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ORIGINAL PAPER

Expressed sequence-tag analysis of ovaries of *Brachiaria brizantha* reveals genes associated with the early steps of embryo sac differentiation of apomictic plants

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Abstract In apomixis, asexual mode of plant reproduction through seeds, an unreduced megagametophyte is formed due to circumvented or altered meiosis. The embryo develops autonomously from the unreduced egg cell, independently of fertilization. *Brachiaria* is a genus of tropical forage grasses that reproduces sexually or by apomixis. A limited number of studies have reported the sequencing of apomixis-related genes and a few *Brachiaria* sequences have been deposited at genebank databases. This work shows sequencing and expression analyses of expressed sequence-tags (ESTs) of *Brachiaria* genus and points to transcripts from ovaries with preferential expression at megasporogenesis in apomictic plants. From the 11 differentially expressed sequences from immature ovaries of sexual and apomictic *Brachiaria brizantha* obtained from macroarray analysis, 9 were preferentially detected in ovaries of apomicts, as confirmed by RT-qPCR. A putative involvement in early steps of *Panicum*-type embryo sac differentiation of four sequences from *B. brizantha* ovaries: *BbrizHelic*, *BbrizRan*, *BbrizSec13* and *BbrizSti1* is suggested. Two of these, *BbrizSti1* and *BbrizHelic,* with similarity to a gene coding to stress induced protein and a helicase, respectively, are preferentially expressed in the early stages of apomictic ovaries development, especially in the nucellus, in a stage previous to the differentiation of aposporous initials, as verified by in situ hybridization.

Keywords Apomixis · EST · Forage grass · Ovaries · Plant reproduction

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Introduction

In angiosperms, sexual reproduction begins with the formation of sexual spores via meiosis, producing microspores and megaspores, a phenomenon called sporogenesis. At gametogenesis, through successive mitoses, multicellular gametophytes are formed: in the anther, the microgametophyte or pollen grain, and in the ovule, the megagametophyte or embryo sac. The pollen grain contains one vegetative cell and two sperm cells, all haploids (n). The embryo sac contains seven cells: three antipodals, two synergids and one egg cell, haploids, and a diploid (2n) central cell. Double fertilization occurs in the ovule when one sperm cell fertilizes the egg cell and the other sperm cell fertilizes the central cell

giving rise, respectively, to the diploid zygote, the precursor of the embryo, and the triploid endosperm. Together with the surrounding maternal sporophytic tissue, they form the seed.

Among the asexual modes of plant reproduction, gametophytic apomixis comprises the autonomous development of embryos from an unreduced gamete. Gametophytic apomixis differs from sexual reproduction by formation of an unreduced embryo sac due to circumvented or altered meiosis during megasporogenesis (apomeiosis), and by embryo development without fertilization of the egg cell (Nogler 1984). The unreduced embryo sac can be originated from nucellar cells, in apospory, or from the megaspore mother cell, in diplospory (Nogler 1984).

Brachiaria is the most widely cultivated and economically important forage grass in Brazil (Araújo et al. 2008) which is the largest meat exporter country (CREMAQ 2010) and has most of its cattle herd raised under pasture conditions. Reproduction in *Brachiaria* is mainly by aposporic apomixis (Miles et al. 1996).

Apomixis is an agronomically desirable trait for modern agriculture, since it can be used to fix relevant multigenic characteristics in important crops. The financial benefit from cloning hybrids through apomixis, in rice only, was estimated at 2.5 billion US dollars per year (McMeniman and Lubulwa in Spillane et al. 2004). In contrast, switching apomixis to sexuality in *Brachiaria* is desirable for breeding programs, considering that it would contribute to increasing the genetic variability of this forage grass by allowing the use of hybridization techniques to generate new varieties. However, apomixis is still poorly understood, hindering efforts to develop apomictic cultivars in crops, such as maize, rice and wheat and to manipulate apomixis and sexuality in *Brachiaria* (Pinheiro et al. 2000; Araujo et al. 2005).

In aposporic *Brachiaria decumbens* and *Brachiaria brizantha*, a somatic cell of the nucellus, the aposporous initial (ai), after two successive mitoses, gives rise to an unreduced *Panicum*-type embryo sac, containing four cells: two synergids, one egg cell and a central cell, all 2n (Dusi and Willemse 1999; Araujo et al. 2000). Only the fertilization of the central cell is required to form the seed (Ngendahayo 1988; Alves et al. 2001). Autonomous embryo development occurs from the unreduced egg cell, generating identical progeny from maternal origin. Deregulation of genes associated with megasporogenesis could redirect the gametophyte development pathway towards apomixis. One evidence that supports this idea is that characteristics of ovary and embryo formation of apomicts were found in mutated sexual plants (Grossniklaus 2001; Tucker et al. 2003) and associations between important genes related to seed development and apomixis were made in *Arabidopsis thaliana* mutants (Ohad et al. 1996; Chaudurhy et al. 1997). An important process analogous to the first steps of diplospory was described in the *A. thaliana dyad* mutant where the megaspore mother cell forms an embryo sac without reduction by meiosis, apomeiosis (Ravi et al. 2008). Also in *A. thaliana*, a triple mutant genotype called *MiMe* had meiosis replaced by mitosis, forming an unreduced embryo sac, similarly to diplospory (d'Erfurth et al. 2009). Clonal reproduction through seeds, achieved by crossing mutants that form unreduced embryo sacs, *MiMe* and *dyad*, with a strain that eliminate chromosomes after fertilization (Marimuthu et al. 2011), raised expectations for engineering apomixis in important crops. In a process more similar to apospory, it was reported that *ago9* mutants (mutation on gene *ARGONAUTE 9*) differentiated into multiple cells in the nucellus, being able to initiate gametogenesis (Olmedo-Monfil et al. 2010). Recently, Singh et al. (2011) demonstrated that a maize mutant on a gene orthologous of the *Arabidopsis AGO9* (*AGO 104*) is defective in chromatin condensation during meiosis and produces unreduced viable megagametophytes.

Apomictic plants have common developmental characteristics (Ozias-Akins 2006) and the identification of megagametophyte related genes of apomicts can contribute to discover the gene expression programs involved in the regulation of this mode of reproduction. In apomictic plants, the main steps and the genes involved in the formation of megagametophytes are still poorly understood. Only a limited number of studies have been carried out on ESTs analysis of apomictic species, such as *Poa pratensis* (Albertini et al. 2004), *Eragrostis curvula* (Cervigni et al. 2008), *Panicum maximum* (Yamada-Akiyama et al. 2009), *Paspalum simplex* (Polegri et al. 2010) and *Boechera* spp. (Sharbel et al. 2010).

In *B. brizantha*, our group identified 11 ESTs, ten of them differentially expressed in ovaries of the apomictic BRA 000591, cv. Marandu, and the sexual BRA 002747 accessions (Rodrigues et al. 2003). Among these sequences, specificity to megasporogenesis and megagametogenesis was detected. Three of them were expressed mostly in synergids at late stages of embryo sac development; *BbrizMYO*, in apomictic and sexual plants, and *BbrizAQP* and *BbrizMAPK*, in apomictic plants. Their involvement in autonomous embryo development, a characteristic of apomicts, was suggested (Alves et al. 2007).

In this work, ESTs of ovaries before anthesis of sexual and apomictic *B. brizantha* were sequenced and annotated. For a more detailed insight into gene expression in the female development of *B. brizantha*, we examined gene expression patterns of 166 ESTs in immature ovaries, comparing sexual and apomictic *B. brizantha* using macroarray, Rt-qPCR and in situ hybridization. The putative role of the differential sequences during apomixis development in *B. brizantha* is discussed.

