queensway) at the ADIS, Max Planck Institute for Plant Breeding Research, Cologne, Germany and transferred into 96-well and 384-well microtiter plates with 150 µl and 50 µl freezing medium (LB supplemented with 36 m*M* K_2HPO_4, 13.2 m*M* KH_2PO_4, 1.7 m*M* sodium citrate, 0.4 m*M* $MgSO_4$, 6.8 m*M* $(NH_4)SO_4$, 4.4% (v/v) glycerol and 12.5 µg/ml chloramphenicole), respectively. After overnight growth at 37°C, the plates were stored at −70°C.

For estimating BAC clones containing organellar DNA, BAC colony filters were prepared on 22.2×22.2-cm nylon membranes Q-Filters (Genetix) using a 96-pin gridding head of the Genetix QPix robot. Five percent (4,608 colonies) of the BAC library was gridded in a 3×3 double spot offset using one guide spot. Filters were incubated overnight at 37°C with the colony side up on LB/chloramphenicol (12.5 µg/ml) plates. Processing and hybridization of the filters were done as recommended in the QPix manual. As probes, [^{32}P]-labeled tobacco chloroplast pXX1.6 and *Oenothera* mitochondrial *cox*I sequences (kindly provided by K. Krupinska, University of Kiel) were used. Filters were exposed to imaging plates for 1–3 h and analyzed using the FLA-5000 phosphor imager (Fujifilm, Kanagawa).

Pooling of the library for PCR-based screening

The 428 96-well format microtiter plates constituting the library were organized into three series of pools, with each pool constructed from four plates. The plate pools comprised 384 single BAC clones; the rows and columns sub pools comprised 48 and 32 single clones, respectively. For pooling, clones from four plates were replicated onto Nunc OmniTrays containing 2× LB agar supplemented with 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside, 25 µg/ml isopropylthio-β-D-galactoside and 12.5 µg/ml chloramphenicol. For subpooling, the clones were plated onto trays containing 2× LB agar supplemented with 12.5 µg/ml chloramphenicol and incubated overnight at 37°C. To start a liquid culture from the plate pools, we inoculated all of the colonies into 500 ml 2× LB supplemented with 12.5 µg/ml chloramphenicol and cultured them at 37°C and 220 rpm to a cell density of 3–4×10^9 cells/ml. The subpools of the rows and columns were inoculated in 250 ml 2× LB with 12.5 µg/ml chloramphenicol under the same conditions. The bacterial pools were centrifuged and stored at −20°C until DNA extraction. BAC pool DNA was prepared by a modified alkaline-lysis method using the QIAGEN Large-Construct kit according to manufacturer's instructions (QIAGEN). BAC subpool DNA was isolated using the QIAGEN Plasmid Maxi kit. The only modification to the QIAGEN protocols was that the plated colonies were directly suspended in 500 ml/250 ml liquid medium without an additional starter culture. BAC DNA was dissolved in 300 µl 1× TE and stored at 4°C.

BAC library screening with AFLP and STS markers

BAC DNA (75 ng) from pools and subpools was used as the template for amplified fragment length polymorphism (AFLP)-based screening. Nine sex-linked AFLP markers were selected based on a preliminary genetic map of the L5 sex chromosome (Table 1). The AFLP protocol has been described previously (Reamon-Büttner et al. 1998). Electrophoresis was carried out on an 8% LongRanger polyacrylamide gel (Biozym, Oldendorf) with a Li-Cor DNA sequencer (Li-Cor Biosciences, Bad Homburg).

For sequence-tagged site (STS)-based screening of the BAC library, two sex-linked STS markers derived from the corresponding AFLP markers were selected on the basis of a genetic map of the L5 sex chromosome (Table 1). STS-PCR was carried out in 96-well reaction plates (Sarstedt, Nümbrecht) with a Perkin Elmer 9600 thermocycler (Foster City, Calif.) using 25 ng BAC pool or BAC subpool DNA as template (Reamon-Büttner and Jung (2000). PCR products were analyzed on a 3% Metaphor agarose gel (BMA, Rockland, Md.).

BAC-derived marker development

Terminal ends of positive clones identified with AFLP and STS markers were sequenced by DLMBC (Berlin). Sequence data were edited with Lasergene software (DNASTAR, Madison, Wis.). Primers were designed with the OLIGOS 9.6 program (Kalendar 2002).

