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A genetic map of *Asparagus officinalis* based on integrated RFLP, RAPD and AFLP molecular markers

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Abstract An integrated genetic map of the dioecious species *Asparagus officinalis* L. has been constructed on the basis of RFLP, RAPD, AFLP and isoenzyme markers. The segregation analysis of the polymorphic markers was carried out on the progeny of five different crosses between male and female doubled-haploid clones generated by anther culture. A total of 274 markers have been organized to ten linkage groups spanning 721.4 cM. Since the haploid chromosome number of asparagus is ten, the established linkage groups probably represent the different chromosomes; however, the only group associated with a specific chromosome is the one which includes sex, whose determinant genes have been located on chromosome 5. A total of 33 molecular markers (13 RFLPs, 18 AFLPs, 2 RAPDs and 1 isoenzyme) have been located on this chromosome. The closest marker to the sex determinant is the AFLP SV marker at 3.2 cM.

Key words Asparagus · Dioecious species · Genetic map · Molecular markers

Introduction

Genetic maps represent different molecular markers linked to loci of interest and can be used in marker-facilitated breeding programs (Tanksley et al. 1989, 1992; Gebhardt and Salamini 1992; Rafalski and Tingey 1993; Kurata et al. 1994; Becker at al. 1995). Identified loci involved in plant resistance to diseases (Martin et al. 1993; Nodari et al. 1993; Paran and Michelmore 1993; Adam-Blondon et al. 1994; Balint-Kurti et al. 1994; Dixon et al. 1995), or in physiological processes like ripening (Giovannoni et al. 1995) and in agronomic characters, such as seed or fruit quality (Ahn et al. 1992), have also been located on genetic maps.

The number of cultivated species for which a genetic map is available is increasing and includes not only the most important crop plants like cereals (Ahn and Tanksley 1993; Gardiner et al. 1993; Becker et al. 1995; O'Donoughue 1995; Loarce et al. 1996), legumes (Vallejos et al. 1992; Adam-Blondon et al. 1994; Shoemaker and Specht 1995) and oil and sugar producing plants (Barzen et al. 1995; Berry et al. 1995; Foisset et al. 1996) but also fruit plants (Lodhi et al. 1995; Rajapakse et al. 1995; Sondur et al. 1996), spice plants (Lefebvre et al. 1995; Lenaud et al. 1995) and forest plants (Byrne et al. 1995; Polmion et al. 1995; Devey et al. 1996; Gocmen et al. 1996).

The dioecious species *Asparagus officinalis* is an important crop plant which is cultivated in many countries world wide. A linkage map with 48 RFLP markers and a combined RFLP and RAPD map have recently been reported. These maps were constructed with the double-pseudocross mapping strategy (Lewis and Sink 1996; Jiang et al. 1997). In a previous paper we have reported the linkage arrangement of RFLP loci in progenies derived from crosses between doubled-haploid asparagus clones (Restivo et al. 1995); however a saturated map of asparagus is not yet available. In the present study we report on a genetic map derived from the integration of RFLP markers with RAPD (Williams et al. 1990) and AFLP (Zabeau and Vos 1993; Vos et al. 1995) molecular markers. The validity of AFLP markers for map construction has been

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shown by Becker et al. (1995) who integrated a RFLP map of barley with AFLP markers.

The construction of a map for the dioecious species *A. officinalis* also allows the identification of markers linked to sex. The sex-determining genes of asparagus have been associated with chromosome 5 (Loptien 1979), but nothing is known about these genes, which as yet have not been isolated. A saturated map of the sex chromosome is the first step to attempt the isolation of the sex-determining genes by positional cloning. In a previous paper (Restivo et al. 1995) we reported three sex-linked RFLP markers; in the present work we have enriched the map of the sex chromosome 5 with several other markers, both RFLPs and AFLPs.

Materials and methods

Plant material

The *A. officinalis* genotypes utilized in the present work have been described previously (Maestri et al. 1991; Restivo et al. 1995). Parental lines are doubled-haploid clones generated by anther culture at the Research Institute for Vegetable Crops, Section of Montanaso Lombardo. Since the male parents are homozygous, including the sex determinants, and the F_1s are all male, linkage analysis has been carried out on BC_1 of progeny of 40–80 plants from five different BC_1s (crosses A, B, D, E and S).

