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Genome rearrangements derived from autopolyploidization in *Paspalum* sp.

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Abstract

Rapid sequence rearrangements along with alterations in cytosine-directed methylation patterns follow allopolyploid formation in several plant species. The occurrence of similar changes is yet to be examined in detail during the generation of autopolyploids. The objective of this work was to investigate the presence of nascent variation in genome sequence after autopolyploidization in two *Paspalum* species. The increments of ploidy level were produced either by hybridization involving unreduced gametes or colchicine treatment. Genetic modifications affected 15–23% and 9.55% of the genomic loci in *Paspalum rufum* and *Paspalum notatum*, respectively. In all cases the frequency of band loss from the progenitors was significantly higher than that of novel band gain. In random amplified polymorphic DNA (RAPD) experiments the majority of polymorphisms were detected by particular sets of primers, suggesting that repetitive domains were preferentially targeted. Several *P. notatum* polymorphic fragments were cloned, sequenced and used as probes in genomic hybridization analyses. Some of them corresponded to interspersed middle-repetitive sequences with unknown function. Methylation-sensitive genomic hybridization showed that sequence modifications occurred invariably in cytosine-methylated regions, which remained methylated after polyploidization.

Keywords: Autoploidy; Genome evolution; Genome variation; Molecular markers; Ploidy level; Polyploidy

1. Introduction

Newly synthesized polyploids provide the opportunity to investigate genetic and epigenetic events occurring immediately following genome duplication (reviewed in Ref. [1]). The strategy of generating such plants to analyze genetic/epigenetic modifications was employed in a number of species, including *Arabidopsis* sp. [2,3], *Brassica* sp. [4], cotton [5] and wheat and its wild relatives [6–8]. Wheat in particular was the object of exhaustive molecular studies that generated novel insights into the nature of the genomic changes associated with polyploid formation (reviewed in Ref. [9]).

In general, results indicated that rapid sequence deletion was a major cause of genome variation in wheat synthetic allopolyploids [6–8,10–16]. Sequence elimination appeared to be a common, non-random, highly reproducible event, whose direction was determined by the genomic combination of the amphiploids, although some of the changes occurred in a random fashion [13]. Sequence elimination was not affected by the parental genotype, cytoplasm or ploidy level and did not result from intergenomic recombination [13]. The same pattern of sequence loss was detected in spontaneously produced allopolyploids, as well as in those obtained by colchicine treatment or through tissue culture [13]. In addition, transcriptional silencing/activation of particular retrotransposons and rapid epigenetic modifications were reported [15,16].

Similarly, genetic/epigenetic changes were observed in allopolyploids of *Arabidopsis* [2,3,17], *Brassica* [4] and triticale [8]. In contrast, a quiescent genome behavior was reported for allopolyploid cotton [5] and *Spartina anglica* [18], in which a strict additivity of the parental sub-genomes was

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evident. However, assays of homoeologous transcript representation in natural and synthetic tetraploid cotton revealed a transcription bias, with altered gene expression induced either by silencing or unequal expression of the duplicated genes [19]. In some cases, similar homoeologous expression and silencing patterns were displayed in both natural and synthetic tetraploids, suggesting common immediate and long-term responses to polyploidization [19]. The overall data from newly formed cotton tetraploids implicate epigenetic rather than genetic changes as the mechanism causing variation.

While in allopolyploids genetic and epigenetic alterations might be associated with both the merger of two divergent genomes in the same nucleus and a general increase in gene copy number, the formation of autopolyploids only implies an enlargement in gene copy number, being the duplicated genomes of identical or very similar nature. The occurrence of genomic modifications in recently formed autopolyploids was not extensively investigated yet. Recently, genetic polymorphisms were reported for a 'back-and-forth' ploidy altered series of weeping lovegrass (*Eragrostis curvula*) consisting of a natural tetraploid, its dihaploid derivative (obtained by tissue culture treatment) and two tetraploids obtained from the dihaploid by colchicine treatment [20]. Twenty-eight percent of the detected loci demonstrated polymorphism during the polyploidization events. Curiously, most of the polymorphisms were of a 'revertant' type, that is the fingerprinting profiles were characteristic of a given ploidy level [20].

