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A comprehensive analysis of gene expression alterations in a newly synthesized *Paspalum notatum* autotetraploid

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Abstract

Although the causes of novel variation in polyploids are not well understood, they could involve changes in gene expression through dosage-regulation, altered regulatory interactions and rapid genetic/epigenetic changes. The objective of this work was to contribute to the elucidation of the patterns and mechanisms of gene regulation during the early stages of polyploid formation. The genome-wide expression of ~10,000 genes was monitored by using differential display transcript profiling on flowers of a diploid genotype and a newly-formed autotetraploid derivative. Differential expression of 64 clones was validated by reverse-Northern blot. Sequence analysis showed that 42 were homologous to 26 distinct genes of known function while the remaining 22 were classified as novel sequences. Genes detected were involved in processes of DNA repair, chromatin structure modification, regulation of transcription, proteolysis, protein folding, carbohydrate and lipid metabolism and signal transduction. Our results show evidence for gene expression alterations occurring immediately after polyploidization in *Paspalum*.

Keywords: Autopolyploidy; Differential display; Gene expression regulation; *Paspalum notatum*; Ploidy-regulated expression

1. Introduction

Although polyploidy has long been recognized as a prominent speciation and adaptation process in plants [1–6], recent investigations have shown that genome doubling has been also significant in the evolution of all vertebrates and in many other eukaryotes [7–11]. Angiosperms in particular have been the subject of considerable speculation about the frequency of polyploidy occurrence. Estimates vary regarding the proportion of them that have experienced one or more episodes of chromosome doubling at some point in their evolutionary history, but it is certainly 50% and might be more than 70% [12]. This prominence of polyploidy in flowering plants might imply some adaptive significance [13–15]. An increase in the number of genomic complements is often associated with the emergence of novel phenotypes not present in diploid progenitors [16–19]. Some of these traits, such as increased drought tolerance, apomixis (asexual seed production), pest resistance, flowering time, organ size and biomass, could allow polyploids to enter new niches or enhance their chances of being selected for use in agriculture [18].

The duplication of genomes, either of the same (autopolyploidy) or more frequently of divergent (allopolyploidy) origin, is therefore a major force of evolution that affects both genome size and gene copy number [14,16]. In connection with this, polyploidy raises a problem for gene regulation. The amount of product expressed is often critical for proper cellular function, and with all genes copied, the complex regulatory network may be modified in peculiar ways [20]. One of the best examples of such regulation is the silencing of the rRNA genes of one of the parental sets in

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amphiploids, termed ‘‘nucleolar dominance’’ (reviewed in [21,22]). Pioneer works on transgenic plants [23–25] showed that silencing occurred in duplicated genes. Moreover, it was also shown that a change in ploidy might affect transgene silencing in *Arabidopsis* [26].

Several investigations have addressed the response of the transcriptome to a change in the ploidy level. Galitski et al. [27] performed a systematic cDNA microarray survey on the *Saccharomyces cereviciae* transcriptome that anticipated the ploidy-regulated differential expression of several genes related to cell growth and cell development. In plants, Guo et al. [28] analyzed the expression of 18 specific genes in leaf tissue of a maize ploidy series from monoploid to tetraploid. Two works performed on synthetic and natural *Arabidopsis* allotetraploids ([29,30], respectively) involving a genome-wide analysis of gene expression using cDNA-amplified fragment length polymophisms (AFLP), reported on epigenetic changes and on gene silencing. In a similar survey Kashkush et al. [31] analyzed the events that affect gene structure and expression in the early stages of allopolyploidy in wheat. They observed rapid gene loss, either in the F_1 intergeneric hybrid or after chromosome doubling, gene silencing (in part associated with cytosine methylation) and transcriptional activation of retroelements. In a recent study Adams et al. [32] showed that, although many homoeologs contribute approximately equally to the transcriptome, a surprisingly high percentage of genes exhibit silencing or biased expression that is developmentally regulated, both in natural and synthetic polyploids. Furthermore, they showed that in a few cases alternative homoeologs had been reciprocally silenced in different organs, suggesting subfunctionalization. Analysis of differential expression patterns in diploid, autotetraploid and allotetraploid lines derived from *A. thaliana* and *A. arenosa* showed that the silencing of progenitors’ genes is rapidly and/or stochastically stablished [33]. A subset of genes present locus-specific susceptibility to ploidy-dependent gene regulation [33]. General evidence points to a commotion of genetic and epigenetic events that include genome reshuffling, (retro)transposon activity modulation and gene silencing affecting the recently formed polyploids.

The objective of this work was to analyze the transcriptome response at the early stages of autopolyploidy in the perennial grass *Paspalum notatum* to contribute to the understanding of the mechanisms involved in ploidy-mediated gene regulation. This species constitutes an attractive system for this study, since it presents natural diploid races reproducing sexually and autotetraploid ones reproducing by aposporous apomixis [34]. Particularly, analysis and comparison of the flower transcriptome representation in diploid and tetraploid plants may contribute to a better understanding of the molecular scenario where these alternative modes of reproduction (apomixis/sexuality) take place.

