Theor Appl Genet (1997) 94:439–444

S. C. Pessino · J. P. A. Ortiz · O. Leblanc
C. B. do Valle · C. Evans · M. D. Hayward

Identification of a maize linkage group related to apomixis in *Brachiaria*

Received: 9 September 1996 / Accepted: 20 September 1996

Abstract A bulked segregant analysis using RFLPs and RAPDs was carried out to identify molecular markers co-segregating with apomixis in a *Brachiaria* F_1 population. The test population used was a cross between sexual *B. ruziziensis* R44 and the aposporous apomictic *Brachiaria brizantha* cv Marandu. The *Brachiaria* genome was systematically scanned using 61 cDNA and genomic maize clones detecting 65 loci located at 40 cM, on average, one from each other in the maize genome. The finding of a clone that presented a polymorphic band co-segregating with apomixis (umc147) led to the identification of another marker within the same area (umc72). The clones belong to a duplicated linkage group that maps to the distal part of maize chromosome-1 long arm and chromosome-5 short arm. RAPD analysis using 184 primers from Operon sets yielded one more marker (OPC4) significantly linked to the trait mapping the same locus. OPC4 had been previously reported as a potential marker for apospory in *Pennisetum*. A map of the region was constructed using additional clones that belong to the same maize linkage group. Since that was the only genomic region that presented an apomixis-linked polymorphism our observations support the existence of a single locus directing apospory in *Brachiaria*.

Key words Apomixis · Bulked segregant analysis · Comparative mapping · Molecular markers · *Brachiaria*

Communicated by F. Salamini

S. C. Pessino (✉) · J. P. A. Ortiz · O. Leblanc
C. Evans · M. D. Hayward
Institute for Grassland and Environmental Research,
Plas Gogerddan, Aberystwyth, SY23 3EB, Wales, UK
C. B. do Valle
EMBRAPA/CNPGC, C.P. 154, Campo Grande, MS, 79002-970,
Brazil

Introduction

The ability of flowering plants to confine a new individual into a seed is not necessarily linked to sexuality. Some angiosperms commonly reproduce asexually through seeds, by a process called apomixis (Nogler 1984a). Apomixis gives rise to fertile seeds whose embryos derive directly from maternal cells rather than from the fusion of male and female gametes. These embryos therefore have a genetic constitution identical to that of the female parent. This trait has been observed in more than 300 plant species belonging to 35 families, the most well-represented including the Gramineae, Compositae, Rosaceae and Rutaceae (Richards 1986).

Apomictic reproduction is generally attributable to two possible mechanisms, adventitious embryony and gametophytic apomixis. In adventitious embryony embryos directly form from nucellar cells. In gametophytic apomixis the embryo arises from the egg cell of an unreduced embryo sac (female gametophyte) that can originate either (1) from an archesporial cell by mitosis after omission or failure of meiosis (diplospory) or (2) from a nucellar somatic cell (apospory). The developmental details of apomictic processes have been extensively reviewed (Nogler 1984 a; Asker and Jerling 1992; Koltunow 1993; Naumova 1993).

Apomixis has been widely reported to be genetically inherited even though the degree of the trait could be influenced by stimuli such as the identity of the male parent and environmental factors like photoperiod or temperature (for a review see Nogler 1984 a). Analysis of the offspring of apomictic vs sexual crosses has provided evidence that the different apomictic mechanisms are controlled by single Mendelian factor(s). Apospory in *Panicum maximum* (Savidan 1982), *Ranunculus auricomus* (Nogler 1984 b), *Cenchrus ciliaris* (Sherwood et al. 1994) and *Brachiaria* (do Valle and Miles 1994) is probably controlled by a single dominant gene. Studies of nucellar embryony in *Citrus*

presented similar results (Parlevliet and Cameron 1959; Iwamasa et al. 1967). Genetic regulation of diplospory in *Taraxacum* (Mogie 1988) suggested that the control of female meiosis resides on a single chromosome and probably at a single locus. Finally, Leblanc et al. (1995) reported the identification of three RFLP markers linked to diplospory in maize-*Tripsacum* hybrids that were also found to be linked in the maize genetic map, supporting the single-locus hypothesis.

