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ORIGINAL PAPER

A molecular map of the apomixis-control locus in *Paspalum procurrens* and its comparative analysis with other species of *Paspalum*

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Abstract Since apomixis was first mapped in *Paspalum*, the absence of recombination that characterizes the related locus appeared to be the most difficult bottleneck to overcome for the dissection of the genetic determinants that control this trait. An approach to break the block of recombination was developed in this genus through an among-species comparative mapping strategy. A new apomictic species, *P. procurrens* (Q4094) was crossed with a sexual plant of *P. simplex* and their progeny was classified for reproductive mode with the aid of morphological, embryological and genetic analyses. On this progeny, a set of heterologous rice RFLP markers strictly co-segregating in coupling phase with apomixis was identified. These markers were all located on the telomeric region of the long arm of the chromosome 12 of rice. In spite of the lack of recombination exhibited by the apomixis-linked markers in *P. procurrens*, a comparative mapping analysis among *P. simplex*, *P. malacophyllum*, *P. notatum* and *P. procurrens*, allowed us to identify a small group of markers co-segregating with apomixis in all these species. These markers bracketed a chromosome region that likely contains all the genetic determinants of apomictic reproduction in *Paspalum*. The implications of this new inter-specific approach for overcoming the block of recombination to isolate the genetic determinants of apomixis and gain a better comprehension of genome structure of apomictic chromosome region are discussed.

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Introduction

Sexual reproduction is the most important source of genetic variability in the plant kingdom. However, avoidance of sexual pathways and hence of genetic segregation might be an advantageous tool for plant breeders to guarantee genetic uniformity and stability of cultivars. Apomixis is defined as "asexual (agamic) reproduction by seeds" (Nogler 1984a) because it allows the production of individuals that are genetically identical to the mother plant by circumventing meiosis and fertilisation of the egg cell. In this view, harnessing apomixis into crop species would be an excellent option because it could be used to fix over generations, the yield advantage provided by the heterosis characteristic of hybrid seeds (Hanna and Bashaw 1987; Koltunow et al. 1995). This asexual reproduction pathway currently occurs in a small proportion of higher plant species, constituted mainly by wild tropical grasses and few minor crops. Its potential use in major crops such as corn and rice will be dependent on the possibility to introduce this trait on these species and control its expression so that high yields of genetically pure crops can be obtained. Unfortunately although apomictic reproduction seems to be genetically controlled in wild species, these are not amenable for applying the tools of molecular biology commonly used in other model biological systems such as *Arabidopsis* or *Petunia*. On the other hand, although impressive progress has been recently made in *Arabidopsis* to reproduce some features of apomictic reproduction, i.e. apomeiosis

(Ravi et al. 2008; d′Erfurth et al. 2009; Olmedo-Monfil et al. 2010), and parthenogenesis (Ravi and Chan 2010) by mutagenesis, no full apomictic *Arabidopsis* mutants have been produced to date. Furthermore, it is still unclear whether these phenotypes are related to apomixis in wild species (Garcia-Aguilar et al. 2010). Recently, an artificial apomictic *Arabidopsis* has been produced by combining the aforementioned apomeiotic and parthenogenetic mutants by crossing (Marimuthu et al. 2011). However, since this plant still relies on crossing to express full maternal inheritance, it cannot be defined as a genuine apomictic genotype as it stands. Therefore, there is a need to get insights into the genetic mechanisms underlying apomictic reproduction in wild species to understand how an apomictic system could have evolved from the sexual background and to verify whether an ectopically induced apomixis system in *Arabidopsis* could serve at developing of a stable and universal apomixis system to be introgressed in crop species. With the ongoing progress in large-scale sequencing, the genetic dissection of the genetic determinants of apomictic reproduction is becoming approachable in its natural biological background. Several candidate genes have been identified via transcriptional profiling (Albertini et al. 2005; Chen et al. 2005) or gene mapping (Schallau et al. 2010), but none of these have been proven to be involved in apomictic development by complementation or functional analysis. In this view, we are using two species of the genus *Paspalum* (*P. simplex* Morong and *P. notatum* Flügge) as model biological systems to identify the genetic determinants of apomictic reproduction in the perspective to develop an apomictic system to be introgressed in monocots, such as rice and maize.

Paspalum is a large grass genus that includes many valuable species used as natural pasture mainly in warm regions of the Americas. This genus is characterized by an extraordinary variety of reproductive systems including allogamy (due to self incompatibility), autogamy (including cleistogamy) and apomixis. In general, diploid species are sexual, whereas most polyploids are apomictic (Quarin 1992). The main type of apomixis in *Paspalum* is apospory in that one or more nucellar cells give rise to unreduced embryo sacs by two or three mitotic divisions thus, avoiding meiosis completely. The unreduced egg cell gives rise to a functional embryo by parthenogenesis avoiding paternal contribution, whereas fertilisation of the central cell by one sperm nucleus is necessary for the correct development of the endosperm (Cáceres et al. 2001).

Molecular markers linked to apomixis have already been found in three *Paspalum* species. Of these, *P. simplex* and *P. malacophyllum* Trinius belong to the subgenus *Anachyris*, which consists of an agamic complex of six closely related species of which some are formed by both sexual diploid and apomictic polyploid races (Urbani et al. 2002; Hojsgaard et al. 2008). We previously established that the apomixis controlling locus (ACL) in *P. simplex* is genetically defined by a single dominant allele characterized by a strong repression of recombination and a significant synteny of markers with the telomeric region of the long arm of chromosome 12 of rice (Pupilli et al. 2001). Its physical dissection, followed by sequencing of large DNA fragments showed that the ACL is mainly formed by a large "sea" of repetitive and transposable elements and non-coding DNA where rare genes are embedded (Calderini et al. 2006). Finally, FISH analysis of an apomixis-linked BAC showed that the ACL is located in a non-pericentromere and non-heterochromatic chromosome area indicating that the genes that are contained in this locus could be transcriptionally active (Calderini et al. 2006).