2. Materials and methods

2.1. Plant material

Plants used for gene expression and RAPD analysis were several different clonal individuals (obtained by vegetative propagation through rhizomes) of a *Paspalum notatum* diploid genotype (C4-2x, 2n = 2x = 20) and a newly synthesized autotetraploid derivative (C4-4x, 2n = 4x = 40). C4-2x and C4-4x were obtained by Quarin et al. [35] after colchicine treatment of diploid calluses induced in vitro from young inflorescences (see Fig. 1). Ploidy levels and complete euploidy was confirmed in C4-2x and C4-4x by chromosome counting in root tips [35]. The mode of reproduction was determined to be sexual for both genotypes by the study of megasporogenesis and embryo sac development [35]. All plants were grown in a greenhouse

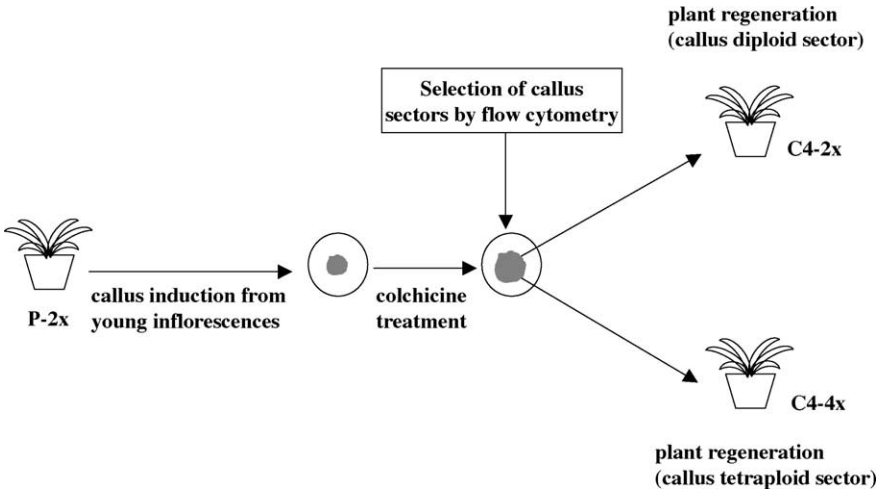

Fig. 1. Schematic representation of the experimental procedure used for the generation of the C4-2x and the C4-4x genotypes of *Paspalum notatum*. A detailed description of the methods used can be consulted in Quarin et al. [35].

at IBONE, Corrientes, Argentina, and maintained in identical conditions to assure reproducibility of the differential display analysis. A reproductive calendar defined by the microscopical analysis of several different pollen development stages was carefully established. Flowers from both genotypes (C4-2x and C4-4x) were collected when the anthers of the central spikelet in the raceme showed pollen mother cells (PMC stage).

2.2. Differential display assays

Total RNA was obtained from flowers by using the SV total RNA Isolation kit from Promega. Differential display experiments were conducted under the general protocol reported by Liang and Pardee [36] with minor modifications [37]. The anchored oligonucleotides used were named DDT1, DDT2, DDT3, and DDT4 and corresponded to the sequence $5'T_{(12)}$(ACg)X3′,where X was A, C, G, or T, respectively. One hundred and twenty decamers from the British Columbia University RAPD Primer Synthesis Project (sets 3 and 4) were used in combination with the anchored oligonucleotides to create primer pairs. Reverse transcription was performed using Superscript II reverse transcriptase (Gibco-Life Technologies) as indicated by the manufacturers. PCR reactions were prepared in duplicate in a final volume of 25 μl containing 1X Taq activity buffer (Promega), 1.5 mM $MgCl_2$, 50 μM dNTPs, 0.70 μM arbitrary primer, 2.5 μM anchored primer, 2 U of Taq DNA polymerase enzyme (Promega) and 2.5 μl of the reverse transcription reaction (previously diluted 1/20). Negative controls were carried out including total RNA non reverse-transcribed, to verify the absence of chromosomal DNA in the RNA preparations. The cycle program consisted of an initial step of 3 min at 94 °C, 40 cycles of 20 s at 94 °C, 20 s at 42 °C and 30 s at 72 °C, followed by a final step of 5 min at 72 °C. Samples were mixed with denaturing loading buffer, treated for 3 min at 95 °C and separated in a 6% polyacrylamide gel. Amplification products were silver-stained following the DNA silver staining system procedure (Promega) and recorded in a silver sequence APC film (Promega). Differential fragments were excised, eluted in a buffer of 0.5 M ammonium acetate and 1 mM EDTA pH 8, precipitated in ethanol, and re-amplified using the same PCR conditions described above. Several bands showing identical intensity between the diploid and tetraploid lines in both duplicates were selected, to be used as controls of equal expression. All fragments were cloned with the aid of the pGEM®-T Easy Vector System (Promega). Clones were named by indicating the anchored and the random primers used, the ploidy level of the individual presenting the band (2x or 4x) and a number indicating the relative position within the lane (if more than one band had been isolated from the same primer pair). Sequencing of the differential display cDNA clones was performed by Macrogen Inc. (Korea). Analysis of DNA similarity was carried out using the BLAST package 2.0 on the National Center of Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Reverse-Northern experiments