DNA extraction

Phylloclades were collected and frozen immediately in liquid nitrogen and then stored at −80°C. They were the source of DNA for all analyses. DNA of single individuals was extracted from 1–2 g of phylloclades, following the procedure described by Dellaporta et al. (1985) with minor modifications.

RFLP analysis

Anonymous DNA probes (500–2000 bp) from a *Pst*I genomic library of asparagus cloned into plasmid pUC 19 were utilised for RFLP analysis. The preparation and characteristics of the library have been described previously (Restivo et al. 1995). DNA digestion with restriction enzymes (Boehringer, Mannheim), separation, blotting onto hybond N+ membranes (Amersham) and hybridization were all performed following the manufacturer's instructions. Radioactive probes were labelled using the Boehringer or Promega random priming labelling kit. Blots, wrapped in Saran Wrap, were exposed to Kodak XAR films with an intensifying screen for 1–7 days at −80°C. Blots were prepared for re-use by dipping in boiling 0.5% SDS and leaving them in the solution until it reached room temperature.

RAPD analysis

Ten-base oligonucleotide primers of arbitrary sequences (Operon Technologies Inc, Alameda Calif.) were emploiyed for RAPD analysis. The reaction mixture (25 µl) was prepared as follow: Tris HCl 10 mM pH 8.3, KCl 50 mM, $MgCl_2$ 2 mM, dATP, dCTP, dGTP, dTTP each 100 µM, primer 5 pmol, genomic DNA 15 ng, *Taq* polymerase (Boeringer Mannheim) 0.5 U.

Amplifications were run in a DNA Thermal Cycler 480 (Perkin Elmer Cetus) set for 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C followed by a final extension of 5 min at 72°C. Amplification products were analyzed after electrophoretic separation on a 1.4% agarose gel containing 0.1 µg/ml of EtBr.

AFLP analysis

AFLP marker analysis was performed essentially as described by Zabeau and Vos (1993) and Vos et al. (1995). Total genomic DNA of asparagus (1 µg) was restricted with two enzyme combinations, 5 U *Eco*RI and 5 U *Mse*I or 5 U *Pst*I and 5 U *Mse*I (*Eco*RI and *Mse*I from Boehringer-Mannheim; *Pst*I from New England Biolabs), in 50 µl of 10 mM Tris-acetate buffer, 10 mM magnesium acetate, 50 mM potassium acetate, 50 mM DTT, pH 7.5 for 2 h at 37°C. To the restricted-DNA 10 µl of a mixture containing 5 pmol of *Eco*RI adapter or 5 pmol of *Pst*I adapter, 50 pmol of *Mse*I adapter, 1 U of T4 DNA ligase (Boehringer-Mannheim) and 1.2 mM of ATP in the same buffer as before were added. The ligation reaction was incubated at 37°C for 3 h.

A first round of amplification by PCR was performed with 5 µl of template DNA using a primer pair based on the sequences of the *Eco*RI and *Mse*I adapters, or of the *Pst*I and *Mse*I adapters, that had one additional selective nucleotide at the 3′ end (*Eco*RI + 1 primer, *Mse*I + 1 primer, *Pst*I + 1 primer) as described by Vos et al. (1995). The final round of PCR amplification was performed using primers based on the same sequence as the *Eco*RI + 1, *Mse*I + 1, *Pst*I + 1 primers but with two additional selective nucleotides at the 3′ end for *Eco*RI-digested samples (*Eco*RI + 3 primers), and one selective nucleotide at 3′ end for *Pst*I-digested samples (*Pst*I + 2 primers); the second primer was *Mse*I with two additional nucleotides for both combinations (*Mse*I + 3 primers). The *Eco*RI + 3 or *Pst*I + 3 primers were labelled by phosphorylating the 5′ end with [^{33}P] ATP.

The PCR products were mixed with an equal volume of 98% formamide, 10 mM EDTA, 0.05% bromophenol blue and xylene cyanol as tracking dyes, denaturated at 90°C for 3 min and quickly cooled on ice. Aliquots (3 µl) of sample solution were loaded onto a denaturating 4.5% polyacrylamide gel (20 : 1 ratio acrylamide:bisacrylamide) in 0.5 × TBE buffer (50 mM Tris, 50 mM boric acid, 1 m MEDTA, pH 8.0), 7.5 M urea. 1 × TBE was used as the electrophoresis buffer. Gels were run at constant power (equivalent to 40–50 V/cm), fixed, dried as for sequencing gels and then exposed to X-ray film (Kodak X-OMAT AR5), for 48–72 h.

