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ORIGINAL PAPER

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Molecular cytogenetics and DNA sequence analysis of an apomixis-linked BAC in *Paspalum simplex* reveal a non pericentromere location and partial microcolinearity with rice

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Abstract Apomixis in plants is a form of clonal reproduction through seeds. A BAC clone linked to apomictic reproduction in *Paspalum simplex* was used to locate the apomixis locus on meiotic chromosome preparations. Fluorescent in situ hybridisation revealed the existence of a single locus embedded in a heterochromatin-poor region not adjacent to the centromere. We report here for the first time information regarding the sequencing of a large DNA clone from the apomixis locus. The presence of two genes whose rice homologs were mapped on the telomeric part of the long arm of rice chromosome 12 confirmed the strong synteny between the apomixis locus of *P. simplex* with the related area of the rice genome at the map level. Comparative analysis of this region with rice as representative of a sexual species revealed large-scale rearrangements due to transposable elements and small-scale rearrangements due to deletions and single point mutations. Both types of rearrangements induced the loss of coding capacity of large portions of the "apomictic" genes compared to their rice homologs. Our results are discussed in relation to the use of rice genome data for positional cloning of apomixis genes and to the possible role of rearranged supernumerary genes in the apomictic process of *P. simplex*.

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Introduction

Gametophytic apomixis is an alternative route of reproduction that in plants gives rise to seeds that are genetically identical to the mother plant via the intermediate formation of an unreduced gametophyte (Nogler 1984). Several types of apomixis are described in nature differing in origin and development of the maternal embryo (see Asker and Jerling 1992 for details). In aposporic apomixis, a somatic cell of the nucellus enlarges and gives rise by mitotic divisions to an unreduced (2n) embryo sac. The unreduced egg cell develops parthenogenetically into an embryo whereas the central cell gives rise to the endosperm either parthenogenetically (autonomous apomixis) or through the fusion with a sperm nucleus (pseudogamous apomixis). Sexual development is usually present in apomictic plants but sexual megagametophyte development is frequently arrested at the stage of the functional

megaspore. Male gametophyte development in apomictics is identical to that of sexual plants.

The introduction of apomictic reproduction in cultivated plants would revolutionize the seed market especially for those crops of which the production of hybrid seeds is important (Spillane et al. 2004). Unfortunately, although apomictic reproduction has been shown to be under genetic control (Savidan 2000), the trait is present only in wild species for which common approaches of molecular biology can only be applied with some difficulty. In addition, no genuine apomictics have been recognized so far in sexual model systems such as *Arabidopsis* or *Petunia*. Nevertheless, several mutations in gametophytic development in *Arabidopsis* have been reported that at least resemble some aspects of apomictic reproduction (Koltunow and Grossniklaus 2003). With the ongoing progress in large-scale sequencing, comparative mapping and reverse genetics, the study of apomixis is becoming approachable in its natural biological background. Therefore the isolation of the genetic determinants of apomixis in wild apomictics and their transferring in sexual crops through genetic engineering is now a useful strategy. As an example, interesting candidate genes for apomixis have been recently isolated and characterized in *Poa pratensis* (Albertini et al. 2005).

Paspalum simplex is an aposporic apomictic subtropical grass. This species has a number of characteristics that make it amenable for finding gene(s) involved in apomictic reproduction through map-based strategies. The most important ones are the existence of sexual diploid races that are cross-compatible with polyploid apomictic counterparts and a relatively small genome size of 0.75 pg per haploid genome (Caceres et al. 1999). However, *P. simplex* presents a strong repression of recombination in the vicinity of the Apomictic Controlling Locus (ACL; Pupilli et al. 2001) as was also reported in other apomictic systems such as *Pennisetum squamulatum*, *Tripsacum dactyloides*, and *Brachiaria decumbens* (see Grossniklaus 2001 for review). Lack of recombination is particularly problematic because it markedly reduces the power of the map-based cloning strategies and no lines isogenic for apomixis can be obtained. In the absence of recombination, physical dissection of the ACL through generation and screening of large-insert genomic libraries is an essential step toward the isolation of the genetic determinants of apomixis (Roche et al. 2002). A strategy aimed at restricting the area of the locus to be sequenced, has been reported recently for *Paspalum*. This strategy involves comparison of the ACL of several *Paspalum* spp. with a common set of markers to identify conserved areas containing genetic determinants of apomixis (Pupilli et al. 2004). Within the large ACL of *P. simplex*, only a relatively short region spanning 6–8 cM is conserved among the different apomictic species analysed. However, even if the part of the ACL that is conservatively linked to apomixis in *Paspalum* is significantly narrowed down, the recombination suppression at this locus is still a major drawback for the genetic dissection of the single components of apomixis that in *P. simplex* are: (1) apospory, (2) parthenogenetic development of the embryo and (3) deviation from the normal 2:1 ratio to 4:1 ratio for female : male genome contribution in the endosperm. The hypotheses formulated to explain recombinational suppression around the apomixis locus one deals with its hypothetical position in heterochromatic pericentromere areas (Ozias-Akins et al. 1998; Pupilli et al. 2001). The regions near to the centromere are known to be dense in heterochromatin and almost devoid of crossovers as reported for wheat (Delaney et al. 1995), tomato (Sherman and Stack 1995) and barley (Künzel et al. 2000). In contrast, on the basis of the syntenic relationships with rice and maize the position of the ACL is thought to be (sub)distally located in *Paspalum* (Pupilli et al. 2001) and *Brachiaria* (Pessino et al. 1997) respectively. Alternatively, it has been suggested that the cause of the repressed crossovers may be related to heterozygosity for an inversion or other chromosomal rearrangements (Goel et al. 2003; Ozias-Akins et al. 1998; Pupilli et al. 2001, 2004). In such rearranged regions chromosomes can not pair, and when they pair crossovers give rise to dicentric anaphase bridges or unbalanced chromosome segregation and hence lead to sterility. Different hypotheses about the functionality of genes contained in the apomixis locus can be drawn in the two cases: if the ACL is embedded in an heterochromatic region then the genetic determinants of apomictic reproduction are likely to be inactive (Bender 2004), alternatively as a result of a rearrangement, a gene or group of genes related to reproduction could be re-located in a different chromosomal location thereby mis-regulating genes responsible for the proper sexual development (Goel et al. 2003). According to the mechanism hypothesized, appropriate strategies should be adopted to identify critical genes for apomixis first and induce apomictic reproduction in sexual systems thereafter. In a recent paper, Goel et al. (2003) reported for the first time the FISH–aided localization of the apomixis locus on metaphase chromosomes and they claimed that its vicinity to the centromere in *Cenchrus ciliaris* and to centromeric repeats in *P. squamulatum* could cause the repression of recombination at the same locus in these species. Further FISH analyses on pachytene chromosome preparations of the same species are in favour of these assumptions (Akiyama et al. 2004; Akiyama et al. 2005; Ozias-Akins et al. 2003). FISH using pachytene chromosomes as targets offers superior spatial resolution of adjacent probes compared to metaphase chromosomes and display clearly the dense heterochromatic regions on the chromosomes and so allow the assignment of a probe position in relation to the structural organization of its surrounding chromosome context (de Jong 2003). In addition, FISH of DNA probes on late pachytene or even meiotic pro-phase stages provide information about the chromosome pairing and chiasma formation of the area in which the corresponding locus is located.

Comparative mapping efforts in *P. simplex* and related species have shown that the ACL is highly syntenic with the distal part of the long arm of chromosome 12 of rice (Pupilli et al. 2001, 2004). If this is correct down to the level of individual genes, it would considerably simplify identification of putative apomixis genes taking advantage of the molecular tools available in rice.

