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Partial isolation of the genomic region linked with apomixis in *Paspalum simplex*

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Abstract Apomixis is a form of asexual reproduction through seed and has the potential to be applied, to great benefit, to agriculture. Understanding the genetic control of apomixis has proven to be a challenging task because the trait is mainly present in wild species and genetic mapping is often impaired by a block of recombination. A physical mapping approach has therefore been undertaken to unlock the genetic control of apomixis in *Paspalum simplex* Morong, a species with a relatively small genome and which exhibits a degree of genetic synteny with rice. In this paper, we report on the construction of a bacterial artificial chromosome library for *Paspalum simplex* with a coverage of approximately three genome equivalents and an average insert size of 94 kb. The BAC library was screened with 19 sequence characterized amplified region markers which were 100% linked to apomixis and a recombinant SCAR marker, all developed through a bulked segregant analysis strategy. A mini-sequencing procedure reported in the literature greatly aided the direct development of SCAR markers from amplified fragment length polymorphism bands. Several BAC clones linked to apomixis were identified and assembled into seven contigs and 18 singletons. Two of the BAC clones identified contained independently isolated markers. This is the first such report in an apomictic model that lacks recombination at the locus. We believe that extension of the contigs coupled to high-throughput sequencing will help the understanding of the genomic structure of the apomixis locus in *P. simplex*.

Keywords Apomixis · *P. simplex* · BAC clones · SCAR markers

Abbreviations

ACL	Apomixis controlling locus
AFLP	Amplified fragment length polymorphism
BAC	Bacterial artificial chromosome
HB	Homogenization buffer
HMW	High molecular weight
SCAR	Sequence characterized amplified region
SSR	Simple sequence repeat
TE	Transposable element

Introduction

Apomixis is a form of asexual reproduction widely spread among wild species but, with the exception of some forages and fruit trees, almost completely

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absent among crop plants. The outcome of apomixis is the formation of an embryo and seed without meiosis or fertilization of the egg cell. The opportunity to obtain a seed genetically identical to the mother plant potentially makes apomixis an extremely valuable agronomic trait, and therefore major efforts have been carried out to unravel its genetic control. For example, an understanding of apomixis could allow the development of a system to fix heterosis in those crops for which the production of hybrid seed is important (Hanna 1995; Spillane et al. 2004).

There are two main forms of apomixis: (1) sporophytic and (2) gametophytic. Sporophytic apomixis involves the direct formation of an embryo from a somatic cell in the ovule. In gametophytic apomixis the embryo sac is obtained via: (1) aposporory, where the nucellar cell divides autonomously and forms an unreduced gametophyte; and (2) diplospory, where a functional megaspore undergoes incomplete meiosis and forms an unreduced embryo sac through three rounds of mitosis. In both cases, the unreduced egg cell develops parthenogenetically into an embryo. At the same time, endosperm development in apomictic species may be independent of fertilization (autonomous endosperm formation) or may need the fusion of the polar nuclei with a pollen nucleus (pseudogamy) (Koltunow and Grossniklaus 2003). In most of the species studied to date, the inheritance of gametophytic apomixis was often associated with either a single locus or a small number of loci (Bicknell and Koltunow 2004; Ozias-Akins 2006). In the case of aposporous apomixis, a few studies have proved that it behaves as a dominant trait exhibiting Mendelian inheritance, with a strong block of recombination at the locus (Ozias-Akins and Van Dijk 2007 and references therein). Clear exceptions are in *Poa pratensis* and *Hypericum perforatum* where apospory and parthenogenesis were shown to recombine (Albertini et al. 2001; Matzk et al. 2005; Schallau et al. 2010). Contrastingly, in diplosporous apomixis, diplospory and parthenogenesis were shown to recombine in several cases (Ozias-Akins and Van Dijk 2007); nevertheless even in such systems more detailed analysis revealed suppression/reduction of recombination at the locus controlling one of the components of apomixis, e.g. diplospory in *Erigeron* (Noyes and Rieseberg 2000) and parthenogenesis in *Taraxacum* (Van Dijk et al. 2009).

There is a need to overcome the possible genetic constraints caused by the recombinational block by physically dissecting the apomixis locus. Several strategies have been undertaken including deletion mapping (Catanach et al. 2006) and the sequencing of physically isolated apomixis-linked bacterial artificial chromosomes (BACs) (Conner et al. 2008). The present paper reports on the partial isolation of the apomixis controlling locus (ACL) in *Paspalum simplex* Morong. This species is a warm season grass mainly grown in South America that was chosen as a model species to study apomixis because of the existence of sexually compatible apomictic and sexual cytotypes, its small genome size and relatively high seed production (Pupilli et al. 2001). *P. simplex* exhibits apospory followed by parthenogenesis, and it is necessary for the male gamete to fuse with the central cell in order to trigger endosperm development (pseudogamy).

