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Inheritance of parthenogenesis in *Poa pratensis* L.: auxin test and AFLP linkage analyses support monogenic control

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Abstract Gametophytic apomixis in Kentucky bluegrass (*Poa pratensis* L.) involves the parthenogenetic development of unreduced eggs from aposporic embryo sacs. Attempts to transfer the apomictic trait beyond natural sexual barriers require further elucidation of its inheritance. Controlled crosses were made between sexual clones and apomictic genotypes, and the parthenogenetic capacity of (poly)diploid hybrids was ascertained by the auxin test. A bulked segregant analysis with RAPD and AFLP markers was then used to identify a genetic linkage group related to the apomictic mode of reproduction. This approach enabled us to detect both an AFLP marker located 6.6 cM from the gene that putatively controls parthenogenesis and a 15.4-cM genomic window surrounding the target locus. A map of the *P. pratensis* chromosome region carrying the gene of interest was constructed using additional RAPD and AFLP markers that co-segregated with the parthenogenesis locus. Highly significant linkage between parthenogenesis and a number of AFLP markers that also appeared to belong to a tight linkage block strengthens the hypothesis of monogenic inheritance of this trait. If a single gene is assumed, apomictic polyploid types of *P. pratensis* would be simplex for a dominant allele that confers parthenogenesis, and this genetic model would be further supported by the bimodal distribution of the degree of parthenogenesis exhibited in the (poly)diploid progenies from sexual x apomictic matings. The molecular tagging of apomixis in *P. pratensis* is an essential step towards marker-assisted breeding and map-based cloning strategies aimed at investigating and manipulating its mode of reproduction.

Key words Apomixis · Auxin test analysis · Bulked segregant analysis · Gene mapping · Kentucky bluegrass · Parthenogenesis · Molecular markers

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Introduction

Apomixis means asexual reproduction through the seed. It involves the formation of embryo sacs without meiotic reduction and embryos without egg-cell fertilization (Nogler 1984a). In particular, gametophytic apomixis occurs with the parthenogenetic development of unreduced eggs from apomeiotic embryo sacs that arise from either a somatic cell of the nucellus (apospory) or a megaspore mother cell with modified or no meiosis (diplospory). Specific genotypes carrying complex traits, as well as hybrids impossible or difficult to perpetuate through a sexual system of reproduction, can be maintained and multiplied under an apomictic mode of reproduction.

Kentucky bluegrass (*Poa pratensis* L.) is one of the most important forage and turf grasses found in temperate climates (Bashaw and Funk 1987). While it reproduces mainly through facultative aposporous parthenogenesis, it has an extremely versatile mode of reproduction which ranges naturally from nearly obligate apomixis to complete sexuality (Mazzucato et al. 1995; Barcaccia et al. 1997a). Apomictic reproduction in *P. pratensis* is pseudogamous (i.e. it requires the

fertilization of polar nuclei for endosperm development) and leads to progenies of maternal origin. Sexual reproduction through either outcrossing or selfing is also possible in *P. pratensis*. A convenient nomenclature used to define progenies of aberrant origin is B_{II} for n + n (poly)diploid hybrids and B_{III} for 2n + n (poly)triploid hybrids (i.e. fertilization of reduced and unreduced egg cells, respectively).

A lack of knowledge on the inheritance of the apomictic trait hinders attempts to transfer it beyond natural sexual barriers. The targeting of apomixis in *P. pratensis* would increase the chances of cloning the gene(s) involved and thus of transferring this valuable trait to species that naturally propagate through sexuality, not only other grasses (e.g. *Lolium* and *Dactylis*) but also dicot crops. For instance, in polyploid sexual complexes that mainly reproduce through cross pollination, such as *Medicago* and *Solanum*, apomixis would provide a unique opportunity for developing superior monogenotypic cultivars with permanently fixed heterosis (Jongedijk 1991; Barcaccia et al. 1997b). Although the mode of reproduction is known to be under genetic control, detailed genetic analysis is extremely difficult in *P. pratensis*, due to the association of facultative apomixis with a high and irregular ploidy ($x = 7$, $2n = 28$–147) and a high and variable level of heterozygosity (Speckmann and van Dijk 1972). Cyto-embryological progeny tests have indicated that only a single gene or a few tightly linked dominant genes are required for the genetic transmission of parthenogenesis and that apomixis is a simply inherited system in *P. pratensis* (Matzk 1991a).