The specific objectives of the work presented here were: (1) to locate the ACL on *P. simplex* pachytene chromosomes in relation to its heterochromatin/euchromatin structural context, (2) to get information about the kind of genes present on the ACL by partial sequencing of the apomixis related BAC clone used as a probe and (3) to verify whether the synteny between the ACL of *P. simplex* and the distal part of the long arm of rice chromosome 12 observed at the map level is also conserved at the sequence level.

Materials and methods

Apomictic and sexual plants of tetraploid *P. simplex* ($2n = 4x = 40$) used in this study belonged to a backcross population (BC1) generated by crossing an apomictic hybrid as pollen donor with a colchicine-induced sexual tetraploid (Pupilli et al. 2001). Genomic DNA extraction, Southern blotting and hybridisation procedures were those reported by Pupilli et al. (2001). The bulks of DNA for AFLP analysis were prepared for each of the apomictic or sexual phenotypes by mixing together an equal amount of genomic DNA isolated from each of ten plants. AFLP procedures and conversion of AFLPs into RFLP probes were those reported by Labombarda et al. (2002). To enlarge the size of the apomixis-linked fragments, the Genomewalker™ (BD Biosciences Clontech, CA) procedure was used according to the instructions' manual.

Sequencing and contig assembly of the BAC H10 was performed by Greenomics™ (Wageningen, NL). Gene structure and predicted protein sequences were obtained from assembled contigs using the FGENESH program at the SoftBerry site (http://www.softberry.com/berry.phtml) with the "monocot" option. Predicted proteins were compared for the most closely annotated rice proteins using the BLASTP function (Altschul et al. 1997) at the TIGR (http://www.tigr.org/tdb/) database. TBLASTN comparisons were also performed to search for alternative rice gene homology using the same database. Rice-*Paspalum* sequence comparisons were performed with the "BLAST 2 SEQUENCES" tool of the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Expression of putative genes was determined using BLASTN analysis against the dbEST at NCBI.

Pollen mother cells at pachytene and metaphase I stages in immature flower buds were used in all FISH experiments. Anthers containing meiocytes at late pachytene to metaphase I were fixed in freshly prepared ice-cold 96% ethanol : glacial acetic acid (3:1) for at least 3 hours at 20°C. Anthers were then rinsed in distilled water twice and once in 10 mM citrate buffer, pH 4.5, respectively. Cell walls were digested in a pectolytic enzyme mixture containing 0.3% (w/v) cellulase RS, 0.3% (w/v) pectolyase Y23 and 0.3% (w/v) cytohelicase in citrate buffer for 2 h at 37°C. After two washes in water we carefully transferred each anther to a grease-free slide. The material was squashed with a fine needle, covered with acetic acid (60%) and then stirred and baked on a slide warmer at 45°C to spread chromosomes on slides. Cells were rinsed with ice-cold acetic acid fixative and left to air-dry for at least 2 h. DNA of the 346H10 BAC (1∼μg) was labelled with biotin-16-dUTP with a nick translation kit using the manufacturer's protocol (Roche) and FISH was performed according to the methods of Zhong et al. (1996). Chromosomes were counterstained in 5 μg/ml DAPI in Vectashield anti-fade (Vector laboratories). Slides were examined with a Zeiss Axioplan 2 Imaging Photomicroscope equipped with epifluorescence illumination, filters sets for DAPI and TRITC fluorescence. Selected images were captured by a Photometric Sensys 1,305×1,024 pixel CCD camera. Image capturing and tresholding was performed with the Genus Image Analysis software (Applied Imaging Corporation). DAPI images were compressed to a 210 grey level (Red = 210, Green = 210, Blue = 210) and were separately enhanced with a 7×7 Hi-Gauss high pass spatial filter to accentuate minor details and heterochromatin patterns on the chromosomes. All fluorescence images were pseudo-coloured and improved for optimal brightness and contrast in Adobe Photoshop CS.

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Results

Development of molecular probes

The first step of this investigation consisted of the isolation of apomixis-specific (i.e. hemizygous for apomixis) sequences to develop molecular probes for FISH analysis. A bulked segregant analysis, combined with the cross hybridisation strategy of Labombarda et al. (2002) was followed. The AFLP pattern of two bulked samples of DNA for each of the apomictic and sexual phenotypes was compared with 159 primer combinations to select apomixis-related polymorphisms. Thirty-three AFLPs of lengths between 95 and 330 bp were selected as present in apomictic and absent in

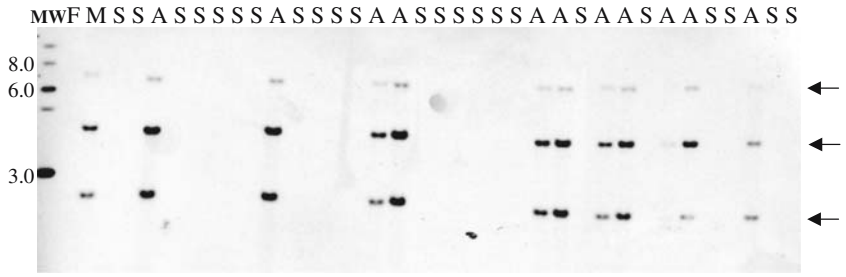

Fig. 1 Hemizygosity of the B11 probe. Hybridising banding pattern of the probe B11 with the *Eco*RI-digested DNAs of 11 apomictic (*A*) and 23 sexual (*S*) individuals representative of the mapping population of *P. simplex*. F is the female (sexual) and M is the pollinating (apomictic) parent; arrows indicate three hemizygous fragments co-segregating with apomixis; Molecular weight (*MW*) standards are expressed in kb

sexual bulked samples and therefore these were assumed to be tightly linked to apomixis. Since in most cases a single band excised from the gel resulted in several amplicons of almost identical molecular weight with different sequence, the cross-hybridisation procedure of Labombarda et al. (2002) was used to group the multiple fragments arising from the transformation of a single band into *E. coli*, according to the sequence. Shortly, for each transformation, from 40 to 60 PCR-positive clones were blotted and probed with an insert randomly chosen among those blotted over from the same transformation. The hybridised probe was washed out and blots were probed again with one of the other clones that did not hybridise with the previous probe. The procedure was repeated until all of the inserts could be grouped according to their sequence homology. Sixty-seven groups of fragments were obtained from the transformation of 33 apomixis-linked bands. A fragment representative for each of the 67 groups was used as probe against blots containing digested DNA of 5 apomictic and 5 sexual plants. Eight of them proved to be hemizygous as they showed 1 to 3 hybridizing fragments in apomictic plants only. Since the original intent was to use the hemizygous fragments as probes for FISH analysis, we extended the longest 5 of these 8 with the genome walker procedure to reach the minimum size of 1.5–2.0 kb that is required to get reliable signals. Only one (B11) of the fragments conserved its specificity for apomixis when enlarged to 1.5 kb while the others proved to be short hemizygous regions (< 540 bp) interspersed with regions sharing homology with sexual genotypes. To verify the tight linkage of B11 with apomixis in *P. simplex*, this fragment was hybridized with DNAs of the entire mapping population. The hybridising banding pattern of B11 with the DNAs of a subset of 34 individuals is reported in Fig. 1. Three bands of approximate lengths 2.8, 4.5 and 7.0 kb were inherited from the apomictic parent (M) and strictly co-segregated with apomixis without any other alleles present in the sexual genotypes. This trend was observed in all the 89 individuals of the mapping population confirming both hemizygosity and tight linkage with apomixis. However when used as probe for FISH analysis, B11 did not show a clear and reproducible signal (not shown) and therefore we decided to use a longer fragment encompassing the B11 probe by screening an available BAC library of apomictic *P. simplex* (O. Calderini, in preparation). Primers were designed on the original B11 sequence to amplify an apomixis-specific SCAR (Fig. 2). The same primers were then used to screen the BAC library arrayed according to a 3-D pooling strategy as reported by Donnison et al. (2005). Three positive clones were identified and one of them (346H10) was confirmed to include the B11 probe by Southern analysis.