A single dominant 'locus' is required for apomeiosis and parthenogenesis in the aposporous species *P. simplex* and *notatum*. Strongly distorted segregation ratios in favour of sexual genotypes have been reported in both species, suggesting that this gene either has a lethal pleiotropic effect with incomplete penetrance, or is linked to a lethal factor which causes gamete death in homozygotes (Pupilli et al. 2001; Martínez et al. 2001). The ACL of *P. simplex* is partially hemizygous, lacks recombination, spans approximately 15 cm as estimated by a comparative mapping approach, and exhibits a strong synteny with the distal region of the long arm of chromosome 12 of rice (Pupilli et al. 2001). The ACL of *P. simplex* is physically located in a non-heterochromatic region of the genome and 10× sequencing of an apomixis-linked BAC (Calderini et al. 2006) and unpublished results have shown that the region has undergone extensive rearrangements, as demonstrated by InDels and single nucleotide polymorphisms (SNPs), and that it has accumulated during its evolution many transposable elements (TEs) and pseudogenes. BAC clone sequencing also further supported synteny of this region with rice chromosome 12 based on genic sequences identified. However, numerous rearrangements due to TE insertions or deletions has led to the conclusion that the apomixis-associated genes probably lack coding capacity. Nevertheless it is possible that the pseudogenes which originated from the rearrangements are transcribed, and preliminary

transcriptomic studies suggest this (Calderini et al. unpublished). Such transcribed pseudogenes could act as trans-acting factors responsible for the dominant, plastic and incomplete penetrance of apomixis in most systems, as suggested by (Ozias-Akins et al. 2003). A complementary approach, based on transcriptional profiling of apomictic and sexual flowers of *P. simplex,* has revealed the presence of alleles at the locus that appear to have lost their coding capacity. These non-coding alleles could interact with their 'sexual' homologues, resulting in non-optimal expression in apomictic genotypes (Polegri et al. 2010). A recent report in *Pennisetum* which involved extensive sequencing of BAC clones from the ASGR region identified several genes and transposable elements. Even though the mutational load in the sequenced region does not necessarily mean that no protein-coding regions exist, it is possible that the *Pennisetum/Cenchrus* apomixis locus has undergone rearrangements that have a negative influence on the gene-coding capacity similarly to *Paspalum* (Conner et al. 2008; J. Conner and P. Ozias-Akins, personal communication).

The present paper reports (1) the construction of a *P. simplex* BAC library to enable the physical isolation of the apomixis locus in this species; (2) the isolation of 19 sequence characterized amplified region (SCAR) markers which are 100% linked to apomixis in *P. simplex* and four recombinant markers; and (3) the use of the SCAR markers to identify BAC clones from the apomixis locus in *P. simplex*. The BAC library also represents a valuable tool for the isolation of genes related to other important traits for a genus that is widely grown as a forage crop in South America.

Methods

Plant material

Leaf material for preparation of plant nuclei was harvested from an apomictic plant (18) which is part of a segregating population of *P. simplex* (Pupilli et al. 2001). Plants were maintained in an unheated glasshouse and young leaves were harvested after spring regrowth for high molecular weight (HMW) DNA extraction. Nuclei were isolated from freshly harvested leaves or after storage at −80°C.

HMW DNA isolation

The protocol of Farrar and Donnison (2007) was followed with minor modifications. A total of 15 g of *P. simplex* leaves were ground in liquid nitrogen and resuspended in homogenization buffer. The suspension was filtered with two layers of miracloth and major debris removed by centrifugation at low speed (57*g* for 5 min). The supernatant was then centrifuged at high speed (1,500*g* for 10 min) to pellet the nuclei. Nuclei were washed twice in wash buffer and finally resuspended in 1 ml of homogenization buffer (HB). The nuclear suspension was embedded in low melting agarose in a 1:1 ratio and used to prepare agarose plugs that were settled at 4°C overnight. The plugs were washed/stored for one week in 70% ethanol in a −20°C freezer according to the method of Luo et al. (2001). Subsequently plugs were washed with TE 20:50 buffer and then transferred into lysis buffer for 24 h. After lysis, plugs were washed with EDTA (0.5 M for 1 h, 0.05 M for 1 h, in both cases on ice with shaking). This was followed by further washes on ice: 3 × 1 h in TE (10:1) plus 0.1 M PMSF, 3 × 1 h in TE (20:50) on ice. Before restriction, plugs were pre-electrophoresed to improve the efficiency of the digest (Farrar and Donnison 2007).