PCR analyses were carried out in 25-µl volumes, each containing 25 ng of genomic DNA, 2.5 m*M* dNTPs, 5 pmol of primers forward and reverse, 2.5 µl 10× PCR-buffer and 1.0 U *Taq* DNA polymerase (Amersham Biosciences, Freiburg). All of the PCR analyses consisted of an initial denaturation of 30 s at 94°C;

Table 1 Results from screening a subset of the BAC library (41,088 clones) with AFLP and STS markers tightly linked to the *M* locus of asparagus. Map positions were calculated with 206 plants from the F_2 population

Marker	Genetic distance between marker and *M* locus (cM)	Identified BAC clones[a] (kb)
EM4150	0.00	364C6 (45)
STS4150.1	0.00	364C6 (45)
STS3156	0.00	73F6 (100), 77E7 (85)
EM3156	0.00	73F6 (100), 77E7 (85)
EM3353	0.00	407A8 (220)
EM4447.1	0.15	45B2 (85), 347D2 (95)
EM3950	0.16	385C3 (50)
PM3551	0.41	46C7 (90), 381C5 (93), 383B6 (75)
PM3159	0.52	385C3 (50)
EM4654	2.38	115G11 (100), 373B5 (6)
EM4447.2	3.63	151F5 (90)

[a] The insert sizes of identified BAC clones are given in brackets

amplification for 32 cycles of 30 s at 94°C (denaturation), 30 s at 50–60°C (primer annealing) and 40 s at 72°C (extension); a final extension for 5 min at 72°C. The PCR products were visualized on a 1% agarose gel electrophoresis. In the case of no polymorphism, PCR products were subjected to digestion with different restriction enzymes.

Map construction

Mapping of BAC end-derived markers was carried out on 802 plants of the segregating population, with the exception of marker Asp8-T7, which was analyzed in 127 plants that showed recombination between flanking markers EM4447.2 and EM3251. Markers Asp1-T7, Asp4-SP6 and Asp8-T7 were scored dominantly, whereas marker Asp2-SP6 was scored codominantly. Marker positions were determined with the mapping software JOINMAP 3.0 (Van Ooijen and Voorrips 2001) using high stringency conditions with REC and LOD threshold values 0.25 and 8, respectively. For map construction, Kosambi's mapping function was used (Kosambi 1944).

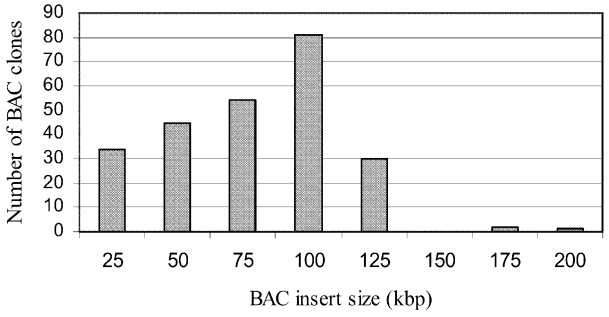

Fig. 1 Insert size distribution of the asparagus *Hin*dIII BAC library. Insert sizes of 247 BAC clones are plotted against frequency. BAC inserts were released by digestion of the DNA minipreps with *Not*I, fractionated on a pulsed-field gel and estimated for sizes by comparison with the low-range molecular-weight marker (New England Biolabs, Frankfurt)

Results

Construction and characterization of the library

To provide a resource for map-based cloning of the sex gene in asparagus, we constructed a *Hin*dIII-BAC library from a *MM* male plant. The library consists of 86,784 clones arrayed in 428 96-well and 119 384-well microtiter plates. To assess the quality of the library, BAC DNA of 270 randomly selected clones was isolated, digested with *Not*I and analyzed by CHEF electrophoresis. Of these 270 clones, 247 (91.5%) contained an insert. The sizes of these inserts ranged from 25 kb to 200 kb, with an average size of approximately 82 kb (Fig. 1). Among the 247 clones, 32% contained inserts in the range of 25 kb to 50 kb. On the basis of this average insert size and a haploid genome size of 1,323 Mb (Bennett and Leitch 2003), the coverage of the library was about 5.5 genome equivalents, thereby rendering a greater than 99% probability of finding a specific sequence from this library.

To determine the representation of organellar DNA in the BAC library, we gridded 5% of the clones on filters and hybridized these to [^{32}P]-labeled chloroplast (cp) and mitochondrial (mt)DNA probes. The results from this screening showed that approximately 0.6% of the cloned inserts contained cpDNA and 2.0% contained mtDNA. The low proportion of clones containing organellar DNA sequences is due to the use of nuclear DNA for the library construction.

