Theor Appl Genet (1995) 90:124–128

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Linkage Arrangement of RFLP loci in progenies from crosses between doubled haploid *Asparagus officinalis* L. clones

Received: 5 April 1994 / Accepted: 28 July 1994

Abstract A preliminary genetic map of the dioecious species *Asparagus officinalis* L. (2n = 20) has been constructed on the basis of restriction fragment length polymorphism (RFLP) and isozyme marker data. With DNA samples digested with either *Eco*RI or *Hin*dIII 61 out of 148 probes (41%) identified RFLPs in six families of doubled haploid lines obtained through anther culture. A higher level of polymorphism (65%) was observed when a single family was screened for RFLPs using six distinct restriction enzymes. Segregation analysis of the BC progenies (40–80 individuals) resulted in a 418-cM extended map comprising 43 markers: 39 RFLPs, three isozymes and one morphological (sex). These markers are clustered in 12 linkage groups and four of them exhibited significant deviations from the expected 1:1 ratio. One isozyme and three RFLP markers were assigned to the sex chromosome.

Key words *Asparagus officinalis* L. · Genetic map RFLP

Introduction

Genetic maps are useful tools for plant breeding (Tanksley and Rick 1980; Soller and Beckman 1983). Restriction fragment length polymophisms (RFLPs), a new class of genetic markers, have several advantages over morphological and isozyme markers and are currently contributing greatly to the construction of detailed genetic maps. The level of allelic variation for RFLP markers in plant populations is much greater than that for morphological or isozyme markers (Tanksley 1983; Helentjaris et al. 1985). Furthermore, RFLP markers usually behave in a co-dominant manner, are apparently free of epistatic effects, and are developmentally stable (Beckman and Soller 1983). RFLP linkage maps have been constructed for several crop species, including tomato, corn, potato, lettuce, rice, lentil, cabbage, common bean and soybean (Bernatsky and Tanksley 1986; Helentjaris et al. 1986; Landry et al. 1987; McCouch et al. 1988; Gebhardt et al. 1989; Havey and Muehlbauer 1989; Slocum et al. 1990; Vallejos et al. 1992; Lark et al. 1993).

Asparagus officinalis is an important crop plant for which a genetic map is not yet available. Interest in this species is not only agronomical but also theoretical. In fact, *A. officinalis* is a dioecious species (haploid chromosome number 10) in which sex determination is probably controlled by two sex-determining genes (Marks 1973) which have been located by Loptien (1979) on chromosome 5. No information is presently available, however, on the nature or the mode of action of these genes. A detailed RFLP map could lead to the identification of new genetic markers that are tightly linked to sex-determining genes. In turn, such markers could allow early sex recognition – asparagus males are more productive – and could be the start point for the isolation of DNA fragments tightly linked to sex-determining genes. The achievement of this goal may be greatly facilitated by the small genome size of asparagus (1.8×10^6 bp) (Galli et al. 1988).

In a previous paper, we analyzed isozyme polymorphisms in the progeny of different crosses between doubled haploid male and female individuals and found one malate dehydrogenase locus on a sex chromosome (Maestri et al. 1991). Here, we report on a further characterisation of this genetic system by RFLP analysis and on the identification of three RFLP markers that appear to be linked to sex-determining genes.

Communicated by F. Salamini

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Materials and methods

Plant material

The *Asparagus officinalis* genotypes used in the present work have been described previously by Maestri et al. (1991). Parental lines were obtained from the Research Institute for Vegetable Crops (Section of Montanaso Lombardo) and are doubled haploid clones generated by anther culture. Since male parents are homozygous for sex determinants and all F_1s are male, linkage analysis has been carried out on BC progeny (40–80 individuals) from six different families (cross A, cross B, cross D, cross E, cross G and cross S).

Phylloclades, frozen in liquid nitrogen immediately after collection and stored at − 80 °C, were used as a source of DNA for RFLP analysis.

Preparation of an asparagus genomic library

Anonymous DNA probes (500–2 000 bp) from a *Pst*I genomic library of asparagus cloned into plasmid pUC 19 were utilized for RFLP anlaysis.