Molecular mapping of apomixis in *P. notatum*, a species that is distantly related to those belonging to the subgenus *Anachyris* showed a similar block of recombination at the ACL as observed in the other *Paspalum* species (Stein et al. 2007). However, the ACL in *P. notatum* shows important differences when compared with that of *P. simplex* and *P. malacophyllum*, such as chromosome structural alterations as judged by the interruption of synteny of apomixis-linked markers (Pupilli et al. 2004). The comparative mapping of the ACL in the three species, conducted with a common set of rice heterologous probes, showed that although all the apomixis-linked markers in *P. simplex* and *P. malacophyllum* were syntenic to the same portion of the chromosome 12 of rice, the ACL of *P. notatum* was localized in a hybrid chromosome which carries markers mapped to both rice chromosomes 2 and 12. In all these species, the lack of recombination is a common feature of the ACL and it is considered an obstacle for positional cloning of the key genes. Comparative molecular mapping of apomixis among several species within the same genus could contribute to narrow down the portion of the ACL that should be targeted for mining genes involved in apomictic reproduction in *Paspalum*. The adding of other species to this analysis could reduce further the size of the ACL worth to be considered for gene mining.

Paspalum procurrens Quarin is another species that belongs to the *Anachyris* subgenus and shows cross compatibility with *P. simplex* at both diploid (Espinoza and Quarin 1998) and tetraploid levels (Hojsgaard et al. 2008). The aim of this research was to identify a set of molecular markers that are linked to apomixis in *P. procurrens* among those previously found to be conservatively linked to this trait in other *Paspalum* species with the perspective to narrow down the target area of the ACL used to mine the genetic determinants of apomixis. For this purpose we have (1) generated and characterized a *P. simplex* × *P. procurrens* progeny by its reproduction mode; (2) characterized the ACL in *P. procurrens* by using rice and *Paspalum* probes,

and (3) compared the ACL findings in *P. procurrens* with other species of *Paspalum* and aligned the resulting map to that of rice.

Materials and methods

Plant material

A mapping population segregating for apomixis was generated at IBONE, Corrientes, Argentina, by crossing a sexual tetraploid ($2n = 4x = 40$) plant of *P. simplex* (C_1B_2), with a natural tetraploid ($2n = 4x = 40$) aposporous apomictic plant (Q4094) of *P. procurrens* collected at El Salvador, Chuquisaca, Bolivia (Hojsgaard et al. 2008). The female parent C_1B_2 was a S_1 plant of *P. simplex* derived from a colchicine-induced autotetraploid (Cáceres et al. 1999) produced at the Institute of Plant Genetics, CNR Research Division of Perugia, Italy. This genotype was previously classified as 100% sexual on the basis of (1) cyto-embryological analysis of mature embryo sacs, (2) molecular analysis based on an apomixis-specific marker, and (3) progeny tests using DNA fingerprints (data not shown). The autopolyploidy of apomictic *P. simplex* has been proposed through cytological analysis by Caponio and Quarín (1987) and confirmed by tetrasomic inheritance of molecular markers (Pupilli et al. 1997). Similarly, the autopolyploid nature of the induced sexual tetraploid *P. simplex* was proven by molecular markers (Pupilli et al. 2001) and, that of *P. procurrens* by cytological analysis (Hojsgaard et al. 2008). The crosses between parental genotypes were carried out in two summer seasons: 2003/2004 and 2005/2006. Despite the high level of self-incompatibility of the female parent (data not shown), emasculation and subsequent pollination were accomplished in an artificial fog chamber to prevent occasional self-pollination as described by Burton (1948). Seeds were germinated in flats with sterilized soil; seedlings were transplanted into individual pots in a greenhouse, and then transplanted into a space-planted field nursery.

Characterization of the F_1 progeny

The hybrid origin of progenies was first determined based on the presence of stolons, a specific trait of *P. procurrens*, and further confirmed with a molecular marker-aided progeny test. One of the main morphological differences between *P. simplex* and *P. procurrens* is the growth habit. While *P. simplex* plants exhibit tufted upright tillers, *P. procurrens* has stoloniferous growth habit with decumbent stems rooting at the branch nodes. As the stoloniferous habit is a dominant trait of the male parent (Espinoza and Quarin 1997; Hojsgaard et al. 2008), all hybrids are expected to develop stoloniferous branches. To confirm their hybrid origin, a random sample of 20 individuals of the offspring was fingerprinted with RAPD markers and their amplification patterns were compared with those of both parents. Because RAPDs are dominant markers, any homozygous male-specific amplicon would be highly informative to confirm the hybrid origin of a given progeny. Genomic DNA was extracted from the parents of the mapping population, and from 20 F_1s according to methodology described by Martínez et al. (2003). A total of 19 arbitrary decamers from the RAPD Primer Synthesis Project of the British Columbia University were assayed according to Martínez et al. (2003). The PCR products were separated by electrophoresis along 5% denaturing polyacrylamide gels and visualised by silver staining using the Silver Sequence™ DNA sequencing system (Promega) following the manufacturer's instructions.

The apomictic or sexual reproductive phenotypes were assigned to each F_1 individual of the entire hybrid population. The first criterion to discriminate apomictic from sexual offsprings was the presence or absence in each F_1 individual of an apomixis-specific marker (SCAR, Sequenced Characterized Amplified Region) previously isolated from *P. simplex*, and linked to apomixis in that species (Calderini et al. 2006). To test the robustness of the SCAR marker results, cyto-embryological analyses of mature ovules were carried out in all SCAR-positive as well as in a random sample of 14 SCAR-negative F_1s. Spikelets at blooming were fixed in FAA (70% ethanol:formaldehyde:acetic acid, 18:1:1, v/v) for 24–48 h. Fifty to seventy pistils were dissected from each plant and transferred to a plaque with clearing fluid (Herr 1971), composed of lactic acid (85%):chloral hydrate:phenol:clove oil and xylene (2:2:2:2:1, by weight), for at least 24 h. Pistils were then distributed on a slide drenched with clearing fluid, protected with a cover glass and observed with a Leica DiaStar phase contrast microscope. Mature embryo sacs displaying an egg cell and two synergids at the micropylar end, a large 2-nucleated central cell and a mass of proliferated antipodal cells at the chalazal end (embryo sac of *Polygonum*-type) were considered to have originated from functional reduced megaspores. Conversely, aposporous embryo sacs could be discriminated from meiotic ones on the basis of the absence of antipodals cells. A plant was considered sexual when its ovules carried exclusively a single embryo sac of *Polygonum*-type. Those plants that frequently showed mature ovules with multiple aposporous embryo sacs (two to four per ovule) were considered to reproduce by apomixis. Plants producing ovules with mainly aposporous sacs, together with some meiotic embryo sacs in the same or in other ovules were classified as apomictic because of their genetic ability for apospory. Additionally, two putatively apomictic and two sexual F_1

plants, as classified by both SCAR and cyto-embryological analyses, were selected for progeny tests aided with RAPD markers. F_2 progenies were developed from these four F_1s by harvesting seeds from open-pollinated inflorescences, and they were tested with RAPDs to investigate whether progenies exhibited maternal (from apomictic F_1s) or non-maternal (from sexual F_1s) fingerprint patterns. RAPD analysis was performed on 15 F_2 individuals for each F_1 mother plant using 14 decamers.