The genus *Paspalum* (Poaceae, subfamily Panicoideae, tribe Paniceae) includes around 400 species, many of which are important natural forage resources in the tropical and subtropical areas of Central and South America. There is a wide range of ploidy levels in the genus, from 3*x* [21] to 16*x* [22]. The basic number of chromosomes for most of its species is $x = 10$, but some of them present $x = 6$ or 9 [23,24]. Polyploidy occurs in approximately 80% of the *Paspalum* species. Around 50% of the polyploids are tetraploids. Several of the tetraploid species present an autoploid origin [25–30]. While diploid genotypes are sexual and self-incompatible, polyploids can be self-incompatible sexuals or self-fertile pseudogamous apomicts, depending on the species. Most tetraploids reproduce by aposporous apomixis [31], but diplosporous apomixis is present at low frequency in the genus [31]. Most of the species are perennial, easy to cultivate and can be propagated vegetatively by stolons or rhizomes [31].

The objective of this work was to analyze and compare the variation of genome structure following an increment in the ploidy level using two different systems of *Paspalum*: (1) a small family of four *Paspalum rufum* individuals, consisting of two tetraploid parental genotypes along with one hexaploid and one tetraploid offspring (offspring were derived from controlled hybridizations of the parental genotypes) and (2) a *Paspalum notatum* diploid and its colchicine-induced autotetraploid counterpart. Molecular marker profiling followed by polymorphic band cloning, sequencing and genome hybridization analysis were used to investigate the occurrence of genetic/epigenetic modifications associated with an increase in the number of identical genomic complements within the nucleus.

2. Methods

2.1. Plant material

Two different tetraploid (4*x*) genotypes of *P. rufum* (4082 and 3785, $2n = 4x = 40$) were initially crossed to generate a hybrid progeny. Genotype 3785 was an individual plant collected from a native population, 39 km north of Paso de los Libres, Argentina. It reproduces by facultative apomixis of the aposporous type (unpublished data), and was used as the pollen donor for crosses. Plant 4082 was a highly sexual plant, with some potential for apomictic reproduction. It was obtained by colchicine treatment from a diploid individual. This 4*x* plant was the female parent in our controlled crosses. Two individual plants of the 4082 × 3785 progeny were chosen for study: one tetraploid and one hexaploid. The hexaploid probably arose as a consequence of the fertilization of an unreduced egg cell from the mother (4082) by reduced pollen from the pistillate donor (3785) to form a BIII ($2n + n$) hybrid (a relatively frequent event in apomicts). Both parents, the hexaploid offspring (PR5) and a tetraploid offspring (PR9) were selected to conduct molecular marker studies.

Also a *P. notatum* diploid (C4-2x, $2n = 2x = 20$) and its related autotetraploid (colchiploid) (C4-4x, $2n = 4x = 40$) were studied [32]. To produce the diploid and tetraploid genotypes, embryogenic calli were induced from young inflorescences of a natural diploid genotype (P2) and then treated with colchicine [32]. Sections of different ploidy levels were selected from the same callus by flow cytometry and sub-cultured to plant regeneration medium. Ploidy levels and complete euploidy of the regenerated plants were confirmed by chromosome counting in root tips squash [32].

2.2. Random amplified polymorphic DNA (RAPD) analysis

Genomic DNA was extracted from 4 g of fresh leaves by using the method of Dellaporta et al. [33] including the modifications introduced by Ortiz et al. [34]. Sample quality was checked by measuring UV absorbance ratios at 260 nm/280 nm and running 1% (w/v) agarose gels, to confirm DNA integrity and the absence of RNA contamination. Each RAPD amplification was performed in a reaction volume of 25 μl containing 30 ng of the primer, 20 ng of genomic DNA, 1X Taq polymerase reaction buffer (Promega), 15 μM each dNTPs, 1.5 mM magnesium chloride and 1 U of Taq polymerase (Promega). The decamers from the University of British Columbia (series 3 and 4) were used to generate DNA profiles. Amplifications were carried out in a MJ Research Thermocycler programmed as follows: an initial denaturation at 93° for 1 min, 45 cycles of 1 min at 92°, 1 min at 36°, 1 min 30 s at 71° and a final extension step of 5 min at 72°. Negative controls were included to discard contamination of the reagents. Reliability and repeatability were assessed by running duplicate reactions. Amplification products were separated in denaturing 5% polyacrylamide gels, electrophoresed in 1X TBE and silver-stained. Gel images were digitized or recorded in APC film (Promega). Polymorphic bands were excised from gels, eluted, re-amplified and cloned in pGEMTeasy vectors (Promega).