Apomixis makes possible the production of clonal seed from a particular genotype, allowing hybrid vigour to be maintained indefinitely and avoiding the variability distinctive of sexual reproduction. Introduction of the trait into major food crops would be of great value since it would ensure an increased production in developing countries through the continuous propagation and exploitation of heterotic hybrid combinations (Savidan and Dujardin 1992; Jefferson 1994). Considerable effort has therefore been centred on introducing the trait into crops such as pearl millet (Dujardin and Hanna 1989) or maize (Berthaud and Savidan 1989; Savidan et al. 1995). If additional knowledge on the molecular basis of apomixis were obtained, the character could be directly transferred to other crop species by genetic transformation. This procedure would avoid the difficulties generated by large breeding programs, the lack of wild apomictic relatives, and the breeding barriers that prevent introgression of the character from unrelated species (Koltunow 1995).

The mode of reproduction in the forage grass *Brachiaria* is closely related to its ploidy level. Diploid genotypes are sexual, but polyploid ones reproduce through the formation of aposporous embryo sacs followed by parthenogenetic development of the egg cell and pseudogamous development of the endosperm (Brown and Emery 1958). We have used an F_1 population derived from a cross between apomictic *Brachiaria brizantha* and sexual *Brachiaria ruziziensis* that segregated 1 : 1 for the mode of reproduction for the detection of genetic markers linked to the gene(s) responsible for apospory. The strategy adopted is similar to that presented by Leblanc et al. (1995) and is based on probing with molecular markers of known map position in maize, taking advantage of the genomic synteny observed among grass genomes (Moore et al. 1995). Additionally, RAPD analysis was used to detect more markers. We report here the identification of two RFLPs and one RAPD marker significantly linked to the apomictic trait. A possible map was constructed by screening the region using additional maize markers.

Materials and methods

Plant material

Genetic data are based on 43 tetraploid *Brachiaria* plants derived from a cross between sexual *B. ruziziensis* R44 (2n = 4x = 36) and apomictic *B. brizantha* cv Marandu (2n = 4x = 36) generated at CNPGC, Campo Grande, Brazil. Modes of reproduction were cytoembryologically determined (methyl-salicilate-cleared ovaries observed under differential interference contrast) in Campo Grande (do Valle and Miles 1994) and confirmed at IGER by a second analysis using a benzyl-benzoate/dibutyl phthalate series as the clearing agent. The criteria for distinguishing unreduced and reduced embryo sacs were: (1) the embryo sac structure (number and distribution of the nuclei), (2) the number of embryo sacs per ovary, and (3) the presence of antipodal cells.

DNA extraction

DNA extractions were performed according to Dellaporta et al. (1983) with the following modifications: plant tissue (6 g of leaves) was frozen in liquid nitrogen, powdered with a mortar and pestle and transferred into 50-ml O-ridge tubes. Then 15 ml of extraction buffer were added (100 mM Tris-HCl pH 7.5; 50 mM EDTA pH 8.0; 500 mM NaCl; 2% SDS; 1% polyvinylpirrolidone). The mixture was incubated at 68°C for 20 min followed by the addition of 4 ml of 5 M KAc. Samples were incubated in ice for 1–2 h and then centrifuged (20 min, 11 000 g, 4°C). The supernatant was filtered through a cotton mesh and 25 ml of cold isopropanol was added. Samples were incubated overnight at −20°C and then centrifuged (20 min, 11 000 g, 4°C). Pellets were re-suspended in 700 µl of TE, gently shaken until dissolution and then treated with 5 µl of RNAase (10 mg/ml) for 20 min at room temperature. Samples were then transferred to an Eppendorf tube, phenolized and ethanol-precipitated overnight at −20°C. The DNA was centrifuged (20 min, 15 000 rpm), washed thoroughly in 70% ethanol, dried under an air flux for 1–2 h and dissolved in 400 µl of TE pH 8.0.