DNA was prepared from 15 g of flowers from male and female plants (line UC-157). Extraction and purification of DNA on cesium-chloride gradients was carried out with the method described by Maniatis et al. (1982). Digestion with the restriction enzyme *Pst*I (Boehringer, Mannheim) was performed following the manufacturer's instructions. Following digestion, DNA fragments in a size range between 500–2 000 bp were eluted from an 0.8% agarose gel after electrophoresis (15 h; 35 V). DNA fragments were inserted into pUC 19 and plasmids were introduced into the bacterial strain DH5α using procedures described by Maniatis et al. (1982). After amplification, plasmid extraction and probe preparation were performed according to Maniatis et al. (1982).

We have also utilized a heterologous probe for the *rbcs* (rubisco small subunit) gene of tomato, kindly supplied by Dr. G. Giuliano (ENEA, Rome), and a heterologous probe for the *cab* (chlorophyll a/b binding light harvesting complex protein) gene of maize kindly supplied by Dr. L. Bogorad (Harvard University).

DNA extraction, blot preparation and hybridization

DNA for RFLP analysis was extracted from 1–2 g of phylloclades following the procedure of Dellaporta (1985) with minor modifications. DNA digestion with restriction enzymes (Boehringer, Mannheim), DNA separation, blotting onto hybond N+ membranes (Amersham), and hybridization were performed following the manufacturers instructions. Radioactive probes were labelled using the Boehringer or Promega random primer labelling kit. The blots were wrapped in Saran Wrap and 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.

Linkage analysis

Linkage analysis was done by means of the MAPMAKER computer package (Lander et al. 1987), with the following conditions: (1) recombination frequency lower than 0.3; (2) LOD score greater than or equal to 3, using the map function of Kosambi (1944). The schematization shown in Fig. 3 was obtained with the program DrawMap (Van Oojien 1994).

Results

Library characterization

In spite of its rather small genome, *A. officinalis* has a high content of highly- and middle-repeated sequences (Galli et al. 1988). To obtain a library enriched for single-copy sequences, the six-base cutter *Pst*I was used to digest genomic DNA.

Clones analyzed for polymorphisms were classified into three categories according to their relative copy number. The definition of these categories, based on the number of discrete bands that could be detected in a given lane, was as follows: (1) single-copy sequences: 1–3 bands; (2) multiple-copy sequences: 4–6 bands; (3) repeated sequences: more than six bands.

According to this designation, 71.3% of the clones were classified as single copy, 22.9% as multiple copy and 5.8% as repeated copy. These results thus indicate that our asparagus genomic library is a good source of anonymous, mainly single-copy, DNA probes for RFLP analysis.

RFLP analysis

For RFLP analysis, 146 random genomic clones and two cDNA clones (*cab* and *rbcs*) were tested on both parental and F_1 DNA digested with either *Eco*RI or *Hin*dIII.

A high level of polymorphism was found in the parental population, with 61 probes (41%) detecting polymorphisms in at least one cross. However, the frequency of polymorphism among distinct crosses was different. Crosses A and D exhibited the highest rate of polymorphism (31%), whereas the lowest rate (21%) was found in cross B. Some probes detected polymorphisms in all crosses upon digestion with either *Eco*RI or *Hin*dIII. In this respect, cross D was prominent. Indeed, when cross D was screened for RFLPs using six distinct restriction enzymes (*Eco*RI, *Hin*dIII, *Dra*I, *Xba*I, *Bgl*II, *Eco*RV) (Fig. 1), the percentage of "good probes" (i.e., those detecting a polymorphism) increased from 41% to 65%. The "one cross/six enzymes" approach appears to be superior to the "five crosses/two enzymes" approach from several point of views:

(1) since a reduced number of plant genotypes has to be examined and less filters for hybridization need to be prepared, the former approach is technically simpler,

(2) using one cross and six distinct restriction enzymes one can obviate the loss of precision caused by the segregation of two RFLP markers in different crosses,

(3) since under the conditions of this experimental approach an increased number of probes turns out to be "good probes", a larger portion of the genome can be screened and a more extensive map can thus be constructed.

Nevertheless, in order to better integrate the previously identified isoenzyme markers in the asparagus genetic map both approaches were followed.