RFLP analysis

Genomic DNA (8–9 μg) was digested overnight with 20 units of *Eco*RI or *Hin*dIII (New England Biolabs, NEB) on a final volume of 50 μl to detect informative polymorphisms between the parental lines and with the appropriate enzyme for the analysis of the whole segregating population. The restricted DNA fragments were electrophoresed in 1% w/v agarose gels overnight at 50 mA and blotted onto Hybond-N^+ membranes (GE healthcare), according to the capillary transfer method (Southern 1975). Blots were saturated using fragmented salmon sperm DNA, hybridized overnight with ^{32}P-labelled probes (Ready-to-Go DNA labelling Kit, GE healthcare), washed according to the membrane instruction manual, replacing SSPE with SSC and omitting the last two high-stringency washes. Filters were exposed for at least 16 h to X-ray films at −80°C to obtain a clear autoradiographic image. Probed membranes were stripped in 30% v/v formamide, 2× SSC and 0.1% w/v SDS buffer at 74°C for 60 min and reprobed up to ten times. Both rice anchor markers (belonging to the New Landmarker Set) and *Paspalum* homologous probes (Calderini et al. 2006) were used. Markers linked to apomixis in other *Paspalum* species together with an additional set of rice markers mapped to chromosome 12 (http://rgp.dna.affrc.go.jp/publicdata/estmap2001/Chr12.html) were used to detect co-segregation with apomixis in *P. procurrens.*

Data analysis

The crosses were made between a colchicine-induced autotetraploid plant of *P. simplex* and a natural tetraploid plant of *P. procurrens*, a species that likely originated by autopolyploidization (Hojsgaard et al. 2008). According to Allard (1960), in autotetraploids there are two possible modes of allele inheritance depending upon random chromosome (RO) or random chromatid (RA) assortment segregation. For an allele in single dose (single-dose restriction fragment, SDRF), segregation ratios of 1:1 for RO or 0.87:1 for RA (presence:absence, respectively) are expected in the gametes of the parental plant. Conversely, segregation ratios of 5:1 for RO or 3.7:1 for RA (presence:absence, respectively) are expected when the segregating alleles are in double-dose (double-dose restriction fragment, DDRF) and 27:1 (presence:absence, respectively) for alleles in triple dose (triple-dose restriction fragment, TDRF) for RA only. Considering the size of the segregation population available, only SDRFs inherited from the apomictic parent were considered for linkage analysis (Wu et al. 1992). Association between loci in coupling phase was tested by generating an input file coding both presence (1) and absence (0) markers from segregation pattern of parental genotypes and 134 F_1 individuals. The coupling phase file was recoded by inverting the presence and absence data set to test for loci associated in repulsion phase (Al-Janabi et al. 1993). Linkage analysis was performed including both data sets together in the same input file and analysed with the computer program MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992), using the "F2 backcross" option and a minimum LOD score threshold of 3. Since recombination frequencies detected in this study were either 0 or nearly 20%, crossing over interference is negligible in both cases and therefore the Haldane mapping function was used to convert them into map distance (cM) (Liu 1998). Data regarding the putative function of predicted genes located in the rice chromosome region bracketed by the two markers C1069 and C996 were retrieved from the http://rice.plantbiology.msn.edu/ site using the "genome browser" and "GO retrieval" functions for gene position and GO annotation, respectively.

Results

Interspecific crosses and hybrid progeny identification

The first cross between *P. simplex* (C_1B_2) and *P. procurrens* (Q4094) was made in summer 2003. Two hundred and eighteen emasculated spikelets were pollinated and 70 seeds were obtained. A second cross between the same parental plants was accomplished in the same period of 2005 when more than 270 spikelets were crossed and 120 seeds were obtained. Overall, only 150 seeds were able to germinate, from which 134 plants were recovered. After 2–3 months all plants developed stoloniferous branches, a dominant character present only in the male parent *P. procurrens*, indicating that the whole progeny had a hybrid origin. To corroborate the hybrid nature of the F_1 population, a random sample of 20 individuals were fingerprinted with RAPD markers. Twenty random primers were tested on the DNA of the parental plants and eight were selected because they generated one or more bands specific of the *P. procurrens* parent. In all of the 20 selected F_1 individuals it was possible to amplify those bands that were specific to the male parent *P. procurrens*, thus confirming the hybrid origin of the progeny.

Mode of reproduction of the F_1 hybrids

The entire progeny of 134 hybrids was investigated for mode of reproduction. The PCR amplification of the apomixis-linked SCAR showed a band of the expected molecular weight in the *P. procurrens* parent and no bands in the female *P. simplex* parental line (Fig. 1a). Out of all the 134 hybrids, only eight showed the diagnostic amplicon whereas the large majority of the others (116) showed a non parental band of about 800 bp and 10 F_1s showed no bands. In those F_1s that showed the diagnostic band, the non-parental one was either absent (# 41, Fig. 1a) or very faint (# 47, Fig. 1a). These non-parental bands may have originated from heteroduplex molecules formed between alleles or sequences showing partial matching coming from both parents (Ayliffe et al. 1994). Ovule analysis was conducted only in six of the eight SCAR-positive plants (#29, #31, #41, #47, #95 and #114), because two of them died before flowering. Multiple embryo sacs in at least one ovule were observed in all the six plants analysed (Fig. 1b), though a single embryo sac was observed in some ovules of these six plants. Out of the 126 SCAR-negative hybrid group, 14, randomly selected for embryological analysis, showed exclusively ovules bearing a single embryo sac with antipodal cells of meiotic origin, thereby confirming the attribution of reproductive phenotype inferred by the presence/absence of the SCAR marker. To further confirm the results obtained with the apomixis-specific marker screening and the embryological observations, we carried out progeny tests on two putative sexual (#13 and #25) and two putative apomictic F_1s (#29 and #31). The eight primers previously selected to verify the hybrid nature of the F_1 plants were tested on sixty F_2 progenies, 15 for each F_1 individual. All F_2 descendents from the F_1 plants #29 and #31 showed a PCR banding pattern identical to that of their respective F_1 mother plants, whereas several segregating bands were observed among the F_2 descendents from the F_1 plants #13 and #25 (Fig. 1c). Progeny tests confirmed the results obtained with the SCAR marker and embryological analysis. As a consequence, 8 F_1 plants were definitely classified as apomictic and 126 as sexual.