FISH analysis

The FISH analysis of pachytene chromosomes of apomictic *P. simplex* using the BAC 346H10 as a probe is shown in Fig. 3. The overall organization of the *P. simplex* pachytene chromosomes shows densely stained heterochromatic blocks in the pericentromeric regions with a large number of smaller knobs dispersed along the proximal and distal euchromatin regions. Figs. 3a and b display the hybridising pattern of the same pachytene chromosome preparation of apomictic *P. simplex* probed with the BAC 346H10 captured at different threshold levels of the fluorescence signals. At a high level weak background signals were truncated, leaving a single signal on one of the chromosomes clearly detectable (arrow on Fig. 3b), whereas at a low

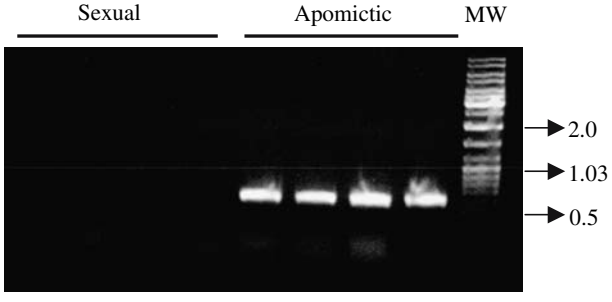

Fig. 2 Apomixis-specific SCAR in *P. simplex*. Primers designed on an hemizygous region of *P. simplex* amplified an apomixis-specific fragment of 650 bp. The same primers were used to select the BAC 346H10. Molecular weight (*MW*) standards are expressed in kb

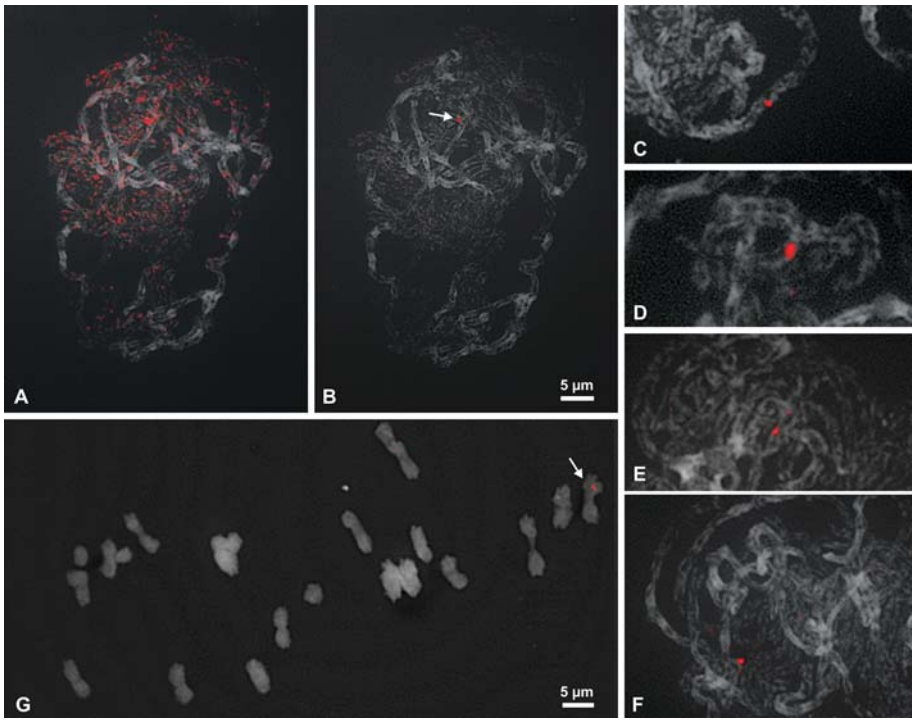

Fig. 3 Fluorescence in situ hybridisation of meiotic chromosomes of apomictic *P. simplex*. Chromosomes are counterstained in DAPI and pseudocoloured in *grey*. The brightly fluorescing blocks are the pericentromere regions. **a** The detection threshold level is low, capturing even the weakly fluorescing spots of the probe signals in the euchromatin regions and the strong signal of the 346H10 target. Note the absence of the background signals in the pericentromere regions. **b** Higher threshold levels displays only the single 346H10 signal. **c–f** are examples of 346H10 signals on the pachytene complement. The figures clearly show hemizygosity for the 346H10 region. Partial asynapsis mostly in the late pachytene complements (especially **e**, **f**). **g** Metaphase I complement with 346H10 on one of the half bivalents (*arrow*). The *double dots* show the signals on sister chromatids

level, many minor foci were observed all along the chromosomes (Fig. 3a). These minor signals were consistently seen all along the chromosomes but not in the cytoplasm and therefore interpreted as repetitive sequences in the BAC 346H10 that have related sequences across the chromosomes. The Fig. 3c–f show additional pachytene preparations confirming the uniqueness of the apomixis-related signal and a probable small stretch of asynapsis (Fig. 3e, f). Two pieces of evidence are provided by the FISH analysis of pachytene chromosomes of apomictic *P. simplex*: (1) the single signal confirms at the cytological level the partial hemizygosity already noted at the genetic level and (2) the ACL of *P. simplex* is located on a distal region of a chromosome rather than on the densely DAPI stained heterochromatic pericentromeric regions. However, due to the presence of many minor heterochromatic chromomeres dispersed along the chromosomes in this species, the vicinity to or even the embedding of the ACL in one of these knobs cannot be ruled out. In Fig. 3g a small doublet is clearly detectable on one of the bivalents (arrow). Since this signal is adjacent to a chiasma but not directly involved in crossing-over (in that case it would be in the middle), it is concluded that the ACL is located on two chromatids of an unbound arm of one of two chromosomes paired at the bivalent group.

Sequence analysis

The *P. simplex* BAC clone 346H10, was shotgun-sequenced at 10X coverage. There are some gaps that could not be closed because of the repetitive DNA that was also very AT rich. However the result was a total of 129,046 bp of sequence representing around 99.3% of the total BAC length (Table 1). Some of the sequences could be assembled in contigs of up to 23 kb. The sequence data are organized in 20 contigs whose lengths range from 1,463 to 23,166 bp. Four of these, summing up to 13,033 bp, did not show any predicted peptide when processed with the FGENESH program using the "monocot" option. Thirteen peptides related to mobile elements were identified in nine contigs. Among transposon-related elements, peptides belonging to ping/pong/SNOOPY (2), En/Spm (2) and mariner (1) subclasses were identified together with a single hAT domain related to the HOBO/AC/TAM3 subclass. Among retrotransposons, peptides related to ty3–gypsy (2), and ty1-copia (2) subclasses were identified together with two other proteins related to unclassified classes of retrotransposons and one polyprotein of general type. The sequences related to transposable elements may be responsible for the background signals in FISH (Fig. 3a). Four genes were identified as unrelated to transposable elements and whose e-values were e^{-34} or

Table 1 Characteristics and homology analysis of the predicted genes found in the BAC H10