Materials and methods

Plant material

Two accessions of *B. brizantha* (Syn. *Urochloa brizantha*) from Embrapa's germplasm collection were used in this work: BRA 002747 (B105), a sexual diploid ($2n = 2x = 18$), and BRA 000591 (B030), a facultative apomictic tetraploid ($2n = 4x = 36$) named *B. brizantha* cv. Marandu, with up to 98% of apospory (Araujo et al. 2000). Both were cultivated in the field at Embrapa Genetic Resources and Biotechnology (Brasília-DF, Brazil).

RNA extraction and EST library construction

Four different EST libraries were constructed, two from ovaries of the sexual and two from ovaries of the apomictic *B. brizantha* at two different developmental stages: megasporogenesis and late megagametogenesis, as previously categorized (Araujo et al. 2000). Megasporogenesis includes stages I and II of ovary development, from megaspore mother cells to functional megaspore differentiation in sexuals and, in apomicts, it comprises differentiation of ai and degeneration of the tetrad. In late megagametogenesis, at stage IV, before anthesis, the embryo sacs are cellularized.

For each cDNA library, 70–90 μg of total RNA was extracted with TRIZOL® (Invitrogen™) according to Rodrigues et al. (2003). Poly $(A)^+$ RNA was purified using Dynabeads (Dynal®), according to the manufacturer's protocol. Samples were diluted in 2 μl of DEPC-treated water and the first strand cDNA synthesis was conducted.

The four directional cDNA libraries were constructed using the Creator SMART cDNA Library Construction Kit (Clontech), according to the User Manual, in pTriplex2 vector. After insert ligation into pTriplex2 vectors, the resulting plasmids were transformed by electroporation in *Escherichia coli* Xl1Blue strain. The white colonies grown on LB/Xgal/IPTG solid media were picked at random for sequencing. The libraries were named as follows: B030_IeII, of ovaries of apomictic plants at megasporogenesis; B030_-IV-, of ovaries of apomictic plants with mature embryo sacs; B105_IeII, of ovaries of sexual plants at megasporogenesis; B105_-IV-, of ovaries of sexual plants with mature embryo sacs.

EST library sequencing and analysis

After plasmid DNA isolation of inserts, the 2,976 purified plasmids were sequenced using the oligonucleotides: PT2 F2 (5′GCGCCATTGTGTTGGTACCC3′) and PT2R2 (5′ CCGCATGCATAAGCTTGCTC 3′ (Proite et al. 2007), respectively, and the Dye Terminator chemistry, on automated sequencers ABI 377 and ABI 3700 (Applied Biosystems) at Embrapa Genetics Resources and Biotechnology′s DNA sequencing platform. The first clones obtained, used for validation of libraries (768 EST) were sequenced with 5′ and 3′ end primers. The other 2,208 were sequenced with the 3′ end primer only. Base calling and quality analysis of individual bases of the ESTs were performed using the program PHRED (Ewing et al. 1998). For insert size determination, 192 clones were picked and, after PCR reaction using PT2F2 and PT2R2, analyzed by electrophoresis in 1% agarose gel.

Sequence analysis and annotation

Sequence trimming was done as described by Telles and Silva (2001), which resulted in removal of low-quality sequences, Poly$(A)^+$ RNA tails, ribosomal RNA, vector and primer regions. The sequences from the four libraries were then assembled into clusters using the CAP3 assembler (Huang and Madan 1998) to form unigenes. Sequence comparison using BLAST (Basic local alignment search tool; Altschul et al. 1997) was performed locally. The cutoff *e*-value of $<e^{-5}$ was used to define the similar homologues and the unigene sets that did not meet this requirement were annotated as unknown. The following databases were used in the annotation step: GenBank nr (Benson et al. 2008), MIPS *A. thaliana* (Schoof et al. 2004) and SwissProt (Gasteiger et al. 2001). Predicted protein sequences were aligned with BLASTx against KOG-Eukaryotic Orthologous Groups (Tatusov et al. 2003) and Gene Ontology (Ashburner et al. 2000). The output was parsed by locally developed PERL scripts where the EST assembly and the result of several alignments were available for manual curation. Results from BLAST against five databases were used in the manual curation for all the unigenes: these were NR, MIPS, KOG and SwissProt. In addition, other sequences that have already been described in the literature as related to plant reproduction development were used to align against the sequences from this work in order to find *Brachiaria* sequences that can be related to reproductive development.

Comparative analysis

For comparison of *Brachiaria* ESTs to rice pistil ESTs available in the genebank, a Digital Differential Display was performed by forming clusters for these two EST groups with TGILC (Pertea et al. 2003). This software uses the following parameters for the cluster assembly: 40 bp overlap, minimum of 95% of ID, maximum of 30 bp of mismatch. The clusters are executed using CAP3 (Huang and Madan 1998) for generation of consensus sequences. A second round of clustering and trimming was performed according to Pappas et al. (2008). In this step, clustering of 2,634 reads were submitted to a pre-processing pipeline

that starts with the base calling program PHRED (Ewing et al. 1998) followed by cloning vector removal with cross match (http://www.phrap.org), repeat masking with Repeat Masker (http://www.repeatmasker.org) and quality trimming with Lucy (Chu and Holmes 2001). The assembled ESTs were compared to *Brachypodium distachyon* ESTs (http://www.modelcrop.org/) and *Sorghum bicolor* gene models (http://genome.jgi-psf.org/Sorbi1/Sorbi1.home.html) by Bidirectional Best Hit according to Hulsen et al. (2006).

Macroarray preparation and analysis

To select the sequences to be blotted on to the membrane for hybridization, 166 ESTs were chosen mostly for their similarity to sequences already related to important processes of gamete differentiation. BLASTX and BLASTN results and analysis of their orthologous expression in *The Arabidopsis Information Resource (TAIR,* http://www.arabidopsis.org) were used. The sequences are listed in Supplementary Table 1. The clones were obtained from the cDNA libraries, except for clone 21 (Rodrigues et al. 2003). Three clones were chosen as controls: clone 21, actin and tubulin. These sequences were PCR-amplified using the same oligonucleotides used for sequencing the libraries, and blotted in duplicate onto Hybond N^+ membranes (Amersham Biosciences).

Four probes were prepared from 1 μg of Poly $(A)^+$ RNA from leaves and immature ovaries of both sexual and apomictic *B. brizantha*. Total RNA and Poly $(A)^+$ RNA were prepared as described for cDNA library construction, and probes were prepared according to Hotscribe first-strand cDNA labeling kit protocol (Amersham™).

Hybridization

The hybridization protocol and data analysis were previously established by Valle and Carvalho (personal communication). Membranes were pre-hybridized in 5× SSPE solution (20× SSPE = 3 M NaCl; 200 mM NaH_2PO_4; 20 mM Na_2·EDTA.$2H_20$, pH 7.4); 5× Denhardt's solution; 0.5% SDS, for 2 h, at 65°C. Hybridization was performed with the same solution, except for the addition of the probes. Membranes were hybridized for 16 h, at 65°C and washed in 2× SSPE solution/0.1% SDS and 1X SSPE/ SDS 0.1%, for 30 min each, at 65°C. Membranes were then exposed to high sensitivity films and then scanned for image analysis.

Image and statistical analysis

The images were obtained using Agfa DuoScan T1200, generating high-resolution images. Signal intensity was quantified using Zero-Dscan version1 (Scanalytics Division of CSPI, MA, USA), according to the manufacturer's instructions. Data were exported to MS Excel (Microsoft, Redmond, WA, USA), for statistical analysis, which was done according to Nogueira et al. (2003).