RFLP mapping

Some of the anchor markers previously proved to be linked to apomixis in other *Paspalum* species (Pupilli et al. 2001, 2004; Calderini et al. 2006) together with an additional set of rice markers mapped on chromosome 12 were tested in this study. These markers were used as hybridising probes against the genomic DNA digested with either *Eco*RI or *Hin*dIII as these restriction enzymes were the most effective on revealing informative polymorphisms in *Paspalum* (Pupilli et al. 2001). In total, 13 probes were used in this study of which nine mapped on rice chromosome 12, one to chromosome 2 and three were *Paspalum* markers. Of the ten rice probes used, three were not homologous to the *P. simplex* × *P. procurrens* population and one did not show any informative polymorphisms (i.e. bands specific of the apomictic *P. procurrens* parent). Conversely, all three *P. simplex* probes showed at least one informative polymorphism. On average, the *P. procurrens* parent showed nearly twice RFLP bands (mean = 3.46 bands per probe/enzyme combination) than *P. simplex* (mean = 1.77 bands per probe/enzyme combination) as expected on the basis of the different level of heterozygosity content of the two plants (see "Materials and methods" section for pedigree information). As an indirect confirmation of this difference, we noticed that while fragments segregating from the apomictic parent showed either 1:1 or 5:1 (presence:absence) segregation ratios as it can be accounted for tetrasomic inheritance of SDRFs or DDRFs, respectively, all the bands specific of the sexual parent segregated as DDRFs. The proportion of segregating SDRFs is positively correlated to the heterozygosity content in autotetraploids (Wu et al. 1992). Rice probes C1069, C996, R1759 and C901, all located on a specific region of the rice chromosome 12 (Fig. 2), showed at least one fragment linked 100% in coupling phase with the ACL, similarly to what it was reported in *P. simplex* and *P. malacophyllum* (Pupilli et al. 2004). The probes C1069 and C901 revealed each one apomixis linked fragment with approximate length of 1.8 and 1.6 kb, respectively; C996 revealed a doublet of 5.8 + 5.9 and R1759 two fragments co-segregating with apomixis of 1.4 + 4.8 kb (Fig. 2). All these probes showed apomixis-linked fragments of identical size in *P. simplex* (Pupilli et al. 2001, 2004) while in *P. malacophyllum* C901 was unlinked to apomixis and only C996 and C1069 showed linkage with apomixis in *P. notatum* (Pupilli et al. 2004). The hybridising banding pattern of the telomeric probe C901 revealed a 4.8 kb band (C901a, Fig. 2) that showed an extent of recombination with the ACL of *P. procurrens*. A similar recombination rate was noticed for the same probe in a linkage group likely allelic to the ACL in *P. simplex* (Pupilli et al. 2001). This suggests that the strong synteny of the ACL of *Paspalum* with one telomere of chromosome 12 of rice extends beyond the recombinationally repressed core area of the ACL including flanking regions in which recombination block is less stringent. A gradual relaxation of recombination block in flanking regions of the ACL in *Paspalum* was already noticed by Calderini et al. (2010).

The molecular weight of the apomixis-linked fragment revealed by the probe C1069 was of 1.8 kb in all the *Paspalum* species investigated indicating high level of conservation of the area of the ACL in the close nearness of this marker. Similarly, apomixis-linked fragments were revealed by the probe C996 in all the four