Detailed linkage maps of specific chromosomes or genomic regions have been developed by analysing the segregation of randomly selected molecular markers in single populations. For instance, the identification of DNA traits associated with specific genes based on the use of markers randomly distributed in the genome coupled with the bulked segregant analysis, BSA (Michelmore et al. 1991), appears very promising, even in a remarkably complex species like *P. pratensis*. It is based on pooling the DNAs from individuals sharing the same genetic background but showing extreme classes of a given trait, and then screening for differences between genetic classes using molecular markers. This approach provides a method for rapidly identifying markers linked to a specific genetic trait and accurately locating genes in a saturated genetic map. In particular, molecular differential screening of plants with contrasting modes of reproduction is considered one of the most powerful tools for identifying, isolating and using the gene(s) underlying the expression of apomixis. Up to now, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers have been mostly used for identifying specific genes, including mutations of the sexual system of reproduction (Delourme et al. 1994) and those controlling the apomictic mode of reproduction in *Pennisetum* (Ozias-Akins et al. 1993; Gustine et al. 1997), *Tripsacum* (Leblanc et al. 1995) and *Brachiaria* (Miles et al. 1994; Pessino et al. 1997). However, for this purpose, amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995) have recently provided a novel and robust technique that combines the reliability of the RFLP technique with the power of the polymerase chain reaction (PCR) RAPD technique.

This report describes the use of a bulk segregant analysis to detect a genetic linkage group related to auxin-tested parthenogenesis in apomictic types of *P. pratensis*.

Materials and methods

Plant materials, auxin test analysis and bulks' composition

Controlled crosses were carried out between completely sexual clones (S1, S2 and S4) and highly apomictic genotypes (RS9, RS18 and RS20) of *Poa pratensis* with a chromosome number ranging from 44 to 58 ($2n \cong 6x$ up to $8x$, with $x = 7$). Matings between sexual plants ($S1 \times S2$ and reciprocal), apomictic plants ($RS7 \times MM12$ and reciprocal) and apomictic by sexual crosses ($RS7 \times S1$ and $RS18 \times S4$) were also performed (Barcaccia et al. 1997a). The sexual clones were selected from a Dutch cultivar (Matzk 1991a) and the apomictic genotypes from an Italian natural population (Mazzucato 1995). Reproductive characteristics of the plant material used are given in Table 1.

The genetic capacity for parthenogenesis was estimated in 69 F_1 plants from all progenies by the auxin test (Matzk 1991b); i.e. a synthetic auxin treatment of inflorescences can induce ovaries to develop by parthenocarpy so that fertilization is no longer possible nor required for seed formation. In this case, it is thought that the egg cell can develop autonomously if it carries the genetic capacity for parthenogenesis (Matzk 1991b). The auxin treatment of panicles and classification of induced grains (i.e. grains where the endosperm is lacking) were according to Mazzucato et al. (1996) using 20–30 specimens per plant. For cytoembryological analyses, caryopses were dissected and examined under a stereo-microscope. The degree of parthenogenesis was calculated as the frequency of caryopses showing one or more embryos but lacking the endosperm.

This approach enabled the selection of 48 F_1 plants, characterized as B_{II} hybrids on the basis of hybrid DNA fingerprints and nuclear DNA contents (Barcaccia et al. 1997a), from progenies $S1 \times RS9$, $S2 \times RS20$, and $S4 \times RS18$ segregating for the mode of reproduction, with a contrasting (i.e. null or very low versus medium or high) degree of parthenogenesis. For bulked segregant analysis, two parthenogenetic and two non-parthenogenetic bulks of 13 and 11 plants each were prepared by combining the DNA from B_{II} hybrids that scored the lowest and the highest rates of embryos autonomously formed in the B_{II} progenies.

Molecular markers and polymorphism types

All bulked DNA samples were investigated by RAPD and AFLP markers using the parental types of *P. pratensis* as controls. Total genomic DNA was isolated following the procedures described by Barcaccia and Rosellini (1996) for RAPD analysis and Dellaporta et al. (1983) for AFLP analysis. RAPD analysis and electrophoresis were performed according to the protocol reported in detail by Barcaccia (1994) and Mazzucato et al. (1995). A total of 48 different 10-*mer* primers selected within the P, Q and R kits (Operon Technologies) were used for the detection of RAPD markers.