Linkage analysis

Each marker was tested for a fit to a 1 : 1 Mendelian segregation ratio by χ^2 analysis ($\alpha = 0.005$). The JOINMAP 1.4 (J.M.) package was used to perform linkage analysis. Loci were assigned to linkage groups with a two-point analysis based on a lod score $\geqslant 4$. Ordering of the markers within each linkage group was performed at a lod score $\geqslant 3$. Map distances were based on Kosambi's (1944) mapping function.

Results and discussion

As shown in Table 1 the number and type of polymorphic markers employed for segregation analysis was not the same for all crosses. The strategy followed for each type of marker is described below.

Table 1 Frequency of polymorphic markers analyzed in the progeny of different asparagus backcrosses

Backcross	Number of individuals	Marker type				
		Morphological	Isoenzyme	RELP	RAPD	AFLP
A	35	1	0	17	0	0
B	40	1	1	10	0	0
D	76	1	0	90	8	210
E	40	1	4	10	0	0
G	48	1	5	4	0	0
S	48	1	3	15	10	0

Morphological markers

The genetic control of the following three morphological markers has been established for *A. officinalis*: persistent green (g/g, Irizarry et al. 1965), red stalk (p/p, Basset et al. 1971), anthocyanin-less (Peirce 1982) and sex. The sex-determining genes (locus M) have been located on chromosome pair number 5 (Loptien 1979). Among these markers we included only the sex locus which is shared by all crosses.

Isoenzyme markers

The isoenzyme markers have been analysed in all crosses by the technique of Restivo et al. (1995).

RFLP markers

As described in a previous paper (Restivo et al. 1995) RFLP analysis was conducted with two different strategies: the individuals of cross D were analysed after DNA digestion with six different restriction enzymes (*Eco*RI, *Hin*dIII, *Dra*I, *Xba*I, *Bgl*II, *Eco*RV), while the other 4 crosses were analysed after digestion with only two enzymes (*Eco*RI and *Hin*dIII). With the two combined approaches 51% of the analysed probes detected a polymorphism between the parental DNAs of different crosses. However 13% of the polymorphisms detected between the parental DNAs were not amenable to segregation analysis in the BC progenies because the female and the corresponding F_1 displayed the same RFLP pattern. In the BC progenies we have analysed the segregation of 91 polymorphic probes from the asparagus genomic library and the cDNA probe pYu109 of *TS2*, a gene involved in sex differentiation of the maize inflorescence (DeLong et al. 1993).

The RFLP markers represent the backbone of the present map because at least two of them are found in the linkage groups shared by two or more crosses. This allows the construction, using the Join Map package, of an integrated map with data coming from the different analysed crosses.

RAPD markers

RAPD analysis was conducted on crosses D and S for the high level of variability previously exibited and the number of backcross individuals available (75 for cross D and 48 for cross S). We have analysed the amplification products obtained with 220 primers (the average number of products per primer was 4.5) and identified 36 polymorphic bands. Due to the dominant type of inheritance of the polymorphism (presence versus absence), only 50% of the polymorphisms were amenable to segregation analysis.

AFLP markers

AFLP analysis was conducted on cross D. We analysed 46 primer combinations: 35 based on *Eco*RI + 3 and *Mse*I + 3 and 11 based on *Pst*I + 2 and *Mse*I + 3 (Table 2). The average number of bands visible on the gel for the two different primer combinations was 105; 10.3% of the observed bands were polymorphic. As already tested for RAPDs, only 50% of the polymorphisms were amenable to segregation analysis. An example of an AFLP experiment is shown in Fig. 1, where an amplified fragment scored as present in male, but not in female, is clearly visible.

RFLP, RAPD, AFLP analysis: which one is more convenient?