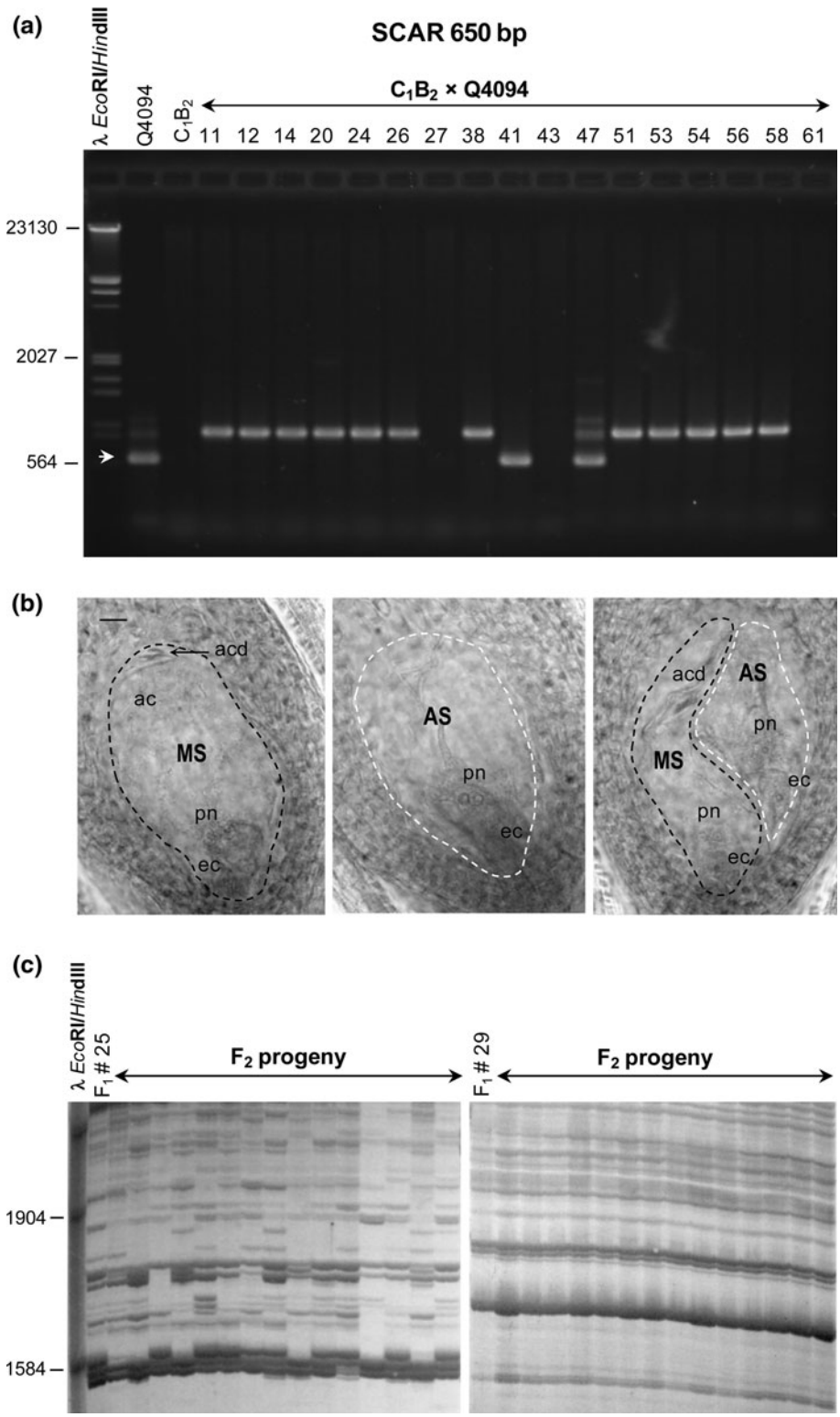

Fig. 1 Characterization of the reproductive mode of the hybrid population *Paspalum procurrens* (Q4094) × *P. simplex* (C_1B_2). **a** Image showing the migration pattern for SCAR marker in both parentals and in a sample of the descendants after electrophoresis in agarose gel (1%). Only the male parental (Q4094) and F_1 plants # 41 and # 47 showed the 650 bp fragment specific for apomixis in *P. simplex* (*arrow*). **b** Images of the different embryo sacs observed in the F_1 progeny. *Left* ovule from one of the F_1 individuals which do not amplify the SCAR showing the presence of unique meiotic embryo sacs. *Centre and right* ovule showing one and two embryo sacs, respectively, belonging to those F_1 individuals which amplified the SCAR. **c** Image showing the amplification banding pattern obtained with the primer UBC470 on F_2 descendants of a sexual (#25) and an asexual (#29) F_1 plant. *ac* antipodal cells, *acd* antipodal cells deteriorated, *pn* polar nuclei, *ec* egg cell, *MS* meiotic sac, *AS* Aposporous sac. *Bar* 30 µm

Paspalum species analysed even though the molecular weights of these fragments varied among these species (this study; Pupilli et al. 2001, 2004). Unexpectedly, C454 did not show any apomixis-linked fragments in *P. procurrens* although this marker mapped very close to C1069 in rice and it was linked to apomixis in *P. simplex*, *P. malacophyllum* and *P. notatum*. To explore further the telomeric side of the rice region marked by the two loci C1069 and C454 (http://rgp.dna.affrc.go.jp/publicdata/estmap2001/Chr12.html), four additional probes

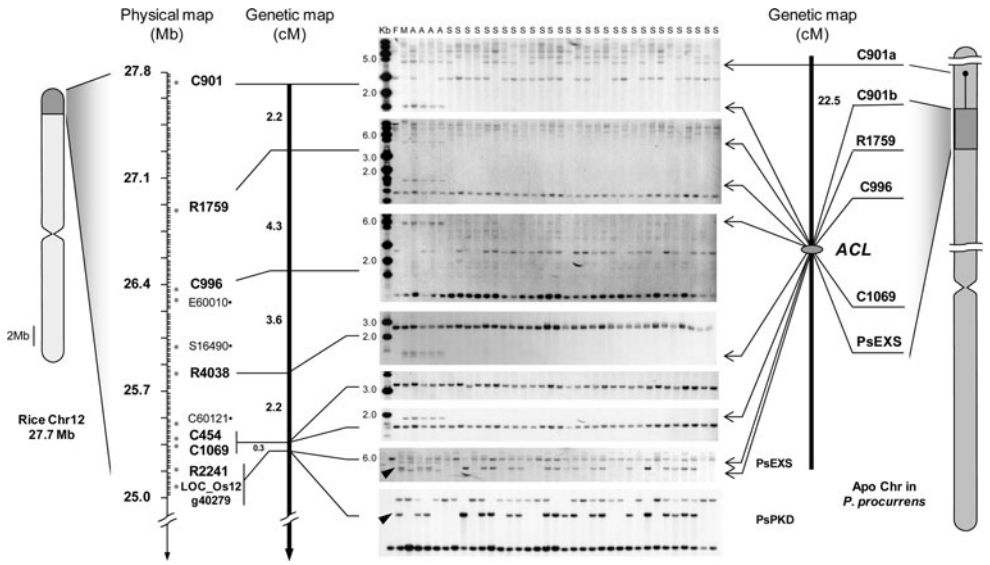

Fig. 2 Alignment of the telomeric region of the long arm of rice chromosome 12 (*left*) with the ACL of *P. procurrens* (*right*). In the centre, the hybridising banding patterns of the digested DNAs of sexual (F) and apomictic (M) parental lines and 34 segregant apomictic (A) or sexual (S) individuals are reported. *Arrows* indicate apomixis-linked bands. *Arrowheads* indicate two tightly linked fragments unrelated to apomixis revealed by the homologous *Ps*EXS and *Ps*PDK clones that are linked to apomixis in *P. simplex*. *Closed circle* non homologous probes, *astrerisk* non polymorphic probes

(C60121, R4038, S16490, E60010) were tested for linkage to apomixis in *P. procurrens*. Of these, three were not homologous (C60121, S16490, E60010) and R4038 showed a doublet co-segregating with apomixis (Fig. 2). Since the *P. simplex* clone *Ps*EXS showed a clear linkage with apomixis in *P. procurrens* and the same clone was homologous to the rice marker R2241 that maps very close to C454 in rice, we conclude that a deletion of a small area surrounding C454 occurred at the ACL of *P. procurrens* after this species diverged from the other *Paspalum* species. The marker C932 located on rice chromosome 2 and linked to apomixis in *P. notatum* did not show any informative polymorphism between parents. Other than *Ps*EXS, another *Paspalum* homologous probe detected linkage with apomixis in *P. procurrens*. This marker was the RFLP-converted SCAR marker used to discriminate sexual from apomictic individuals in the F_1 population under study and revealed a poorly hybridising fragment of around 8.7 kb linked to apomixis in *P. procurrens* (not shown). The marker *Ps*PKD developed in *P. simplex* as a PCR-generated probe of a portion of a gene located on an apomixis linked BAC in *P. simplex* (Calderini et al. 2006) was not linked to apomixis in *P. procurrens* but revealed a fragment that co-segregated with a 5.4 kb band detected in the hybridising banding pattern of the probe *Ps*EXS (Fig. 2). This indicated that the two *P. procurrens* homologs of *Ps*EXS and *Ps*PKD that in *P. simplex* are located on the same BAC (Calderini et al. 2006) are also closely linked in *P. procurrens* perhaps on the sexual chromosome counterpart of the ACL. Of these, only the probe *Ps*EXS showed an apomixis-linked band. None of the fragments detected by the used probes were linked to apomixis in repulsion phase, since apomixis-bearing chromosome seems to have no homologous region.