Inserts cloned in pGEM®-T Easy vector were re-amplified by using the M13 forward and reverse general primers as indicated in Pessino et al. [37]. Fifty microliters of the amplification reactions and 50 μl of DOT 2X solution (0.8 M NaOH, 20 mM EDTA) were mixed. Samples were denatured 5 min at 90 °C and put on ice. A piece of Hybond N + nylon membrane (Amersham-Pharmacia) was placed on pile of absorbent papers and covered with a dot blot plaque. Denatured samples were loaded in each well and allowed to be absorbed by capillary action. Ten microliters of Na(OH) 0.4 N were added to each well and filtered. Membranes were placed on an absorbent paper soaked in 2X SSC for 1 min and baked at 80 °C for 2 h. Total RNA (25 μg) was labeled with α^{32}PdATP by performing a reverse transcription with the enzyme Superscript II (Gibco-Life Technologies) in a total volume reaction of 50 μl. Four different labelling reactions were set up for the four anchored primers. Membranes were hybridized overnight with 10^7 cpm of the denatured radiolabeled probe (consisting of an mix of the four labelling reactions) and washed as indicated in Pessino et al. [37]. Biomax Kodak films were exposed overnight or longer and the signal intensity was evaluated by phosphorimaging (Storm Phosphorimager® Amersham Biosciences).

2.4. Genomic Southern blots

DNA extraction was performed as follows: approximately 6 g of fresh leaves were frozen in liquid nitrogen, powdered with a mortar and pestle and transferred to 50-ml polypropylene tubes containing 20 ml of extraction buffer (100 mM Tris–HCl pH 7.5; 700 mM NaCl; 50 mM EDTA pH 8; 1% (w/v) CTAB; 140 mM 2-mercaptoethanol). The mixture was incubated with gentle agitation at 65 °C for 60 min and then, 10 ml of chloroform/octanol (24:1) were added. Samples were mixed for 10 min and centrifuged at 5000 × *g* for 10 min. The aqueous phase was collected into a new tube and the extraction with chloroform/octanol was repeated. The aqueous phase was collected and DNA was precipitated by adding an equal volume of chilled isopropanol. Genomic DNA was collected with a glass hook, washed in a solution of 76% ethanol, 0.2 M sodium acetate for 20 min, air dried, resuspended in 700 μl of TE pH 8 (1 mM Tris–HCl, 0.1 mM EDTA) and incubated with 5 μl of a RNAase solution (10 mg/ml) at 37 °C for 30 min. Samples were re-precipitated with 5% (v/v) of 5 M NaCl and an equal volume of chilled isopropanol. Finally, DNA was centrifuged (20 min at 10,000 × *g*), washed thoroughly in 70% ethanol, dried in oven at 37 °C for 1 h and dissolved in 700 μl of ultrapure water. DNA samples were quantified by

reading absorbance at 260 and 280 nm using an Spectronic Genesys 5 spectrophotometer. 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 1% agarose gels/1× TAE and blotted onto nylon membranes using 10× SSC buffer. Non-radioactive (digoxigenin) labelling, hybridization and detection procedures were performed as indicated in Pessino et al [37].

3. Results

3.1. Detection of gene expression variations in flowers

Differential display banding patterns from floral samples of a diploid individual (C4-2x) and an autotetraploid counterpart (C4-4x) were compared in duplicated tests. Out of a total of 120 oligonucleotide combinations originally assayed, 80 were selected for further analysis based on the clear profiles they generated. Fig. 2 shows an example of the banding patterns detected in flowers of the diploid and the autotetraploid plants. Bands were scored only in the middle portion of the gel, where resolution was maximal and profiles were fully reproducible. Ploidy-altered candidate transcripts were distinguished by its clear differential signal between 2x and 4x samples, whereas surrounding bands were roughly equivalent. Most of the isolated bands showed a presence–absence pattern. Only in a few cases bands showing clear quantitative differences were cut, eluted and amplified. Table 1 summarizes the preliminary number of alterations detected in the polyacrylamide gels. The molecular weight of the segments obtained ranged from 100 to 600 bp. Out of the 129 candidate bands, only 90 were successfully eluted, reamplified and cloned.

3.2. Reverse-Northern blot validation of the detected expression changes

Clones isolated from DD experiments were subjected to reverse-Northern validation to discard false positives. We hybridized replica membranes (where identical amounts of the clone PCR amplifications had been loaded) either with labeled total RNA which originated from the C4-2x or the C4-4x genotypes. Because equal amounts of total RNA were used to probe the replica membranes, the intensity levels on the blots provide a measure of the specific representation of a given transcript per μg of total RNA. Several controls of identical expression between the diploid and tetraploid genotypes were taken from the DD gels and included in the blots.