RFLP analysis

Probes

The RFLP clones (csu and umc) used in this study were 38 cDNAs and 56 genomic maize probes, originally from the University of Missouri-Columbia, that were kindly provided by CIMMYT, Mexico.

Filter preparations

For bulked segregant analysis (Michelmore et al. 1991) both apomictic and sexual bulks were made of ten F_1 plants each. A first set of membranes was prepared using DNA from both bulks, together with five individuals digested with the enzymes *Eco*RI and *Hin*dIII. The five hybrids were included to check the inheritance of the fragments detected in the bulks. A second set of membranes was then prepared using DNA from the bulks and the complete F_1 progeny. Twenty five micrograms of genomic DNA were digested per lane. The digestion reaction included: 1 × PROMEGA-corresponding activity buffer, 1% 0.1 M spermidine, 2.5 U restriction enzyme (*Eco*RI or *Hin*dIII from PROMEGA) per µg of DNA. Digestions were performed overnight at 37°C. Samples were electrophoresed in 1% agarose/1 × TAE gels overnight at 15–25 V and blotted onto nylon membranes (Hybond N, Amersham) by capillarity using 10 × SSC as a transfer buffer. DNA was UV-fixed using a Stratagene UV crosslinker and the filters were finally baked at 80°C for 2 h.

Hybridisation and detection procedures

The non-radioactive DNA-labelling with digoxigenin or fluorescein was made by PCR (25 ng plasmid, sterile distilled water to a final

volume of 100 µl per tube, 1 × *Taq* activity buffer, 50 mM of each dNTP, 0.2 mM oligonucleotides, 5% dig-dUTP or 15% fluorescein-dUTP, 15% glycerol, 1.5 U *Taq* enzyme from BRL). Hybridisation and detection were performed according to the manufacturers' instructions (Boehringer-Mannheim) using a CSPD chemiluminescent substrate. High-performance luminescence detection films (Hyperfilm-ECL, Amersham) were employed. Films were exposed between 2–12 h, depending on the intensity of the signal.

RAPD analysis

For RAPD analysis both apomictic and sexual bulks were made of 10 F_1 plants each and screened using 184 primers from Operon sets A, C, K, F, I, J, L, M and AG. The PCR conditions were as following: 25 µl total volume, approximately 0.8 ng/µl DNA, 2.5 µl 10 × reaction buffer, 100 µM of each dNTP, 1.5 mM Mg^{++}, 1 U *Taq* (BRL) and 1 ng/µl primer. Amplifications were carried out using either a Perkin-Elmer GenAmp PCR System 9600 thermocycler or a MJ Research, Inc. PTC-100 Programmable Thermal Controller. Cycles began with 2 min at 96°C followed by 40 cycles of 1 min at 96°C, 1 min at 37°C and 2 min at 72°C, and a final incubation of 5 min at 72°C. Twelve-point-five microliters of each reaction were electrophoresed in 2% agarose/1 × TBE at 40 V during 2.5 h.

Linkage analysis

Linkage analysis was conducted with JOINMAP Version 1.4 computer software (Stam 1993) using the Kosambi (1943) function. Multipoint analysis was performed using an initial LOD threshold of 3 and lowering the LOD threshold between 2 and 3 to map additional markers. The goodness of fit of segregation was tested by the χ^2 test.

Results

RFLP analysis

The membranes containing DNA from the sexual and apomictic bulks and five individuals in the progeny were screened using 90 RFLP clones detecting loci throughout the maize genome. Sixty one out of the ninety probes hybridised successfully (Table 1), the rest (19 genomic clones and 10 cDNAs) showed smearing or very weak signals probably due to their heterologous origin. The tetraploid F_1 progeny under study was highly polymorphic since 92% of the probes presented segregating bands for both enzymes in the five individuals. Twelve probes which showed differences in the intensity of the bands between both bulks were classified as putatively polymorphic.