BAC vector preparation

The pBeloBAC11-derived vector, pBSBH (O'Sullivan et al. 2001), kindly supplied by O'Sullivan, was used as it contains a stuffer fragment for ease of BAC vector preparation. BAC vector DNA was isolated with the Qiagen miniprep kit according to the manufacturer's instructions. A total of 10 µg of vector was digested with *Hin*dIII, dephosphorylated and electrophoresed on an agarose gel (O'Sullivan et al. 2001). The pBeloBac11 vector DNA was recovered from the agarose gel slice by dialysis (Farrar and Donnison 2007). A concentration of up to 15 ng of vector was used for ligation.

BAC library construction

A pre-restriction digest was performed to determine the optimal restriction enzyme concentration (Farrar and Donnison 2007). A total of 12 plugs, containing *P. simplex* HMW DNA, were then pre-incubated in

single tubes containing 400 µl of restriction buffer B (Roche) for 2 × 1 h on ice. The restriction reaction comprising 400 µl per plug of restriction buffer B plus BSA containing 0.2 U of *Hin*dIII was incubated for a further 4 h on ice, and then transferred to a 37°C waterbath for 1 h. The reaction was stopped by adding 1 ml of 0.5× TBE on ice. Restricted plugs were loaded onto a 1% agarose gel 0.5× TBE and electrophoresed overnight (16 h) using a pulsed field gel electrophoresis (PFGE) system (BIORAD CHEF DR-II) set to 5.2 V cm^{-1}, 120° included angle, linear pulse ramp from 0.5 to 40 s. Gel slices containing HMW DNA from 100 to 150 kb were recovered and were subject to dialysis as described by Farrar and Donnison (2007). A minimum amount of buffer was used to avoid over-dilution of the HMW DNA. After dialysis, the DNA was quantified on an agarose gel and 250–350 ng of restricted *P. simplex* DNA was used for ligation to 6–15 ng of pBeloBAC11 vector previously restricted with *Hin*dIII. Two ligations were set up overnight at 16°C, one for each DNA size range selected. The ligation reactions were drop-dialysed against TE (10:0.5) for 2 h (Farrar and Donnison 2007). A total of 5 µl of the ligation was used for electroporation of 20 µl of electrocompetent cells (DH10B, Invitrogen). A BioRad Gene Pulser II was used with the following settings: 100 ω, 25 mF and 1.5 kV. After 1 h recovery in SOC medium at 37°C, transformed cells were plated onto LB-agar containing 12.5 µg ml^{-1} of chloramphenicol. The plates were incubated at 37°C overnight (16–20 h) and the resulting BAC colonies were picked by hand using cocktail sticks into 96-well microtiter plates containing liquid LB plus 12.5 µg ml^{-1} of chloramphenicol. The plates were incubated at 37°C overnight and then 1:1 LB/glycerol added and the plates stored at −80°C. The library was stored in 448 plates.

BAC library characterization

To estimate the average BAC clone insert size, 113 randomly selected BAC clones were separately grown in 5 ml of LB liquid medium containing chloramphenicol (12.5 µg ml^{-1}). Plasmid DNA was extracted as described by Farrar and Donnison (2007). BAC DNA was digested with *Not*I and size separated by PFGE at 6 V cm^{-1}, a switch time from 0.5 to 40 s, an angle of 120° and run time of 16 h. Southern blotting and hybridization of selected BAC clones to *P. simplex* genomic DNA was performed according to standard protocols. The chloroplast contamination was evaluated according to the method described by Donnison et al. (2005). Briefly, BAC clones were macroarrayed on nylon filters which were hybridized with labelled chloroplast DNA from three chloroplast genes (*ndhA3*, *psbA2* and *rbcL*).

BACs were pooled in a 3D format and DNA extracted to allow a rapid PCR-based screening of the library as described by Farrar and Donnison (2007). BAC clones were replicated and grown overnight in 96-well microtiter plates, with each well containing 200 µl of LB at a concentration of 12.5 µg ml^{-1}. The content of each plate was pooled in a 50 ml Falcon tube. The plate pools were arranged in a 3D scheme so that each plate was represented in three pools. The 448 plates were thus split into 176 tubes, 56 each for the first two dimensions and 64 for the third dimension. BAC DNA from the pools was extracted by a standard alkaline lysis method and resuspended in 100 µl of water.