Contig no	Bp	FGNESH prediction			TIGR rice database		NCBI	
		Gene no (exons)	Strand	Number of aa	Best homology (e-value)	Chromosome no (BAC/PAC clone)	DBEST, species (e-value)	CDNA library generated from
469	15,234	1(3)	+	376	Transposon protein, putative, ping/pong/SNOOPY sub-class (3.4×10^{-117})	1 (P0491F11)	CD901980, wheat (4×10^{-70})	Grains
		2(1)	+	441	Transposon protein, putative, ping/pong/SNOOPY sub-class (3.7×10^{-197})	1 (P0491F11)	AJ470575, barley (10^{-145})	Callus
		3(10)	–	906	Protein kinase domain, putative (1.2×10^{-140})	12 (OSJNBa0056D07)	CA214513, sugarcane(10^{-109})	1 cm long inflorescences
497	6,700	1(1)	–	285	Retrotransposon protein, putative, Ty3-gypsy sub-class (0.060)	4 (OSJNBb0054B09)	No significant similarity found	
852	4,450	1(5)	–	281	Protein kinase domain, putative (8.4×10^{-65})	12 (OSJNBa0056D07)	BE406366, wheat (8×10^{-40})	Roots of etiolated seedlings
869	5,695	No reliable predictions						
103	7,995	1(1)	+	43	Hypothetical protein (0.34)	9 (OSJNBb0095I04)	CA240703, sugarcane(6×10^{-11})	Calli submitted to low and high temperature stress
201	2,872	1(4)	–	503	Transposon protein, putative, mariner sub-class (1.3×10^{-166})	4 (OSJNBa0091C07)	CA240703, sugarcane(6×10^{-11})	Calli submitted to low and high temperature stress
236	1,749	1(4)	+	264	Transposon protein, putative, CACTA, En/Spm sub-class (7.4×10^{-2})	11 (OSJNBa0019A16)	CV136109, loblolly pine (0.004)	Embryos
491	9,545	1(3)	–	445	Hypothetical protein (2.0×10^{-34})	**12** (OSJNBa0002L05)	CA257457, sugarcane (9×10^{-10})	Developed inflorescence and rachis (20 cm-long)
617	2,141	No reliable predictions						
642	3,734	No reliable predictions						
874	2,993	1(4)	–	457	Retrotransposon protein, putative, Ty1-copia sub-class (6.1×10^{-77})	12 (unknown)	CA173581, sugarcane (3×10^{-37})	First apical stalk internodes of adult plants
950	6,458	1(11)	–	963	Protein kinase domain, putative (7.1×10^{-230})	12 (OSJNBa0056D07)	CA167090, sugarcane (10^{-162})	Stalk bark from adult plants
025	2,461	1(3)	–	74	ERD1/XPR1/SYG1 family, putative (1.2×10^{-36})	12 (OSJNBa0056D07)	CN130500, *sorghum* (1×10^{-90})	Acid- and alkaline-treated roots
212	1,807	1(7)	–	332	Transposon protein, putative, CACTA, En/Spm sub-class (2.0×10^{-09})	9 (P0698G06)	CV035745 loblolly pine (1.1)	Roots of 1-year-old loblolly pine
387	6,620	1(3)	+	243	Retrotransposon protein, putative, Ty3-gypsy sub-class (0.0041)	2 (P0476C12)	CA193081, sugarcane (2×10^{-21})	Seedlings inoculated with *Gluconacetobacter diazotroficans*
		2(1)	+	89	Similar to auxilin-like protein (0.12)	1 (P0458A05)	CB329645, maize (2.3)	2 mm ear tissue
534	1,741	1(3)	+	159	ERD1/XPR1/SYG1 family, putative (1.1×10^{-40})	12 (OSJNBa0056D07)	CD432092, *sorghum* (10^{-111})	Ethylene-treated seedlings
776	1,463	No reliable predictions						
891	12,365	1(2)	+	279	Putative polyprotein (1.4×10^{-120})	3 (OSJNBa0032H19)	CB637644, rice (6×10^{-19})	3 week old leaves
		2(4)	–	789	hAT family dimerisation domain, putative (4.8×10^{-214})	1 (OJ1174_D05)	CO524891, maize (3×10^{-23})	Multiple tissues

Table 1 (Contd.)

Contig no	Bp	FGNESH prediction			TIGR rice database		NCBI	
		Gene no (exons)	Strand	Number of aa	Best homology (e-value)	Chromosome no (BAC/PAC clone)	DBEST, species (e-value)	CDNA library generated from
983	23,166	1(3)	+	464	Retrotransposon protein, putative, Ty1-copia sub-class (5.2×10^{-145})	5 (P0636E04)	CF572445, sugarcane (4×10^{-86})	Internodes 6–11 from 12 month old plants
		2(3)	+	187	Hypothetical protein (0.045)	5 (OJ1122_B08)	CV478128, flax (0.005)	Outer fiber-bearing tissues
		3(12)	–	816	Protein kinase domain, putative (3.6×10^{-184})	12 (OSJNBa0056D07)	CA208453, sugarcane (10^{-135})	Stalk Bark from adult plants
		4(2)	+	331	Retrotransposon protein, putative, unclassified (1.7×10^{-104})	2 (P0030G11)	CO085082, *Gossypium raimondii* (0.002)	Whole seedlings with first true leaves
		5(1)	+	467	Retrotransposon protein, unclassified (3.3×10^{-238})	6 (P0012B02)	BU049357, maize (10^{-162})	Unigene III from Maize Genome Project
		6(5)	–	662	Protein kinase domain, putative (1.3×10^{-115})	12 (OSJNBa0056D07)	BJ463684, barley (6×10^{-21})	Germinating shoots
T7s	9,857	1(8)	+	835	FAR1 family, putative (4.8×10^{-94})	3 (OSJNBb0096H12)	CA906116, bean (0.095)	Suspensor region of globular-stage embryos
		2(2)	+	110	Dof domain, zinc finger, putative (0.90)	7 (OSJNBa0060O17)	CN820196, oat (0.047)	6 day old etiolated leaves

less. The presence of two of them in the BAC under study was further validated with previously annotated genes and by their conservation in the orthologous region of the rice genome. The resulting predicted proteins of these two genes showed significant homology with a protein of the EXS family (PsEXS), and to a protein kinase domain (PsPKD). These two genes were not arrayed as a continuum but were split in two (PsEXS) or several (PsPKD) fragments that did not show any homology between them. When the predicted translated sequences of these two genes were blasted against the TIGR database with the rice training set option we identified the position of the putative orthologous rice genes which were both located on the BAC clone named OSJNBa0056D07 in opposite orientation, and separated by 25,845 bp. To test whether a better similarity could be found elsewhere in the rice genome we compared the FGENESH-translated protein and the rice genome using TBLASTN. No hits were obtained with e-values lower than those observed for the putative orthologous rice genes found with the previous analyses. These results confirm that the EXS and PKD genes of rice (OsEXS and OsPDK, respectively) are the orthologs of *P. simplex* genes. Moreover, significant homology with ESTs of *Sorghum* for PsEXS and sugarcane, wheat and barley, for PsPKD were found indicating that the genes identified are functional and widespread among the grasses. The rice BAC clone OSJNBa0056D07 was adjacent (6 bp overlapping) to the other BAC OSJBBa0001B02 that harboured three ESTs (C454, C1069 and C60087), which were conservatively linked to apomixis in several *Paspalum* spp. (Pupilli et al. 2004). The corresponding rice EST of OsEXS, (R2241) was previously tested for its capacity to detect apomixis-linked alleles in *Paspalum* spp.: although it revealed apomixis-linked alleles in the closely related species *P. simplex* and *P. malacophyllum*, the same probe gave unclear results in the distantly related species *P. notatum*. To verify if the *Paspalum* homolog of R2241was linked to apomixis also in *P. notatum*, we amplified a portion of the ORF of the PsEXS and hybridised it against the digested DNA of the mapping population of *P. notatum*. The resulting hybridising pattern was almost identical to that obtained with R2241 but in this case an apomixis-linked allele was clearly detectable (not shown). Similarly, a probe amplified from the sequence of PsPKD yielded an apomixis-linked allele in *P. notatum*. This indicates that the BAC clone under study belongs to the region conservatively linked to apomixis in several *Paspalum* spp. previously identified (Pupilli et al. 2004) and then likely to contain candidate genes for apomixis in this genus. To verify if the non overlapping contigs of PsEXS and PsPKD are different fragments of the same genes and to evaluate the level of sequence conservation between *Paspalum* and rice, their intron/exon structure was compared in the two species. The OsPKD (locus name in TIGR database: LOC_Os12g40280.1) is a large gene spanning more than 25 kb, containing 49 exons and coding for a protein of 4,262 aminoacids. The