A second set of membranes containing DNA from the bulks and the complete F_1 progeny was hybridised against the clones detecting potential polymorphisms. Eleven out of the twelve probes either showed polymorphisms not linked to the trait or were no longer confirmed. One of the probes (umc147) presented a polymorphic band that appeared to be linked to apomixis on *Eco*RI-digested samples (Fig. 1). Further analysis of the flanking regions based on the maize genome yielded another marker linked to apomixis detected by umc72 (Fig. 1). Both markers detected restriction fragments present in the apomictic parent but not in the sexual one (Fig. 2) and segregated in the progeny according to a 1:1 ratio ($\chi^2_1 < 0.025$, $0.8 < P < 0.9$). The segregation of the bands analysed in the 43 individuals of the F_1 progeny permitted the detection of a highly significant linkage between both markers and apospory (for umc147 $\chi^2_1 = 13.58$, $P < 0.001$, $r = 0.20$; for umc72 $\chi^2_1 = 11.94$, $P < 0.001$, $r = 0.25$). Clones umc147 and umc72 belong to the same duplicated linkage group of maize present in the distal part of chromosome-1 long arm and chromosome-5 short arm (according to the maps reported by Heredia-Díaz et al. 1994 and Ribaut et al. 1996). Several additional markers that belong to the same linkage group in maize were assayed on the *Brachiaria* population to produce a probable map of the region. Out of six total RFLP clones tested one gave a smeared pattern (csu33) and another presented only one segregating band showing a severely distorted segregation (umc84) and was not therefore included in the analysis. The remaining four probes (csu149, umc90, csu134, csu137) were linked to the same group carrying the apospory locus in *Brachiaria*. A possible map of the *Brachiaria* linkage group carrying the apospory gene is shown in Fig. 3 together with the comparative region of the short arm of maize chromosome 5. The *Brachiaria* map displays partial similarity to the maize one, but the position of some markers is altered.

Table 1 RFLP probes used in the scanning of the *Brachiaria* genome detecting loci at 40-cM intervals, on average, in the maize genome

Maize chromosome	Probes detecting a locus in a given chromosome[a]
1	umc128, umc84, csu3, csu61, csu110*, umc115, umc76, umc106
2	umc36, csu29*, umc34, csu154, csu148, umc6
3	umc121, csu29*, csu58, csu32, umc39, umc32, csu56*, umc16, csu96
4	umc31, csu36*, csu100, csu91
5	umc147, umc43, csu36*, umc141, umc104, csu134, csu108, umc68, csu26
6	csu146, umc28, umc85, umc62, umc65, csu56*, csu116, umc38
7	csu81, umc35, csu27, csu129, csu5, umc80
8	csu110*, csu165, umc120, umc89, umc103, umc48
9	umc109, umc81, csu54, umc94, umc95
10	umc130, csu48, umc44, csu46

[a] As indicated in the map reported by Heredia-Díaz et al. (1994)
* Probe for which duplicate loci are indicated

RAPD analysis

Out of the 184 primers analysed in the bulks, 31 did not amplify or else amplified poorly. Two primers (OPC15