Out of the total 90 transcripts assayed, 11 were undetectable. Fifteen cloned cDNAs that seemed likely to represent up- or down-regulated genes on the bases of the differential display analysis were shown by reverse-northern to be equally represented between 2x and 4x samples. The differential pattern of these cDNAs during differential display analysis could have arisen from any of several causes. For example, these cDNAs could have been subject to undetermined PCR artifact or they could represent minor background cDNAs that were co-electophoresed and

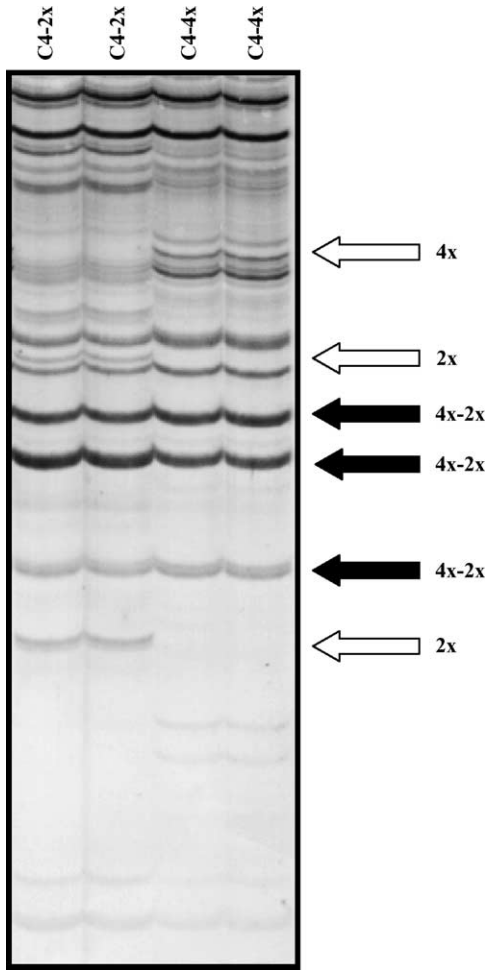

Fig. 2. Patterns of differential display amplification from flower tissue of the diploid and tetraploid plants. Assays were conducted by duplicate. White arrows mark polymorphic bands of the presence/absence type between C4-2x and C4-4x. Black arrows point bands equally represented in both samples, of the type of those that were isolated and cloned to be used as controls of identical expression in the reverse-Northern assays.

Table 1
Preliminary number of polymorphic bands that were scored in the differential display gels

Anchored primer[a]	Total bands analysed	Total alterations (%)
DDT1 [5′-$T_{(12)}$(ACg)A-3′]	2030	32 (1.57)
DDT2 [5′-$T_{(12)}$(ACg)C-3′]	2040	35 (1.71)
DDT3 [5′-$T_{(12)}$(ACg)G-3′]	2332	29 (1.24)
DDT4 [5′-$T_{(12)}$(ACg)T-3′]	3215	33 (1.02)
All primers	9617	129 (1.34)

[a] Each anchored primer was combined with 20 different random decamers to obtain amplicons.

co-eluted from the gel band. Alternatively, they might integrate a family of transcripts of similar sequence with only some members showing differential expression, which might obscure variation in the hybridization experiments. Only 64 of the original 90 cDNAs were confirmed to represent up- or down-regulated genes in the autotetraploid. Out of the clones obtained from presence–absence isolations, 71% survived the reverse-Northern validation. Meanwhile, only 20% of the clones obtained from differential bands were confirmed as true positives. Examples of the reverse-Northern experiments are shown in Fig. 3.

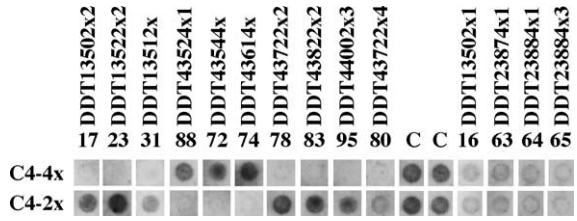

Fig. 3. Reverse-Northern blot hybridization patterns of the differentially expressed cDNA fragments. Each of the fragments was PCR amplified and dotted onto nylon membranes. Replicate membranes were prepared and hybridized to ^{32}P-labeled RNA from flowers of C4-2x and C4-4x (PMC stage). Clone number is indicated at the top of the figure. C: controls.

3.3. Molecular characterization of the isolated cDNAs

Sequencing of the 64 confirmed clones showed that 42 of them were homologous to 26 genes of known function (see Table 2). We found redundancy for some of the sequences, which were represented several times. The remaining 22 clones represented novel sequences. Comparisons were established from data bank information by using the BLAST package 2.0 and/or performing internal similarity searches among all cDNAs isolated.