Discussion

The apomixis phenotype and the linked molecular markers showed a significant deviation from Mendelian inheritance in *P. simplex* × *P. procurrens* population in that apomixis was strongly disfavoured compared with sexuality. The ratio 1 (apomicts):15.7 (sexuals) does not fit any obvious model of inheritance where apomixis is dominant with a proposed allelic configuration of Aaaa, where A is the allele carrying the locus, unless strong segregation distortion from the expected ratios of 1:1 or 0.87:1 (in case of random chromosome or random chromatid assortment, respectively) is invoked. Similar biased segregation distortion towards sexual progeny has been observed in interspecific cross of sexual *Pennisetum glaucum* × apomictic *Pennisetum squamulatum* and this distortion was exacerbated when apomixis was transmitted through the female gametes (Roche et al. 2001). Similarly, segregation distortion against apomixis was also reported in *Tripsacum*-maize hybrids when the female parent was apomictic (Grimanelli et al. 1998) and among non-grass agamic systems, in *Hieracium* (Catanach et al. 2006), *Ranunculus* (Nogler 1984b), *Taraxacum* (Vijverberg et al. 2004) and *Erigeron*

(Noyes and Rieseberg 2000). In *Paspalum*, segregation distortions against apomixis have been reported in intraspecific crosses (Martínez et al. 2001; Pupilli et al. 2001) and to a much greater extent in interspecific crosses (this study; Pupilli et al. 2004). In particular, in *P. simplex* it is possible to compare the extent of segregation distortion against apomicts in a population obtained by crossing a natural tetraploid apomictic plant with a colchicine-created sexual autotetraploid plant of the same species (1 apomicts:1.6 sexuals; Pupilli et al. 2001) with those obtained when an S_1 descendant of the same sexual genotype was crossed with apomictic *P. malacopyllum* (1 apomicts:5 sexuals; Pupilli et al. 2004) and *P. procurrens* (1 apomicts:15.7 sexuals; this study) used as pollen donors. Therefore, the excess of sexual plants in segregating populations was remarkably higher in interspecific crosses compared with intraspecific ones. Several hypotheses have been formulated to explain the low transmission rate of apomictic phenotype in segregating populations. Nogler (1982) was the first to postulate the existence of a mechanism that eliminates the apomixis-bearing chromosome when transmitted through haploid pollen as an explanation for the absence of natural diploid apomictic plants. The existence of a recessive lethal factor causing the death of the haploid gametes harbouring the apomixis locus has been considered later on as a possible reason for this phenomenon in *Ranunculus auricomus* (Nogler 1984b), *Erigeron annuus* (Noyes et al. 2007), *Taraxacum officinale* (van Dijk 2003), and demonstrated with the aid of molecular markers linked to the trait in *Tripsacum dactyloides* (Grimanelli et al. 1998) and *P. simplex* (Martínez et al. 2007). The presence of a lethal factor linked to the apomixis locus acting at either gametophytic or sporophytic levels has also been considered as an explanation for the distorted transmission of apomixis through polyploid gametes in *Pennisetum squamulatum* (Roche et al. 2001) and *P. notatum* (Martínez et al. 2003). The genetic consequence of the meiotic drive is that one of the allelic alternatives is transmitted in excess to the progeny (Lyttle 1991). This mechanism has been proposed to cause segregation distortion of apomixis in *Tripsacum dactyloides*-maize hybrids (Grimanelli et al. 1998) and *Pennisetum squamulatum*-pearl millet hybrids (Roche et al. 2001). However, according to the view of Harlan et al. (1964) "apomixis and sexual reproduction are not alternative modes of reproduction either genetically or operationally but are independent and simultaneous phenomena", apomixis and sexuality are not mutually exclusive in Mendelian terms but particularly in the case of aposporic apomixis the two reproductive modes co-exist in the same ovary and their proportion in segregating populations depends on post meiotic factors favouring one or the other mode. Therefore, as Bicknell et al. (2000) proposed for *Hieracium*, lethality at the zygotic level is a more likely explanation for segregation distortion when apomixis is inherited through polyploid pollen. On a recent paper Polegri et al. (2010) reported that out of nearly 200 genes differentially expressed between apomictic and sexual lines of *P. simplex*, only 10% were genetically linked to apomixis. This means that the transferring of the apomixis locus from an apomictic "donor" to a sexual "receiver" implies the reprogramming of the regulation of a *plethora* of genes that act downstream of the apomixis-linked factors. This reprogramming could be related to a delicate network of gene-to-gene communications likely based on sequence homology. If even few of these interactions are not working properly, apomictic embryos could be lethal or at least disadvantaged compared with sexual ones. Failures of these interactions are more frequent as the higher is the genetic distance between the parents because the sexual "receiver" genotype might be not able to adapt itself to the new apomictic condition if the apomictic locus to be introgressed comes from a genetically unrelated genotype. To sum up, we believe that sporophytic lethality (or a co-existence with gametophytic lethality) could explain the low number of apomictic hybrids in *P. simplex* × *P. procurrens* population and the striking differences on segregation distortion of apomixis between interspecific and intraspecific crosses in *P. simplex*.