Development of apomixis-linked SCARs from AFLP markers

The mapping population used to develop markers linked to apomixis in *P. simplex* was described in Pupilli et al. (2001). To isolate amplified fragment length polymorphisms (AFLPs) linked to apomixes, bulked segregant analysis was performed (Michelmore et al. 1991). Genomic DNA was extracted from 500 mg of fresh leaves of 10 apomictic and 10 sexual plants using the method of Pupilli et al. (2001). An equal quantity of the DNA of each plant was pooled in 2 × 5 plant bulks for each phenotype. Pools contained 500 ng of DNA as required by the method of Vos et al. (1995) and as modified by Labombarda et al. (2002). After selective PCR amplification, the AFLP amplicons were separated on a 6% denatured polyacrylaminde gel and visualized in the Genomyx SC Scanner (Beckman) as described by Labombarda et al. (2002). The image was saved as a TIFF file and analysed with Adobe Photoshop CS2 version 9.0 software. The position of the AFLP bands present in apomictic plant bulks and absent in their sexual counterparts was recorded with the aid of the coordinate system of the Genomyx scanner. The gel sectors containing the informative bands were excised from the gel using a scalpel, soaked in

100 µl distilled water, incubated at −80°C for 30 min, then vigorously vortexed for 30 s, spun down and stored overnight at 4°C as described in Brugmans et al. (2003). A total of 5 µl of the supernatant was used as template for PCR amplification using the same primer combinations and thermocycling conditions as in the pre-selective amplification. After gel analysis to check molecular weights, PCR products were purified using a PEG-based precipitation protocol. A 15 µl aliquot of PEG solution (20% polyethylene glycol, 2.5 M NaCl) was added to an equal volume of PCR product and incubated at 37°C for 15 min. After centrifugation at maximum speed for 15 min, the resulting pellet was rinsed twice with chilled 80% ethanol and centrifuged for 2 min. The supernatant was discarded and the pellet dried in a Speedvac for 3 min and resuspended in 8 µl TE buffer. After gel quantification, 10–20 ng of purified fragments were directly sequenced bidirectionally using the same primer combinations as in pre-selective amplification with the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The reactions were run on an ABI Prism 3730 sequencing apparatus (Applied Biosystems). For those amplicons for which a clear single sequence was obtained, several primer combinations (PerPrimer v1.1.14) were screened to select apomixis-specific SCARs. From one to four forward and reverse primers were designed so as to generate a minimum of 80-bp fragment size and minimize the number and the stability of potential primer dimers. These primer pairs were screened using the genomic DNA of two apomictic and two sexual plants to select those primers capable of amplifying a single band in apomictic plants but not sexual plants. To confirm linkage of the selected SCARs with apomixis, the primers were used to screen a further 100 plants (50 apomictic plus 50 sexual). When multiple sequences were encountered in the re-amplified fragments, the procedure of Brugmans et al. (2003), known as AFLP-mediated mini-sequencing, was used to select the apomixis-linked sequence. Briefly, a set of degenerate *Mse*I primers was used for the selective amplification from the 100-fold-diluted selective amplification product in combination with the same fluorescein-labelled *Eco*RI primer. SCAR markers specific for apomictic fragments were developed and validated as described above.

Development of SCAR markers from SSRs

To improve the coverage of the apomixis locus we developed further SCAR markers from heterologous simple sequence repeat (SSR) markers of seashore paspalum (*P. vaginatum*) (Wang et al. 2005). A total of 27 primers pairs, kindly suggested by H.L Wang, USDA-ARS, USA (Table 1), were tested for their transferability to *P. simplex*. SSRs were amplified according to the reported protocol (Wang et al. 2005) except that the annealing temperature used was 55°C. PCR amplification products were electrophoresed on a 3% agarose gel. The markers were tested for amplification on DNA bulks of apomictic and sexual plants; the primer pairs detecting polymorphisms were then used to amplify DNA extracted from single plants within the segregating population.

Construction of BAC contigs

Single BAC clones were grown overnight at 37°C in 1.5 ml 2× YT medium and plasmid DNA isolated. The BAC was fingerprinted using SNaPshot labelling (Luo et al. 2003). A total of 42 µl of BAC DNA was added to the restriction digest reaction. The restriction digestion mix of 9.0 µl consisted of five units each of *Bam*HI, *Eco*RI, *Xba*I, *Xho*I, and *Hae*III restriction endonucleases; 1× NEBuffer 2; 5 µg BSA; 5 µg DNase-free RNase A (Sigma R-6513) and 0.1% β-mercaptoethanol. DNA was digested at 37°C for 3 h. The digested DNA was added to 10 µl of SNaPshot labeling solution (1 µl of SNaPshot Multiplex Ready Reaction Mix, Applied Biosystems), 2 µl 1× NEBuffer 2, 2.5 µl 100 mM Tris, pH 9.0, and 4.5 µl ddH2O. The reaction was incubated at 65°C for 60 min. To precipitate the DNA, 6 µl of 2.5 M sodium acetate (pH 5.2) and 100 µl of prechilled 95% ethanol (−20°C) were added to the labelled reactions and they were incubated at −80°C for 15 min. DNA was resuspended in 10 µl of Hi-Di formamide (Applied Biosystems) and 0.2 µl of ABI internal size standard LIZ-500 (ABI No. 4322682, size range 35–500 bp) was added to each sample. Capillary electrophoresis was performed on an ABI Prism 3130 using the E5 chemistry module according to the ABI User Bulletin 2005. Fragment data were collected by the ABI program Genemapper and processed with the program Genoprofiler