Table 3 shows the amount of polymorphism detected with the three techniques utilized and the percentage of markers amenable to segregation analysis. The highest level of polymorphism, and the highest percentage of markers which may be utilized for segregation analysis, were detected with the RFLP technique. The RAPD technique is a very simple non-radioactive method which should analyse a large part of the genome. With our system, however, it appears to be less convenient both for the extremely low rate of polymorphism detected and for the high number of markers which cannot be utilized. The major advantage of the AFLP technique compared to RAPD and RFLP analysis is the

Table 2 Frequency of polymorphism detected in cross P with the two AFLP primer combinations

	Primer combination	
	*Eco*RI + 3/Mse + 3	PstI+2/Mse + 3
Number of analyzed combinations	35	11
Average number of bands per gel	90	120
Maximum number of polymorphisms per gel	22	18
Minimum number of polymorphisms per gel	2	1
Total number of polymorphisms	151	59
Average number of polymorphisms per gel	8.6	10.6

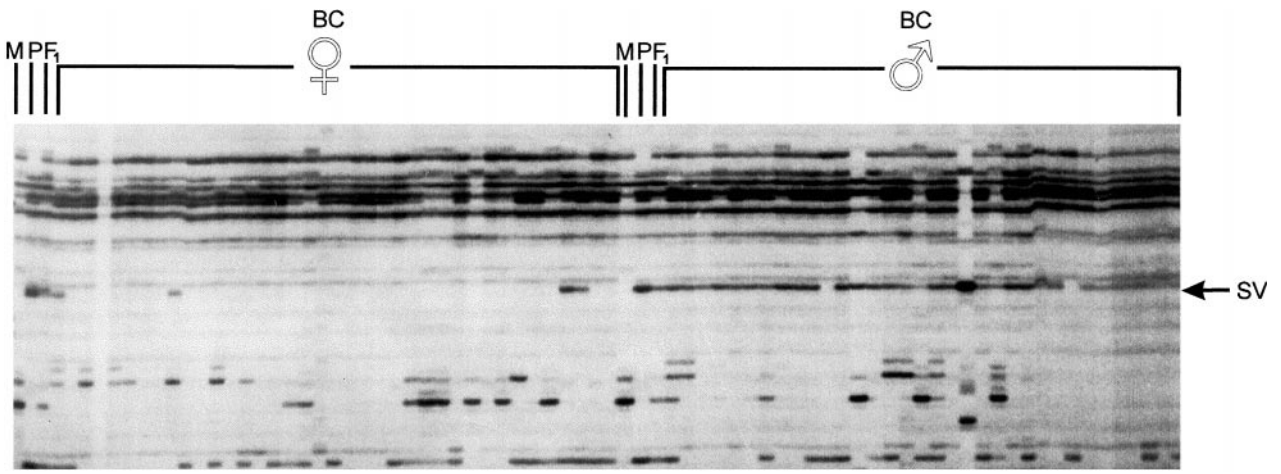


Fig. 1 An AFLP autoradiograph showing segregation of the sex-linked band SV. The band is present in the male parent (*P*) and absent in the female parent (*M*). Among the BC_1s it is present in the male but not in the female: four recombinants are visible

capacity to reveal many polymorphic bands in one lane. For that reason, even if in our system the AFLP markers do not offer the highest rate of polymorphism and 50% of them cannot be utilized for segregation analysis, they appear extremely efficient because they allow the simultaneous analysis of a large number of bands per gel.

Construction of the asparagus map

Data derived from RFLP, RAPD and AFLP analysis conducted during this work have been integrated with data formerly obtained to construct a revised genetic map of asparagus. A total of 341 polymorphic loci have been analyzed: nine isoenzymes, 104 RFLP, 17 RAPD, 210 AFLP and one morphological marker (sex). Among these markers, 274 are organized into ten groups spanning 721.4 cM, with an average distance of 2.6 cM (Fig. 2). Eight markers form four linkage groups of two markers only (AF195-AF201, 22.7%; AP2- AP3, 2.5%; OA120.8-OB051.7, 10.7%; CAT-OE070.8, 14.2%;

Table 3 Frequency of polymorphism detected and analyzed by the three molecular techniques employed