RT-qPCR

Oligonucleotides pairs were designed using Primer 3.0 program (Melting temperatures, Tm, varying between 59 and 60°C and primer lengths of 20–23 bp) for the 11 sequences with differential expression, detected by macroarray analysis (Supplementary Table 2). For RT-qPCR reaction, RNA was extracted from young leaves; from a pool of ovaries at the four stages before anthesis (I, II, III and IV) and from a pool of anthers at the corresponding stages of sexual and apomictic *B. brizantha* and 30 cycles were performed for each sample according to Silveira et al. (2009). PCR reactions were performed in 96-well plates with the Chromo4 Real-Time PCR Detector System (Bio-Rad®) using SYBR® Green to detect dsDNA synthesis. Reactions were done in 20 μL volumes containing PCR Buffer (Invitrogen™), 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 U Taq Platinum (Invitrogen™), 0.1X SYBR Green (Amersham™), 200 nM of each primer and 10 μl sscDNA (corresponding to 5 ng of total RNA). Reactions were run in a BioRad RT-qPCR device using the following cycling parameters: 94°C for 5 min, 40 cycles of 94°C for 15 s, 60°C for 10 s, 72°C for 15 s and 60°C for 35 s. No-template controls (NTC) were included for each primer pair, and each PCR reaction was performed in triplicate. Dissociation curves for each amplicon and agarose gel were then analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 40 to 100°C and reading at each °C. Two biological replicates for each of the samples were used for RT-qPCR analysis, and three technical replicates were analyzed for each biological replicate. Relative gene expression was calculated using qBase software version 1.3.4 (http://medgen.ugent.be/qbase/). The reference gene used was *BbrizUBI*, previously described as the best reference gene for these samples in *B. brizantha* (Silveira et al. 2009). Also, for four sequences, to which the expression ratio between apomictic versus sexual ovaries was 2.5 times higher, RT-qPCR was performed using cDNAs synthesized from RNA extracted from ovaries at developmental stages I, II (megasporogenesis) and IV (mature embryo sac) (Araujo et al. 2000) for both sexual and apomictic *B. brizantha*, according to Silveira et al. (2009).

5′ Rapid amplification of cDNA ends (RACE)

A Marathon™ cDNA library from ovaries of *B. brizantha* (Alves et al. 2007) was used for 5′-RACE experiments.

Two specific oligonucleotides were designed for *BbrizSti1* and *BbrizHelic* in a first round of RACE reaction. A second round was done for *BbrizSti1* with two new specific oligonucleotides that anchored to elongate the 5′ region of these sequences. Specific oligonucleotides used are listed on Supplementary Table 3. The PCR cycle condition was: 94°C, 1 min; 5 cycles of 94°C, 30 s and 72°C, 4 min; 5 cycles of 94°C, 30 s and 70°C, 4 min and lastly 35 cycles of 94°C, 20 s and 68°C 4 min. *E. coli* was transformed with pGEM vector (Promega) containing the PCR products, and plasmid DNA containing insert was purified (Sambrook et al. 1989) and sequenced. The sequences obtained were analyzed and contigs were formed using phred, phrap and CAP3 programs.

In situ hybridization

In situ hybridization (ISH) was performed in sections of ovaries from the sexual and the apomictic accessions at all stages of female gametophyte development. Tissue preparation, embedding and in situ hybridization and post hybridization were carried out as previously described (Dusi 2001). The RNA probe was synthesized using the DIG RNA labeling kit (Roche) according to the manufacturer's protocol. PCR fragments of 150–300 bp from 3′ coding regions of *BbrizSti1* and *BbrizHelic* were cloned into pGEM-T Easy Vector System I (Invitrogen™ life technologies) and used as a template for in vitro transcription with T7 and SP6 polymerases, used as sense and antisense probes, respectively. Sections were hybridized with 600 ng/mL DIG-labeled RNA probe, overnight, at either 42, 55 or 60°C. After washing and mounting, sections were observed with a Zeiss Axiophot light microscope. Before hybridization, a few samples were stained with acridine orange and examined under UV light for RNA integrity checking.

Results

Analysis of EST libraries

For bioinformatics analysis, the four EST libraries which were sequenced separately were gathered as a unique library of *B. brizantha* ovaries, containing ESTs from ovaries of the very early stage of development until ovaries before anthesis, with a bias for early-stage ovaries of apomictic plants. From a total of 2,976 clones sequenced, 2,198 (73.9%) showed high quality after trimming and were analyzed for function prediction. The majority of high quality EST reads (1,647) were derived from the library of ovaries of apomictic plants at megasporogenesis (B030_IeII), while 196 ESTs were from the library of ovaries containing mature embryo sacs of apomicts (B030_-IV-), 215 from the library of ovaries of sexual plants at megasporogenesis (B105_IeII) and 140 from ovaries of sexual plants with mature embryo sacs (B105_-IV-). These ESTs were assembled as 1,832 putative transcripts (contigs): 240 clusters and 1,592 singletons. The quality of the resultant combined libraries was confirmed by insert sizes. Of these, 28.6% corresponded to clones with inserts smaller than 500 bp, 69.2% with inserts between 500 bp and 1 Kb, and 4% with inserts larger than 1 kb. The most abundant ESTs contained six to 14 reads. The two contigs with the highest number of reads are similar to a hypothetical protein and a no-hit sequence. The encoded protein shows high similarity to hypothetical proteins of *Medicago trunculata* (gb|ABO81861.1|) *and Oryza sativa* (gb|DQ167400.1). Out of 1,236 contigs annotated, the majority (71.6%) had no hits to well characterized proteins described in five public databases (NR database, MIPS database, KOG, Swiss-Prot and Pfam) and thus could not be classified into protein family groups. The remainder of the contigs (28.4%) showed significant similarity to already described proteins and were divided into categories in relation to their predicted function in KOG (Fig. 1). According to the eukaryotic orthologous group database (KOG), 257 contigs encoded predicted hypothetical proteins. From the five main KOG groups, ESTs from ovaries of *B. brizantha* showed high similarity to proteins related to processes and signaling (35.8%), as shown in Fig. 1. These groups include 25 functional categories (available in KOG), and these libraries have contigs distributed among 23 of them, ranging from 0.4 to 16.7% (Fig. 1).

An extensive search of sequences already described in the literature was performed in order to create a database of sequences related to reproductive development and to compare it to the sequences generated in this library. Among the annotated ESTs, some encoded proteins were similar to proteins known to be involved in sexual and apomictic megagametophyte development. Ortholog candidates of these sequences were selected in order to check their involvement either in sexual or apomictic development (Supplementary Table 1). Some *B. brizantha* contigs were similar to sequences related to plant reproductive development, especially in *A. thaliana*. Information available in the literature and, mostly, in *The Arabidopsis Information Resource (TAIR;* http://www.arabidopsis.org) were used to make this relation between *Brachiaria* sequences and their possible involvement in reproductive development.

Seven contigs showed similarity between 82 and 95% with MADS-Box genes from monocots using BLASTN and BLASTP. One of those contigs represented a full-length coding sequence with 1,159 bp and 384 deduced amino acids (B030_IeII001_G05; Supplementary Table 1).