2.3. Amplified fragment length polymorphisms (AFLP) analysis

The AFLP procedure was performed following the manufacturers' instructions for the AFLP Analysis System I (Life Technologies, GIBCO BRL) with minor modifications. About 900 ng of genomic DNA were simultaneously digested with *Eco*RI and *Mse*I. Fragments were ligated to *Eco*RI and *Mse*I adapters and constituted the template for further amplifications. Pre-amplification primers had one selective nucleotide. Pre-amplification products were diluted (1:10) in Tris 10 mM/EDTA 0.1 mM and used as templates for selective amplification. Twenty-nine combinations of the *Eco*RI and *Mse*I AFLP primers supplied by the manufacturer (containing three selective nucleotides) were used for selective amplification: M37E43, M37E40, M37E33, M37E36, M37E35, M33E40, M33E33, M33E36, M33E35, M35E36, M41E43, M41E40, M41E33, M41E36, M41E35, M40E43, M40E40, M40E33, M40E36, M40E35, M43E40, M43E36, M43E35, M38E40, M38E36, M42E40, M42E36, M42E35 and M39E40. An MJ Research Thermocycler programmed for the cycling profile as indicated in Vos et al. [35] was used for both pre-amplification and selective amplification. Reliability was assessed by the use of duplicate samples. Following amplification, the PCR products were mixed to the correct proportion with 3X loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95 °C for 5 min and immediately placed on ice. Aliquots of 5 μl of the denatured samples were loaded onto denaturing 5% (w/v) polyacrylamide gels. Amplification products were visualized by using the Silver Staining-System from Promega, and digitized. Polymorphic bands were excised from the gels, eluted, re-amplified and cloned in pGEMTeasy (Promega).

2.4. Sequencing and bioinformatic analysis

Sequencing of the RAPD and AFLP derived clones was done by Macrogen Inc. (Korea). Sequence similarity analysis was performed using the BLASTN and BLASTX packages of the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/). The position of related sequences onto the rice genome was determined after a BLASTN analysis with the SEQUENCES-BLAST tool on the Gramene web page (http://gramene.org/) with a maximum *E*-value for reported alignments of 0.0001 and using the 'allow some local mismatch' option.

2.5. Genomic Southern blots

DNA extraction was performed as indicated above (Section 2.2). DNA samples were quantified by measurement of UV absorbance at 260 and 280 nm. Genomic DNA (25 μg) was digested overnight with 2.5 U/μg of the restriction enzymes: *Eco*RI, *Hin*dIII, *Msp*I and *Hpa*II. Samples were electrophoresed in 0.8% agarose gels with 1X TAE and blotted onto nylon membranes using 10X SSC buffer. Non-radioactive digoxigenin labeling, hybridization and detection procedures were performed as indicated in Pessino et al. [36].

3. Results

3.1. Genome alterations during ploidy changes (4x to 6x) in P. rufum

Random amplified polymorphic DNA analysis [37] was initially used to compare the genomic structure of four related *P. rufum* genotypes with different ploidy levels. We analyzed the genetic structure of two tetraploid parental genotypes (4082 and 3785), a hybrid presenting the parental ploidy level (PR9, tetraploid) and a second hybrid showing an increased ploidy level with respect to the parents (PR5, hexaploid). Since the mother plant (4082) is a facultative aposporous apomict, the hexaploid possibly originated from pollination of a non-reduced (4*x*) egg cell with reduced pollen (2*x*) (a polyploidization pathway widely recognized for long time in apomicts). However, an eventual fertilization of a reduced egg cell with unreduced pollen cannot be excluded, and therefore this possibility was taken into account when analyzing the data.