The characterization of the apomixis-controlling-locus (ACL) in *P. procurrens* shows that additional small-scale rearrangements have taken place in this region compared with other species belonging to the *Anachyris* subgenus of *Paspalum*. The *Anachyris* complex is formed by six species, which diverged from each others at the diploid sexual level (Urbani et al. 2002) to develop tetraploid aposporic counterparts by autotetraploidization (Hojsgaard et al. 2008). Comparative molecular genetic analysis of the ACL in these species was initiated by Pupilli et al. (2004) who showed that a restricted *Paspalum* genome region homologous to a specific chromosome area of rice was conservatively linked to apomixis in several *Paspalum* species even though some extents of small-scale rearrangements took place among the species analysed. The ACL of *P. procurrens* fits well to this picture and gives us an integrative view of evolutionary pathway of the genomic region encompassing apomixis within the *Anachyris* subgenus. Of all the rice markers screened for informative polymorphism between sexual and apomictic individuals in *Paspalum* (Pupilli et al. 2001), only those located on a specific telomeric region of rice chromosome 12 showed co-segregation with apomixis in three *Anachyris* species (this study; Pupilli et al. 2004). Not all the markers that were linked to apomixis in *P. simplex* were so in *P. procurrens*. The rice probe C454 (LOC_Os12g4560) and the *Paspalum* marker *Ps*PKD (rice homolog LOC_Os12g40279) were not linked to the ACL in *P. procurrens* as these were in *P. simplex*, *P. malacophyllum*

and *P. notatum*. Notwithstanding the markers C454 and C1069 (LOC_Os12g40530) and the homologue of the gene *Ps*PKD are located in a region of rice genome spanning around 174 kb (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) corresponding to 0.3 cM (http://rgp.dna.affrc.go.jp/publicdata/estmap2001/Chr12.html), the hybridization pattern of C1069 revealed a clear apomixis-linked fragment in *P. procurrens* whereas C454 and *Ps*PKD were unlinked to apomixis. Of the markers located at both sides of C454 on rice map, *Ps*EXS, an homologue of rice EST R2241 (LOC_Os12g40340) that mapped toward the centromeric side, showed 100% linkage with the apomixis locus in *P. procurrens*. To the telomeric side, three tested markers (C60121, S16490 and E60010) mapped in a window of 5.8 cM (1,062 kb) comprised between C1069 and C996A (LOC_Os12g42180) did not show homology with *P. procurrens* DNA and one (R4038; LOC_Os12g41290), located in the same area revealed an apomixis-linked fragment (Fig. 2). The fact that markers linked to apomixis in some *Paspalum* species were not so in other closely related species is probably due to deletions and/or insertions of genes related to transposable elements, as Calderini et al. (2006) observed when comparing the intron/exon structure of apomixis-linked genes in *Paspalum* with that of their rice homologs. Such mutations could interrupt locally the gene collinearity and linkage with apomixis. Data presented here show that the apomixis locus in *P. procurrens* underwent a deletion event that spans 2.4 cM in rice corresponding to around 490 kb comprised between the markers C1069 and R4038 when compared with the same region of *P. simplex* and *P. malacophyllum*. In any case, the gene corresponding to the EST C454 (an interesting KH domain RNAse) is unrelated to apomictic reproduction in *P. procurrens* and likely in the whole *Paspalum* genus.

According to Urbani et al. (2002), all the species of the subgenus *Anachyris* underwent polyploidization after they diverged from the common *Paspalum* ancestry at diploid sexual level. Pupilli et al. (2004) compared the collinearity of anchor markers between sexual and apomictic chromosomes and noticed that although strict collinearity with rice chromosomes 2 and 12 was observed for sexual chromosomes of *Paspalum notatum*, the ACL on the apomictic chromosome co-segregated with markers of both rice chromosome 2 and 12 indicating that an heterozygous translocation occurred as a consequence of polyploidization. Such a translocation caused local sequence divergence and then loss of pairing ability and of recombination at the ACL in this species. Similar rearrangements in the form of inversion has also been hypothesized to have occurred at the ACL in *P. simplex* (Pupilli et al. 2001) *Pennisetum squamulatum* (Ozias-Akins et al. 1998) and *Cenchrus ciliaris* (Goel et al. 2003). Therefore, we believe that in the evolutionary pathway of the ACL some *Paspalum* species evolved the potentiality to express apomixis at diploid level in an unstable chromosome area prone to be easily rearranged. Subsequently, full expression of apomixis could have taken place after polyploidization that generated, in short-term, large-scale rearrangements (in terms of chromosomal inversions or translocations) and in a long-term perspective small-scale rearrangements such as indels and point mutations. An experimental evidence to support this pathway was provided by Quarin et al. (2001) who noticed that artificial polyploidisation induced apomixis in a sexual diploid genotype of *P. notatum*.

In this scenario, the relationship between the homologues of the markers *Ps*PKD and *Ps*EXS in *P. procurrens* is worth noting. The corresponding two genes were located on the same 130 kb BAC in *P. simplex* although their physical distance could not be assessed as these were located on different contigs (Calderini et al. 2006). Similarly, these genes were on the same rice BAC (OSJNBa0056D07) spanning 25 kb apart and, finally, they were also closely linked in the *P. procurrens* genome as they showed two strictly co-segregating fragments inherited from the apomictic parent (Fig. 2) without any recombination event detected across all the 134 F_1s analysed. Although these two genes were both linked to apomixis in *P. simplex*, *P. malacophyllum* and *P. notatum* (Pupilli et al. 2004) only *Ps*EXS showed an apomixis-linked fragment in *P. procurrens*. Either large-or small scale rearrangements could be responsible for the lack of the apomixis-linked allele for *Ps*PKD. In this view, we hypothesize two possible mechanisms to have caused the lack of linkage with apomixis of the marker *Ps*PKD i) the region bracketed by *Ps*PKD and *Ps*EXS could be the break point of a chromosomal inversion that affected the ACL of *P. procurrens* or ii) the *Ps*PKD apomixis-linked allele was lost after the formation of the ACL by a deletion or a point mutation generating a new or disrupting a pre-existent restriction site. Of all the rice apomixis-linked markers located on the telomere of the long arm of chromosome 12, the homologue of *Ps*PKD was the closest to the centromere. None of the other tested probes that were located more centromerically showed linkage with apomixis (Pupilli et al. 2001). This may indicate that the area of the ACL delimited by the markers *Ps*PDK, *Ps*EXS and C1069 represent its former "centromeric" border and that the same area encompass one of the breakpoints of the inversion that originated the rearranged ACL of the species under study. Alternatively, loss of apomixis-linked alleles could be related to general mechanisms of genome remodelling by chromatine elimination as observed in newly formed polyploids including *Paspalum* (Martelotto et al. 2007) as a means to restructure the genomes towards the restoration of diploid–like behaviour (Parisod et al. 2010, and references therein).