Table 1 SSR primers derived from seashore paspalum tested in *Paspalum simplex* (Wang et al. 2005)

Primer name	Amplification in *P. simplex*
W5	+
W12	+
W20	+
W21	+
W22	–
W26	+
W29	–
W31	–
W34	–
W35	+
W39	+
W40	+
W41	+
W45	+
W46	–
W49	+
M2	+
M4	+
M5	+
M25	+
M26	+
M38	+
M39	+
M41	+
M42	+
M45	+
M50	+
S9	+
S10	+
S12	+
S13	+
S15	–
S16	+
S21	+
S23	+
S24	+
S25	+
S26	+
S29	+
S30	+

(http://wheat.pw.usda.gov/PhysicalMapping/). Files generated by Genoprofiler were imported into the FPC (version 9.3, http://www.agcol.arizona.edu/software/fpc/ at the University of Arizona). FPC parameters were set at tolerance = 7 and stringency = 10^{-12}.

Results

BAC library construction and characterization

The *P. simplex* BAC library was the result of two ligations of two different size-selected HMW DNA fractions into the pBeloBAC vector. A pre-incubation in 70% ethanol was used as suggested by Luo et al. (2003) and may have helped to increase the efficiency of the ligation. In total 43,008 BAC clones were picked and stored in 448 × 96-well plates. The average insert size of the library was estimated, by *Not*I restriction of 113 randomly selected BAC clones (Figs. 1 and 2), to be 94 kb. The percentage of empty clones was 0.8% and because the DNA content of

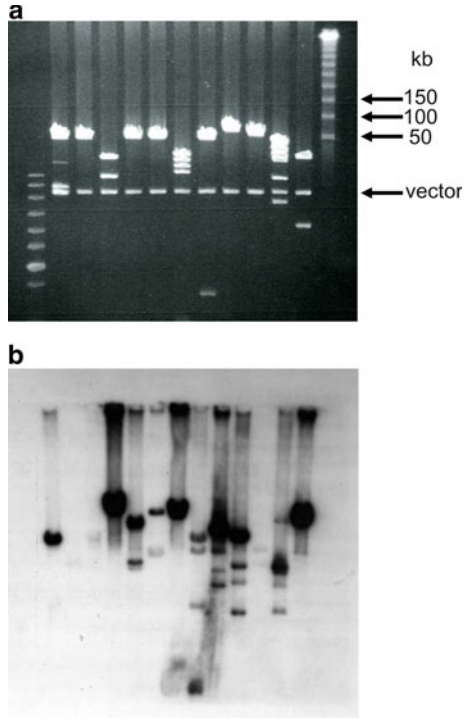

Fig. 1 Pulsed field gel electrophoresis (PFGE) of inserts from randomly selected BAC clones: **a** DNA from BAC clones was restricted with *Not*I and separated on agarose gel following PFGE; **b** Southern blot hybridization of *Not*I restricted clones with *P. simplex* genomic DNA. The size of some reference bands from the DNA size marker (Lambda Ladder PFG Marker, New England Biolabs, USA) are indicated in kb

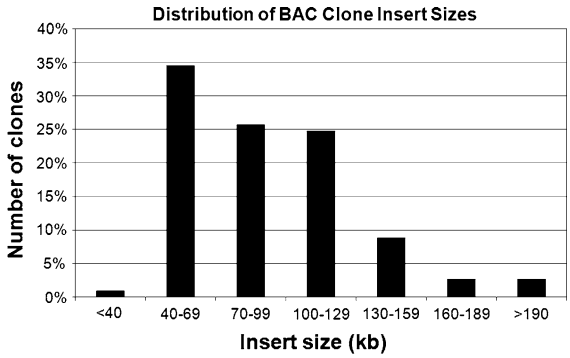

Fig. 2 Insert size distribution of 113 BAC clones from the *P. simplex* BAC library. Insert sizes in kb were calculated from *Not*I digest of BAC DNA after separation with pulsed field gel electrophoresis as in Fig. 1a

P. simplex (2C) has previously been estimated as 1.4 pg (Caceres et al. 1999) the BAC library represents approximately three-fold genome coverage for this species. Southern hybridization of 13 randomly selected BAC clones revealed that most contained at least one positive hybridizing fragment when probed with total genomic DNA of *P. simplex* (Fig. 1). The organelle contamination was evaluated by Southern analysis with three chloroplast-genome derived genes and was estimated to be 1.7%.