Four different decamers generated 83 total RAPD markers, out of which 35 were polymorphic between samples. Banding profiles are schematized in Table 1. The tetraploid offspring (PR9) showed a typical profile, with 10 bands originated from the mother, 5 bands originated from the father, 18 bands originated from either one or both parents but absent in the offspring (involving loci heterozygous in both parents) and 50 monomorphic bands. The hexaploid offspring (PR5) showed an aberrant pattern of amplification. Since the hexaploid was necessarily generated either by: (1) fertilization of an unreduced egg cell with reduced pollen, or (2) fertilization of a reduced egg cell with unreduced pollen, the profile should obligatory adjust to only one of this situations. If it was the case that the hexaploid was generated by fertilization of an

Table 1
Genetic comparison of *Paspalum rufum* genotypes 4082 (4*x*), 3785 (4*x*), PR5 (6*x*) and PR9 (4*x*)

Q4082	Q3785	PR5	PR9	Pattern type	No.	T^a (%)	P^b (%)
+	+	+	+	1/1/1/1 (a)	48	57.83	–
+	+	–	+	1/1/0/1 (b)	2	2.41	5.71
+	–	–	–	1/0/0/0 (c)	3	3.61	8.57
+	–	+	–	1/0/1/0 (d)	1	1.20	2.85
+	–	–	+	1/0/0/1 (e)	5	6.02	14.28
–	+	–	–	0/1/0/0 (f)	3	3.61	8.57
+	–	+	–	1/0/1/0 (g)	2	2.41	5.71
+	–	+	+	1/0/1/1 (h)	5	6.02	14.28
–	+	+	+	0/1/1/1 (i)	5	6.02	14.28
–	+	+	–	0/1/1/0 (j)	4	4.82	11.42
+	+	+	–	1/1/1/0 (k)	5	6.02	14.28
Total					83		

Number of RAPD fragments of each banding pattern type and percentage of polymorphic bands.

[a] Percentages were calculated with respect to the total number of bands (83).

[b] Percentages were calculated with respect to the number of polymorphic bands (35).

unreduced egg cell with reduced pollen, all bands originated in the female should be present in the progeny plant. Patterns like b, c, e, i and j (see Table 1) were not compatible with this model. Alternatively, if the hexaploid had been generated from the fertilization of a reduced egg cell with an unreduced pollen grain, all bands originated in the male should be present in the progeny plant. Profiles like b, d, f–h did not fit this hypothesis. Therefore, amplification profiles were incongruent and did not correlate with those expected for a classic sexual process involving a uniparental non-reduced gamete and a quiescent genome behavior. The unexpected absence of bands should be imputed to genetic modifications associated with the ploidy level increase. If the unreduced gamete had been the egg cell, 19 bands had presented an anomalous behavior (22.89%). If the unreduced gamete had been the generative nucleus of pollen, 13 bands had presented an anomalous behavior (15.66%). Therefore, genetic polymorphisms ranged from ∼15 to 23%, depending on which one really was the unreduced gamete (male or female). Polymorphisms involved band loss. Novel bands absent in the parental genotypes but present in the progeny were not detected.

3.2. Genome alterations during ploidy changes (2x to 4x) in *P. notatum*

For analyzing the *P. notatum* diploid and its related autotetraploid (colchiploid), 27 decamer primers were used in RAPD amplifications. An average of 21 bands per primer was scored. Results obtained are presented in Table 2. Out of a total of 565 bands, 52 (9.20%) were variable between genotypes. Of this group, 88.5% were bands present in the diploid and absent in the tetraploid (1/0 type or bands that were missed during tetraploidization), while 11.5% were bands present in the tetraploid and absent in the diploid (0/1 type or bands that were gained during tetraploidization). Differences in the incidence of 1/0 versus 0/1 polymorphisms were highly significant ($\chi^2 = 30.76$, d.f. = 1, $p < 0.01$), based on an expectation of equal occurrence.

Interestingly, the majority of polymorphisms were revealed by four specific decamer primers (designated UBC 258, 271, 280, 355), which suggests that at least some of the regions rearranged during the polyploidization event may show a repetitive structure and be represented at numerous genomic loci. The polymorphism detection rate for each one of these four primers was ∼30–40%. Out of the remaining 23 primers, one revealed ∼20% differential bands, three detected polymorphism rates of ∼10–15%, another three, lower than 10% and 16 revealed no variation.

The genetic variation between the diploid and tetraploid (colchiploid) genotypes of *P. notatum* was also evaluated using amplified fragment length polymorphisms markers [35]. An example of a gel displaying the bands obtained by AFLP is

Table 2
Genetic comparison of *Paspalum notatum* genotypes C4-2x and C4-4x

C4-2x	C4-4x	RAPD			AFLP		
		No.	T^a (%)	P^b (%)	No.	T^a (%)	P^b (%)
+	–	46	8.14	88.46	79	8.23	83.16
–	+	6	1.06	11.54	16	1.67	16.84
+	+	513	90.80	–	865	90.10	–
Total		565	–	–	960	–	–

Number of RAPD and AFLP fragments of each banding pattern type and percentage of polymorphic bands.