Comparative mapping of apomictic reproduction indicates that the ACL did not undergo large-scale rearrangements during its evolution from *P. simplex* to *P. procurrens* although small deletions were locally detected without changing remarkably the strong synteny of markers with the telomere of the long arm of rice chromosome 12. On grass genome evolution, small-scale rearrangements are much more common than the large-scale rearrangements presented in the comparative circle map (Bennetzen and Freeling 1997). The structural changes of the ACL among the three *Anachyris* species studied to date are depicted by small rearrangements and *P. simplex* seems to represent the ancestral situation as marker synteny with the homoeologous genome region of rice was much more significant in this species compared with the other species analysed. The use of single copy gene sequence of model species as anchor markers and the exploitation of synteny make it possible to look for and identify specific genetic features in non model species. Local genomic cross-referencing using cloned collinear segments of different grass genomes have uncovered the conserved segments (genes) hidden in blocks of otherwise not conserved repetitive and mobile DNAs (Avramova et al. 1996; Chen et al. 1997). In spite of the lack of recombination that hampers apomixis gene isolation in *Paspalum*, comparative mapping approach showed that of all markers present over the rice regions related to asexuality, only a few are conservatively linked to apomictic reproduction across four different species. Three *Anachyris* genotypes representing *P. simplex*, *P. malacophyllum* and *P. procurrens* share at least one apomixis-linked fragment revealed each by the probes *Ps*EXS (R2241), C1069, C996A and R1759, that bracketed in rice a chromosome region of 13 cM corresponding to 1,675 kb. This region is further narrowed down in *P. notatum*, a distantly related

Table 1 Characteristics and homology analysis of some of the predicted genes located in the region bracketed by the markers C1069 and C996 of chromosome 12 of rice that could be involved in the apomixis process in *Paspalum*

Locus name (marker)	Start–end position (bp)	Putative function	Molecular function		Biological process	
			GO slim ID	GO name	GO slim ID	GO name
LOC_Os12g40530 (C1069)	25, 045, 341–25, 048, 935	MuDR family transposase	No hits		No hits	
LOC_Os12g40770	25, 202, 409–25, 207, 146	Ankyrin-1, putative, expressed	GO:0000166	Nucleotide binding	GO:0007275	Multicellular organismal development
LOC_Os12g40890	25, 277, 210–25, 282, 627	OsIAA30—Auxin-responsive Aux/IAA gene family member, expressed	GO:0003700	Transcription factor activity	GO:0009719	Response to endogenous stimulus
LOC_Os12g41060	25, 380, 937–25, 385, 134	AP2 domain containing protein, expressed	GO:0003677	DNA binding	GO:0009719	Response to endogenous stimulus
LOC_Os12g41350	25, 589, 064–25, 594, 650	Meiotic asynaptic mutant 1, putative, expressed	GO:0003677	DNA binding	No hits	No hits
LOC_Os12g41680	25, 767, 338–25, 772, 947	No apical meristem protein, putative, expressed	No hits		GO:0007275	Multicellular organismal development
LOC_Os12g41900	25, 933, 830–25, 942, 444	SET domain containing protein, expressed	GO:0005488	Binding	GO:0006351	Transcription
LOC_Os12g42120	26, 084, 259–26, 086, 439	PPR repeat containing protein	GO:0016740	Transferase activity	No hits	
LOC_Os12g42150	26, 094, 981–26, 097, 170	WD domain, G-beta repeat domain containing protein, expressed	GO:0000166	Nucleotide binding	No hits	
LOC_Os12g42160	26, 097, 399–26, 102, 591	Kinesin motor domain containing protein, putative, expressed	GO:0003774	Motor activity	No hits	
LOC_Os12g42180 (C996)	26, 105, 879–26, 108, 174	Ribosomal protein, putative, expressed	GO:0005198	Structural molecule activity	GO:0006412	Translation

species in which only two markers out of the above mentioned (C996A and C1069), showed apomixis-linked fragments. These markers spun 5.8 cM on the rice map that correspond to a physical distance of around 1,062 kb. These distances should not be of many orders of magnitude greater in the ACL of *Paspalum* as witnessed by the co-localisation of the two genes *Ps*EXS and *Ps*PKD in rice and *Paspalum* genomes. The genomic region of *Paspalum* delimitated by the two markers C1069 and C996 likely includes all the genetic determinants of apomictic reproduction in this species. As an example of a possible candidate gene located in this area, the clone C996A in rice is an EST of the gene LOC_Os12g42180 that is the homologue of the *Arabidopsis* gene *HUELLENLOS* (*HLL*) encoding for a ribosomal protein that is essential for normal ovule development (Skinner et al. 2001). Additional examples of genes that are located in this area which, on the basis of their predicted molecular function, could have a role on apomixis process in *Paspalum* are listed in Table 1. Among these genes worth of noting are the ankyrin-1, auxin responsive, meiotic asynaptic mutant 1 that are putatively involved in processes related to cell differentiation. Furthermore an AP2-containing homolog of embryogenesis-related gene of *Arabidopsis BABY BOOM*, was found in the apospory locus of *Pennisetum* (Conner et al. 2008). On the other hand, the inclusion of *P. procurrens* in the apomixis comparative mapping strategy allowed us to discard the KH domain protein corresponding to the EST C454 from the list of candidate genes of apomixis in *Paspalum*.

The comparative mapping approach to identify the genes responsible for apomictic reproduction in *Paspalum* is conceptually similar to the deletion mapping approach of Catanach et al. (2006) in *Hieracium*. Spontaneous deletions occurring during speciation have screened out all those genes that are dispensable for apomictic reproduction. The genus *Paspalum* is well suited for this purpose because it includes many agamic complexes differentially related each other and formed each by apomictic polyploid species with crossable sexual counterparts (Quarin 1992). The small-scale rearrangements detected at the ACL of *P. procurrens* with respect to *P. simplex* contributed to narrow down the size of the ACL that is conservatively linked to apomixis in several *Paspalum* species. This gives us a core of markers linked to apomixis between closely related species as well as distantly related species within the *Paspalum* genus. Such interspecific recombination helps to overcome the intra-specific block of recombination that severely hampers the isolation of apomixis genes in these species by genetic means, restricting the genomic window to mine the genetic determinants of apomixis.

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