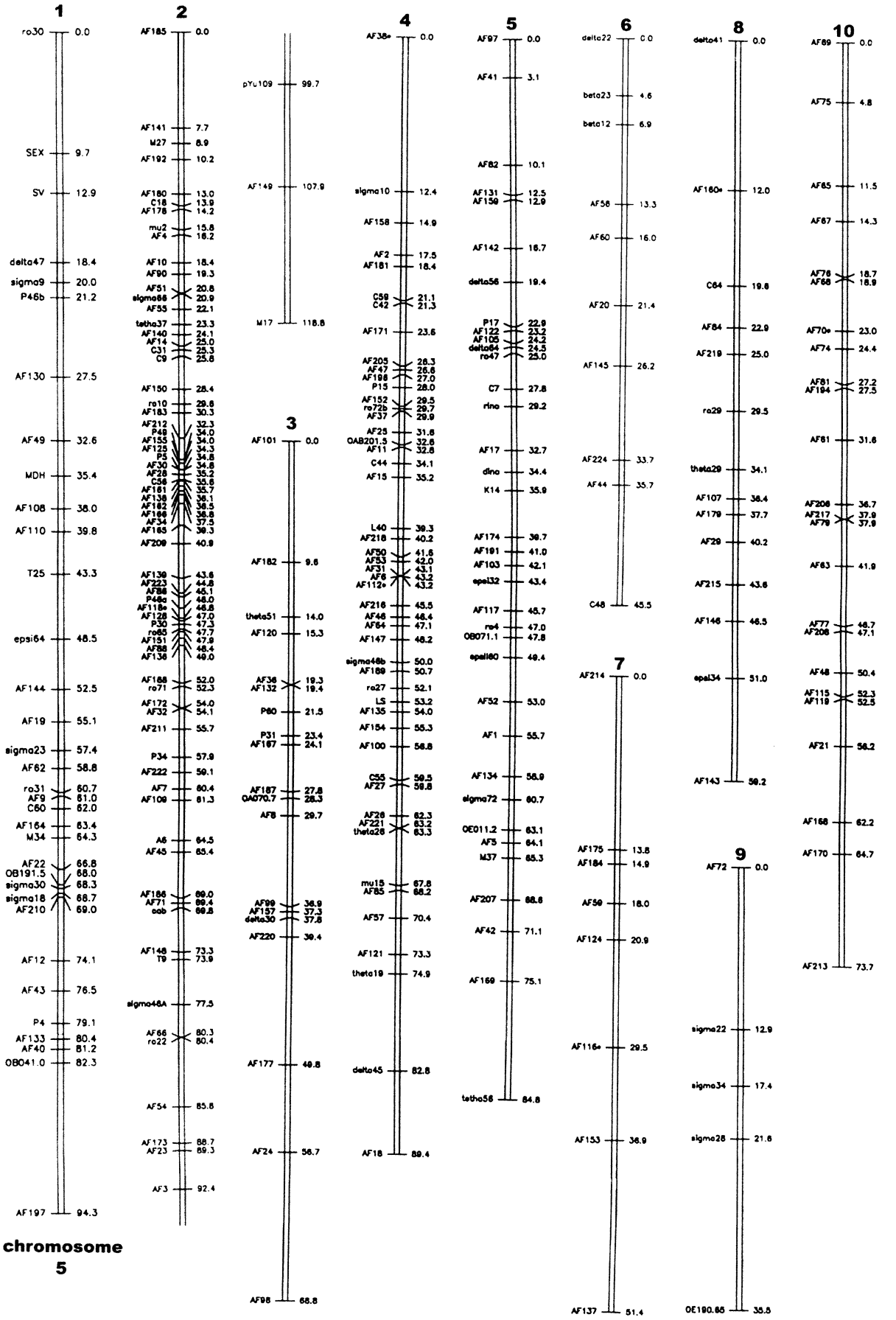
Technique	Number of analysed loci	Polymorphic loci	Scoreable markers
RFLP	271	139 (51%)	121[a] (87%)
RAPD	990	36 (3.6%)	17 (47%)
AFLP	4500	410 (9.1%)	210 (51.1%)

[a] The segregation analysis was carried out on 104 RFLP markers

where % is the estimated percentage of recombination); the remaining 58 markers are unlinked.