3.4. Genomic Southern blots

Four cDNAs (corresponding to clones DDT13512x, DDT13522x2, DDT32844x1 and DDT32754x1) were used as probes for Southern hybridization with/without methylation-sensitive restriction enzymes, to address whether there has been any detectable modification in the genome around some of the genes that show expression alterations (Fig. 4). Genomic DNA from both plants (C4-2x and C4-4x) was

Table 2
Clones detected by differential display and validated by Northern analysis that showed homology to sequences stored in the data banks in the BLAST search

Clone ID	C4-2x[a]	C4-4x[a]	GeneID[b]	Gene name	*E*-value[c]
DDT12532x	+	0	AY108856	His kinase 1 Cytokinin receptor	4 e^{-71} (n)
DDT13502x2	+	0	AY110765	Carboxypeptidase D-like protein	8 e^{-32} (n)
DDT13512x	+	0	NP_911373	MutT-like protein	1 e^{-4} (x)
DDT13522x2	+	0	AK065618	Putative glucose 6P/P translocator	8 e^{-33} (n)
DDT13582x	+	0	AY106380	Putative anthranilate synthase	2 e^{-73} (n)
DDT13682x	+	0	AY104632	Putative F-box protein FBL4	1 e^{-98} (n)
DDT13702x1	+	0	AY111855	Putative DHHC-type zinc finger	1 e^{-43} (n)
DDT13702x2	+	0	AY110181	Histone H1-like protein	1 e^{-59} (n)
DDT23732x1	+	0	NM_185079	Hypothetical protein	4 e^{-21} (n)
DDT23732x2	+	0	AY109470	tetrafuctional protein of glyoxysomal fatty acid beta-oxidation	2 e^{-35} (n)
DDT23742x1	+	0	NM_191995	Putative receptor protein kinase-like protein	4 e^{-97} (n)
DDT23742x3	+	0	AJ295156	Putative nucleotide-glucose dehydratase	1 e^{-58} (n)
			AB079064	UDP-glucuronic acid decarboxylase	1 e^{-46} (n)
DDT23742x4	+	0	AY032604	ADP-glucose pyrophosphorylase small subunit	7 e^{-17} (n)
DDT23742x5	+	0	BA000029	Mitochondrial tRNA-Cys, tRNA-Asn and tRNA-Tyr	1 e^{-73} (n)
DDT23832x1	+	0	AY103975	Enolase	1 e^{-11} (n)
DDT23884x1	0	+	YP_095850	Acyl CoA dehydrogenase	5 e^{-65} (x)
DDT32834x2	−	+	AY082604	23S chloroplast ribosomal RNA gene	2 e^{-92} (n)
			AY096798	*Arabidopsis thaliana* At2g07707 mRNA sequence	5 e^{-71} (n)
DDT32844x1	−	+	AB014412	ubiquitin-conjugating enzyme	2 e^{-17} (n)
DDT32844x3	−	+	AY104730	cellulose synthase	1 e^{-11} (n)
DDT32844x4	−	+	AY105537	putative L34 ribosomal protein	6 e^{-5} (n)
DDT43524x1	0	+	NP_567403	TMS membrane family protein	4 e^{-07} (x)
DDT43614x	0	+	AY105892	Fts-H like protein	2 e^{-87} (n)
DDT43722x2	+	0	CAA71759	hypothetical protein	5 e^{-23} (x)
			XP_478243	chitinase III-like protein	4 e^{-22} (x)
			XP_476385	zinc finger and C2 domain protein-like protein	2 e^{-11} (x)
DDT43722x4	+	0	NM_197393	unknown protein	1 e^{-14} (n)
DDT43822x2	+	0	NP_916777	unknown protein	3 e^{-38} (x)
			XP_213249	ubiquitin-like protein	1 e^{-18} (x)
DDT43964x1	0	+	AY109456	DNA J related protein	7 e^{-51} (n)

[a] Characters 0/+ indicate a pattern of the presence-absence type, while +/− indicate clear quantitative differences in the reverse-Northern experiments.
[b] Gen-Bank accession numbers of the homologous sequences (only the best hits were listed).
[c] (n) and (x) indicate that homology was found with blastn or blastx, respectively. DNA sequences found by blastn searches were further characterized by blastx analysis.