PCR-based screening of the BAC pools

Since the BAC library was constructed from a single plant of the mapping population, it was possible to screen the library with AFLP and STS primer combinations using pools of BAC DNA as templates. Our pooling strategy utilized three different pool types involving four 96-well plates: (1) pools of 384 single BAC clones, (2) rows of subpools containing 48 single clones and (3) columns of subpools containing 32 single clones. A total of 267 pools—107of the 384pools of single BAC clones and 160 subpools derived from rows and columns—were screened with nine sex-linked AFLP and two STS markers. For each marker, at least one BAC could be found. Altogether, 13 positive clones were obtained (Table 1). Four clones were detected in duplicate by two different primer combinations.

Development of BAC end-derived markers

The SP6 and T7 ends of 13 positive BAC clones were sequenced and 26 primer combinations were designed. The copy number of BAC end sequences in the asparagus

Table 2 PCR-based markers developed from *M*-linked BAC clones. Each PCR started with denaturation at 94°C for 30 s, followed by 32 cycles and terminated with an additional extension at 72°C for 5 min

Markers	Forward and reverse primers	Annealing temperature (time)	Restriction enzymes
Asp1-T7	5′-CTTGGCGTGAATACGTTGC-3′ 5′-TCTCTTGTTCAATATACTC-3′	59°C (30 s)	–
Asp2-SP6	5′-GCTCTTTGAGGGTGTTT-3′ 5′-TGCTCCTCCACTCTCA-3′	59°C (30 s)	*Mse*I, *Tru*I
Asp4-SP6	5′-AGGCCTCTCAAGTTTCA-3′ 5′-AGCAGATCCCACATTGA-3′	60°C (30 s)	*Taq*I
Asp8-T7	5′-AGATCTGAGATCCGGTTCC-3′ 5′-AATAGTTTCATGGAGGAAGG-3′	50°C (30 s)	*Mse*I, *Tru*I