Table 1 Reproductive characteristics of the sexual clones and apomictic genotypes

Parental plants	Chromosome number[a]	Degree of apospory[a,b]	Degree of parthenogenesis[b,c]	Degree of apomixis[c,d]
S1	48	0.0	0.5	0.0
S2	44	0.0	2.6	0.0
S4	58	0.0	0.7	0.0
RS7	50	–	57.1	86.7
RS9	56	66.6	66.6	90.0
RS18	54	60.0	55.0	92.8
RS20	46	–	62.5	89.9
MM12	42	74.3	71.4	80.0

According to [a]Barcaccia et al. (1997), [b]Mazzucato (unpublished), [c]Matzk (1991), [d]Mazzucato (1995)

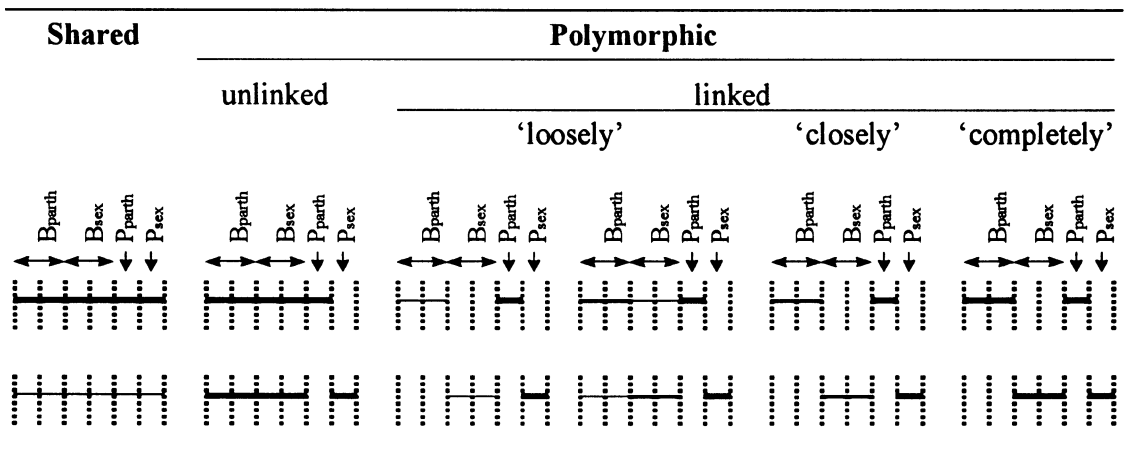

Fig. 1 Schematic classification of DNA polymorphisms: B_{parth} and B_{sex} indicate the bulked progeny plants with and without the target gene, respectively; P_{parth} and P_{sex} are the apomictic and sexual parental types carrying and lacking the target gene, respectively (Captions *loosely* and *closely* indicate a quantitative polymorphism whereas *completely* indicates a qualitative polymorphism

Photographs (K65HM video copy processor paper, Mitsubishi) of the randomly amplified DNA fragments were taken after staining of the 1.4% agarose (Gibco, BRL) gels with ethidium bromide. AFLP fingerprinting was performed using the original protocol of Vos et al. (1995) with the modifications described by Cnops et al. (1996). AFLP analysis was based on the detection of *Eco*RI/*Mse*I genomic restriction fragments by PCR amplification with 36 different primer combinations having three selective nucleotides (Life Technologies). Labelled selected DNA fragments separated on standard 4.75% polyacrylamide (SEQUAGEL-4, National Diagnostics) gels were visualized by autoradiogram (BIOMAX MR-1 film, Kodak) after a 36-h exposure at $-80°C$ using intensifying screens.

Three types of polymorphic markers were scored between bulked DNA samples. They were considered 'loosely' or 'closely' linked to the target gene when polymorphisms proved to be quantitative, i.e. of unequal intensities with respect to the signal of the related parent, and 'completely' linked to the target gene when qualitative polymorphisms were detected, i.e. the presence of intensity was equal to that of the related parent versus absence of the signal (Fig. 1). Selected polymorphic markers were then checked on individual DNA samples of the B_{II} progenies of known reproductive behaviour.

Linkage analysis

B_{II} hybrid progenies of *P. pratensis* were scored for dominant markers that segregated not only from the apomictic genotypes carrying the target gene, but also from the sexual lines. Only those polymorphic markers that were shared by both apomictic and sexual parents were considered. The observed segregation ratio of RAPD and AFLP markers was tested by chi-square analysis for goodness of fit to the expected 1 : 1 segregation ratio (presence versus absence of the marker) and also by the 2×2 contingency test for independent assortment of two allelic pairs (frequency of parthenogenesis versus zygotic embryogenesis). Segregation data of markers were analysed with JOINMAP version 2.0 (Stam and Van Ooijen, 1995) using the 'cross pollination' (CP) population-type option, i.e. an F_1 population resulting from a cross between two heterogeneously heterozygous and homozygous parents. As far as the genotype code option is concerned, presence of marker and apomictic phenotype were assigned to "ab" = heterozygous dominant, and absence of marker and sexual phenotype were assigned to "aa" = homozygous recessive. The target locus was recorded as a putative marker fully co-segregating with the trait being mapped. The identification of linkage groups was performed using a minimum LOD score of 3 and a maximum recombination value of 30%. The map distances were calculated by the Kosambi function. Individual RAPD and AFLP loci describe the 10-*mer* primer nomenclature and the *Eco*RI/*Mse*I selective bases' combination, respectively, and the relative marker size (kb).