[a] Percentages were calculated with respect to the total number of bands (565 or 960).

[b] Percentages were calculated with respect to the number of polymorphic bands (46 + 6 = 52 or 79 + 16 = 95).

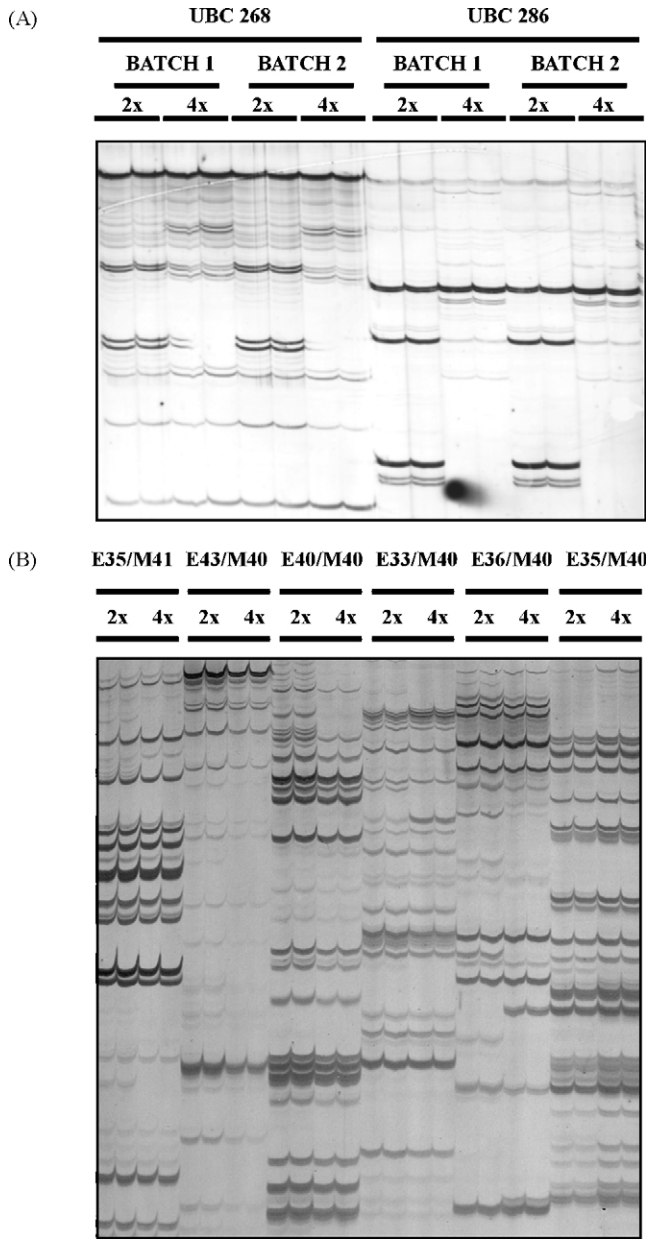

Fig. 1. RAPD and AFLP banding profiles obtained from the diploid (C4-2x) and tetraploid (C4-4x) genotypes. RAPD (panel A) and AFLP (panel B) reactions were run by duplicate. In the case of RAPDs, two different batches of each UBC oligonucleotides were used for amplification to test repeatability. Bands observed in both duplicate reactions from only one of the genotypes were selected, re-amplified, eventually sequenced and/or used as probes in genomic hybridization assays.

shown in Fig. 1. AFLP bands were scored as present or absent in each genotype for every primer combination. Polyacrylamide gels yielded an average of 80 bands per primer combination used. In total, 960 markers were reckoned and classified into the three types of banding patterns: 1/0, 0/1 or 1/1 (corresponding to bands present only in the diploid, only in the tetraploid or in both plants, respectively) (see Table 2).