comparison of the exon/intron structures between *Paspalum* and rice PKDs is reported in Fig. 4. The four *Paspalum* contigs containing sequences homologous to OsPKD almost completely spanned its sequence. The longest contig (Fig. 4a), corresponding to the 23,166 bp fragment no 983 (Table 1), showed two major rearrangements compared to the related region of OsPKD consisting on the insertion of two nested genes related to unclassified transposable elements in central region and of some elements related to the ty1-copia subclass retrotransposon together with a gene coding for a hypothetical protein at one extreme. All the inserted genes were in the opposite orientation with respect to PsPKD and interrupted locally the colinearity between *Paspalum* and rice homologues. As a consequence of these insertions the structure of the gene was split into two main parts both exhibiting a high level of homology with OsPKD but apparently evolving as independent genes because of the presence, in both parts of putative sites for initiation of transcription and polyadenylation. The overall sequence between the two homologs showed portions of strong homology between coding regions (black to black relationships), alternated with others in which OsPKD coding regions were significantly homologous with non-coding *Paspalum* regions (black to dashed relationships) and others in which no homology was detected. The region of PsPKD that was homologous to the start of the OsPKD was formed by five exons, the first of which has a start codon located 86 bp downstream of a putative TATA box site and a final exon containing a stop codon 253 bp upstream of a putative polyadenylation site. The first coding region of rice was not present in the PsPKD sequence and the third exon of rice exhibited two large portions of homologous DNA (74 and 76%, respectively) separated by an internal region of non-homologous DNA. The related exon of PsPDK showed a more severe destruction of colinearity in the region more proximal to the insertion site of the two retrotransposon-related elements. The predicted protein of this portion of PsPKD (662 aminoacids) showed good identity (59%) with the rice homolog and conserved, among other sequences, an apparently functional tyrosin-kinase domain. Similarly the second putative gene, included between a retrotraspososn-related element 122 bp upstream its putative transcription initiation site and a gene coding for an hypothetical protein 352 bp downstream the polyadenylation site, showed a significant identity (41%) with the homologous region of the rice gene. A relatively large region of PsPKD (712 bp), between the insertions of the Ty1-copia sequence and the gene coding for the hypothetical protein appears to have lost its coding

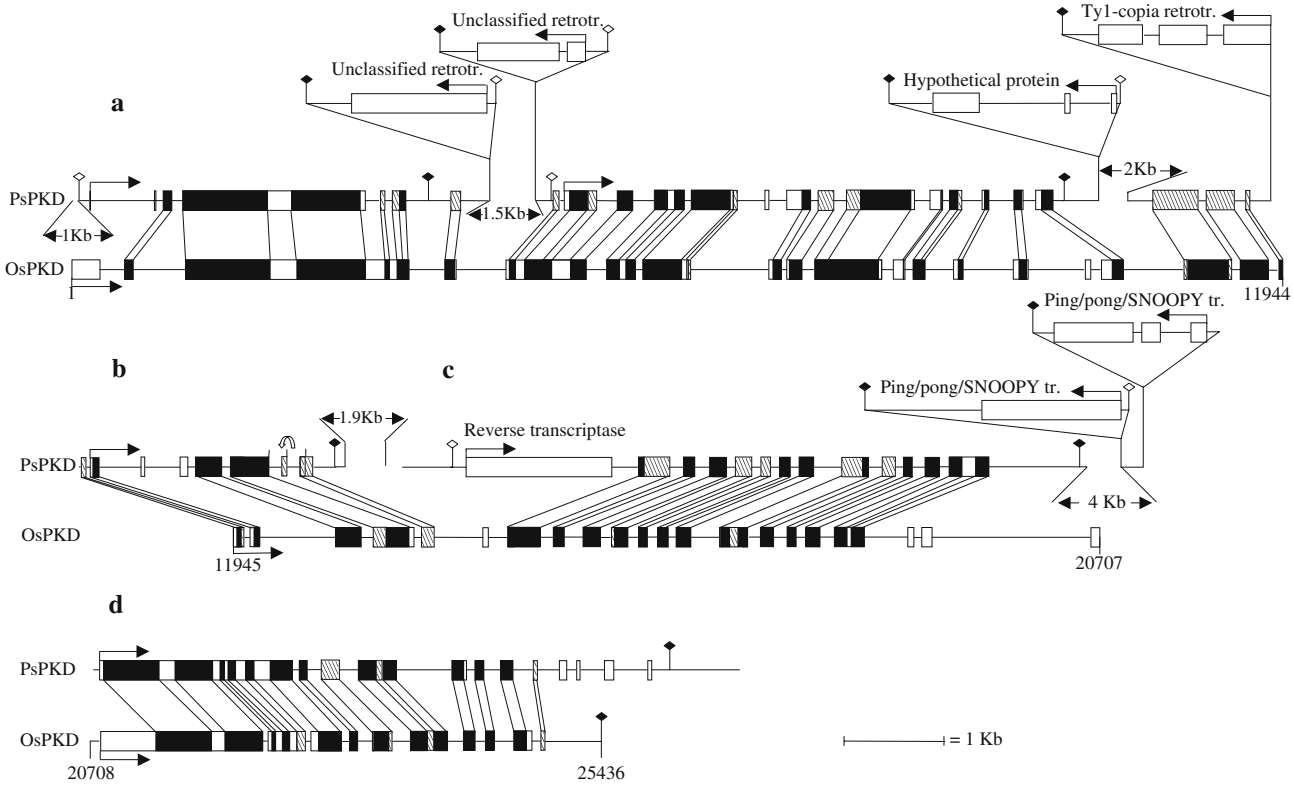

Fig. 4 Microcolinearity of the Protein Kinase Domain gene of *P. simplex* (PsPKD) with the rice homolog (OsPKD). **a–d** These parts refer to the contigs nos 983, 852, 469 and 950 of Table 1, respectively. Homologous areas are connected by *vertical lines*. *Arrows* indicate gene orientation and position of the first exon. *Black and white boxes* indicate homologous coding and non-homologous coding regions respectively while *dashed boxes* indicate homologous non coding regions. *Open and black rhombuses* indicate putative initiations of transcription and polyadenylation sites, respectively