Table 2 Segregation of the degree of parthenogenesis expressed by single F_1 plants in progenies from different cross combinations (*M* maternal · B_{II}, (poly)diploid hybrid · B_{III} (poly)triploid hybrid · *H* (poly)haploid · *S* self)

Cross-combinations	Number and genetic origin of F_1 plants	Number of plants with parthenogenesis		
		< 3%	11–50%	51–97%
Sexual × sexual				
S1 × S2	3 (B_{II})	3	0	0
S2 × S1	3 (B_{II})	3	0	0
Apomictic × apomictic				
MM12 × RS7	3 (B_{II})	0	1	2
	1 (M)	0	0	1
	2 (S)	0	1	1
RS7 × MM12	2 (B_{II})	0	1	1
	1 (M)	0	0	1
	2 (H)	0	1	1
Apomictic × sexual				
RS9 × S1	1 (B_{III})	0	0	1
	1 (M)	0	0	1
RS18 × S4	1 (B_{II})	0	0	1
	1 (M)	0	0	1
Sexual × apomictic				
S1 × RS9	20 (B_{II})	10	6	4
S2 × RS20	13 (B_{II})	6	5	2
S4 × RS18	15 (B_{II})	6	4	5

Results and discussion

Auxin test analyses and segregation of parthenogenesis in the (poly)diploid progenies

The auxin test enabled the selection of plants in progenies from 'sexual × apomictic' matings segregating for mode of reproduction. Of the 48 (poly)diploid hybrids accurately checked at the cyto-embryological level, 22 lacked any appreciable parthenogenetic activity (< 3%), and 26 exhibited a parthenogenetic capacity ranging from 11% to 97% (Table 2). Although the variable chromosome number of the parents could have altered segregation patterns, crosses of sexual clones with apomictic genotypes yielded segregation ratios that were compatible with an inheritance model which postulates that the expression of parthenogenesis requires a dominant allele of a single polysomic locus, assuming random assortment of chromosomes. In fact, the overall segregation ratio between plants showing a null or very low degree of parthenogenesis (on average, 0.4%) and plants scoring a medium or high degree of parthenogenesis (on average, 52.6%) did not differ significantly from the 1 : 1 ratio expected in the case of dominant genetic control (Matzk 1991a), as can be seen from the bimodal distribution of the trait observed in the B_{II} progenies (Fig. 2). The skewed distribution of frequencies in the B_{II} hybrids with a medium or high degree of parthenogenesis (Fig. 2) may reflect an asexual embryo development influenced by modifying genes, as earlier hypothesised by Matzk (1991a). Moreover, it has to be noted that the expression of parthenogenesis in such B_{II} hybrids is not complete but, on average, comparable with the levels scored by the apomictic parents (Tables 1 and 2). Despite the small size of individual B_{II} progenies, the segregation patterns of parthenogenesis were comparable even within each single cross combination (Table 2). The dominant expression of parthenogenesis over obligatory zygotic embryogenesis was further confirmed by the fact that none of the B_{II} hybrids from sexual × sexual crosses was parthenogenetic and that all maternal plants from apomictic × apomictic crosses had a parthenogenetic capacity comparable to parental values (Table 2). Moreover, selfs and (poly)haploids from apomictic parents also scored a medium or high degree of parthenogenesis.

The auxin test proved to be an efficient method for examining a major step of the whole apomictic process in detail and providing a reliable estimate of the genetic capacity for parthenogenesis in *P. pratensis*. However, it is still not known whether the extent of parthenogenesis reflects usable apomixis. Because haploid parthenogenesis usually occurs at low frequencies, it seems reasonable to consider the degree of parthenogenesis a predictive estimate of aposporous parthenogenesis potentialities, and thus of the final extent of apomixis of single plants. In the present study, crosses of sexual clones with apomictic genotypes resulted in both non-parthenogenetic and parthenogenetic F_1 hybrids. Thus, the parthenogenesis was under strong genetic control, as previously reported in *P. pratensis* by Matzk (1991a). Assuming a single gene, apomictic polyploid types of *P.*