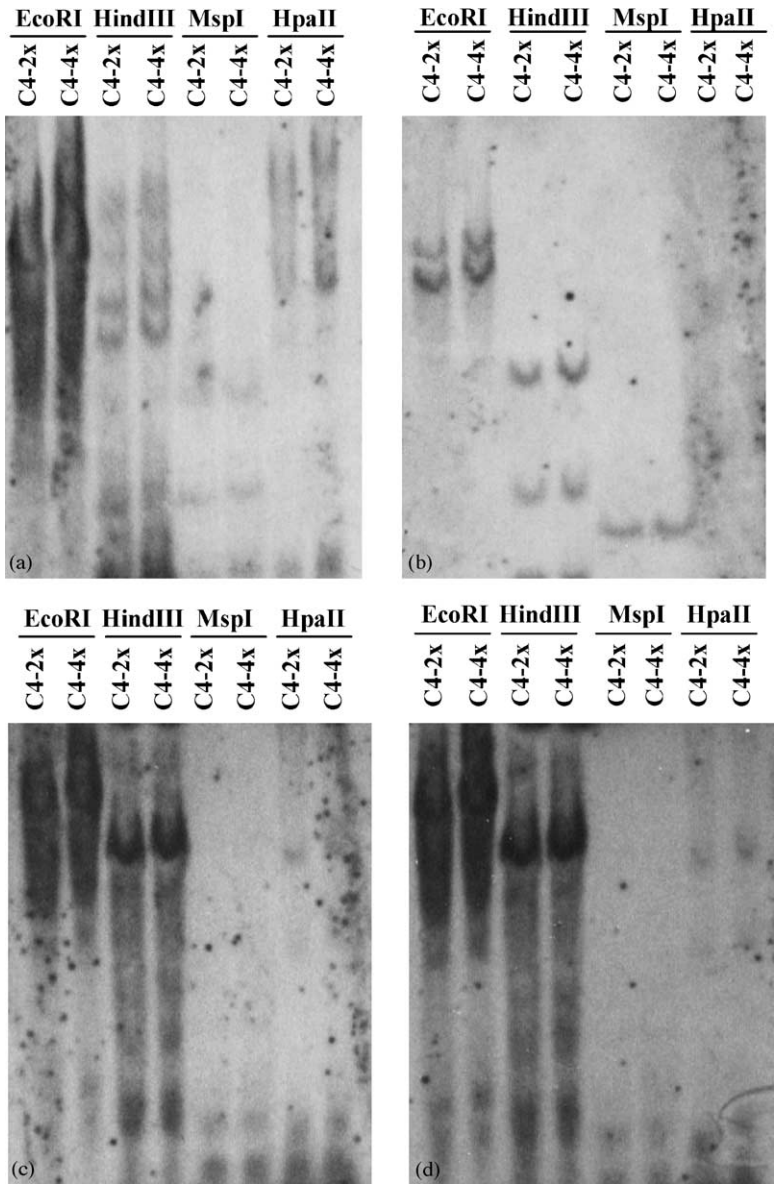

Fig. 4. Southern blot analysis of four genes that showed an alteration in gene expression in the autotetraploid plant. DNA samples of C4-2x and C4-4x were digested with *Eco*RI, *Hin*dIII and the two methylation sensitive isoschizomers *Msp*I and *Hpa*II, loaded on the gel side by side and hybridized with digoxigenin-labelled DDT13512x (a), DDT13522x2 (b), DDT32844x1 (c), and DDT32754x1 (d). Lack of bands in the *Hpa*II lanes hybridized with DDT13522x2 (panel b) might be related to the generation of high molecular weight fragments homologous to the probe that are usually not effectively transferred from the gel to the nylon membrane. Probe DDT32752x1 had presented an altered expression of the +/− type in the reverse-Northern experiments and shown no homologies in the blast searches. However, Southern blot hybridization using this probe and DDT34844x1 revealed an almost identical hybridization pattern (see panels c and d), suggesting that both clones might be detecting different sectors of the same gene.

digested with enzymes *Eco*RI, *Hin*dIII, *Msp*I, and *Hpa*II. Since all fragments assayed were low copy genes, Southern analysis was fully informative. We found that hybridization patterns were identical in all cases for both plants, suggesting that gene expression alteration in these cases is not associated with DNA rearrangements and/or changes in methylation at the restriction sites analysed. This observation suggests that, for these particular clones, dosage-regulation and/or altered regulatory interactions must be considered as a possible cause for expression changes.

4. Discussion

4.1. Functional classes involved in differential regulation

We report here a full survey of the flower transcriptome for the isolation of candidate genes that are differentialy expressed at 2x and 4x levels in *Paspalum notatum.* Out of the total 9617 transcripts analysed by differential display, the contrasting expression of 48 (26 known + 22 unknown) was finally validated in the reverse-Northern blot experiments, suggesting that at least ∼0.49% of the genes have

considerably altered their expression immediately after autotetraploid formation (~0.11% up-regulated whereas ~0.38% down-regulated). The estimate for down-regulated genes is of the same order of magnitude as found in *Arabidopsis* allotetraploids for silenced genes [29,30]. Although sequence analysis allowed us to assign identities for some of the ploidy-regulated transcripts, further studies should be performed in order to determine or at least infer, the biological significance of these observations and to associate a contrasting representation of the transcripts with particular characteristics of either genotype. The spectrum of the up/down-regulated genes did not show a trend for a particular class. However, several groups of genes could be defined in terms of functional specification. Genes differentially expressed are involved in processes as diverse as DNA/chromatin structure modification, regulation of transcription, proteolysis, protein folding, carbohydrate and lipid metabolism and signal transduction.