capability. Homology between two non-coding regions was detected in contig No 852 (Fig. 4e); part of the *Paspalum* homologous region was duplicated close to an exon (curved arrow). Still, the predicted protein showed significant homology with rice (identity level 49%). Two different kinds of insertion characterized contig no 469 (Fig. 4c): a putative reverse transcriptase, probably a remnant of retrotransposon insertion, was present 156 bp downstream of a putative initiation site and two nested transposons of the ping/pong/SNOOPY class were present in the opposite orientation 4,081 bp downstream of the polyadenylation site. Since no stop codons were detected within the coding region of the reverse transcriptase, we argue that its ORF is transcribed as a continuum together with the PsPKD exons until the next stop codon located in the last exon before transposon insertion. Although maintaining a significant homology (48% identity) with its rice counterpart, contig no 469 showed dramatic rearrangement in terms of loss of coding capacity (nearly half of the *Paspalum* regions related to rice coding regions turned out to be non coding). Conversely, the two ping/pong/SNOOPY transposons did not appear to affect the structure of the final part of the *Paspalum* gene probably because of the relatively long distance of their insertion site with respect to the last exon. The last three exons of the rice gene close to the 20,707 bp position, appear to have no homologous counterpart in *Paspalum* or alternatively these could be in the gap between contigs nos 469 and 950. This last contig (Fig. 4d) showed a high level of identity (53%) with the terminal part of OsPKD; the exon/intron structure was similar between the two genes although PsPDK had 4 additional exons in the final part not present in the rice gene and, an internal exon of 162 bp has lost its coding capacity while conserving homology with its rice coding counterpart. The loss of the coding capacity of *Paspalum* sequences with respect to their homologs in rice was mainly due to small deletions (1–5 nucleotides) and point mutations that introduced new stop codons. By comparing the sequences of the EXS predicted proteins between *Paspalum* and rice (Fig. 5), we noted an overall high level of homology for both contigs (96 and 66% identity for contigs nos 025 and 543, Table 1 respectively). The existence of two putative polyadenylation sites on both contigs suggests that the correspondent mRNA is transcribed separately in the two stretches even though no transcription initiation sites were detected. A significant homology (93% identities, e-value: 1×10^{-42}) was detected between two non coding regions of 120 bp (Fig. 5a). Three coding regions of PsEXS lost their coding capacity in contig 534 (Fig. 5b) as a consequence of two small deletions and a point mutation that, as already noted for PsPKD introduced new stop codons. Three other exons of OsEXS (locus name in TIGR database: LOC_Os12g40340.1) were not present in *Paspalum* homologs probably because of their location in the unsequenced portion of PsEXS. Neither insertion of transposable elements nor of other genes were detected in the PsEXS.

To summarize, both large- and small-scale rearrangements were noted in the structure of PsPKD and PsEXS when compared with their rice homologs taken as a reference for the sexual homolog of apomictic *Paspalum*: large scale rearrangements included insertions of components of transposable elements. When these were inserted in the opposite orientation with respect to PsPDK, they induced the formation of multiple genes that appeared to be transcribed independently from each other. Conversely when an element is inserted in the same direction, its coding region appears to be transcribed as a continuum with the other exons of the PsPKD gene. Small-scale rearrangements included a 110 bp duplication, frequent small deletions and single point mutations that inducing premature stop codons, caused the loss of coding capacity of many exons.

Discussion

Taking advantage of the existing partial hemizygosity of the ACL in *P. simplex* (Labombarda et al. 2002), we have isolated an apomixis-linked BAC clone and used it as a probe in FISH analysis of pachytene chromosomes to characterize cytogenetically the genetic location of the apo locus in this species.

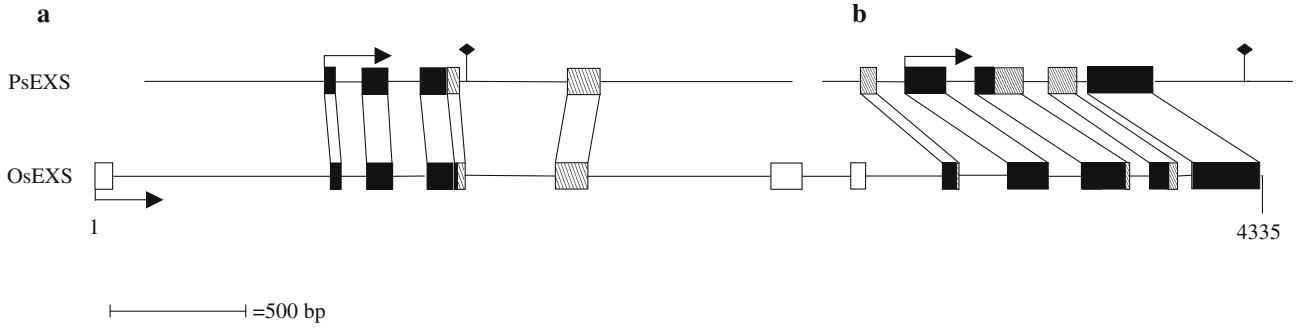

Fig. 5 Microcolinearity of the ERD1/XPR1/SYG1 gene of *P. simplex* (PsEXS) with the rice homolog (OsEXS). **a, b** These parts refer to the contigs numbers 025 and 534 of Table 1, respectively. Symbols are as in Fig. 4

Are the hemizygous regions of the ACL key regulatory factors for apomictic reproduction in *P. simplex*?

Hemizygosity has already been reported in several apomictic systems either of the aposporic type such as *P. squamulatum* (Ozias-Akins et al. 1998), and *C. ciliaris* (Goel et al. 2003) or diplosporic type (*T. dactyloides*, Grimanelli et al. 1998). The presence of sequences that are strictly apomixis-specific (i.e. sequences that do not hybridise to DNA of sexual relatives nor to that of the rest of the genome of apomictic genotypes) may suggest the existence of apomixis-specific genetic factors and/or a possible role of supernumerary chromatin in the control of apomictic reproduction (Roche et al. 2001). Alternatively a functional role of the apomixis-specific sequences is suggested: a proposed model hypothesizes that these regions act as a sink to sequester proteins that regulate genes involved in megagametogenesis thereby inducing mis-regulation of sexual process (Koltunow and Grossniklaus 2003). Finally the apomixis-specific sequences could have arisen as a direct consequence of the recombinational block that has caused sequence divergence and then hemizygosity at the apomixis locus. In this latter view, hemizygosity appears to be a consequence of the recombination block rather than having a functionally active role in the control of apomixis. On a small scale analysis of the conservation of apomixis-specific SCARs among several apomictic *Paspalum* species, we found that some of these SCARs isolated from *P. simplex*, including that used to isolate the BAC clone under study, when converted into RFLP probes, were conserved in closely related species whereas the same sequences were not homologous or lost their apomixis-specificity when hybridised to the DNA of more distantly related species (A. Busti, unpublished results). Hemizygosity of apomixis-related sequences was also not maintained when hemizygous and apomixis-related probes of *P. squamulatum* were cross-hybridised with the DNA of the related apomictic species *C. ciliaris* (Roche et al. 1999). Conversely apomixis-linked markers that are related to functional alleles are conserved and linked to apomixis in several *Paspalum* species (Pupilli et al. 2004). If the apomixis-specific sequences are allowed to evolve during speciation of *Paspalum* without affecting apomixis then the genetic factors that are critical for the apomixis reproduction are not likely to be located in these areas. Comparative mapping of apomixis-associated markers provided evidence that apomictic reproduction in *P. simplex* is inherited as a single genetic unit located in a recombinationally repressed area (Pupilli et al. 2001). Several hypotheses have been formulated to explain the causes of the block of recombination described in apomictic systems (Grimanelli et al. 1998; Ozias-Akins et al. 1998; Labombarda et al. 2002; Goel et al. 2003); among these the physical location of the ACL in heterochromatin-dense pericentromeric regions that are known to be recombinationally suppressed appears worthy of investigation. Recent results obtained for *C. ciliaris* seem to confirm this hypothesis since the apomixis locus of this species is located near the centromere in a heterochromatin-rich region (Akiyama et al. 2005). Several characteristics associated with heterochromatin have been observed at the same locus of *P. squamulatum*, although it is not located close to the centromere (Akiyama et al. 2004). In contrast to these results, the ACL of *P. simplex* appeared to be located in a heterochromatic-poor region further away from the centromere. So at least for *P. squamulatum* and *P. simplex* the lack of recombination at the apomixis locus is not directly related with the highly condensed heterochromatic regions close to the centromere or a large asynaptic region. Nevertheless, our FISH results confirm the position of the conserved distal region of the chromosome arm between *P. simplex* and rice chromosome 12 (Pupilli et al. 2001).