Segregation and linkage analysis

As expected, an additive RFLP phenotype was observed in the F_1 progenies as compared to the respective par-

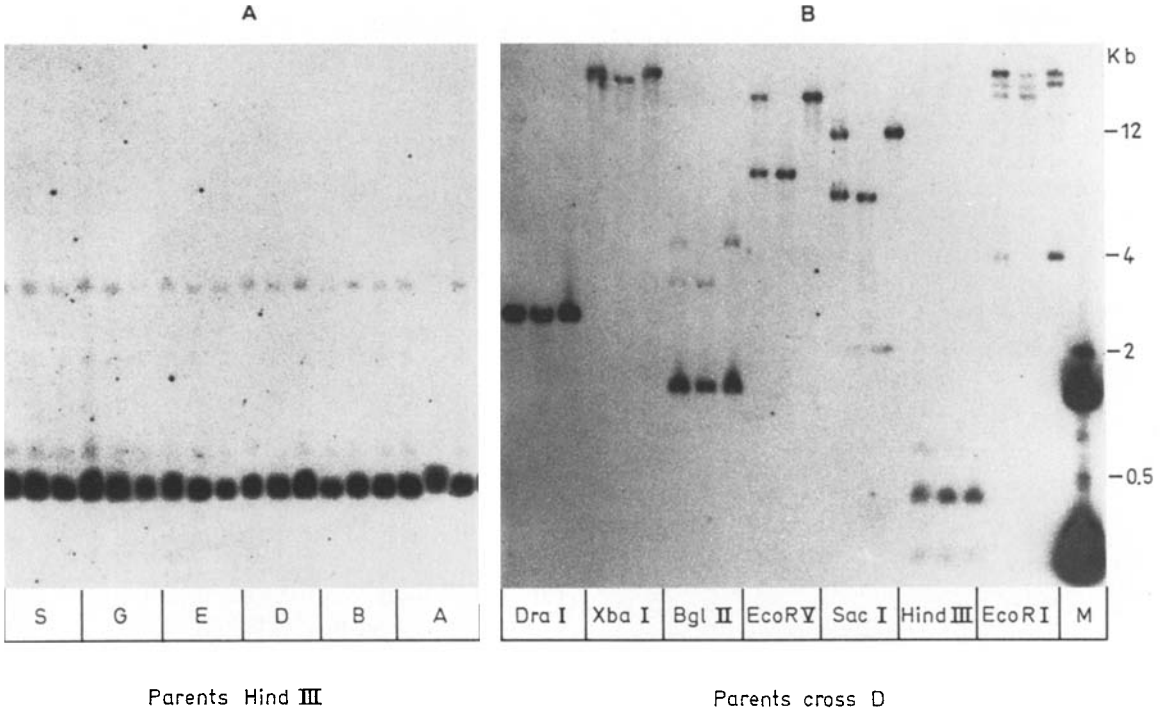

Fig. 1 Southern hybridization pattern of probe M17 with DNA from parents (F_1, ♀, ♂) of **A** six families digested with *Hin*dIII and B cross D digested with different restriction enzymes as indicated

ental phenotypes. Even so, in 65 cases out of 363 (18%) the parental female and the corresponding F_1 displayed the same RFLP pattern and the polymorphism was thus not amenable to segregation analysis.

Some cases of abnormal segregation patterns that cannot be explained on the basis of simple Mendelian co-dominant factors were also observed. These abnormal segregations, either in F_1 or in backcross progenies, were not considered for further analysis.

Differences in the frequencies of abnormal patterns among the various crosses were also apparent. In particular, cross G exhibited a high rate of abnormal patterns (57%) and it was thus discarded. Depending on the type of restriction enzyme used to digest the DNA, the same probe occasionally detected either normal or abnormal patterns in different crosses as well as in the same cross. Possible explanations of these results in terms of either genomic imprinting (Matzke and Matzke 1993) and/or somaclonal variation (Larkin and Scrowcroft 1981) will be considered in a future publication.