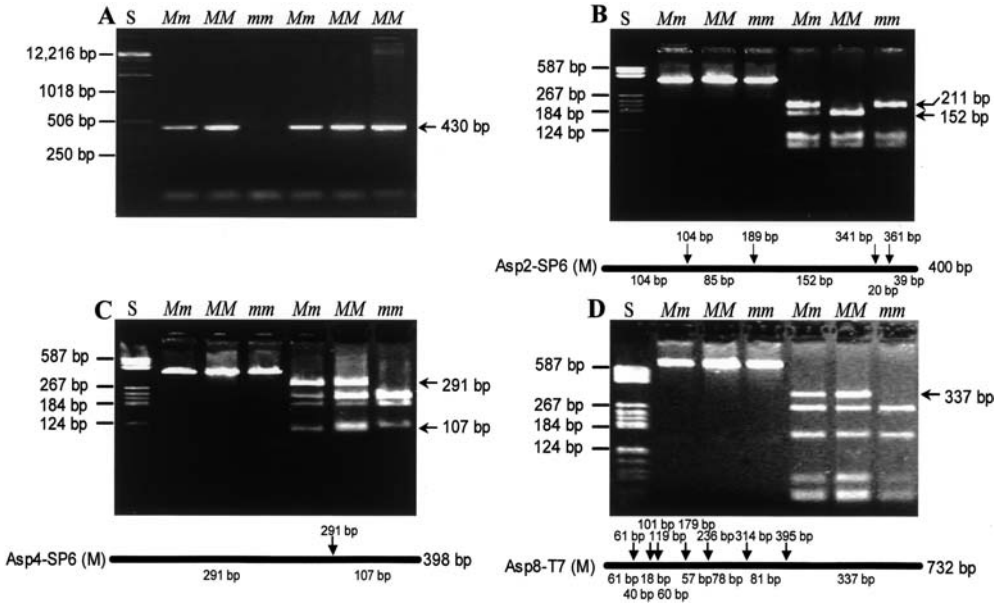

Fig. 2A–D Molecular markers derived from sex-linked BAC clones. PCR fragments were separated on 1% agarose gels. The gels display restriction sites present in the PCR fragments. **A** Marker Asp1-T7 exhibited a singular fragment of 438 bp with DNA from male plants (*Mm*, *MM*) and no fragment with DNA from female plants (*mm*). **B** Asp2-SP6 exhibited a 400-bp fragment with DNA from both male and female plants. Following *Mse*I/*Tru*I digestion, a 152-bp fragment was created from male plants (*Mm*, *MM*) only. A 211-bp fragment was present in female (*mm*) and heterozygous male plants (*Mm*), but absent from homozygous males (*MM*). **C** Asp4-SP6 exhibited a 398-bp PCR fragment with DNA from male and female plants. Following digestion with *Taq*I, a 291-bp fragment was observed in male plants (*Mm*, *MM*) only. **D** Asp8-T7 produced a 732-bp PCR product with DNA from male and female plants. Polymorphisms between males and females became visible after digestion of the PCR products with *Mse*I/*Tru*I. A 337-bp fragment was observed in male plants (*Mm*, *MM*) only

genome was determined in two experiments. In the first experiment, 27 BAC super pools representing 41,088 BAC clones or approximately 2.6 genome equivalents were analyzed by PCR using BAC end-derived primer combinations. The number of positive super pools gave a direct measurement of the copy number of the corresponding sequence. Ten of the BAC end sequences analyzed were regarded as multi/high copy because the corresponding primer combinations yielded PCR fragments in more than four BAC super pools. With one exception, they were excluded from further experiments. Sixteen BAC end sequences were interpreted as single/low-copy sequences because they were present in fewer than four super pools.

In the second experiment, 17 BAC end fragments selected by PCR were used as probes for genomic Southern hybridization. Nine probes showed complex banding patterns, whereas eight probes produced between one and ten bands and thus could be regarded as single/low copy (data not shown).

Combining the results from both experiments, eight BAC ends were found to be single/low copy and were thus useful for marker development. From these, four diagnostic markers could be developed (Table 2) as follows:

– Asp1-T7 was derived from the T7 end of BAC 73F6. A single fragment 438 bp in size was visible only after amplification with DNA from male plants (Fig. 2A).

Hence, the marker is scored in a dominant way, and it can differentiate between male and female plants but not between homozygous and heterozygous ones.

- Asp2-SP6 is a cleaved amplified polymorphic sequence (CAPS) marker derived from the SP6 end of BAC 77E7. The PCR products were 400 bp in size no matter whether DNA from male or female plants was used in the analysis. The sequence has four restriction sites for *Mse*I (TTAA) or its isoschizomere *Tru*I, giving rise to five fragments of 20 bp, 39 bp, 85 bp, 104 bp, and 152 bp, respectively. Polymorphisms were observed after restriction of the PCR products with *Mse*I or *Tru*I prior to separation on a 3% agarose gel. The 152-bp fragment was linked to the *M* allele and therefore present only in males, while the *m*-linked 211-bp fragment was present in heterozygous males and females (Fig. 2B). Thus, this marker can be scored in a codominant way, and it can discriminate between homozygous and heterozygous males.
- Asp4-SP6 was derived from the SP6 end of BAC 384C6. On the basis of sequence analysis, a 398-bp fragment was expected. While a band in this size range was observed following electrophoresis on a 3% agarose gel, subsequent *Taq*I digestion uncovered that this band was a mixture of different fragments: apart from the expected fragments (291 bp, 107 bp), two more fragments in the 200-bp size range were observed. Obviously, those fragments are not linked to the *M* gene because they were present in both the male and female plants, whereas the 291-bp fragment cosegregated with the *M* locus. The absence of the Asp4-SP6 fragment from the female plants can be explained by a deletion of the corresponding primer binding sequence. Thus, this marker has to be scored in a dominant way, clearly differentiating between males and females.
- Asp8-T7 was derived from the T7 end of BAC 407A8. This marker can distinguish between male and female plants after *Mse*I digestion of the 732-bp PCR product. For the PCR, the annealing temperature had been adjusted to 50°C. As in the case of Asp4-SP6, the bands are a mixture of different PCR products. Sequence analysis revealed seven *Mse*I/*Tru*I restriction sites for the male haplotype (Fig. 2D). Hence, restriction with *Mse*I/*Tru*I should yield eight fragments of 18 bp, 40 bp, 57 bp, 60 bp, 61 bp, 78 bp, 81 bp and 337 bp, respectively. However, due to the limited resolution capacity of a 3% agarose gel, only the 337-bp fragment was clearly separated from the other ones that were clustered in two groups, one in a size range between 18 bp and 61 bp and the other between 78 bp and 81 bp. Two non-specific bands of 170 and 250 bp were present in all genotypes. Linkage analysis revealed that the corresponding alleles are not linked to the sex gene.