Is the recombination block at the ACL caused by perturbations of chromosome pairing in *P. simplex*?

It is well known that a wide range of chromosome rearrangements such as translocations and/or inversions can cause local lack of chromosome pairing (asynapsis) or lack of chiasma formation (desynapsis). Although inversions or translocations involving large chromosome segments may cause gamete sterility and are not likely to be transmitted to the progeny, a smaller rearrangement can cause perturbations of chromosome pairing without affecting gamete viability. FISH analysis of pachytene complements showed that the ACL is located in the vicinity of a small asynaptic region. Therefore the lack of recombination at the ACL of *P. simplex* is likely caused by the missing capacity of a local pairing probably as a consequence of a local rearrangement (i.e. inversion and/or translocation). Asynapsis involving a single chromosome arm can also explain the lack of detection of molecular markers allelic to, or even linked in repulsion phase to, apomixis in the form of both heterologous or homologous co-dominant RFLPs (Pupilli et al. 2001) or AFLPs (Labombarda et al. 2002).

Is the map synteny of the ACL between rice and *Paspalum* also confirmed at the sequence level?

In a comparative study of the organization of the ACL in several *Paspalum* spp., we previously showed that out of the large chromosome region involved in apomixis, only a core region marked by three rice ESTs located in the telomeric part of rice chromosome 12 is conserved in all apomictic species analysed (Pupilli et al. 2004). On the basis of the results presented here we have extended this core region so as to include two additional markers corresponding to two genes PsEXS and PsPKD. The rice EST related to PsEXS was mapped at 0.3 cM distance from the other apomixis-linked markers, a distance that corresponded approximately to 124 kb in the rice map. This core region underwent intense

rearrangement that in the species *P. notatum* was indentified as a translocation that locally interrupted synteny of rice markers. On a cytological study of the same species, a meiotic abnormality typical of an inversion was detected (Stein et al. 2004). Although translocation and a subsequent inversion might have occurred simultaneously or even at different times, a common feature in the apomictic *Paspalum* species analysed is that the ACL has been inherited as an alien and highly rearranged chromosome region. Whereas no assumptions can be made on the possible role of PsPKD on apomictic reproduction in *Paspalum*, the possible function of PsEXS may deserve attention. The EXS family is named after ERD1/XPR1/SYG1 and proteins containing this motif include: (1) the C-terminus of SYG1, a G-protein associated signal transduction protein in *Saccaromices cerevisiae*, (2) sequences that are thought to be murine leukaemia virus receptors (XPR1, Battini et al. 1999) and (3) the ERD1 proteins that in *S.cerevisiae* are involved in the localization of endogenous endoplasmic reticulum proteins (Hardwick et al. 1990). The function of the total EXS domain as a whole is unknown although it is worth noting that the SYG1 protein is involved in the pheromone-mediated mating signal transduction in yeast. Deletion mutants of this protein can suppress the cell cycle arrest and differentiation in both mating types and the suppression capacity is related to the loss of specific portions of the gene (Spain et al. 1995). The same authors hypothesized that one of these deletion mutants (Syg1Δ340p), has the capacity to promote cell division in otherwise arrested (differentiated) cells. In this view, if we compare the gene structure of PsEXS with that of OsEXS, we note deletion of some rice exons and loss of coding capacity of others suggesting a possible mechanism according to which the existence of deleted (or rearranged) supernumerary copies of genes involved in sexual development can induce re-programming of differentiated cell into the apomictic developmental process.

Comparative map-based cloning is referred to as the possibility to isolate a gene of interest in model species once the homologous region in which the gene is located in relation to anchor markers is known (Chen et al. 1997; Feuillet and Keller 1999). The effectiveness of this strategy would be confirmed if the colinearity of gene order on the chromosomes is conserved at the sequence level. In most cases the colinearity between rice and other genomes is frequently interrupted by chromosomal rearrangements such as inversions, duplications in addition to changes in gene content, order and orientation (Bennetzen and Ramakrishna 2002). More recently, investigations on the conservation of microcolinearity between rice and wheat showed variable results. Using computational analysis Guyot et al. (2004) demonstrated that interruption of colinearity is frequent between the distal region of wheat chromosome 1S and a 1,219 kb region located on rice chromosome 5S, while strong colinearity for gene order and content was found by Chancret et al. (2004) between the *Hardness* locus of *Triticum monococcum* and its related homoeologous region on rice chromosome 12. In our results a relatively small number of genes appears to be conserved between rice and the ACL of *P. simplex* while interruption of colinearity through migration of genes from other locations appears to be the rule rather than the exception. The migrating genes were mainly related to transposable elements that in the case of PsPKD interrupted locally the colinearity with its rice counterpart and induced the gene to evolve a strategy to counterbalance such invasion thereby retaining perhaps a partial coding capacity. The significant synteny of the ACL of *P. simplex* with a telomere of rice chromosome 12 was confirmed at the sequence level only for two genes for the BAC length analysed. Mosaic patterns of conservation of orthologous loci has already been demonstrated in general terms in the grass family (Song et al. 2002) and exceptions of colinearity observable only at the sequence level in otherwise macrosyntenic regions have been reported and related to gene movement, amplification and inversion (Song et al. 2002 and ref. therein). This consideration raises the question whether the intense gene movements and the frequent colinearity disruption may limit the use of the rice genome sequence for positional cloning of apomixis genes. In addition, as Ozias-Akins et al. (2003) pointed out, there is not a common region on the grass circle (Gale and Devos 1998) to which the aposporous apomixis of the plants for which comparative mapping data are available can be referred to. This suggests a polyphyletic evolution of the locus within the grass family or a localised disruption of synteny caused by rearrangements. On the other hand, several independent studies located loci responsible for spikelet sterility in the same area of chromosome 12 that was homologous to the ACL of *P. simplex* (Wu et al. 1995; Wan et al. 1996; Liu et al. 2001). More recently Kubo and Yoshimura (2004) mapped in the same area the locus *hsa1* responsible for hybrid breakdown in *indica*/*japonica* crosses. In all these cases the spikelet sterility was related to defective development of the embryo sac. These results, together with those reported here seem to strongly indicate that a gene complex responsible for female gametophyte development should exist in this area of chromosome 12 and, among these, the presence of some genes related to apomictic reproduction is conceivable. However, due to the frequent migration of genes from other locations as we noted in our sequenced BAC, the role of additional elements other than those present in chromosome 12 in the expression of the apomictic phenotype should not be ruled out. In any case, large scale sequencing of apomixis-linked BACs, while cumbersome, is perhaps the most promising strategy to identify key genes for apomictic reproduction in wild apomictics.