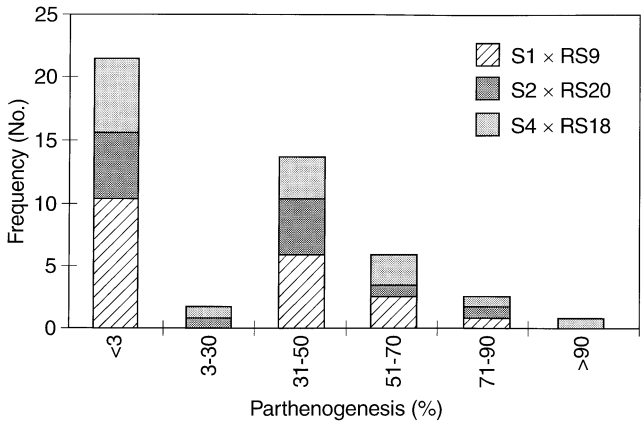

Fig. 2 Bimodal distribution of the degree of parthenogenesis in the three (poly)diploid progenies used for bulked segregant analysis. The $< 3\%$ histogram refers to plants of the non-parthenogenetic bulks with $\bar{x} = 0.4\%$, and the other histograms in the range 11–97% refer to plants of the parthenogenetic bulks with $\bar{x} = 52.6\%$

pratensis would be simplex for a dominant allele able to confer parthenogenesis and sexual types nulliplex, that is lacking all alleles necessary for parthenogenesis in the full set of chromosomes. On the other hand, two dominant alleles can not be involved because the number of apomictic phenotypes would have been much higher than that of sexual ones. Similar results have been obtained for various species: the inheritance of aposporous apomixis appears to be monogenic in *Panicum maximum* (Savidan 1982), *Ranunculus auricomus* (Nogler 1984b), *Cenchrus ciliaris* (Sherwood et al. 1994) and *Brachiaria* (do Valle and Miles 1994; Miles and Escandon 1997).

The putative simple inheritance of parthenogenesis in *P. pratensis* was further investigated by analysing RAPD and AFLP markers co-segregating with the target locus.

Evaluation of bulks and detection of genetic linkages with parthenogenesis

The screening of DNA bulks and controls yielded markers potentially linked to the gene controlling parthenogenesis in *P. pratensis*. In addition to shared and unlinked molecular markers, both quantitative and qualitative polymorphisms were found.

In *P. pratensis*, RAPD and AFLP fingerprints yielded on average 9 and 64 intense and reproducible markers, respectively. Most of these markers were shared between samples or unlinked to the mode of reproduction. Five of the tested $Eco + 3/Mse + 3$ primer combinations, CCA/AGA, CCA/ACA, CCA/AGC, CCA/AGG and CAC/ACA, yielded 8, 5, 4, 3 and 2 polymorphic markers, respectively, that were classified as 'closely' or 'completely' linked to parthenogenesis or zygotic embryogenesis and used to score their segregation in the individual B_{II} progenies. Four of these AFLP markers appeared to be significantly associated with the target locus ($\chi^2 \geqslant 12.10$, $P < 0.001$), as well as 1 additional RAPD marker generated with primer OP/P10 ($\chi^2 = 13.92$, $P < 0.001$) that was registered as 'closely' linked to parthenogenesis (Table 3). A further 11 significant linkages between molecular markers and parthenogenesis were also detected at a lower probability level ($\chi^2 = 6.80$–10.15, $P < 0.01$) (Table 3). Of the 16 markers selected for statistical

Table 3 RAPD and AFLP marker frequency among hybrid progenies and chi-square values for independent assortment of two allelic pairs and for an expected segregation ratio of 1:1

Marker	Progeny	Parthenogenetic		Non-parthenogenetic		χ^{2a}	Total		χ^2
	plants	Present	Absent	Present	Absent		Present	Absent	
CCA/AGA/0.51	48	26	0	8	14	20.38	34	14	4.17*
CCA/AGA/0.45	48	19	7	5	17	10.15	24	24	0.00
CCA/AGA/0.38	48	19	7	6	16	8.27	25	23	0.04
CCA/AGA/0.36	48	25	1	6	16	21.80	31	17	2.04
CCA/AGA/0.27	48	21	5	8	14	8.06	29	19	1.04
CCA/AGA/0.24	48	17	9	5	17	7.10	22	26	0.17
CCA/ACA/0.39	48	13	13	22	0	12.10	35	13	5.04*
CCA/ACA/0.35	48	7	19	17	5	10.15	24	24	0.00
CCA/ACA/0.21	48	2	24	17	5	21.30	19	29	1.04
CCA/AGC/0.31	48	22	4	8	14	9.87	30	18	1.50
CCA/AGC/0.25	48	18	8	5	17	8.55	23	25	0.04
CCA/AGG/0.28	48	18	8	6	16	6.80	24	24	0.00
CAC/ACA/0.18	48	3	23	13	9	10.09	16	32	2.67
OP/P10/0.75	48	21	5	5	17	13.92	26	22	0.17
OP/Q5/0.60	48	16	10	4	18	7.25	18	30	1.50
OP/R3/1.15	48	26	0	15	7	7.29	41	7	12.04*