Development of apomixis-linked SCARs from AFLP markers

To isolate apomixis-linked SCAR markers, a genomic AFLP procedure coupled with a bulked segregant analysis was adopted using 193 primer pair combinations. On the basis of previous analyses, the preselective amplification was performed using *Eco*RI + G/*Mse*I + T combinations, whereas the selective amplifications were performed using *Eco*RI + *n*/*Mse*I + *n* combinations, where *n* was either 3 or 4 in all possible combinations. A total of 16,700 scorable bands were detected with molecular weights of 100–700 bp. Of these, 16,570 fragments were monomorphic in all four bulks, whereas 130 fragments were specific to the apomictic bulks. Neither between-bulk polymorphisms of the same phenotype nor sex-specific polymorphisms were detected. In general the AFLP amplification patterns derived from primer combinations in which at least one primer had four selective bases, were less complex and resulted in the identification of more apomixis-linked polymorphisms, because of a reduction in the occurrence of co-migrating monomorphic bands. All of the 130 putative apomixis-linked fragments were excised from the gel, reamplified and sequenced. Of these, 30 did not amplify or could not be sequenced, 62 generated multiple sequence profiles, and 38 exhibited an unambiguous unique sequence. To overcome the problem of co-migration of amplicons of similar molecular weight but different sequence, the selection procedure of Brugmans et al. (2003) was used. The AFLP amplification mixtures yielding multi-sequence putative apomixis-linked amplicons were re-amplified with *Mse*I primers carrying three, four or five degenerate bases (N) plus each of four additional selective bases at the 3′ end, together with the same AFLP *Eco*RI primer. In such a way, a further 2–3 internal bases from the *Mse*I side were identified. Primers including those at the 3′ end of the *Mse*I side were used to re-amplify the original AFLP mixture and the expected fragments (when present) were excised from the gel, re-amplified and sequenced. This procedure allowed the selection of a further 28 amplicons of unique sequence from the 62 multiple-sequence profiles. For the 66 (38 plus 28) amplicons represented by a single sequence, primer pairs were designed to obtain apomixis-specific SCAR markers. Depending on the size of the amplicons, 1–16 primer pairs were screened. When tested on the two apomictic plus two sexual plants, at least one primer combination per amplicon allowed the amplification of an apomixis-specific fragment. To verify linkage with apomixes, the 22 SCAR- revealing primer combinations were used to amplify the DNA of a population of 100 plants (50 apomictic plus 50 sexual). Sixteen SCARs were identified as being 100% linked to apomixis, two were unlinked and four identified one recombinant event represented by the same sexual plant (map distance = 0.01 cm; according to Liu 1998). This represents the first report of a recombination event scored from a large number of apomixis-linked markers (Pupilli et al. 2001; Labombarda et al. 2002; Pupilli et al. 2004; Calderini et al. 2006). No further recombination events were detected. That four independent markers exhibited a low frequency of recombination with apomixis, as revealed by a single recombinant in the population of 100 plants, indicates that the recombination block extends to a lesser stringency in the ACL in *P. simplex*. The 16 non-recombining SCARs and one recombining SCAR

marker (Psapo7681-211) identified in this study, and two non-recombining SCAR markers from Labombarda et al. (2002) and Calderini et al. (2006), were used to screen the BAC library.

Development of apomixis-linked SCARs from SSR markers

Most of the markers derived from seashore paspalum (*Paspalum vaginatum*) resulted in a distinct amplification product in *P. simplex* (Table 1). For example the primer pair from the marker W5 resulted in a common fragment for both sexual and apomictic DNA bulks, and another fragment of different size specific for the apomictic bulk. Such polymorphism was confirmed by extracting the DNA fragments, cloning and sequencing. The SSR, W5, was originally derived from an expressed sequence tag (EST) from wheat. When aligned to the wheat EST, neither the common fragment nor the apomixis-specific fragment-derived sequences from *P. simplex* exhibited any significant homology to wheat, indicating that the W5 primer pairs amplified a non-homologous region in the *P. simplex* genome. BLASTn analysis with the rice genome revealed that the common fragment between sexual and apomictic plants was homologous to a putative resistance gene (69% identity), and the apomixis-specific fragment was homologous to a retrotransposon (63% identity). A primer pair was designed from the apomixis-specific fragment sequence and was used to amplify DNA from single individuals of *P. simplex*. The same polymorphism was revealed as when using the W5 primer pair. The *P. simplex* primers were then used to screen the BAC library DNA pools.