4.2. Colchicine treatment

The plant material used in this study was produced after colchicine treatment and tissue culture of diploid explants. In fact, nearly all the previous studies on expression and genetic changes directed by a change in ploidy have made use of such treatments in some step of the procedure to obtain the plant material [29–32]. A question that has been considered is whether the use of colchicine and tissue culture techniques might randomly affect genome structure and consequently modify the expression of genes. Previous studies demonstrated that polyploidization involves a shock that profoundly affects the structure of the genome through sequence gain and elimination (reviewed in [18]). In wheat, this phenomenon was found to be independent of the method of chromosome doubling [38]. A comparison of the genomic changes undergone by allopolyploids formed by colchicine treatment, tissue culture and others that arose spontaneously due to the fertilization of unreduced gametes proved that all types of allopolyploids exhibited the same pattern of sequence elimination [38]. Therefore, gene loss is apparently induced by polyploidization itself and might be related to a general phenomenon of stabilization of the new polyploids. Moreover, in other species like cotton, colchicine-induced allotetraploids showed complete genomic stasis [39]. These observations suggest that general genome modification is not associated with the colchicine treatment itself but it is related directly with the change of the ploidy level and involve diverse mechanisms in different taxa.

4.3. Differential expression of genes between C4-2x and C4-4x

A number of genes involved in different general cellular processes were found to be differentially expressed in both plants. Genes related to DNA/chromatin structure modification (like MutT and a histone H1-like protein) are expressed only in the diploid and not in the tetraploid. Molecules involved in protein trafficking and proteolysis (showing homology to ubiquitin carrier E2, ubiquitin itself, F-box ligand, FtsH protease and carboxypeptidase D) are also differentially represented in both transcriptomes. While ubiquitin, F-box ligand and carboxipeptidase D and are expressed only in the diploid, E2 ubiquitin carrier and FtsH protease are expressed only/overexpressed (respectively) in the tetraploid. Interestingly, several cDNAs corresponding to genes related to carbohydrate and lipid metabolism are detected only in one of the lines, for example, glucose 6P/P translocator, ADP glucose pyrophosphorylase, UDP glucuronic acid decarboxylase and a tetrafunctional protein of glyoxisomal fatty acid beta-oxidation are expressed only in the diploid, while cellulose synthase and acylCoA dehydrogenase are activated in the tetraploid. One gene related to cell-cycle regulation (histidine kinase 1 cytokinine receptor) is expressed only in the diploid, while other two (L34 ribosomal protein and TMS membrane protein) are overexpressed in the tetraploid. The molecular chaperone DNAJ is also expressed only in the tetraploid. Several putative transcriptional regulators (like a DHHC-type zinc finger and a zinc finger/C2 domain protein) were found to be expressed only in the diploid. A putative receptor protein kinase is also detected only in the diploid line. Cellular processes where these genes might be involved and possible functional interactions among the different groups are briefly discussed below.

4.3.1. Genes related to DNA/chromatin structure modification

Two transcripts can be included in this category: MutT and histone H1-like protein. The MutT homologs (MutT/MTH) are glycosylases that remove oxidized nucleotide precursors so that they cannot be incorporated into DNA during replication. MutT/MTH (along with MutM and MutY) protects cells from the mutagenic effects of 8-oxoG, the most stable and deleterious product known caused by oxidative damage [40]. This base excision repair pathway (BER) is proposed to protect cells and organisms from mutagenesis and carcinogenesis. Lack of MutT expression in polyploid individuals may either imply a higher potential for mutation or simply be a reflect of a reduced formation of reactive oxygen species (ROS) during metabolism.

Usually transcriptionally active chromatin tends to be deficient in histone H1, while the other core histones have a greater tendency to be modified by acetylation or by the attachment of ubiquitin. Here we detected an absence of transcripts enconding a histone H1-like protein in the tetraploid, and a concomitant alteration in the expression of genes involved in the ubiquitination system (ubiquitin, ubiquitin E2 carrier protein and ligand F-box), which might imply differences in the transcriptional activity of both lines.

4.3.2. Genes related to protein trafficking and proteolysis

An altered expression of several genes that might be involved in the turnover of cellular proteins and its trafficking was observed. Ubiquitination-dependent proteolysis is a major event during regulation of the cell-cycle progression, restructuring of chromatin and induction/execution of cell death. Three genes that belong to the ubiquitination system were isolated in our survey: ubiquitin, ubiquitin carrier E2 and F-box ligand. However, the pattern of expression seems confuse, because while the E2 carrier is overexpressed in the tetraploid, the remaining two are overexpressed in the diploid. By the other hand, the FtsH complex is an ATP-dependent group of proteases located in the stroma-exposed thylakoid membranes that were associated with the repair of photodamaged proteins in thylakoid membranes. FtsH proteases are homologous to proteases characterized in bacteria, and have many isomers in higher plants [41]. Finally, overexpression of carboxypeptidase D, an integral membrane protein that cycles between the trans-Golgi network and the plasma membrane, might be reflecting functional differences in the protein traffic regulation system between the diploid and tetraploid lines. Carboxypeptidase D cleaves C-terminal basic residues from proteins and peptides, functioning in the processing of proteins that transit the secretory pathway [42].