* A significant deviation at $P \leqslant 0.05$ ($df = 1$)
[a]The critical χ^2 at $P = 0.01$ is 6.64 and at $P = 0.001$ is 10.83

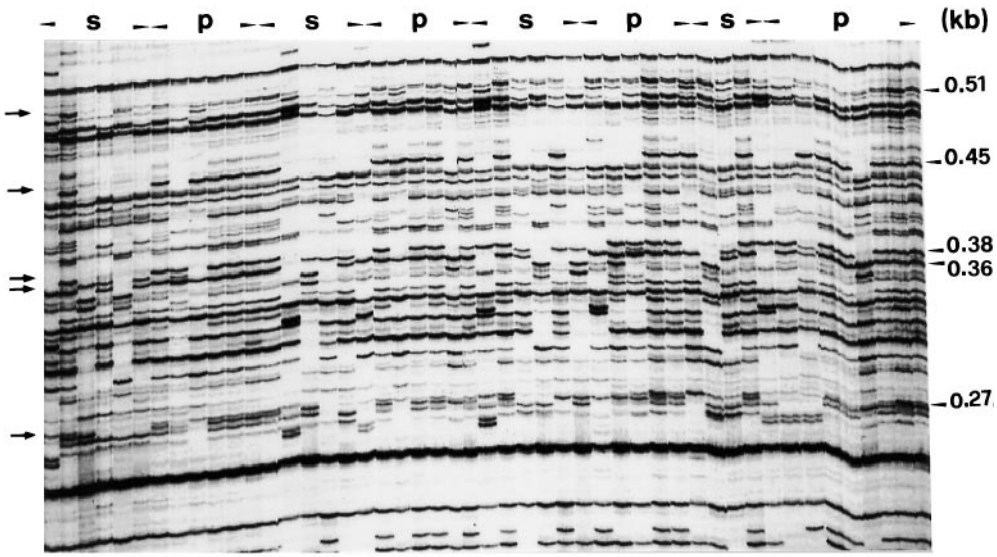

Fig. 3 Segregation of AFLP markers (→) generated by primer combination *Eco* + CCA/*Mse* + AGA in the B_{II} progenies from sexual versus apomictic crosses. *Arrows* indicate markers significantly linked to parthenogenesis. *p* and *s* indicate parthenogenetic and sexual individuals, respectively

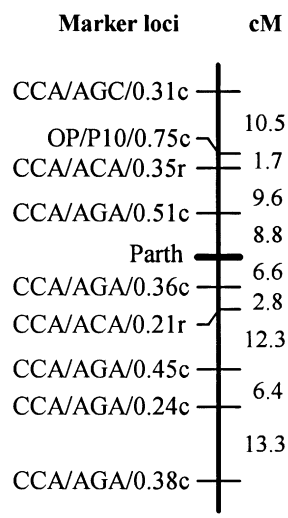

Fig. 4 Genetic linkage group related to parthenogenesis in *P. pratensis*. Map distances are expressed in centiMorgans (cM). Individual RAPD and AFLP loci describe the 10-*mer* primer nomenclature and the *Eco*RI/*Mse*I selective bases' combination respectively, and the relative marker size (kb). The phase of each marker is indicated by a *c* (coupling) or an *r* (repulsion)

dependence on the mode of reproduction, 13 segregated according to a 1 : 1 ratio ($\chi^2 \leqslant 2.04$, $P > 0.05$), while segregation was moderately distorted in 2 and severely so in 1 (Table 3). Twelve AFLP markers and 2 RAPD markers were finally selected for linkage analysis. An example of AFLP fingerprints generated by the primer combination that yielded the highest number of markers significantly co-segregating with the mode of reproduction and fitting a 1 : 1 ratio is given in Fig. 3.