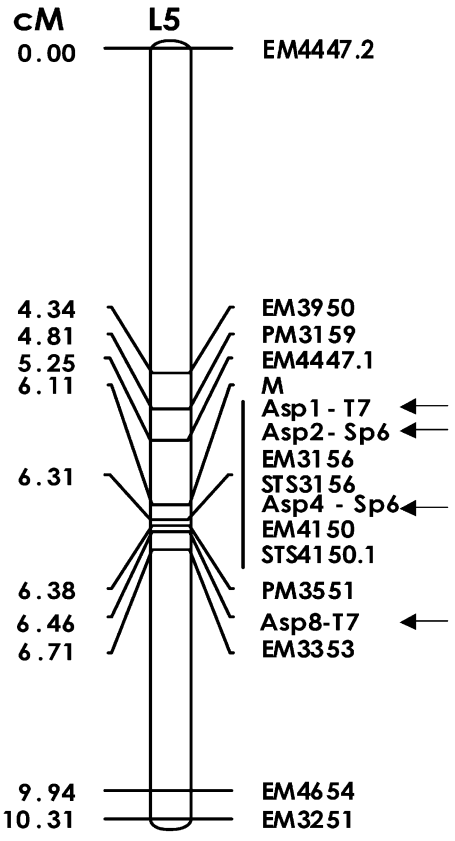

Fig. 3 Integration of four BAC-end-derived markers (*arrows*) into the high-resolution genetic map around the *M* locus of asparagus. The four BAC-end-derived markers mapped in the neighborhood of the markers used to identify the corresponding BAC clones. The map is based on an analysis of 802 plants from the segregating population

Mapping the BAC ends

To confirm whether the identified BACs originated from the *M* region, we analyzed markers Asp1-T7, Asp2-SP6 and Asp4-SP6 using 802 plants of the segregating population. Markers Asp1-T7 and Asp4-SP6 segregated according to the expected ratio of 3:1 (χ^2=3.84; α=5%), while marker Asp2-SP6 segregated 1:2:1 (χ^2=5.99; α=5%). These markers were integrated into the existing genetic map (Fig. 3). Markers Asp1-T7 and Asp2-SP6 co-segregated with marker EM3156, which had been used for selecting the original BAC-clones. Similarly, marker Asp4-SP6 co-segregated with marker EM4150.

To increase the efficiency of the mapping procedure, only informative individuals of the segregating population were investigated with marker Asp8-T7—i.e. individuals showing recombination within the genome region of interest. We selected 127 plants with recombinations between flanking markers EM4447.2 and EM3251. Five of these plants displayed recombination between the *M* locus and marker EM3353. Interestingly, only two of these plants showed recombination between Asp8-T7 and EM3353, which had been used for extracting the corre-

sponding BAC 407A8. Hence, marker Asp8-T7 maps even shorter to the *M* locus than marker EM3353 even though both sequences are located at a physical distance of less than 220 kb from the latter.

Discussion

Large-insert DNA libraries are essential tools for physical mapping and map-based gene cloning in plant species with complex genomes. BAC vectors represent state-of-the-art technology for large-insert DNA library development. We constructed the first large-insert library of *A. officinalis*. The library contains 86,784 clones with an average insert size of 82 kb, which corresponds to 7,377 Mb of cloned DNA or 5.6 haploid genome equivalents. This average insert size of 82 kb is relatively small compared to other BAC libraries. However, the 5.6-fold genome coverage and the low cpDNA and mtDNA content makes this library suitable for map-based cloning of the sex gene and, therefore, of considerable value to asparagus research. For map-based gene cloning or the development of genetic markers for targeted genome regions, only a low representative library is required.

We describe here the first steps of creating a physical map around the sex gene. Because the sex-linked markers used in screening the BAC library are repetitive sequences, they could not be used as probes for filter hybridization. Owing to this limitation, we employed a PCR-based analysis using AFLP and STS markers in combination with a pooling strategy to isolate sex-specific BAC clones. The PCR-based approach is an alternative to filter screening and is an efficient and reliable way to identify specific BAC clones for constructing contigs of overlapping clones and physical maps (Klein et al. 2000; Allouis et al. 2001; Deng et al. 2001; O'Sullivan et al. 2001).