PCR-based screen of the BAC library

The BAC library was screened with 20 SCAR markers (19 AFLP-derived and one SSR-derived, Table 2) and 41 BAC clones were identified. Thus an average of 2.05 clones was identified per SCAR marker. All of the markers resulted in at least one positive BAC clone (Table 3), the number of clones identified ranging from one to four. All the PCR-positive clones were verified by sequencing of the amplification products. Notably, of the 41 BACs identified, the clones 331G4 and 426E10 both included two markers that had been developed independently (Table 3). We believe that this is the first report of multiple hits to the same BAC clone of an apomixis locus. Because the SCAR markers were derived from AFLP fragments using different primer combinations, the occurrence of more than one marker in two apomixis-linked BAC clones suggests that the apomixis locus in *P. simplex* is likely to be smaller than for other species such as for *Pennisetum squamulatum* (Roche et al. 2002). To test if the recombinant BAC 84E7 (Table 3) also contained any of the other three recombinant SCARs, it was used as a template for PCR amplification. No amplification products were observed, indicating that the co-segregation of the recombining SCARs was due to a recombination repression rather than to a biased selection of markers toward the same BAC.

BAC contig production

The 41 BAC clones isolated were fingerprinted at high (10^{-12}) stringency and the results are summarized in Table 3. At the level of stringency used, 22 BACs were arranged into seven contigs and 19 remained as singletons. The contigs contained between two and four clones. No overlap was observed between contigs identified by different markers. In contigs 1, 3, 5, 6 and 7 all the clones isolated with the same marker could be grouped. However, multiple clones derived from markers Psapo7585-180, Psapo6779-249 and Psapo7579-106 remained singletons even at a low level of stringency. The identification of multiple contigs for individual SCAR markers, as also for markers Psapo6487-186 and Psapo6792-234, indicates the occurrence of duplicated regions in the apomixis locus of *P. simplex*. Duplications of hemizygous markers seems to have also occurred in the apomictic species *Pennisetum/Cenchrus* (Roche et al. 2002; Goel et al. 2006) as confirmed at the sequence level (Conner et al. 2008).

Discussion

In this paper we report the construction of the first BAC library in the apomictic species *P. simplex* and for the grass genus *Paspalum*. In a previous work, fluorescence in situ hybridization (FISH) analysis of a BAC clone allowed the elucidation of the physical

Table 2 Primer pairs and the number of BACs isolated for each SCAR marker

SCAR name	Forward primer	Reverse primer	Annealing temperature (°C)	Band size (bp)	Number of BACs identified
Psapo7585-180	CTCGTAGACTGCGTACCAATTCGTA	ATGAGTCCTGAGTAATCGA	59	180	2
Psapo7589-149	ATCTGAAAACTTATCAAACCTTCC	GCGGGCTTTCACAATAATAC	53	149	3
Psapo6779-249	CATGCTGAATTGAGGTAGGC	GAAACAAAAACTCACACCTTCG	55	249	3
Psapo6487-111	TTGCTCACACGTCACCTCTC	TGGTTGAACAACTTTCATGG	52	111	1
Psapo6487-186	AAATATGGCGATGTGCCAAG	GCAATCATCGTAGATACTC AAATCTC	54	186	4
Psapo7579-106	TAGTGTCAACGAGACCTGCT	GGGAGTTACAGAGTTAGTTTT GTTCA	55	106	2
Psapo6582-195	CACAACACAAATCCAACTACGA	CCTGTATTTGCTATTGCTTTCCC	53	195	4
Psapo7090-115	TATTTATGCTTCCAAGGTGCCA	CCACTATTCTATTCAGGTGC	53	115	1
Psapo6989-94	CATGTAAAGAGATCGCACAGG	CATCAGCAGAAATTGTAAG CTACC	57	94	1
Psapo6792-234	CGAGGCAGCATCATCTAAGG	GCATCTGGCATGATTTGGTC	57	234	4
Psapo6592-183	TCACCCTATTACTCATAGGCCA	CATAAACCAATTGATTGGCGTG	61	183	3
Psapo6781-63	TGCTCTTTGGAACTATTGAC	GGATCAGTAGAAACTGATTCCT	58	63	1
Psapo6582-128	GTTTGACTTATTGACTGCATGG	TGGCACATCTGTTTACAGGG	58	128	1
Psapo7687-191	TCTATAACACTGTTTGACACGG	AATTCCACCTGGATGTGAGAG	58	191	1
Psapo6687-194	ACGCGATCATGTGGGATAGC	GCAGCCGCAGGAGAATACAC	58	194	2
Psapo7892-230	GTTGATCACCGTTGAAGTGAG	TTTGTGCATCATCCACTTCC	58	230	2
Psapo7881-124	GGCAACATAACTTAGTGAGGCA	GGGTGTCCAAGCTCTTTACAG	62	124	1
Psapo6677-116	GACTTCTTTACACAGAGGTGG	CTTATCCTGATGGGATACGA	58	116	4
Psapow5	ATGGATAGTGACAATCTGT	TAAGCAAGGAGTAATCAAAG	55	169	2
Psapo7681-211	AGGAGCACCAATTTCTATGGG	TGGAAACCAATGCTAAGGGA	57	160	1