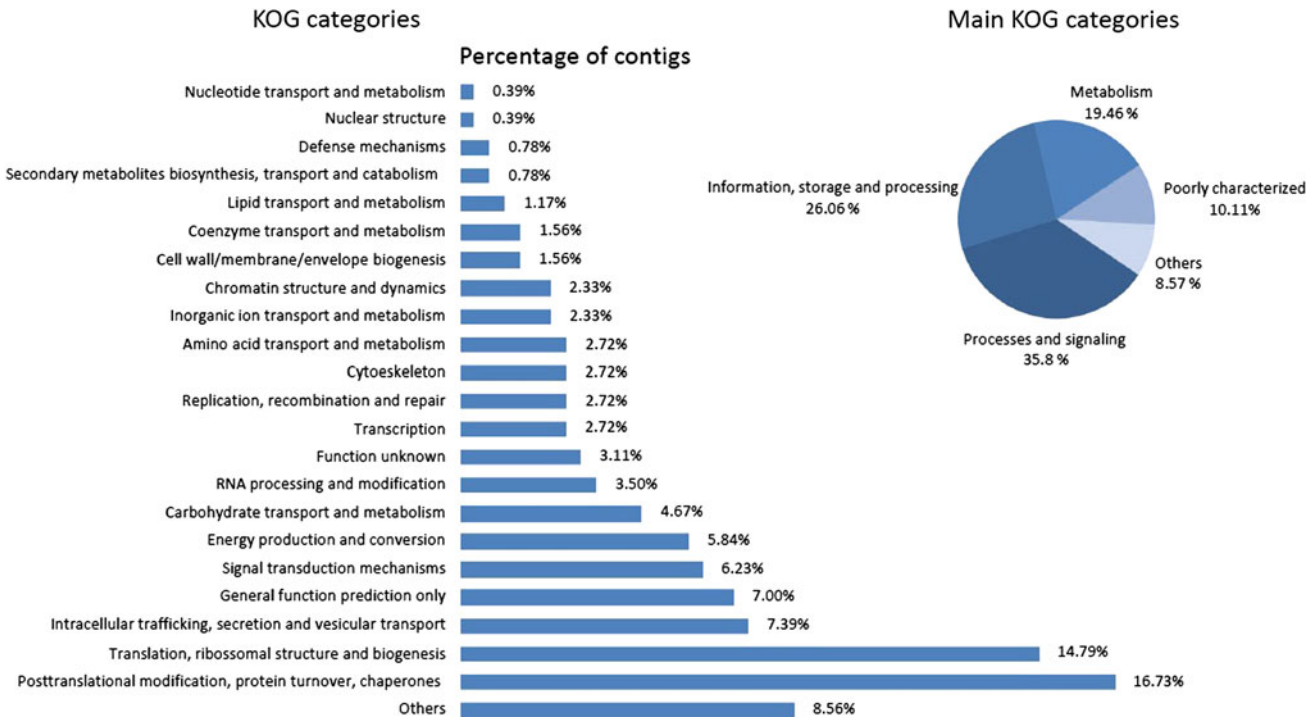

Fig. 1 Functional classification of the 257 annotated contigs among the main KOG categories and subcategories

This sequence showed similarity to the ZAG gene family (for *Zea mays* AGAMOUS), with 51/67 (76%) identity, and whose functional analysis has not yet been described in the literature.

The EST data showed six contigs that have similarity to helicase proteins. One of them is similar to a SNF2/SWI2-like protein of 768 bp sequence, which belongs to a chromatin remodeling family, showing an *e*-value of $2e^{-9}$, and identity of 28/36 (77%) to *O. sativa Os02g0114000* gene (B030_IeII_033_C07). Also, a chromatin remodeling factor family member, of 582 bp, showed the best hit to the *O. sativa Os04g0692700* and a *CHR11* of *Arabidopsis*, with *e*-value of $9e^{-09}$, and identity of 32/70 (45%), (B030_IeII 007_C04; Supplementary Table 1). This gene is involved in haploid nuclear proliferation during megagametophyte development (Huanca-Mamani et al. 2005). Two contigs showed similarity to *SKP1*-like genes and also an *ARGONAUTE* gene, both involved in meiosis. The *SKP1* from *B. brizantha* showed its best hit to *O. sativa* (B030IeII010_D05; Supplementary Table 1) and *Medicago trunculata SKP1*-like genes, with *e*-value of $3e^{-09}$ and $1e^{-11}$, and identity of 43/52 (82%) and 34/46 (73%), respectively. The sequences were from 712 and 673 bp. The putative *Brachiaria ARGONAUTE* gene, a 579 bp sequence, is more similar to *Arabidopsis ARGONAUTE 4* and *9*, with an *e*-value of $1e^{-39}$ and identity of 77/102 (75%) and 78/102 (76%), respectively (B030_IeII007_H05; Supplementary Table 1).

Another gene previously characterized as important for meiosis regulation is the *DMC1* (*disrupted meiotic cDNA*), a highly conserved gene among all clades. The *B. brizantha DMC1* showed high similarity to *OsDMC1*, its best hit on BlastX. It is an 858-bp sequence derived from the B030_IeII library of ovaries of apomicts at megasporogenesis, with an *e*-value of $5e^{-99}$ and identity of 185/187 (98%) with *OsDMC1* protein (B030_IeII002_B09; Supplementary Table 1).

In addition, ESTs of chromosomal modification and transcriptional regulation, hormone-associated genes and stress-related proteins were identified (Supplementary Table 1) and could be related to those already described as being involved in reproductive development in other species.

For the comparison with the *Brachypodium* and *Sorghum* gene models, that would enrich the annotation of *Brachiaria* sequences, 1,576 clusters (219 contigs and 1,357 singlets) were generated by a second round of clustering and trimming according to Pappas et al. (2008). Most *B. brizantha* sequences showed similarity with the genomes of *B. distachyon* and, mainly, of *S. bicolor* (Fig. 2), according to the TGICL software used for this comparison (Pertea et al. 2003).

Macroarray analysis

Among the 166 ESTs encoding proteins putatively involved in megagametophyte development (Supplementary Table 1), a macroarray analysis was performed to check their differential expression in ovaries of sexual and

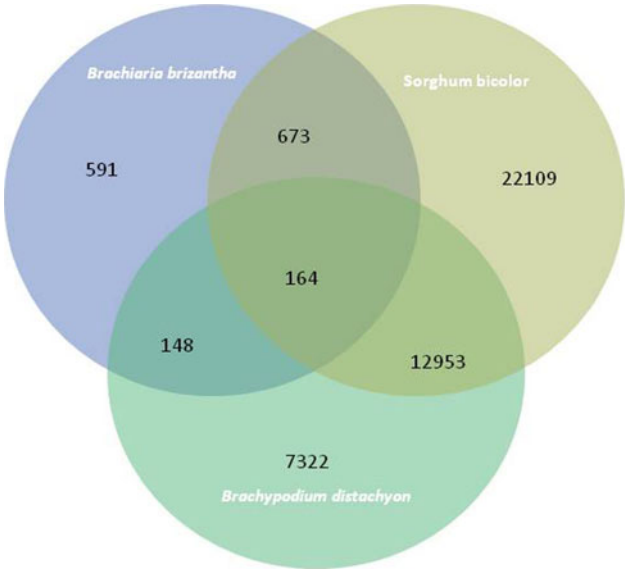

Fig. 2 Venn diagram showing the number of unique and shared genes amongst 1,576 ESTs from *Brachiaria brizantha* and ESTs from *Brachypodium distachyon* and *Sorghum bicolor*

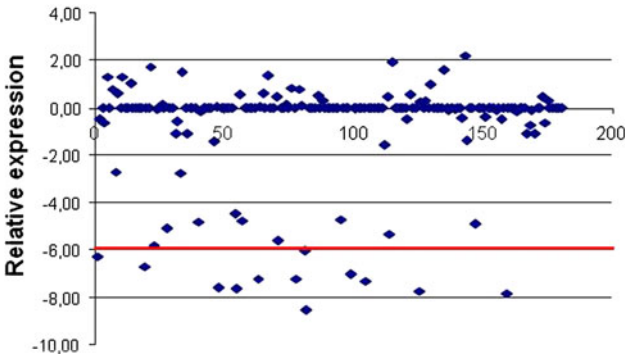

Fig. 3 EST dispersion graphic shows the relative expression in ovaries of sexual versus apomictic *Brachiaria brizantha*. *Dots* with relative expression under −6.00 correspond to the genes considered as differentially expressed

apomictic *B. brizantha* plants. Out of 103 ESTs (61.6%) that fulfilled selection criteria for signal detection, 11 are differentially expressed in sexual and apomictic ovaries of *B. brizantha* (Fig. 3). For sake of simplicity, these ESTs are named according to their best hit on sequence databases (Table 1).