The map of chromosome 5, where the sex determining factors have been located, covers 94.3 cM, with 34 markers at an average distance of 2.8 cM. Among these markers the nearests to the sex locus is the AFLP SV at 3.2 cM. The previously identified markers linked with

Fig. 2 Linkage map of asparagus with groups numbered at the top. Loci are listed on the left, and recombination distances (cM) are listed on the right, of each linkage group. All AFLP markers, with the exception of SV, are indicated as AF followed by a number; RAPD markers are identified by the primer name indicated by the supplier (O followed by another letter and a number from 1 to 20) and by the molecular weight (example: OB19 1.5); MDH is the isoenzyme malate dehydrogenase; all other markers are RFLPs. The map was derived using the program DrawMap (Van Oojien 1994)


1
ro30 0.0
SEX 9.7
SV 12.9
delta47 18.4
sigma9 20.0
P46b 21.2
AF130 27.5
AF49 32.6
MDH 35.4
AF108 38.0
AF110 39.8
T25 43.3
epsi64 48.5
AF144 52.5
AF19 55.1
sigma23 57.4
AF62 58.8
ro31 60.7
AF9 61.0
C60 62.0
AF164 63.4
M34 64.3
AF22 66.8
OB191.5 68.0
sigma30 68.3
sigma18 68.7
AF210 69.0
AF12 74.1
AF43 76.5
P4 79.1
AF133 80.4
AF40 81.2
OB041.0 82.3
AF197 94.3
chromosome 5
2
AF185 0.0
AF141 7.7
M27 8.9
AF192 10.2
AF180 13.0
C18 13.9
AF178 14.2
mu2 15.8
AF4 16.2
AF10 18.4
AF90 19.3
AF51 20.8
sigma66 20.9
AF55 22.1
tetha37 23.3
AF140 24.1
AF14 25.0
C31 25.3
C9 25.8
AF150 28.4
ro10 29.8
AF183 30.3
AF212 32.3
P48 34.0
AF155 34.0
AF125 34.3
P5 34.8
AF30 34.8
AF28 35.2
C56 35.6
AF161 35.7
AF138 36.1
AF182 36.5
AF166 36.8
AF34 37.5
AF165 38.3
AF209 40.9
AF139 43.6
AF223 44.8
AF88 45.1
P46a 46.0
AF118a 46.8
AF128 47.0
P30 47.3
ro65 47.7
AF151 47.9
AF88 48.4
AF136 49.0
AF188 52.0
ro71 52.3
AF172 54.0
AF32 54.1
AF211 55.7
P34 57.9
AF222 59.1
AF7 60.4
AF109 61.3
A6 64.5
AF45 65.4
AF186 69.0
AF71 69.4
eab 69.8
AF148 73.3
T9 73.9
sigma48A 77.5
AF66 80.3
ro22 80.4
AF54 85.6
AF173 88.7
AF23 89.3
AF3 92.4
pYu109 99.7
AF149 107.9
M17 118.8
3
AF101 0.0
AF182 9.6
theta51 14.0
AF120 15.3
AF36 19.3
AF132 19.4
P60 21.5
P31 23.4
AF187 24.1
AF187 27.8
OA070.7 28.3
AF8 29.7
AF99 36.9
AF157 37.3
delta30 37.8
AF220 39.4
AF177 49.8
AF24 56.7
AF98 68.8
4
AF38a 0.0
sigma10 12.4
AF158 14.9
AF2 17.5
AF181 18.4
C59 21.1
C42 21.3
AF171 23.6
AF205 26.3
AF47 26.6
AF198 27.0
P15 28.0
AF152 29.5
ro72b 29.7
AF37 29.9
AF25 31.6
OAB201.5 32.6
AF11 32.8
C44 34.1
AF15 35.2
L40 39.3
AF218 40.2
AF50 41.6
AF53 42.0
AF31 43.1
AF6 43.2
AF112a 43.2
AF216 45.5
AF46 46.4
AF64 47.1
AF147 48.2
sigma48b 50.0
AF189 50.7
ro27 52.1
LS 53.2
AF135 54.0
AF154 55.3
AF100 56.8
C55 59.5
AF27 59.8
AF28 62.3
AF221 63.2
theta28 63.3
mu15 67.8
AF85 68.2
AF57 70.4
AF121 73.3
theta19 74.9
delta45 82.8
AF18 89.4
5
AF97 0.0
AF41 3.1
AF82 10.1
AF131 12.5
AF159 12.9
AF142 16.7
delta56 19.4
P17 22.8
AF122 23.2
AF105 24.2
delta64 24.5
ro47 25.0
C7 27.8
rino 29.2
AF17 32.7
dino 34.4
K14 35.9
AF174 38.7
AF191 41.0
AF103 42.1
epsl32 43.4
AF117 45.7
ro4 47.0
OB071.1 47.8
epsl60 49.4
AF52 53.0
AF1 55.7
AF134 58.9
sigma72 60.7
OE011.2 63.1
AF5 64.1
M37 65.3
AF207 68.8
AF42 71.1
AF169 75.1
tetha56 84.8
6
delta22 0.0
beta23 4.6
beta12 6.9
AF56 13.3
AF60 16.0
AF20 21.4
AF145 26.2
AF224 33.7
AF44 35.7
C48 45.5
7
AF214 0.0
AF175 13.8
AF184 14.9
AF59 18.0
AF124 20.9
AF116a 29.5
AF153 36.9
AF137 51.4
8
delta41 0.0
AF160a 12.0
C64 19.8
AF84 22.9
AF219 25.0
ro29 29.5
theta29 34.1
AF107 36.4
AF179 37.7
AF29 40.2
AF215 43.6
AF146 46.5
epsl34 51.0
AF143 58.2
9
AF72 0.0
sigma22 12.9
sigma34 17.4
sigma28 21.6
OE190.65 35.8
10
AF69 0.0
AF75 4.8
AF65 11.5
AF67 14.3
AF76 18.7
AF68 18.9
AF70a 23.0
AF74 24.4
AF81 27.2
AF194 27.5
AF61 31.6
AF208 36.7
AF217 37.9
AF79 37.9
AF63 41.9
AF77 46.7
AF208 47.1
AF48 50.4
AF115 52.3
AF119 52.5
AF21 58.2
AF168 62.2
AF170 64.7
AF213 73.7