Since segregation analysis was performed on a backcross population, a 1:1 ratio between F_1 and maternal phenotypes was expected. An example of a 1:1 segregation ratio is shown in Fig. 2. Although loci with an aberrant segregation ratio (marked with an asterisk in Fig. 3) may lead to imprecise linkage estimates, they were also included in the map, as has been practiced by other authors (Havey and Muehlbauer 1989).

Altogether, 73 polymorphic loci were considered: 56 RFLP markers provided by the present work, 16 previously identified isoenzymes markers (Maestri et al. 1991), and one morphological marker, i.e., sex.

Linkage between pairs of markers was assessed with the MAPMAKER V3.0 computer program using parameter specified in Materials and methods. As shown in Fig. 3, 43 out of 73 loci have been arranged in 12 linkage groups covering a total of 418 cM. Map distances and the linear order of loci belonging to the same group, were then determined using the MAP function of the

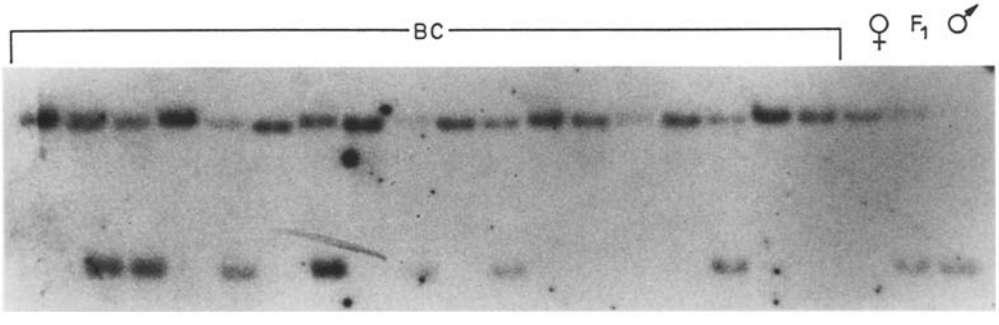

Fig. 2 Segregation analysis of one RFLP pattern in the BC progeny. Parental, F_1 and BC plants DNA from cross D was digested with *Eco*RV and hybridized with probe $\delta 22$