RT-qPCR

To validate macroarray data and to investigate the expression profile of the 11 ESTs differentially expressed in sexual and apomictic ovaries of *B. brizantha*, RT-qPCR experiments were performed using RNA isolated from the reproductive organs (anthers and ovaries) and leaves. Among them, nine ESTs had higher expression in ovaries of apomictic than in sexual plants (Fig. 4), as confirmed by RT-qPCR. For a rigorous comparison of RT-qPCR results, in a previous work, we have identified in *B. brizantha* the best reference genes in samples equivalent to those used here (Silveira et al. 2009). In apomictic plants, a higher expression in ovaries than in leaves was confirmed for all EST tested, with an exception to *BbrizTom1*.

Considering only the four sequences with more than 2.5 times difference on the expression in ovaries of apomicts than of sexual plants, *BbrizHelic*, *BbrizRan, BbrizSec13* and *BbrizSti1,* a more detailed expression analysis throughout different stages of ovary development was performed using RNA from ovaries at megasporogenesis (stages I and II) and at mature embryo sac stage (stage IV), of both sexual and apomictic *B. brizantha* (Fig. 5). The higher expression of the four ESTs in ovaries of apomicts than of sexual plants was confirmed (Fig. 5). Interestingly, all four tested sequences showed higher expression in the early stages of apomictic ovaries development, mainly stage II. Among them, *BbrizSti1* and *BbrizHelic* were not expressed in ovaries of sexual plants, in any of the stages of development tested, suggesting involvement in cell development at the very early stages of apomictic differentiation and analyses of these sequences were continued.

5′ RACE and ISH for BbrizHelic and BbrizSti1

BbrizHelic and *BbrizSti1* were cloned from the EST library of apomictic *B. brizantha* ovaries at megasporogenesis (B030_IeII). *BbrizSti1* consists of a 1,259 bp fragment encoding a 345 amino acid polypeptide with high similarity to STI1 (stress induced 1) proteins, such as STI1 of *O. sativa*, *S. bicolor*, *Zea mays* and *A. thaliana*. *BbrizSti1* shows similarity to a putative stress-induced protein from rice (Os02g0644100), with e-value of $1e^{-54}$, and also with a putative stress-induced protein from *A. thaliana* (AT4G12400) with e-value of $1e^{-47}$. Interpro analysis (Mulder et al. 2003) showed the presence of two conserved domains: STI1 domain that interacts with heat shock proteins and TPR1 domain (D'Andrea and Regan 2003), related to protein–protein interaction.

The full length *BbrizHelic* was obtained also by 5′RACE. It consists of a fragment of 1,154 bp, with high similarity to the helicase-like protein from rice (Os01g0838 100). According to CDD bank analysis (Marchler-Bauer et al. 2002), *BbrizHelic* presented a RPA2_OBF family-like domain, characterized by having a DNA binding site. Members of this family are also associated with DNA replication, recombination and repair (Knoll and Puchta 2011).

The expression profile for *BbrizSti1* (Fig. 6) and *BbrizHelic* (Fig. 7) was analyzed by ISH in the course of ovary development of apomictic and sexual *B. brizantha*.

Table 1 Expressed sequence tags with differential expression determined by macroarray analysis in ovaries of sexual and apomictic *Brachiaria brizantha* plants

EST	e value ID	Description accession number genebank	Expression level (sexual related to apomictic)
*Bbriz*DMC1 B030IeII_002B09	$3e^{-99}$ 185/187 (98%)	DMC1 like protein—*Oryza sativa* (indica cultivar-group) JG437131	Repressed
*Bbriz*EF2 B030IeII_008E11	e^{-113} 201/209 (96%)	Elongation factor 2—*Oryza sativa* (japonica cultivar-group) JG436816	Repressed
*Bbriz*ERD B030IeII_005E11	$8e^{-11}$ 52/138 (37%)	Drought Stress Response Protein type ERD JK145790	Repressed
*Bbriz*Helic B030IeII_007B07	$4e^{-25}$ 55/115 (47%)	Helicase like—*Oryza sativa* (japonica cultivar-group) JG437003	Repressed
*Bbriz*Hyp B030IeII_002B08	$6e^{-06}$ 25/55 (45%)	Hypothetical protein—*Oryza sativa* (japonica cultivar-group) JG437130	Repressed
*Bbriz*MMF B030IeII_003F09	e^{-103} 189/221 (85%)	Mini Chromosome Maintenance Factor—*Triticum aestivum* JG437216	Repressed
*Bbriz*Nup B030IeII_007F07	$2e^{-47}$ 89/113 (78%)	Nucleoporin—*Hyacinthus orientalis* JG437436	Repressed
*Bbriz*Ran B030IeII_003H02	$1e^{-31}$ 67/68 (98%)	Ran related to GTP—*Zea mays* JG437228	Repressed
*Bbriz*Sec13 B030IeII010H12	$3e^{-26}$ 53/59 (89%)	Sec13 type protein—*Oryza sativa* (japonica cultivar-group) JG437606	Repressed
*Bbriz*Sti1 B030IeII_002C02	$8e^{-55}$ 104/116 (89%)	Stress induced protein, Sti1—*Oryza sativa* (japonica cultivar-group) JG437135	Repressed
*Bbriz*Tom1 B030IeII_005F07	$1e^{-70}$ 157/311 (50%)	Putative protein TOM1—*Oryza sativa* (japonica cultivar-group) JG437318	Repressed