sex remained in this linkage group throughout the present analysis. It is interesting to note that among these markers the isoenzyme MDH, which in our map has been located at 25.7 cM from the sex locus (Fig. 2), is also present in the asparagus map presented by Jiang et al. (1997) at a similar distance (22 cM).

The pYu109 probe homologous to *TS2*, the only gene involved in sex differentiation isolated to-date (DeLong et al. 1993), identified a polymorphic band on cross D; this polymorphism, however, is not associated with sex, but is located on linkage group 2. This indicates that the homolog of *TS2* in asparagus is not a sex-determining gene; if it is involved in sex differentiation, it should be under the control of these genes.

Conclusions

The segregation analysis of RFLP, RAPD, AFLP and isoenzyme polymorphic markers in the progeny of five different crosses between doubled-haploid clones, allowed the construction of an integrated map of *A. officinalis*. A total of 274 markers have been organized into ten linkage groups spanning 721.4 cM, the average distance between markers is 2.6 cM. The number of markers per linkage group ranged from 5 to 74. The largest groups are characterized by the presence of regions with a high density of markers . Inside these regions at least two types of markers are present, generally AFLP and RFLP. The cluster in linkage group 2, may correspond to centromeric regions where crossing-over of chromosomal sequences is inhibited (Tanksley et al. 1992)

As the haploid number of chromosomes of asparagus is ten, the linkage groups probably represent different chromosomes; (however, the only linkage group which can be associated with a specific chromosome (chromosome 5, Loptien 1979) is group 1 which includes the sex locus. Up to now no other marker has been associated with a particular chromosome.

A. officinalis is an important crop plant which is susceptible to several diseases and stresses; however, no genes concerned with these problems have so far been cloned. The map presented in this work, with an average map distance of 2.6 cM, could be a useful tool for mapping traits of interest and attempting cloning experiments utilizing bulk segregant analysis (Michelmore et al. 1991).

As for the search of molecular markers associated with sex, we have mapped a total of 33 markers (besides sex) on chromosome 5: 13 RFLPs, 18 AFLPs, two RAPDs and one isoenzyme. These markers are currently utilized for labelling the sex chromosomes and separating them by magnetic sorting. Starting with male and female doubled-haploid clones it should be possible with this method to separate X and Y chromosomes and to construct specific libraries.

In a previous study we have tested the usefulness of the RFLP marker delta 47 (8.7 cM from the sex locus) for early gender diagnosis in asparagus (Biffi et al. 1995), which is very important as male plants are superior to females in terms of longevity, growth precocity and productivity (Benson 1982). The AFLP marker SV identified in this work, whose distance from the sex locus is 3.2 cM, should be more efficient than delta 47 for gender diagnosis in asparagus seedlings.

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