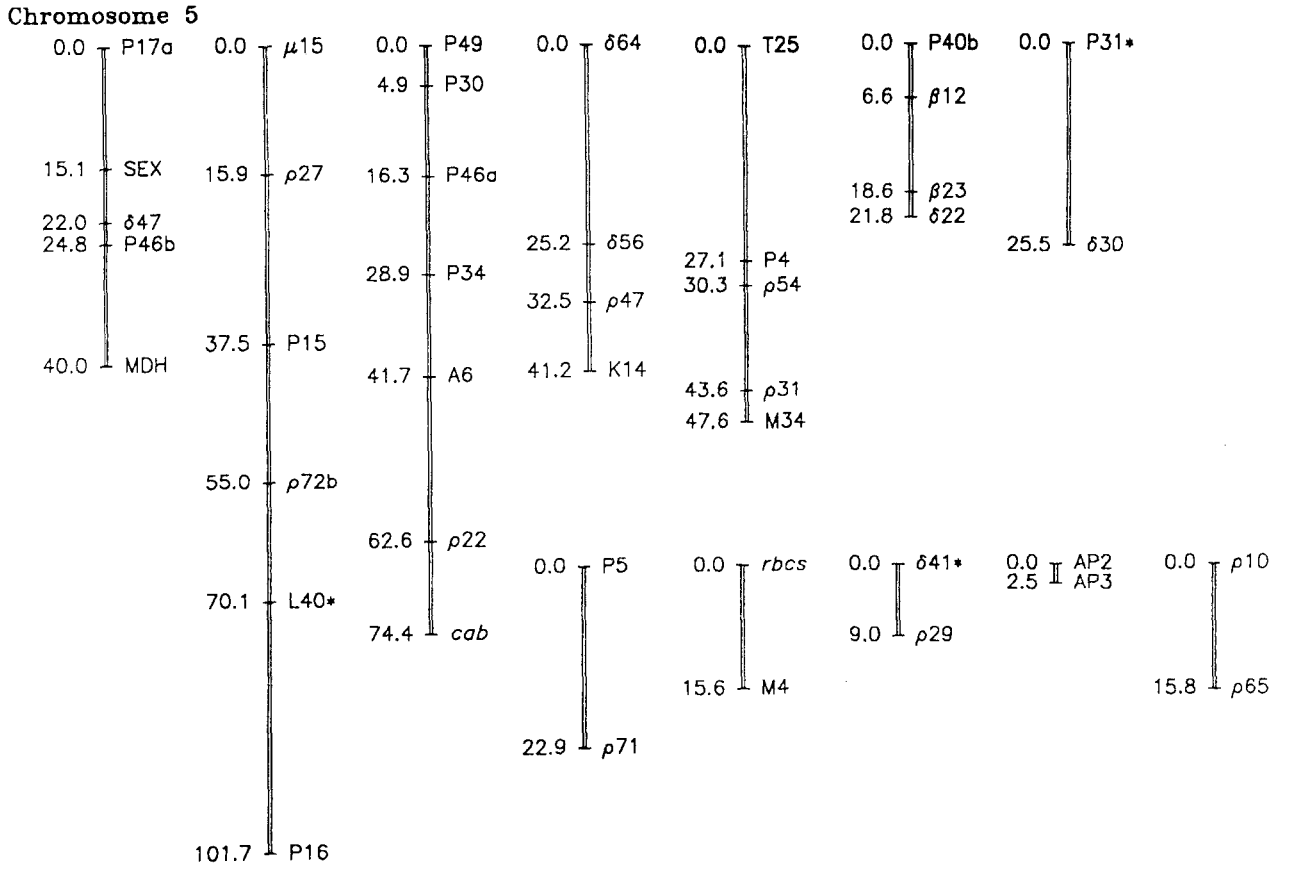

Fig. 3 Linkage groups obtained by integration of RFLP and isozyme marker data from six asparagus crosses. Chromosome 5 is the sex chromosome. (*) indicates probes displaying significant deviation ($P < 0.05$) from the expected BC segregation

program. One linkage group includes sex, one isozyme marker (MDH) and three RFLP markers. This linkage group has thus been assigned to chromosome 5 according to Loptien (1979).

Conclusions

No information is presently available concerning the extent of DNA sequence variability in *A. officinalis*. Although a isozyme marker analysis, previously carried out on seven distinct crosses, suggested a considerable amount of variability at the biochemical level (Maestri et al. 1991), the number of isozymes examined was low and did not indicate a particular cross as being more suitable for further analysis.

We thus set out to construct an asparagus genetic map by applying RFLP analysis to the same previously developed crosses, with the exception of cross C due to the reduced availability of BC individuals.

Using a sample of DNA digested with two distinct restriction enzymes and a set of 148 probes, polymorphisms were detected in at least one cross in 41% of the cases.

Preliminary results indicate, however, that the extent of variability differs significantly among crosses, ranging from 21% in cross B to 31% in cross D. In order to increase this level of variability, DNA from cross D was digested with six restriction enzymes. Following this approach the percentage of positive probes increased from 41% to 65%.

These results strongly support the conclusion that the level of polymorphism that can be detected by individual probes is limited, mainly, by the width of the experimental approach. In fact, it is likely that by increasing the number of restriction enzymes any probe would eventually be able to detect a polymorphism.

The construction of an asparagus map is still in its infancy and a larger number of probes will have to be analyzed. The results obtained so far indicate, however, that a RFLP map of this species is feasible and that RFLP markers can be integrated with isozyme markers. Furthermore, we have shown that a RFLP approach can also lead to the identification of sex-associated markers. In particular, probe δ47 was found to be linked to sex-determining genes at a distance of 6.9 cM. This distance is probably too high to allow one to reach the sex-determining genes by either chromosome walking or chromosome jumping experiments. However, probe δ47 can be utilized to label the sex chromosomes and to separate them by the technique of flow sorting. In turn, this would allow the construction of a sex-chromosome-specific library of doubled haploid plants.

Acknowledgements We thank Prof. C. Soave for helpful discussions, and Mr. A. Grippo for art work. This research was supported by the Ministero Agricoltura e Foreste within the framework of the research project "Development of advanced technologies in agricultural plants."

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