4.3.3. Genes related to carbohydrate and lipid metabolism

Several of the clones show homology to genes involved in the carbon partition pathway: Glucose 6P/P translocator and ADP-glucose pyrophosphorylase are overexpressed in the diploid, while cellulose synthase is overexpressed in the tetraploid. We also found an alteration in the level of expression of a transcript highly homologous to UDP glucuronic acid decarboxylase, the enzyme that catalyzes the conversion of UDP-D-glucuronic acid to UDP-D-xylose. Arabinoxylans in crop plants are the major sugar components of the cell walls, and UDP-xylose is a key substrate in their biosynthesis. Here, again a difference in the biosynthesis of cell wall components is detected between the diploid and tetraploid genotypes.

A tetrafunctional protein of glyoxisomal fatty acid beta-oxidation was found overexpressed in diploids while an acylCoA dehydrogenase is activated in tetraploids, which might indicate a higher rate of lipid oxidation and a lower rate of lipid synthesis in the diploid respect to the tetraploid. The biochemistry of both lines should be examined in the future to determine the validity of these speculations. Interestingly, an *Arabidopsis* mutant (pho3) show an increase in the expression of the plastid glucose 6P/P translocator, the expression of enzymes of starch synthesis and transcription factors/enzymes involved in anthocyanin biosynthesis [43]. Considering the fact that the diploid genotype studied here also overexpresses an anthranilate synthase, pho3 and C4-2x appear to show an alteration in the same biochemical pathways.

4.3.4. Genes related to cell cycle regulation

Three clones differentially expressed might be functionally related with the regulation of cell cycle progression: histidine kinase 1 cytokinine receptor, L34 ribosomal protein and TMS membrane family protein. The primary functions of genes for cytokinin-responsive His-protein kinases are triggering of the cell division and maintenance of the meristematic competence of cells to prevent subsequent differentiation until a sufficient number of cells has accumulated during organogenesis [44]. These genes encodes for primary receptors that directly binds a variety of natural and synthetic cytokinins in a highly specific manner. Histidine kinases can transduce cytokinin signals across the plasma membrane of *A. thaliana* [44]. The recent discovery of the *Arabidopsis* histidine kinase 4 (AHK4)/CRE1/WOL cytokinin receptor in *Arabidopsis thaliana* strongly suggested that the cellular response to cytokinins involves a two-component signal transduction system [45].

On the other hand, ribosomal protein L34 is expressed in actively growing tissues, including various meristems, floral organs and developing fruits. Mechanical wounding and plant growth regulators (cytokinins and auxins) increase the activity of the rpL34 promoter [46]. L34 was identified as a Cdk5-interacting protein that potently inhibits an activator of Cdk5 (p35). L34 also interacts with Cdk4 and, in parallel, inhibits the Cdk4/cyclin D1 activity [47].

4.3.5. Genes related to protein folding

We found a DNAJ-related protein overexpressed in the tetraploid genotype. DNAJ is a molecular chaperone and the prototypical member of the J-protein family. It regulates the activity of 70 kDa heat-shock proteins. Interestingly, the heat-shock protein 70 chaperone machine is functionally connected to the ubiquitin proteasome system (whose expression was found also altered in this work) by the co-chaperon CHIP. Recently, it was proved that DNAJ proteins may represent a further link between the cellular protein folding and degradation machineries. DNAJ proteins contain putative ubiquitin interaction motifs and can modulate the cellular processing of proteins that are targeted for degradation when they are misfolded [48].

4.3.6. Genes related to transcriptional regulation

A putative DHHC-type zinc finger protein and a zinc finger and C2 domain protein-like protein are likely transcriptional regulators whose expression was found altered. Further functional analysis will be necessary to determine which are the particular targets of these molecules.

4.3.7. Genes related to signal transduction

Two genes can be included in this category: histidine kinase 1 cytokinin receptor (already discussed in the cell-cycle regulation section) and a putative receptor protein kinase-like protein. Here again, no functional data is

available regarding the protein involved and specific studies on localization and expression will be required

4.4. Are changes in genome structure and gene expression conserved in different taxa?

Large rapid genetic changes were already reported in re-synthesized allopolyploids of *Brassica* species [49] and wheat [31,38,50–52]. Loss and gain of many DNA fragments were found in the generations immediately following polyploid formation. These changes sometimes occur in the F_1 hybrid generation following formation, prior to any treatment [31,38]. Moreover, the increase of transposable element activity associated with polyploidy raises the possibility of genome-wide modification by de novo insertion of (retro)transposons (reviewed in [16,53]). However, the argument for wholesale genetic modification with gene loss is still in dispute, since there is evidence that, at least for some species, the genome has remained essentially unchanged since polyploid formation [54].

In fact, each polyploidization event might represent a particular case to study genome modification and consequently changes in gene expression levels, which can be modified in a particular ways by insertion/deletion/epigenesis to generate variation. However, at least some general mechanisms and patterns of expression might be conserved among different polyploidization events and even among different taxa. These common changes will be probably identified by comparison of the results of independent research groups characterizing expression in different species.

In future works, we will analyze the expression of the identified genes in natural diploid and autopolyploid lines of *Paspalum notatum* as well as other related species, to determine if each one the differential modulations detected here constitute a general phenomenon and if they are conserved during the course of evolution.

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