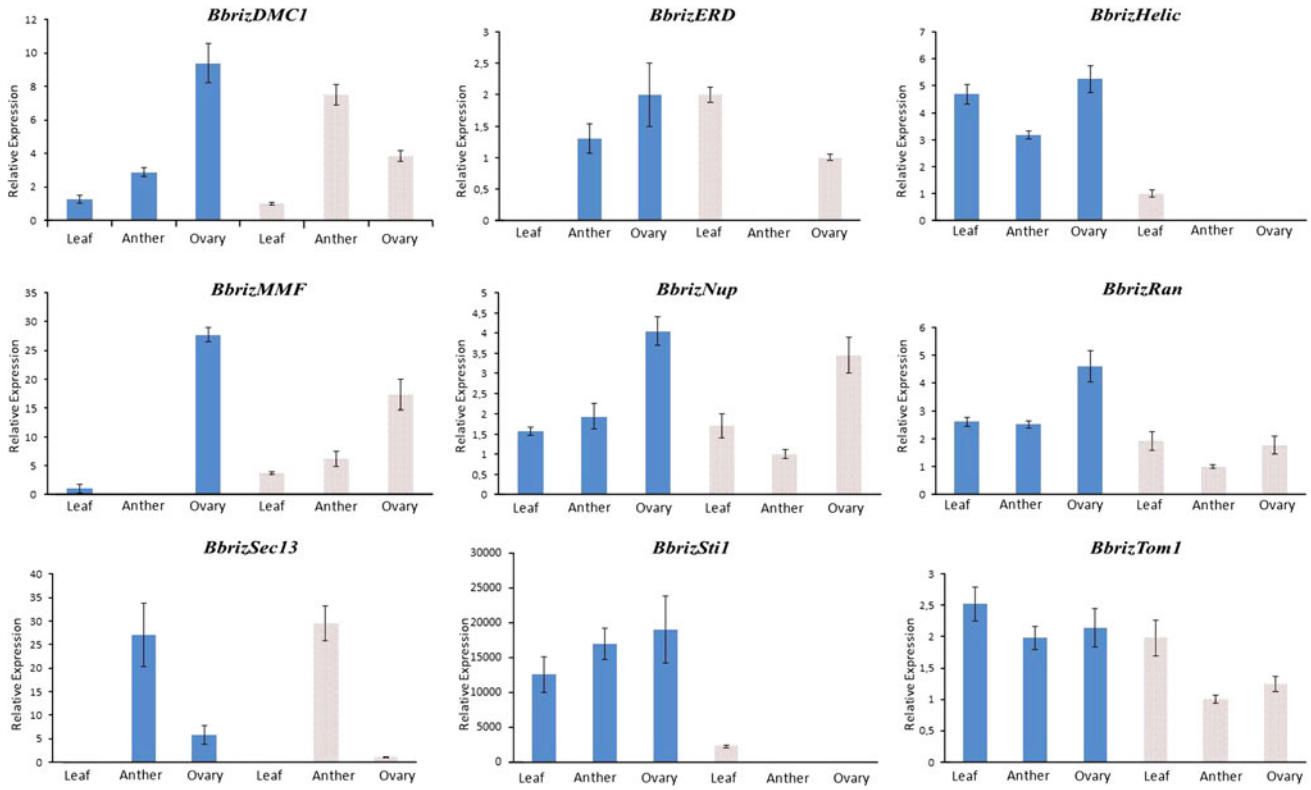

Fig. 4 Relative expression of nine genes in anthers, leaves and ovaries of apomictic (*dark bars*) and sexual (*hatched bars*) *Brachiaria brizantha*. Results represent the mean of three technical replicates of each sample. *BbrizUBI* was used as reference gene (Silveira et al. 2009). *Bar* 2× SEM (standard error of the mean)

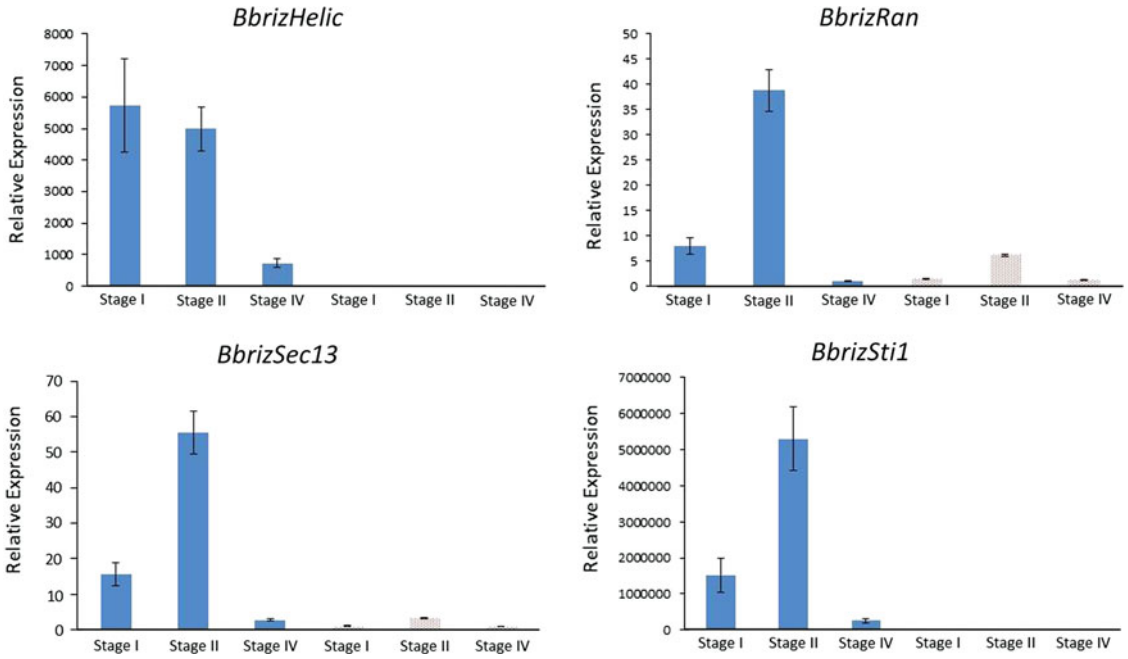

Fig. 5 Relative expression of four genes in ovaries of apomictic (*dark bars*) and sexual (*hatched bars*) *Brachiaria brizantha* at stages I and II of megasporogenesis and IV of megagametogenesis corresponding to mature embryo sac, according to Araujo et al. (2000). Results represent the mean of three technical replicates of each sample. *BbrizUBI* was used as reference gene (Silveira et al. 2009). *Bar* 2× SEM (standard error of the mean)

Acridine orange staining was done to assess proper preservation of RNA in tissue sections. To certify that the signal observed in ISH was not due to probe cross-hybridization, we performed the hybridization step using temperature of 42, 55 and 60°C. Antisense probes of *BbrizSti1* and *BbrizHelic* were detected in ovaries from megasporogenesis to late megagametogenesis (stages I–IV), in apomictic *B. brizantha*. In the most stringent hybridization condition (60°C), the signal was detected mostly in apomictic ovaries in megasporogenesis, corroborating RT-qPCR data. For *BbrizSti1*, at early stages of apomictic development, the hybridization signal was detected in all cells of the ovule but with higher intensity in the megaspore mother cell when compared to the surrounding nucellar cells (Fig. 6a). A stronger signal was detected in surrounding nucellar cells rather than in the ais during differentiation (Fig. 6b). At megagametogenesis, when the embryo sac is already formed, a signal was detected in integuments and nucellar cells, with a weak signal in synergids, central cell and egg cell of the embryo sac (Fig. 6c). At later stages, when the embryo is already formed, no transcript was detected with the antisense probe. No hybridization was observed with the sense probe at any stage of development (Fig. 6d).

In apomictic *B. brizantha*, ISH signal detection of *BbrizHelic* was similar to *BbrizSti1*. At stage I, the early stage of development, transcripts were detected in all the cells of the ovule, including megaspore mother cell and nucellar cells with a weaker signal in the integuments that are at the beginning of their formation (Fig. 7a). At later stages, although there was a slighter lower detection of the probe, it was possible to observe a signal in some cells of the nucellus (Fig. 7b). Sections hybridized with the sense probe did not show any signal (Fig. 7c). At stage IV, in the mature embryo sac, a signal was detected in nucellar cells but not in the inner and outer integuments. The synergids had some signal of hybridization (Fig. 7d). At this same stage, in an ovule with a larger embryo sac, a hybridization signal was weaker in the cells of the embryo sac when compared to the surrounding nucellar cells (Fig. 7e).