The combination of AFLP- and STS-based screening systems has been shown to be a rapid and efficient method for identifying specific BAC clones, although the average number of positive BAC clones per marker was less than expected from the genome coverage of the library. Comparable results have been reported from a sorghum library representing four genome equivalents (Klein et al. 2000). Screening the library with single-copy probes yielded an average of 2.6 positive BACs. We have identified 13 different sex-specific BAC clones by screening 2.5 genome equivalents of the asparagus BAC library with nine AFLP and two STS markers. Only in two cases (EM3156, EM4447.1) was the number of positive clones identified consistent with the predicted genome coverage. The deficit in the number of positive clones may be due to failure of the PCR in some of the DNA pools. In our experiments, a PCR signal was only regarded as a positive one if it was present in all three pool types. It is noteworthy that the two AFLP-derived STS markers identified the same BACs as their corresponding AFLP markers, which is a confirmation that the STS markers are representing their AFLP loci.

The BAC library presented here was constructed for two purposes—first, for development of new diagnostic markers and, second, for identification of the male-determining sex gene. Since male plants outyield female plants on average by 50% (Franken 1970) the production of pure male hybrids is a main target for asparagus breeding. These hybrids can only be produced by crossing homozygous (*MM*) males, which are also referred to as supermales, with females. Supermales are produced by the self-pollination of hermaphroditic flowers on a male plant or by in vitro androgenesis, including anther culture or microspore culture (Feng and Wolyn 1994; Peng and Wolyn 1999). These are time-consuming procedures and in all cases, the efficiency of supermale production was low.

The four PCR-based markers tightly linked to the *M* locus provide important tools for marker-assisted selection in asparagus. Markers Asp1-T7, Asp4-SP6 and Asp8-T7 enable the selection of male plants, but they cannot differentiate between homozygous and heterozygous ones. For this latter purpose, Asp4-SP6 is recommended because the restriction enzyme *Mse*I is considerably cheaper than *Taq*I, which is required for markers Asp2-SP6 and Asp8-T7 with average costs of 0.1 €/unit and 0.0055 €/unit, respectively. Compared with marker Asp1-T7 and four BAC-derived STS markers (data not shown), marker Asp4-SP6 provides more reliable data because the presence of several restriction fragments reduces the danger of misscoring.

Marker Asp2-SP6 can be scored in a codominant way, thus enabling discrimination between homozygous and heterozygous male plants. Compared with PCR-based markers developed from the AFLP-fragment STS3156 (Reamon-Büttner and Jung 2000), the CAPS marker Asp2-SP6 is easy to handle. The informative fragment of marker STS3156 is relatively small (65 bp), and it can only be resolved by using 3% metaphore agarose gels. Metaphore agarose is almost three times more expensive than standard agarose. Moreover, marker STS3156 gives only a yes-or-no result based on a single fragment thereby creating the danger of false negative results. In contrast, the informative restriction fragments of the new marker Asp2-SP6 are substantially longer (211 bp, 152 bp), and they enable differentiation between homozygous and heterozygous males and female plants. The informative fragments resulting following *Mse*I digestion can be analyzed on standard 2% agarose gels. However, the relatively high price (0.1 €/unit) of restriction endonuclease *Mse*I should be considered when applying this marker for practical purposes.

Cloning of the sex gene should give us insights into the male-determination processes at the molecular level and this, in turn, leads us to sex-targeting strategies that would produce only supermale plants. Moreover, the genetic manipulation of male flowering is also of the greatest interest in hybrid breeding because this breeding procedure relies on the availability of male-sterile seed parents. Cloning of the sex gene from asparagus may offer the opportunity for manipulating male flowering even in

monoecious plants, thus facilitating the production of male-sterile plants.