The linkage group that was constructed indicated that the gene that potentially controls parthenogenesis in *P. pratensis* is located between AFLP loci Eco + CCA/*Mse* + AGA/0.36 and *Eco* + CCA/*Mse* + AGA/0.51, 6.6 cM distant from the former ($\chi^2 = 21.80$, $P < 0.001$, $r = 0.07$) and 8.8 cM from the latter ($\chi^2 = 20.38$, $P < 0.001$, $r = 0.09$). Thus, a 15.4-cM genomic window surrounding the target locus was defined (Fig. 4). Additional RAPD polymorphisms detected between DNA bulks were inconsistent when assayed in the progeny using the full set of individuals, and no linkages with the target locus were ultimately determined (Fig. 5a). It is worth mentioning that the AFLP analysis gave quantitative information, since the signal intensity in the selected bulked DNA samples was proportionally correlated to the number of plants that shared a specific genomic template complementary to the oligonucleotide primers used (Fig. 5b).

The 3 AFLP markers found to closely co-segregate with parthenogenesis appear to belong to the same linkage group and show low pair-wise recombinant fractions. Highly significant linkage between parthenogenesis and 3 different AFLP markers agrees with the hypothesis of monogenic inheritance of this trait in *P. pratensis*. This result is also consistent with the bimodal distribution of the degree of parthenogenesis exhibited in the B_{II} progenies. In the case of aposporous apomixis, co-inheritance with molecular markers has been reported in *Pennisetum squamulatum* (Ozias-Akins et al. 1993), and this fact suggested that the factor(s) for apomixis is transmitted by a single chromosome. Moreover, the concept that a single gene directs apospory has been strengthened by the finding, in *Cenchrus ciliaris* and *Brachiaria*, of molecular markers associated with the apomictic trait (Miles et al. 1994; Miles and Escandon 1994; Gustine et al. 1997; Pessino et al. 1997).

The question remains of whether the two essential components of apomixis in *P. pratensis*, *i.e.* aposporic apomeiosis followed by parthenogenesis, are determined by independent or linked genetic factors. Indeed, in Kentucky bluegrass, the cytoembryological steps leading to apomixis appear to be strictly associated: sexual, non-parthenogenetic plants completely lacked aposporic activity in ovules, and apomictic plants were highly aposporic and parthenogenetic (Barcaccia et al. 1997a).

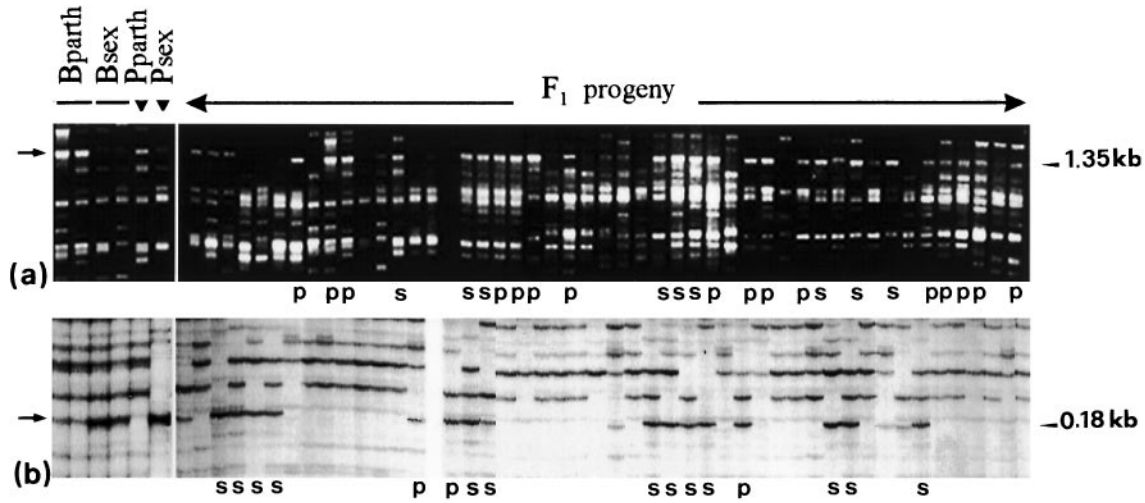

Fig. 5a Qualitative RAPD polymorphism (→) generated with primer OP/Q2 between parthenogenetic (B_{parth}) and sexual (B_{sex}) DNA bulks that appears inconsistent in the progeny present in both parthenogenetic (*p*) and sexual (*s*) B_{II} plants. **b** Quantitative AFLP polymorphism (→) generated with primer combination *Eco* + CCA/*Mse* + ACA between DNA bulks and relative segregation pattern in the progeny. Among the 16 progeny plants showing the marker, the 3 individuals marked with *p* were responsible for the faint signal of the parthenogenetic bulks (B_{parth}), whereas the strong signal of the sexual bulks (B_{sex}) was found in the remaining 13 individuals marked with *s*. P_{parth} and P_{sex} indicate a parthenogenetic and a sexual parent, respectively

Conclusions

Genetic targeting of parthenogenesis in *P. pratensis* appears to be feasible through the BSA approach using available plant DNA materials. In a parental accession of *P. pratensis*, a very high level of heterozygosity was detected, as expected in a wild apomict, and this facilitated the discovery of markers co-segregating with parthenogenesis.