Discussion

The characterization of ESTs from ovaries of apomictic plants will contribute to a better understanding of the differences between sexual and apomictic reproduction pathways. Nonetheless, analyses of ovaries, where these differences occur, are very complex. This is especially true of grasses, due to their reduced size, presence of only one ovary per flower and the difficulty of access and isolation. To date, a few sequenced libraries of megagametophytes of apomicts are available (Albertini et al. 2004; Calderini et al. 2006). This paper shows for the first time the construction and analysis of EST libraries from ovaries of *B. brizantha*. The ESTs analysis combined with

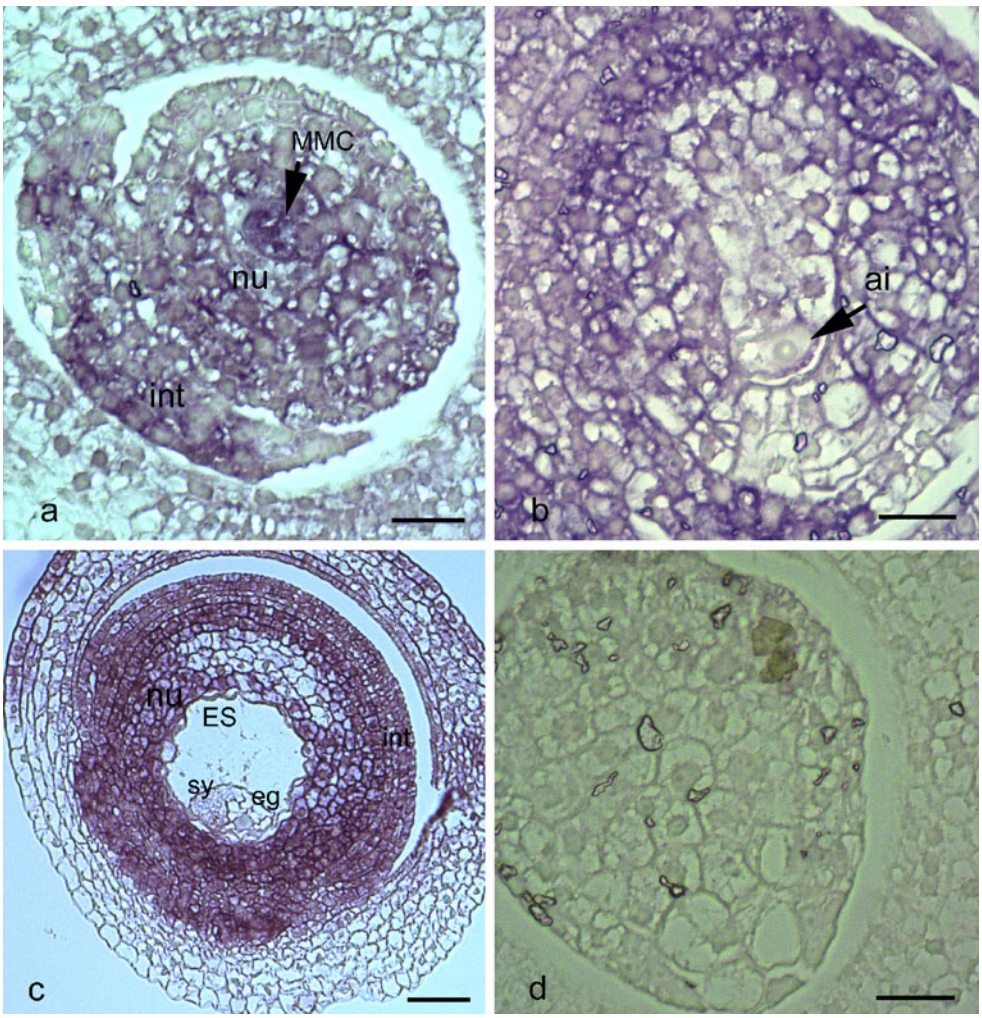

Fig. 6 In situ hybridization for *BbrizSti1* in ovaries from apomictic *Brachiaria brizantha* using antisense (**a**–**c**) and sense (**d**) probes: **a** ovary showing labeling in nucellus and integuments, and mostly in megaspore mother cell, **b** ovary showing no signal in aposporous initial, **c** ovary with mature embryo sac, showing high signal in integuments and nucellus and lower signal in egg cell and synergids, **d** ovary hybridized with sense probe showing no signal. *Bar* 20 µm. *ai* aposporous initial, *eg* egg cell, *ES* mature embryo sac, *int* integuments, *MMC* megaspore mother cell, *nu* nucellus, *sy* synergids

macroarray, RT-qPCR and ISH analyses provide valuable information for those interested in apomixis.

Most of the ESTs described in this work came from ovaries of apomictic *B. brizantha* at megasporogenesis, the point in time at which megaspores degenerate and ai differentiate. The high quality of the libraries produced from ovaries was demonstrated and a higher number of *B. brizantha* ESTs showed similarity to the *S. bicolor* genome when compared to *B. distachyon* genome. This result is in accordance with phylogenetic data, since *Brachiaria* and *Sorghum* belong to the same Panicoideae subfamily, while *Brachypodium* belongs to the Pooideae subfamily. In addition, *Sorghum* shows species with a 2n aposporic-type embryo sac (Hanna et al. 1970; Carman et al. 2011), similarly to *Brachiaria,* and common genes could be involved in this same aposporic process. The *B. brizantha* differential sequences previously described by Rodrigues et al. (2003) were not represented in the EST libraries (data not shown). Interestingly, most of the ESTs showed no hit to sequences already described in public databases. In addition, the two contigs with the highest number of reads are similar to a hypothetical protein and a no-hit sequence, pointing to the potential for novelty in gene discovery in these libraries. These results are in agreement with the data from *Paspalum simplex* (Polegri et al. 2010), in which most apomictic sequences also showed no hits to known sequences from public databases. The distribution into different functional categories on KOG suggests that the library obtained from the combination of four libraries of ovaries before anthesis is representative of the *B. brizantha* transcriptome complexity. The two categories that had the most abundant