The percentage of total polymorphisms detected was 9.90%, a similar figure to that obtained from RAPD analysis (9.20%). This result indicated that even when RAPD and AFLP polymorphisms were generated by different mechanisms (primer annealing site variation and restriction sites variation, respectively), the sequence alterations associated with ploidy changes seem to affect both similarly. The frequency of missing markers originated from the diploid genotype (83.16%) was again significantly higher than the frequency of gained novel bands in the autotetraploid (16.84%) ($\chi^2 = 41.77$, d.f. = 1, $p < 0.01$). However, AFLP markers showed a less pronounced bias in the values of polymorphism occurrence depending on the primer combination used. Considering RAPD and AFLP data together, the general polymorphism rate observed was 9.55%.

3.3. Cloning and sequencing of polymorphic bands

Four of the polymorphic RAPD bands from *P. notatum* (clones 1, 3, 10 and 11) were isolated, cloned and sequenced. BLASTX and BLASTN searches at the NCBI webpage revealed no similarities. A BLASTN analysis using the SEQUENCES-BLAST tool at the GRAMENE website revealed that the sequences derived from clones 3 and 10 displayed homology with non-coding genome sectors of unknown function in rice. They were found repeated 9 and 12 times in the rice genome, respectively (Table 3). The sequences derived from clones 1 and 11 presented no similarities in GRAMENE BLASTN searches and could not be located onto the rice genome (Table 3).

3.4. Genomic Southern blots

Genomic DNAs obtained from C4-2x and C4-4x were digested with four restriction enzymes and subjected to hybridization using the clones mentioned in the previous section (1, 3, 10 and 11) plus three additional AFLP polymorphic fragments (E36M402x, E36M404x and E40M402x) as probes. The enzyme *Hin*dIII is not sensitive to methylation changes of the recognition site, *Eco*RI is partially sensitive (when the next nucleotide flanking the recognition site is a G), while the isoschizomers *Msp*I and *Hpa*II recognize the same target sequence but present different sensitivity to its methylation status, allowing the detection of methylation at the central CG doublet. Hybridization profiles allowed discrimination if the fragments detected repetitive or low-copy genome sectors and if there had been any detectable modification in sequence and/or methylation status of the areas involved.

Informative hybridization patterns were obtained for clones E36M404x, E40M402x, 1 and 3 (hybridization of clones 10 and 11 failed). Clones E36M402x and E36M404x showed an identical hybridization pattern, revealing targeting to the same genomic regions. Both clones had been obtained from amplicons produced by the same primer pair, but while fragment E36M402x appeared only in samples which originated from the diploid plant, E36M404x was solely represented in those corresponding to the tetraploid derivative. The hybridization pattern consisted of several major bands on a smeared background, a common hybridization motif for highly/moderately dispersed repetitive elements (see Fig. 2A). Clone 1 showed discrete bands typical of low-copy sequences (Fig. 2B). Clone 3 presented a uniformly smeared pattern generally associated with the detection of dispersed middle-repetitive sequence elements (not shown).

Polymorphisms between C4-2x and C4-4x were observed when using *Eco*RI as restriction enzyme with both E36M402x and E36M404x. No polymorphisms were detected for clone 1 with the enzymes used. The repetitive nature of clone 3 impaired the detection of polymorphisms.

Methylation-sensitive restriction enzymes allowed the detection of polymorphisms between isoschizomers for all the clones tested, indicating that the genetically modified regions are always methylated. However, C4-2x and C4-4x presented an identical banding profile when patterns produced by the same isoschizomer were compared. Thus, the methylation status at these particular loci remained stable following polyploidization.

Table 3
Characterization of the polymorphic fragments isolated from the RAPD and AFLP experiments

Clone	Sequence accession number	Origin	GRAMENE SEQUENCE-BLAST results[a]	*E*-values[b]	Copy number onto rice genome[c]	Genomic hybridization in *Paspalum notatum*
PPMR1	EF137869	2*x*	Negative	–		Low copy
PPMR3	EF137870	4*x*	Positive	$2.3e^{-9}/8.3e^{-5}$	9	Repetitive
PPMR10	EF137871	4*x*	Positive	$4.3e^{-8}/7.1e^{-5}$	12	Failed
PPMR11	EF137872	4*x*	Negative			Failed
E36M402x	Not sequenced	2*x*	–	–	–	Repetitive
E36M404x	Not sequenced	4*x*	–	–	–	Repetitive

[a] Negative indicates that the sequence presented no significant homologies at the GRAMENE databases; positive indicates significant similarities.
[b] *E*-values informed correspond to the best and the worst hit. The rest of the hits presented intermediate *E*-values (maximum *E*-value for reported alignments used 0.0001).
[c] Total number of hits detected (maximum *E*-value for reported alignments used 0.0001).