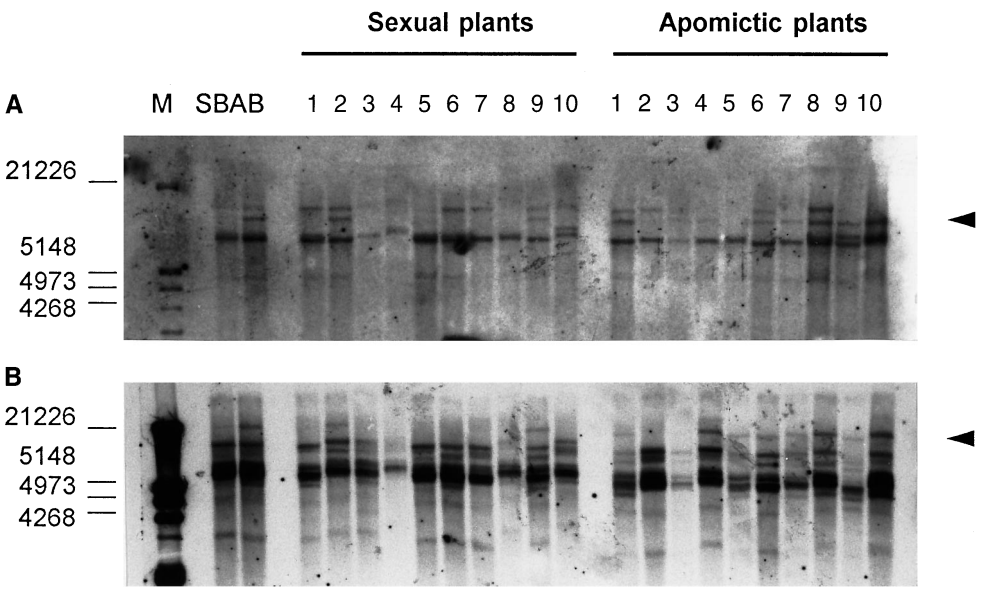

Fig. 1 Hybridization of umc147 **(A)** and umc 72 **(B)** in the progeny of the apomictic vs sexual cross. Samples were digested with *Eco*RI. *M* molecular weight marker (Marker III from Boehringer-Mannheim); *SB* sexual bulk; *AB* apomictic bulk. Classified individual plants that were included in the bulks are shown. The *arrows* indicate fragments that co-segregate with the mode of reproduction

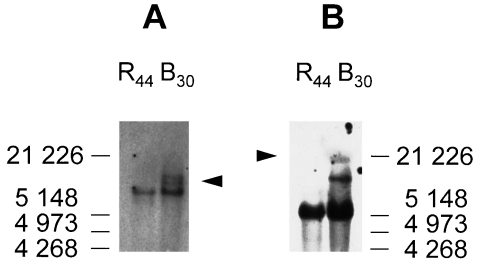

Fig. 2 Hybridization of umc147 **(A)** and umc72 **(B)** in the progeny parents. Samples were digested with *Eco*RI. *R*44 sexual parent (*B. ruziziensis*); *B*30 apomictic parent (*B. brizantha*). *Arrows* indicate the bands that were found to be polymorphic in the progeny. Marker migration is indicated alongside (Marker III from Boehringer-Mannheim)

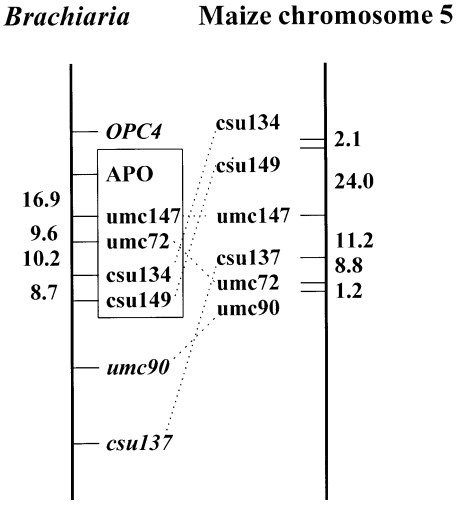

Fig. 3 Comparison of the chromosomal region related to apomixis in *Brachiaria* and the homoeologous maize fragment (Heredia-Díaz et al.1994). For markers *within the box* distances were calculated using the Kosambi function at LOD score 3 (indicated in centi-Morgans). Additional markers (*in italics*) were added using lower LOD scores (2–3)

and OPC4) were finally selected for linkage analysis using DNA of the full set of individuals in the progeny. The polymorphism detected by OPC15 was inconsistent and no linkage was finally determined. However, OPC4 did present a band highly significantly linked to apomixis ($\chi_1^2 = 11.08$, $P < 0.001$, $r = 0.24$) as well as to umc147 ($\chi_1^2 = 10.52$, $P = 0.001$) and to umc72 ($\chi_1^2 = 10.52$, $P = 0.001$). This marker was therefore also included in the same linkage group in the *Brachiaria* genome by JOINMAP (Fig. 3).