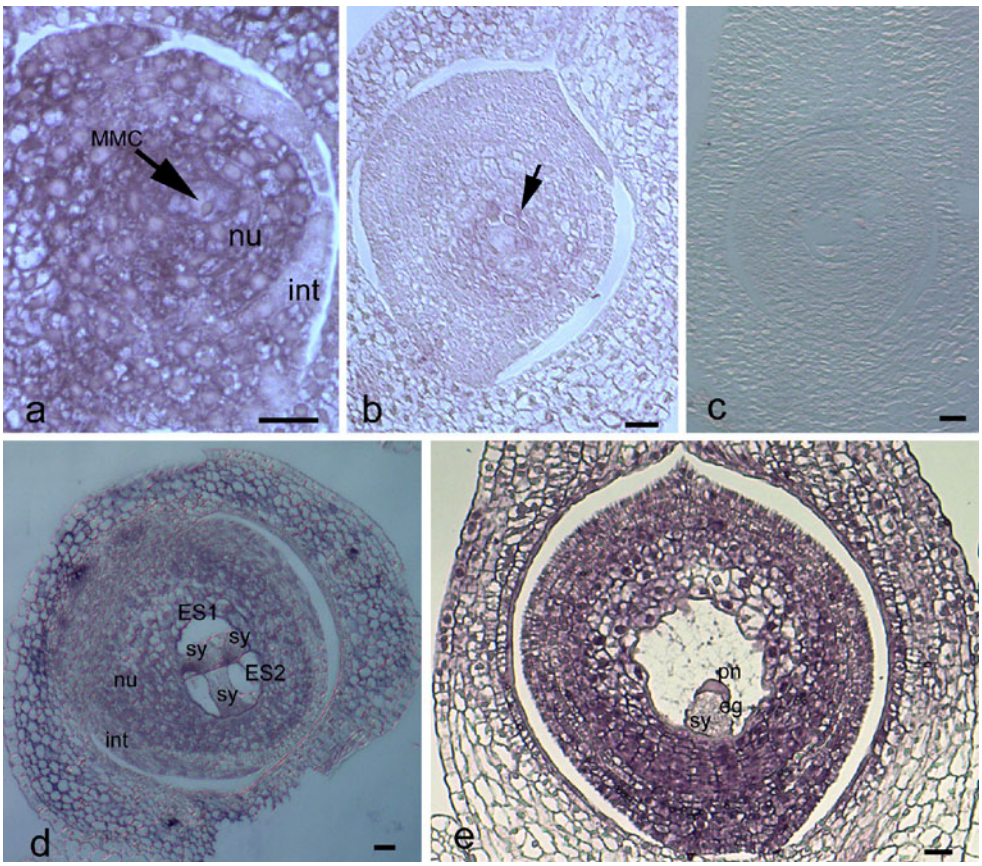

Fig. 7 In situ hybridization for *BbrizHelic* in ovaries from apomictic *Brachiaria brizantha* using antisense (**a**, **b**, **d**, **e**) and sense (**c**) probes: **a** showing labeling in nucellus, and megaspore mother cell, **b** showing stronger signal in some cells of the nucellus (*arrow*), **c** ovary showing no signal, **d** multiple mature embryo sacs showing signals at synergids and at nucellus, **e** mature embryo sac showing strong signal nucellar cells and weaker signal in the synergids, egg cell and in the central cell, near the polar nucleus. *Bar* 20 μm. *eg* egg cell, *ES* mature embryo sac, *int* integuments, *MMC* megaspore mother cell, *nu* nucellus, *pn* polar nucleus, *sy* synergids

transcripts were proteins related to posttranslational modification, protein turnover and chaperones, and the group of proteins with general function prediction only.

The differential expression of the ESTs with potential interest for the study of apomixis was first validated by macroarray and also by RT-qPCR and ISH. The lack of signal from 38% of the macroarray probes could be due to the low expression level or tissue/cell specificity of the genes chosen. Similar levels of elements that could not be evaluated were found with ovaries of *Pennisetum ciliare* (Singh et al. 2007). From the 11 ESTs with higher expression in apomictic *B. brizantha*, one showed similarity to sequences involved in meiosis (*BbrizDMC1*), two in cell cycle regulation (*BbrizMMF*; *BbrizRan*), and two in stress response (*BbrizERD* and *BbrizSti1*). Nine out of 11: *BbrizDMC1*; *BbrizERD*; *BbrizHelic*; *BbrizMMF*; *BbrizNup*; *BbrizRan*; *BbrizSec13*; *BbrizSti1* and *BbrizTom1* were confirmed by RT-qPCR as being more expressed in apomictic ovaries than in sexual ones. Most of them showed higher expression in reproductive (anthers and ovaries) than in vegetative organs (leaves). Staged RT-qPCR for *BbrizHelic*; *BbrizRan*; *BbrizSec13* and *BbrizSti1* showed a higher expression level in apomictic ovaries at megasporogenesis than at mature stage. In addition, the lower level of expression in sexuals, comparing to the apomicts was confirmed. Even though, the expression of these sequences was not ovary specific, the difference between their expression in sexual and apomictic ovaries in the early stages of ovary development, when the main events of differentiation between sexual and apomictic plants occur (Dusi and Willemse 1999; Araujo et al. 2000), suggest their involvement in the female reproductive development of apomictic *B. brizantha*. It is also important to mention that genes, including crucial regulators such as CAPRICE and TRIPTYCHON, may have conspicuous gene expression and have their tissue specific function triggered by the organ specific partners (Schellmann et al. 2002). The putative role of these ESTs during apomictic development is discussed below.

Regarding the putative role of Helicase, this sequence has a DNA binding domain, and members of this family are associated with genetic recombination (Mulder et al. 2003).

Rodrigues et al. (2003) have also described a sequence involved in recombination (clone 20) which is preferentially expressed in ovaries of apomictic *B. brizantha*. The level of expression of *BbrizHelic* in the first stages of ovary development was higher in apomicts than in sexual plants, and this result points to an involvement at the beginning of ai differentiation of apomictic *B. brizantha*, in addition to the megasporogenesis events. In fact, ISH showed higher expression of *BbrizHelic* in nucellar cells positioned in the very region where ais usually differentiate in ovaries of apomictic *Brachiaria* plants (Dusi and Willemse 1999; Araujo et al. 2000).

The high expression of *BbrizSec13* and *BbrizRan* in ovaries of apomictic plants, and more precisely, in ovaries during ais differentiation, could be due to their involvement in the transport of proteins related to specification of the nucellar cells that will form the megagametophyte of apomicts, possibly through an auxin pathway. Alterations in the patterns of auxin transport and synthesis caused identity changes in the constituent cells of the megagametophyte of *A. thaliana* (Pagnussat et al. 2009). Although there is a direct influence of auxin gradient on the formation of the mature embryo sac and its constituent cells, it is possible that this gradient results from a response to an unknown factor that leads to a cascade of cell differentiation (Pagnussat et al. 2009). RAN1 in *Triticum aestivum* participates in auxin signal pathway and its involvement in auxin response pathways and cell division has been suggested; it also has an important role during transport of proteins involved with mitosis and cell cycle (Wang and Liu 2006).

Among the nine *Brachiaria* ESTs with differential expression in ovaries of apomictic compared to sexual plants, two are related to stress response, *BbrizERD* and, notably, *BbrizSti*1. Stress-related genes have already been reported to be associated with apomictic development in other species. In *Boechera* spp., gene expression studies between diplosporic apomictic and sexual ovaries at different developmental stages have shown higher expression of stress-related sequences on apomictic ovaries from the stage of early megaspore mother cell formation to fertilized ovules (Sharbel et al. 2010). Also, the ASG1 (apomixis specific gene) from *P. maximum*, preferentially expressed in ovules of apomicts, presented similarity to proteins associated with drought stress, such as the protein RD22 from *A. thaliana* (Chen et al. 2005). The detection of *BbrizSti1* in the megaspore mother cell, and in nucellar cells previously to ai differentiation leads to speculation on the involvement of stress-responsive genes in the induction of ai from somatic cells of the nucellus. Stress can trigger in vitro developmental processes such as somatic embryogenesis (Ikeda-Iwai et al. 2003; Kikuchi et al. 2006; Kumria et al. 2003). In the development of ais as well as in the development of somatic embryos, somatic cells are reorganized and enter a different pathway, changing their cell fate. In somatic embryogenesis, embryos are formed, while in aposporic apomixis unreduced embryo sacs are formed. Both developmental pathways were previously associated, as was the expression of the *somatic embryogenesis receptor-like kinase* (SERK) gene, involved in the fate of cells towards embryogenesis, which could also participate in the specification of ais in apomicts (Albertini et al. 2005). The mechanism of signal transduction of these phenomena could be similar and comprise auxin and stress-induced genes.

This work reports the first analysis of EST libraries of *Brachiaria* genus. The EST database produced revealed transcripts of *Brachiaria* ovaries especially valuable to identify genes associated to reproduction. It has also proved to be of great value in identifying genes expressed in apomicts as was done by macroarray and RT-qPCR. Among the sequences studied at the present work, *BbrizHelic*, *BbrizRan, BbrizSEC13* and *BbrizSti1* are especially interesting due to their putative involvement in megasporogenesis of apomicts. Further analysis of two sequences, *BbrizSti1* and *BbrizHelic,* showed transcripts localized in nucellar tissues of ovaries of apomictic plants at stages of development previous to ai differentiation, suggesting their involvement on apomixis. The functional characterization of these genes will contribute to new insights on the megagametophyte development of apomicts.

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