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References

Akiyama Y, Conner JA, Goel S, Morishige DT, Mullet JE, Hanna WW, Peggy Ozias-Akins P (2004) High resolution physical mapping in *Pennisetum squamulatum* reveals extensive chromosomal heteromorphism of the genomic region associated with apomixis. Plant Physiol 134:1733–1741

Akiyama Y, Hanna WW, Peggy Ozias-Akins P (2005) High resolution physical mapping reveals that the apospory-specific genomic region (ASGR) in *Cenchrus ciliaris* is located on a heterochromatic and hemizygous region of a single chromosome. Theor Appl Genet DOI: 10.1007/s00122-005-0020-5

Albertini E, Marconi G, Reale L, Barcaccia G, Porceddu A, Ferranti F, Falcinelli M (2005) SERK and APOSTART. Candidate genes for apomixis in *Poa pratensis*. Plant Physiol 138:2185–2199

Altschul SF, Madden TL, Schäffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

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

Battini JL, Rasko JA, Miller AD (1999) A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein–coupled signal transduction. Proc Natl Acad Sci USA 96:1385–1390

Bender J (2004) Chromatin–based silencing mechanisms. Curr Opin Plant Biol 7:521–526

Bennetzen JL, Ramakrishna W (2002) Numerous small rearrangements of gene content, order and orientation differentiate grass genomes. Plant Mol Biol 48:821–827

Caceres ME, Pupilli F, Quarin CL, Arcioni S (1999) Feulgen densitometry of embryo sac permits discrimination between sexual and apomictic *Paspalum simplex*. Euphytica 110:161–167

Chantret N, Cenci A, Sabot F, Anderson O, Dubcovsky J (2004) Sequencing of the *Triticum monococcum Hardness* locus reveals good microcolinearity with rice. Mol Genet Genomics 271:377–386

Chen M, SanMiguel P, de Oliveira AC, Woo SS, Zhang H, Wing RA, Bennetzen JL (1997) Microcolinearity in sh2-homologous regions of the maize, rice and sorghum genomes. Proc Natl Acad Sci USA 94:3431–3435

de Jong H (2003) Visualizing DNA domain and sequences by microscopy: a fifty-year history of molecular cytogenetics. Genome 46:943–946

Delaney D, Nasuda S, Endo TR, Gill BS, Hulbert SH (1995) Cytologically based physical map of the group-3 chromosomes of wheat. Theor Appl Genet 91:568–573

Donnison IS, OSullivan DM, Thomas A, Canter P, Moore B, Armstead I, Thomas H, Edwards KJ, King IP (2005) Construction of a *Festuca pratensis* BAC library for map-based cloning in *Festulolium* substitution lines. Theor Appl Genet 110:846–851

Feuillet C, Keller B (1999) High gene density is conserved at syntenic loci of small and large grass genomes. Proc Natl Acad Sci USA 96:8665–8670

Gale MD, Devos KM (1998) Comparative genetics in the grasses. Proc Natl Acad Sci USA 95:1971–1974

Goel S, Chen Z, Conner JA Akiyama Y, Hanna WW, Peggy Ozias-Akins P (2003) Delineation by fluorescence *in situ* hybridization of a single hemizygous chromosomal region associated with aposporus embryo sac formation in *Pennisetum squamulatum* and *Cenchrus ciliaris*. Genetics 163:1069–1082

Grimanelli D, Leblanc O, Espinosa E, Perotti E, Gonzales de Leon D, Savidan S (1998) Mapping diplosporus apomixis in tetraploid *Tripsacum*: one gene or several genes? Heredity 80:33–39

Grossniklaus U (2001) From sexuality to apomixis: molecular and genetic approaches. In: Savidan Y, Carman JG, Dresselhaus T (eds) The flowering of apomixis: from mechanisms to genetic engineering. CIMMYT, IRS, Montpellier, European Community DG VI, Mexico City, pp 168–211

Guyot R, Yahiaoui N, Feuillet C, Keller B (2004) *In silico* comparative analysis reveals a mosaic conservation of genes within a novel colinear region in wheat chromosome 1AS and rice chromosome 5S. Funct Integr Genomics 4:47–58

Hardwick KG, Lewis MJ, Semenza J, Dean N, Pelham HR (1990) ERD1 a yeast gene required for the retention of luminal endoplasmic reticulum proteins affects glycoprotein processing in the Golgi apparatus. EMBO J 9:623–630

Koltunow A, Grossniklaus U (2003) Apomixis: a developmental perspective. Annu Rev Plant Biol 54:547–574

Kubo T, Yoshimura A (2004) Epistasis underlying female sterility detected in hybrid breakdown in a Japonica-Indica cross of rice (*Oryza sativa*). Theor Appl Genet 110:346–355

Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Labombarda P, Busti A, Caceres ME, Pupilli F, Arcioni S (2002) An AFLP marker tightly linked to apomixis reveals hemizygosity in a portion of the apomixis-controlling locus in *Paspalum simplex*. Genome 45:513–519

Liu YS, Zhu LH, Sun JS, Chen Y (2001) Mapping QTLs for defective female gametophyte development in an inter-subspecific cross in *Oryza sativa* L. Theor Appl Genet 102:1243–1251

Nogler GA (1984) Gametophytic apomixis. In: Jhori EM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 475–518

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127–5132

Ozias-Akins P, Akiyama Y, Hanna WW (2003) Molecular characterization of the genomic region linked to apomixis in *Pennisetum*/*Cenchrus*. Funct Integr Genomics 3:94–104

Pessino SC, Ortiz JPA, Leblanc O, do Valle CB, Hayward MD (1997) Identification of a maize linkage group related to apomixis in *Brachiaria*. Theor Appl Genet 94:439–444

Pupilli F, Labombarda P, Caceres ME, Quarin CL, Arcioni S (2001) The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm of rice chromosome 12. Mol Breed 8:53–61

Pupilli F, Martinez EJ, Busti A, Calderini O, Quarin CL Arcioni S (2004) Comparative mapping reveals partial conservation of synteny at the apomixis locus in *Paspalum* spp. Mol Gen Genomics 270:539–548

Roche D, Cong P, Chen Z, Hanna WW, Gustine DL, Sherwood R, Ozias-Akins P (1999) An apospory-specific genomic region is conserved between Buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen. Plant J 19:203–208

Roche D, Conner JA, Budiman MA, Frisch D, Wing R, Hanna WW, Ozias-Akins P (2002) Construction of BAC libraries from two apomictic grasses to study the microcolinearity of their apospory-specific genomic regions. Theor Appl Genet 104:804–812

Roche D, Hanna WW, Ozias-Akins P (2001) Is supernumerary chromatin involved in gametophytic apomixis of polyploid plants? Sex Plant Reprod 13:343–349

Savidan Y (2000) Apomixis: genetics and breeding. Plant Breed Rev 18:13–86

Sherman JD, Stack SM (1995) Two dimensional spreads of synaptonemal complexes from solanaceous plants VI. High resolution recombination nodule map of tomato (*Lycopersicon esculentum*). Genetics 141:683–708

Song R, Llaca V, Messing J (2002) Mosaic organization of orthologous sequences in grass genomes. Genome Res 12:1459–1555

Spain BH, Koo D, Ramakrishnan M, Dzudzor B, Colicelli J (1995) Truncated forms of a novel yeast protein suppress the lethality of a G protein alpha subunit deficiency by interacting with the beta subunit. J Biol Chem 270:25435–25344

Spillane C, Curtis MD, Grossniklaus U (2004) Apomixis technology development-virgin births in farmers' field? Nat Biotechnol 22:687–691

Stein J, Quarin CL, Martinez EJ, Pessino SC, Ortiz JP (2004) Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. Theor Appl Genet 109:186–191

Wan J, Yamaguchi Y, Kato H, Ikehashi H (1996) Two new loci for hybrid sterility in cultivated rice (*Oryza sativa* L.). Theor Appl Genet 92:183–190

Wu P, Zhang G, Huang N, Ladha JH (1995) Non-allelic interaction conditioning spikelet sterility in an F_2 population of *indica*/*japonica* cross of rice. Theor Appl Genet 91:825–829

Zhong XB, de Jong HJ, Zabel P (1996) Preparation of tomato meiotic pachytene and mitotic metaphase chromosomes suitable for fluorescence in situ hybridization (FISH). Chromosome Res 4:24–28