Detailed linkage maps and suitable DNA markers for *P. pratensis* could address basic questions, such as the genetic control and regulation of apomixis, and also improve the efficiency of plant breeding through marker-assisted selection. To date, the methods most commonly used for investigating apomixis in *P. pratensis* have been cyto-embryological analyses in ovules of the mother plant (Grazi et al. 1961; Naumova et al. 1993; Mazzucato et al. 1996) and progeny tests based on the detection of morphological traits and/or DNA markers and contents (Huff and Bara 1993; Mazzucato et al. 1995; Barcaccia et al. 1997a). Such procedures are often time-consuming and limited in their application. The availability of molecular markers linked to the mode of reproduction would help overcome these limitations by simplifying and accelerating the identification of apomictic individuals. Clearly, the accuracy of marker-assisted selection can be improved if selectable molecular alleles are mapped on both upstream and downstream of the gene of interest. The BSA strategy used in the present study for the identification of clones related to the apomictic pathway detected segregating markers within a 10% recombination on either side of the target locus. Markers within a 30% recombination window could also be detected using bands of unequal intensity (i.e. quantitative polymorphisms). However, it should be noted that the accuracy of the estimates of recombination in a polyploid species such as *P. pratensis* will be influenced by aneuploidy, chromosome instability or aneusomaty and by the mode of pairing. Although meiosis in *P. pratensis* is not regular, the irregularity is not of a degree that might be expected. Brown (1941) reported that in plants with a chromosome number ranging from 42 to 50 pairing was, for the most part, by bivalents, even though univalents, trivalents and quadrivalents were observed. Therefore, the order of the markers in a linkage map should not be consistently affected.

Although PCR-based markers like RAPD and AFLP constitute powerful molecular tools for genome screening and gene mapping, applications of bulked segregant analyses in apomicts have been mainly based on RFLP markers (Leblanc et al. 1995; Pessino et al. 1997). In the past 10 years, grass genomes have been extensively characterized, and comparisons of molecular data have revealed marked similarities in gene composition and linear arrangement (e.g. map syntheny and collinearity) within and shared by *Pooideae* and *Panicoideae* (reviewed by Glaszmann et al. 1997). Although there is no genetic linkage map for *P. pratensis*, the extensive genomic similarity it might share with related taxa should allow common probes/primers to be used for efficient screening and narrowing down of the marked chromosome interval spanning the target gene.

The targeting of the gene(s) encoding for apomixis would increase the chances of manipulating apomictic

reproduction and transferring this valuable trait into sexual crops. At the same time, the conversion of apomixis-nearest flanking markers in sequence-characterized amplified region (SCAR) markers (Paran and Michelmore 1993) could open up new plant selection models in *P. pratensis*. Furthermore, linkage groups related to apomixis components provide a starting point for saturating the chromosome region responsible for conferring apomixis, as well as for isolating and cloning the relative gene(s). It has recently been shown that map-based (or positional) cloning is effective for isolating genes. The technique is based on an increase in genetic resolution and a concomitant decrease in the physical size of the chromosome segment where the target gene must reside until both genetic and physical sizes overlap (Ballvora et al. 1995). High genetic resolution is achieved by mapping closely linked genetic markers relative to the target gene in large segregating populations. The cloning of large genomic DNA fragments (several hundred kilobases) in suitable vectors such as YAC (Yeast Artificial Chromosomes) and subsequent screening with markers that are closely linked to the target gene but can be separated by recombination events can be used to identify clones that carry both flanking markers and the target gene (Martin et al. 1991). Although polyploids may not be an attractive system for gene isolation via positional cloning, the availability of RAPD and AFLP markers linked to the mode of reproduction should prove valuable in species like *P. pratensis* where there is a lack of suitable DNA markers and where considerable time and labour are involved in obtaining genotypes/populations amenable to genetic analysis.

In conclusion, molecular tagging of apomixis appears to be an essential step towards identifying new breeding strategies for investigating and manipulating the mode of reproduction. Not only would DNA tags be applicable to distinguishing between apomictic and sexually reproducing plants early, but they might also allow apomixis to be genetically engineered and introduced into sexual crops. As particular genotypes or hybrid combinations would maintain genetic fidelity with the maternal parent throughout repeated life cycles with apomixis, this reproductive trait could revolutionize agricultural systems and have a global impact on crop production.

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