location and the hemizygosity of the locus (Calderini et al. 2006). With the present work we aimed to increase further the physical isolation of the genomic locus of *P. simplex*. In fact, 17 new SCAR markers tightly linked to apomixis were developed from AFLPs and SSRs through a bulked segregant analysis. A set of co-segregating markers that exhibited a low level of recombination within the apomixis locus was also identified. Several BAC clones were isolated harbouring the selected markers, in particular 40 clones were allocated to the apomixis locus. For several SCAR markers, separate DNA contigs were identified, suggesting duplication at the locus as revealed for other species (Roche et al. 2002). For the first time we were able to identify single BAC clones containing independently isolated markers in a species that holds a strong block of recombination at the locus, thus suggesting the possibility that the apomixis locus in *P. simplex* could be of a relatively more modest size than in other models (Roche et al. 2002). On this basis, a bulked segregant analysis may efficiently saturate the region with molecular markers. In the future, physical mapping of the ACL could be complemented with FISH analysis of the BAC clones and extension of the contigs by chromosome walking coupled with sequencing. A combination of such strategies will also enable an estimation of the size of the locus. Given that the size of the ACL is likely to be smaller in *P. simplex* than in other species, such a strategy would be instrumental in understanding the genetic control of the locus. Comparison with similar data obtained in *Pennisetum/Cenchrus* (Conner et al. 2008) will also be important to identify common mechanisms controlling apomixis across different species.

The emerging model derived from sequence analysis of apomictic plants (Conner et al. 2008) and mutant studies in sexual plants (Ravi et al. 2008; d'Erfurth et al. 2009) is that apomixis arises from deregulation of the sexual pathway (Koltunow and

Table 3 Contig analysis of ACL-linked BAC clones in *P. simplex*

SCAR name	FPC contig no.	BACs			
Psapo7585-180	Singleton	302G3	366H1		
Psapo7589-149	1	298H1	348H10	439A12	
Psapo6779-249	Singleton	385A6	403F1	423D7	
Psapo6487-111	Singleton	215E1			
Psapo6487-186	2	329G8	378H11	391A6	
	Singleton	98D8			
Psapo7579-106	Singleton	43F5	290H2		
Psapo6582-195	3	200G11	288C4	380D2	406C1
Psapo7090-115	Singleton	409G12			
Psapo6989-94	Singleton	331G4			
Psapo6792-234	4	128H3	359B8	410G11	
	Singleton	285A12			
Psapo6592-183	5	129E10	305G8	381G12	
Psapo6781-63	Singleton	331G4			
Psapo6582-128	Singleton	426E10			
Psapo7687-191	Singleton	96G3			
Psapo6687-194	Singleton	17E11			
	Singleton	274F3			
Psapo7892-230	Singleton	282C3	377F10		
Psapo7881-124	Singleton	426E10			
Psapo6677-116	6	127F6	296A7	333G1	312H12
Psapow5	7	34F10	296G9		
Psapo7681-211	Singleton	84E7			

Grossniklaus 2003), through either a genetic or epigenetic mechanism. Given the complexity of apomixis, an understanding of the genomic structure of the apomictic locus in asexual models is likely to be an essential prerequisite for manipulation of the sexual pathway in model or crop plants. Recent reports for the *dyad* mutant and the *MiMe* genotype in *Arabidopsis* (Ravi et al. 2008; d'Erfurth et al. 2009) confirm that elements of apomixis are also probably present in sexual plants. However, as the manipulation of *dyad* and *MiMe* is unlikely to be sufficient (Van Dijk 2008; Meadows 2009), information from sexual models will also need to be integrated with knowledge from asexual plants. Knowledge of the sequence of the apomictic locus is also likely to be of fundamental importance for the epigenetic inheritance of apomixis, the currently favoured model in recent literature (Koltunow and Grossniklaus 2003). There is evidence that specific chromosomal regions transmit the trait in natural apomicts (Ozias-Akins and Van Dijk 2007), and a molecular dissection of such regions will be necessary to test the epigenetic inheritance model; in fact sequence information has also been instrumental for epiallele cloning (Manning et al. 2006).

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