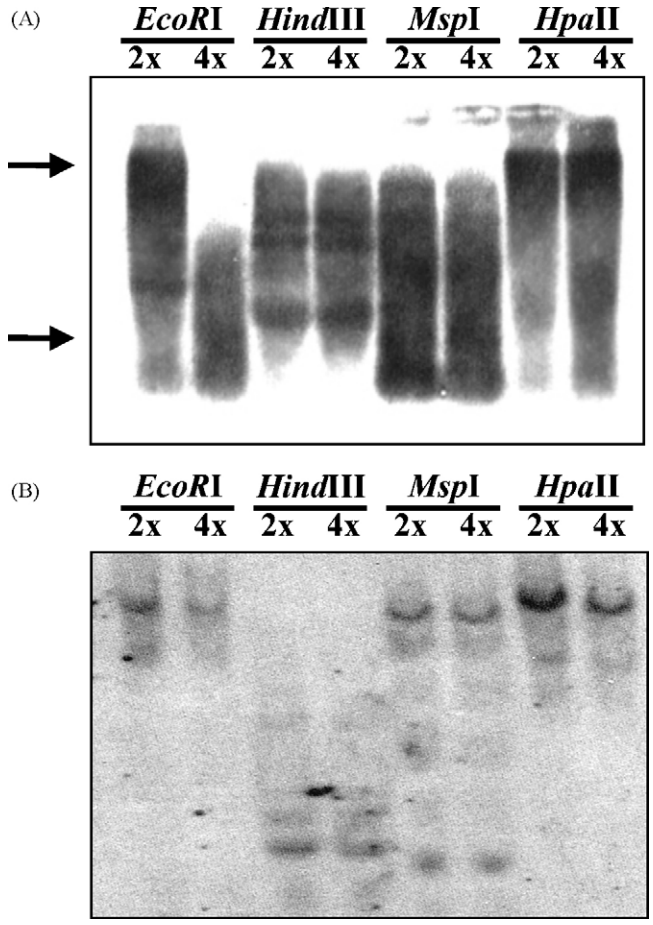

Fig. 2. Genomic hybridization blots of the diploid C4-2x and the tetraploid C4-4x *P. notatum* genotypes. Genomic DNA was digested with the corresponding enzymes (indicated at the top), electrophoresed and blotted onto nylon membranes. Hybridization with the polymorphic fragment E36M402x (Panel A) showed polymorphisms with *Eco*RI (see arrows) and was polymorphic between isoschizomers *Msp*I and *Hpa*II. A replica membrane hybridized with clone 1 (Panel B) revealed discrete bands with no polymorphisms for *Eco*RI and *Hin*dIII. Clear polymorphisms were detected between isoschizomers when using methylation-sensitive restriction enzymes (Panels A and B).

4. Discussion

The results shown here indicate that shortly after polyploidization either by natural or colchicine-induced mechanisms, a substantial fraction of the genome undergoes genetic modification in *Paspalum* sp. In the case of *P. rufum*, the shift from tetraploidy to hexaploidy (caused by fertilization involving an unreduced gamete) generated genome polymorphisms at a rate varying in the interval 15–23%. In the case of *P. notatum*, the conversion of a diploid into an autotetraploid by colchicine treatment originated sequence changes in 9.55% of the detected loci. These results are in agreement (although the percentages of polymorphisms are lower) with those reported for autopolyploids of *E. curvula* [20], where ca. 28% of the loci presented variation after doubling the ploidy level from 2*x* to 4*x*.

Both RAPD and AFLP were suitable and equally efficient to identify and quantify genome variation in the species. Only a small group of RAPD primers allowed detection of polymorphisms, which suggests that at least a part of the sequences affected might be repetitive. However, AFLP markers showed a less pronounced bias in the values of polymorphism occurrence in relation to the primer combination used. The latter might be explained considering the fact that each particular AFLP primer combination can possibly target several loci involving both repetitive and non-repetitive regions (the *Mse*I and *Eco*RI sites are probably represented in both types of sequences), while one specific RAPD decamer detects a longer sequence and therefore could be more selective when directed to one specific region type (alternatively repetitive or non-repetitive, depending on the primer sequence). While AFLP polymorphisms are evenly distributed for all the primers, polymorphisms originated from RAPD would be mainly produced by primers targeted to the affected repetitive regions. Total polymorphism rates would be similar for both techniques, since results originated from each RAPD primer are finally added prior to data analysis.