Discussion

For tagging the apospory gene in *Brachiaria* use was made of the well-known synteny between related Gramineae species. Molecular data of the grass genomes display similarities in gene order and composition as well as map collinearity (Ahn et al. 1993; Bennetzen and Freeling 1993; Moore et al. 1995). Although a genetic linkage map is still not available for *Brachiaria*, this collinearity among genomes allowed us to use the information from the maize genetic maps as support for efficient screening (Leblanc et al. 1995). The set of 61 probes detecting 65 loci systematically selected along the genetic map of maize was assumed to provide a comprehensive scan of the whole *Brachiaria* genome. The bulk segregant analysis originally reported by

Michelmore et al. (1991) was another key aspect in the strategy for the rapid identification of clones related to the apomictic trait. This technique clearly detects segregating markers within a range of 20% recombination on either side of the target locus. Markers within a 30% recombination window would also be detectable at least as bands of unequal intensity (Michelmore et al. 1991). Therefore, intervals of 40 cM, on average, between each detected locus should provide a good coverage of the complete genome by screening it systematically.

In the present study, only one linkage group has been associated with apospory in the complete scanning of the *Brachiaria* genome using both RFLP and RAPD markers, confirming the genetic analysis carried out previously by do Valle and Miles (1994). Both RFLP markers found to co-segregate with apospory belong to the same duplicated maize linkage group that maps to the distal end of chromosome-1 long arm and chromosome-5 short arm. Our finding suggests that there is no other maize linkage group homoeologous to the *Brachiaria* region responsible for apomixis. The RAPD marker is included in the same group. Under the assumption of the existence of a single genetic determinant *B. brizantha* would be simplex for a dominant allele conferring apospory.

Transfer of apomixis to sexual pearl millet from *P. squamulatum* has resulted in an obligate apomictic backcross line BC3 (Dujardin and Hanna 1989). Molecular markers linked to apospory were identified in this BC3 line by RAPD analysis (Ozias-Akins et al. 1993). The authors reported OPC4 as a marker for apospory in *Pennisetum* hybrids. However a Mendelian linkage analysis could not be conducted on this type of material because of the lack of evidence on pairing between the chromosome possibly carrying the factor(s) for apospory and a chromosome from the other parent (Ozias-Akins et al. 1993). In agreement with these results, out of the 184 primers used in our RAPD studies OPC4 was the only one that presented a band linked to apospory in *Brachiaria*. This observation suggests a common genomic location for the gene controlling the trait in both species.

The distal end of chromosome 6 of maize is related to diplospory in maize-*Tripsacum* hybrids (Leblanc et al. 1995). According to our data none of the probes mapping to chromosome 6 of maize presented polymorphisms associated with the character. Nevertheless, one of the probes belonging to the linkage group associated with the character in *Brachiaria* (csu134) was found to map the *Tripsacum* chromosome carrying the apomixis control (Savidan et al. 1995).

The information reported in the present paper provides evidence for the existence of a single locus controlling apospory in *Brachiaria* and on the region of the genome where it is located. A more detailed map is necessary for the isolation of the fragment responsible for conferring the character. Mapping at the diploid level in a larger population, which is currently in progress in our laboratory, will allow the construction of a refined map of this region and of the *Brachiaria* genome as a whole.

Acknowledgements We thank CIMMYT for kindly providing the maize probes used in this study, Dr. Ian Armstead for his helpful comments, and Teresa Montoya and Andrea Bollard for assistance. This work was financed by a grant from the Commission of the European Communities-STD3 program (TS3-CT93-0242). J.P.A.O. was supported by IBONE, Facultad de Ciencias Agrarias UNNE, Corrientes, Argentina. S.C.P. is a recipient of a British Council/ Fundación Antorchas Award. O.L. was supported by a Lavoisier fellowship from the French government (Ministère des Affaires Etrangères).

References

Ahn S, Anderson JA, Sorrells ME, Tanksley SD (1993) Homoeologous relationships of rice, wheat and maize chromosomes. Mol Gen Genet 241:48–490

Asker SE, Jerling L (1992) Apomixis in plants. CRC Press, London

Bennetzen JL, Freeling M (1993) Grasses as a single genetic system: genome composition, collinearity and compatibility. Trends Genet 9:259–261.