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References

Allouis S, Qi X, Lindup S, Gale MD, Devos KM (2001) Construction of a BAC library of pearl millet, *Pennisetum glaucum*. Theor Appl Genet 8:1200–1205

Bennett MD, Leitch IJ (2003) Plant DNA C-values database (release 2.0, January 2003). http://www.rbgkew.org.uk/cval/homepage.html

Deng Z, Huang S, Ling P, Yu C, Tao Q, Chen C, Wendell M, Zhang H-B, Gmitter F Jr (2001) Fine genetic mapping and BAC contig development for the citrus tristeza virus resistance gene locus in *Poncirus trifoliata* (Raf.). Mol Gen Genet 265:739–747

Feng XR, Wolyn DJ (1994) Recovery of haploid plants from asparagus microspore culture. Can J Bot 76:296–300

Flory WS (1932) Genetic and cytological investigations on *Asparagus officinalis* L. Genet Princeton 17:432–467

Franken A (1970) Sex characteristic and inheritance of sex in asparagus (*Asparagus officinalis* L.). Euphytica 19:277–287

Jiang C, Sink KC (1997) RAPD and SCAR markers linked to the sex expression locus *M* in asparagus. Euphytica 94:329–333

Kalendar R (2002) OLIGOS 9.6. Institute of Biotechnology University of Helsinki, Finland

Klein PE, Klein RR, Cartinhour SW, Ulanch PE, Dong J, Obert JA, Morishige DT, Schlueter SD, Childs KL, Ale M, Mullet JE (2000) A high-throughput AFLP-based method for constructing integrated genetic and physical maps: progress toward a sorghum genome map. Genome Res 10:789–807

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Löptien H (1979) Identification of the sex chromosome pair in asparagus (*Asparagus officinalis* L.). Z Pflanzenzücht 82:162–173

Maestri E, Restivo FM, Marziani-Longo GP, Falavigna A, Tassi F (1991) Isozyme gene markers in the dioecious species *Asparagus officinalis* L. Theor Appl Genet 81:613–618

Mozo T, Fischer S, Meier-Ewert S, Lehrach H, Altmann T (1998) Use of the IGF BAC library for physical mapping of the *Arabidopsis thaliana* genome. Plant J 16:377–384

Negrutiu I, Vyskot B, Barbacar N, Georgiev S, Moneger F (2001) Dioecious plants. A key to the early events of sex chromosome evolution. Plant Physiol 127:1418–1424

Okada S, Sone T, Fujisawa M, Nakayama S, Takenaka M, Ishizaki K, Kono K, Shimizu-Ueda Y, Hanajiri T, Yamato KT, Fukuzawa H, Brennicke A, Ohyama K, (2001) The Y chromosome in the liverwort *Marchantia polymorpha* has accumulated unique repeat sequences harboring a male-specific gene. Proc Natl Acad Sci USA 98:9454–9459

O'Sullivan DM, Ripoll PJ, Rodgers M, Edwards KJ (2001) A maize bacterial artificial chromosome (BAC) library from the European flint inbred line F_2. Theor Appl Genet 103:425–432

Peng M, Wolyn DJ (1999) Improved callus formation and plant regeneration for shed microspore culture in asparagus (*Asparagus officinalis* L.). Plant Cell Rep 18: 954–958

Reamon-Büttner SM, Jung C (2000) AFLP-derived STS markers for the identification of sex in *Asparagus officinalis* L. Theor Appl Genet 100:432–438

Reamon-Büttner SM, Schondelmaier J, Jung C (1998) AFLP markers tightly linked to the sex locus in *Asparagus officinalis* L. Mol Breed 4:91–98

Ruas CF, Fairbanks DJ, Evans RP, Stutz HC, Andersen WR, Ruas PM (1998) Male-specific DNA in the dioecious species *Atriplex garrettii* (Chenopodiaceae). Am J Bot 85:162–167

Scutt CP, Jenkins T, Furuya M, Gilmartin PM (2002) Male specific genes from dioecious white campion identified by fluorescent differential display. Plant Cell Physiol 43:563–572

Shibata F, Hizume M, Kuroki Y (2000) Differentiation and the polymorphic nature of the Y chromosomes revealed by repetitive sequences in the dioecious plant *Rumex Acetosa*. Chromosome Res 8:229–236

Shizuya H, Birren B, Kim U-J, Mancino V, Slepak T, Tachiiri Y, Simon M (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc Natl Acad Sci USA 89:8794–8797

Uno Y, Li Y, Kanechi M, Inagaki N (2002) Haploid production from polyembryonic seeds of *Asparagus officinalis* L. Acta Hortic 589:2002

Van Ooijen JW, Voorrips RE (2001) JOINMAP 3.0, software for the calculation genetic linkage maps. Plant Research International, Wageningen, The Netherlands

Wolf DE, Satkoski JA, White K, Rieseberg LH (2001) Sex determination in the androdioecious plant *Datisca glomerata* and its dioecious sister species *D. cannabina*. Genetics 159:1243–1257