The frequent loss of bands observed during polyploidization initially suggested that at least a consistent number of genetic modifications might involve sequence elimination. However, sequence deletion could not be fully confirmed by genomic hybridization blots (see below), probably because several of the probes detected interspersed moderately repetitive patterns. For a RAPD or AFLP band derived from a large dispersed middle-repetitive family, the deletion of one or a few individual member/s would be likely to have minimal impact on the hybridization pattern. Before ruling out a role for sequence elimination during autopolyploidization a higher number of genome loci should be examined in detail using a more resolutive method. An alternative explanation that may account for the frequent loss of bands in both RAPD and AFLP experiments could be the de novo insertion of repetitive elements that are mobilized by ‘genomic shock’ events. Provided that the primer sequence and the *Eco*RI/*Mse*I restriction sites are not represented in the repetitive sequence, de novo insertion would not usually create new primer annealing sites but might separate the existing sites to the point at which the size of the fragment is unsuitable for amplification.

The DNA content of diploid *P. notatum* var. *saurae* was estimated on 0.57 pg/C (or 554 Mbp) [38]. If *P. notatum* holds a typical grass genome, it must be rich in repetitive sequences, and highly methylated. In fact, for all loci examined methylation-sensitive genomic hybridization analyses revealed methylation. The methylation status remained unmodified following polyploidization, at least at the sites targeted by the restriction enzymes used. Methylation-sensitive amplification polymorphism (MSAP) [39] analysis should be used to perform a global characterization of the genome methylation status (analysis here was restricted to specific loci that had been modified in sequence following polyploidization).

In the particular case of the *P. notatum* C4-2x and C4-4x genotypes, an obvious and critical question is whether the use of colchicine and tissue culture techniques might have affected the genome structure and be the origin of at least a proportion of the polymorphisms observed. Several recent reports in the literature indicate that tissue culture techniques/colchicine

treatments used in the generation of autopolyploids do not produce alterations in the genetic constitution of the genotypes; however epigenetic modifications and phenotypic variation can be detected. Analysis of DNA sequence variation in plants generated from embryogenic callus of grapefruit stored in vitro by slow-growth culture methods detected no RAPD polymorphisms with 102 primers used (provided that the ploidy level had remained constant). However, MSAP [39] showed DNA methylation changes in the stored samples compared with the controls [40]. The analysis of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organogenic calli showed that no polymorphic AFLP bands were detected in the derived plants. However, epigenetic changes were detected by MSAP [41]. In *E. curvula*, tissue culture followed by colchicine treatment did not generated genetic polymorphisms between genotypes, since colchiploids obtained from parallel polyploidization events presented an almost identical genetic structure [20]. However, in agreement with the results reported by hop and citrus, the same genotypes presented differences in their general methylation patterns (Silvina Pessino, unpublished results). Moreover, in wheat several synthetic allopolyploids exhibited the same pattern of sequence modifications, independently if they had been generated spontaneously, by tissue culture or colchicine treatment [13]. Altogether, these results suggest that genetic modifications are probably induced by polyploidization itself and appear to involve diverse mechanisms in different taxa, which present variable polymorphism rates. Genetic modifications could be related to a general phenomenon of stabilization of the new polyploids.

The inflorescence transcriptomes of genotypes C4-2x and C4-4x were fully characterized and compared in a former study using differential display analysis [42]. From around 10,000 transcripts analyzed, 64 (representing 48 unigenes) showed differential expression between the diploid and the tetraploid lines. None of the polymorphic bands isolated here corresponded in sequence to those genes whose expression had been found altered between the diploid and tetraploid genotypes [42]. Conversely, the sequence and the methylation pattern remained unmodified around several of the loci (always methylated) presenting differential expression between C4-2x and C4-4x [42]. The overall data reported by Martelotto et al. [42] and here indicate that an increment in the ploidy level gives rise to sequence alterations in methylated regions. These genetic alterations are immediately followed by a partial repatterning of the transcriptome probably mediated mainly by cis- and trans-acting regulatory elements affecting other methylated loci that remain unmodified in sequence.

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