Berthaud J, Savidan Y (1989) Genetic resources of *Tripsacum* and gene transfer to maize. In: Mujeeb-Kazi A, Sitch LA (eds) Review of advances in plant biotechnology, 1985–1988. 2nd Int Symp on Genetic Manipulation in Crops. CIMMYT and IRRI, Mexico DF, Mexico and Manila Philippines, pp 121–130

Brown WW, Emery HP (1958) Apomixis in the Gramineae: Panicoideae. Am J Bot 45:253–263

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: Version 11. Plant Mol Biol Rep 1:19–21

Dujardin M, Hanna WW (1989) Developing apomictic pearl millet – characterisation of a BC3 plant. J Genet Breed 43:145–151

Heredia-Díaz O, Gardiner J, Hoisington D, Chao S, Coe E, Musket T, Xu G (1994) Combined F_2 and IF_2 RFLP map. Maize Genet Coop Newslett 68:30–34

Iwamasa M, Ueno I, Nishiura M (1967) Inheritance of nucellar embryony in *Citrus*. Bull Hort Res Sta Jpn Ser B7:1–8

Jefferson RA (1994) Apomixis: a social revolution for agriculture? The Monitor 19:14–16

Koltunow AM (1993) Apomixis: embryo sacs and embryo formed without meiosis or fertilisation in ovules. Plant Cell 5:1425–1437

Koltunow AM (1995) Apomixis: molecular strategies for the generation of genetically identical seeds without fertilisation. Plant Physiol 108:1345–1352

Kosambi DD (1943) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Leblanc O, Grimanelli D, González-de-León D, Savidan Y (1995) Detection of the apomictic mode of reproduction in maize-*Tripsacum* hybrids using maize RFLP markers. Theor Appl Genet 90:1198–1203

Michelmore RW, Paran I, Kesselli V (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Mogie M (1988) A model for the evolution and control of generative apomixis. Biol J Linn Soc 35:127–154

Moore G, Devos KM, Wang Z, Gale MD (1995) Grasses, line up and form a circle Curr Biol 7:737–739

Naumova TN (1993) Apomixis in angiosperms: nucellar and integumentary embryony. CRC Press, Boca Raton, Florida

Nogler GA (1984 a) Gametophytic apomixis. In: Johri BM (ed) Embryology of angiosperms. Springer Verlag, Berlin, pp 475–518
Nogler GA (1984 b) Genetics of apospory in apomictic *Ranunculus auricomus*. V. Conclusion. Bot Helvetica 94 : 411–422
Ozias-Akins P, Lubbers EL, Hanna WW, McNay JW (1993) Transmission of the apomictic mode of reproduction in *Pennisetum*: co-inheritance of the trait and molecular markers. Theor Appl Genet 85 : 632–638
Parlevliet JE, Cameron JW (1959) Evidence on the inheritance of nucellar embryony in *Citrus*. Proc Am Soc Hort Sci 74 : 252–260
Ribaut JM, Hoisington DA, Deutsch JA, Jiang C, González-de-León D (1996) Identification of quantitative trait loci under drought conditions in tropical maize. Flowering parameters and the anthesis-silking interval. Theor Appl Genet 92 : 905–914
Richards AJ (1986) Plant breeding systems. George, Allen and Unwin, London, pp 403–456
Savidan Y (1982) Nature et hérédité de l'apomixie chez *Panicum maximum* Jacq. PhD thesis, Université of Paris XI
Savidan Y, Dujardin M (1992) Apomixie: la prochaine révolution verte? La Recherche 241 : 326–334
Savidan Y, Grimanelli D, Leblanc O (1995) Transferring apomixis from *Tripsacum* to maize: progress and challenges. In: Taba S (ed) Maize genetic resources. Maize Program Special Report, Mexico D.F.: CIMMYT, pp 86–92
Sherwood RT, Berg CC, Young BA (1994) Inheritance of apospory in buffalo grass. Crop Sci 34: 1490–1494
Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. The Plant Jour 5 : 739–744
Valle CB do, Miles JW (1994) Melhoramento de gramineas do genero *Brachiaria*. In: Simposio sobre manejo pastagens, 11. Fundacao de Estudos Agrarios Luis de Queiroz, Piracicaba, pp 1–23