

BbrizAGL6 Is Differentially Expressed During Embryo Sac Formation of Apomictic and Sexual *Brachiaria brizantha* Plants

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Abstract Species of the genus *Brachiaria* comprise plants with different modes of reproduction, sexual and apomictic. In apomixis, the embryo sac differentiates from an unreduced cell, and the embryo develops in the absence of egg cell fertilisation. In this work, the characterisation and expression analyses of a MADS-box gene from *Brachiaria brizantha*, named *BbrizAGL6*, was described in sexual and apomictic plants. Phylogenetic analyses indicated that *BbrizAGL6* belongs to the AGL6-like subfamily of proteins and clusters together with the AGL6-like protein of other monocots. *BbrizAGL6* and AGL6 show conservation of the protein complex. Furthermore, *BbrizAGL6* expressed preferentially

in reproductive tissues and corresponding transcripts were detected in anthers and ovules. In ovules of *B. brizantha*, where the main differences among sexual and apomictic reproduction occur, *BbrizAGL6* was differentially modulated. Transcripts of *BbrizAGL6* were localised in the megaspore mother cell of ovaries from apomictic and sexual plants and, additionally, in the region where aposporic initial cells differentiate, in the nucellus of apomictic plants. For the first time, a role of an AGL6-like gene in megasporogenesis of apomictic and sexual plants is suggested.

Keywords Apomixis · *Arabidopsis* · *Brachiaria* · MADS-box · Monocot · Plant reproduction

Electronic supplementary material The online version of this article (doi:10.1007/s11105-013-0618-8) contains supplementary material, which is available to authorized users.

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Introduction

The transition between haploid and diploid generations in the life cycle of angiosperms occurs in the ovule. The ovule is also the site of most of the processes relevant to sexual reproduction and to the asexual mode of reproduction through seeds, known as apomixis (Koltunow and Grossniklaus 2003). Sexual reproduction and apomixis diverge in two main steps: meiosis at megasporogenesis and fertilisation (Koltunow and Grossniklaus 2003). In sexual reproduction during the development of the megagametophyte, or embryo sac, after chromosome reduction by meiosis of the Megaspore Mother Cell (MMC), three megaspores degenerate and one functional megaspore is obtained. After three mitotic divisions and differentiation, a seven-celled embryo sac of the Polygonum-type is generated, consisting of: one egg cell, one central cell, two synergids and three

antipodals. Upon double fertilisation, the egg cell and the central cell are fertilised by two spermatic cells, giving rise to the seed embryo and endosperm.

Apomixis is characterised by the omission of meiosis and egg cell fertilisation; it produces a progeny identical to the mother plant by autonomous development of the embryo from an unreduced egg cell. Gametophytic apomixis is classified into apospory or diplospory, depending on the origin of the cell that initiates unreduced embryo sac formation (Nogler 1984; Rodriguez-Leal and Vielle-Calzada 2012). Differentiation of the precursor cell from a MMC that failed to effect meiosis is called diplospory (Nogler 1984). Apospory, observed in *Brachiaria*, a tropical forage grass, is characterised by differentiation of multiple precursors from sporophytic cells, called aposporic initials (AI), in a position other than that occupied by the MMC (Asker and Jerling 1992; Dusi and Willemse 1999; Araujo et al. 2000). Simultaneously, in facultative apomixis, the MMC can form a reduced embryo sac by sexual mode of reproduction. Embryogenesis occurs autonomously without fertilisation of the egg cell in aposporic embryo sacs. Endosperm formation may be autonomous or may require fertilisation (pseudogamy) (Nogler 1984), as in *Brachiaria* (Alves et al. 2001). In *Brachiaria* the unreduced Panicum-type embryo sac differs morphologically from the reduced embryo sac, which is of the Polygonum-type. The Panicum-type embryo sac is composed of just four cells: two synergids, one egg cell and one central cell.

In sexual plants, the formation of the germ line is actively restricted in somatic cells by an epigenetic pathway (Olmedo-Monfil et al. 2010; Singh et al. 2011). In aposporic apomictic plants, somatic cells can originate embryo sacs, thus, its origin is not restricted to the megasporocytes. However, the ability of somatic cells to form embryo sacs in aposporic plants depends on sexual pathway initiation, as shown in apomictic *Hieracium*, where AI appearance requires MMC and tetrad differentiation. These data suggest that an interaction between sporophyte and gametophyte is needed for apomixis (Koltunow et al. 2011). In *Brachiaria brizantha*, AIs are visualised surrounding intact or degenerating tetrads (Araújo et al. 2000). Isolating and characterising genes that are putatively related to the determination of sporophytic and gametophytic fates of the ovule in apomictic and sexual plants can contribute to understanding the molecular basis of these modes of reproduction. Gene expression information is being built in the female reproductive organs, where the main differences between apomictic and sexual plants occur (Pessino et al. 2001; Rodrigues et al. 2003; Polegri et al. 2010; Sharbel et al. 2010; Zhang et al. 2012). In *B. brizantha*, a differential pattern of expression in developing ovaries of apomictic and sexual plants was detected by microarray analysis (Silveira et al. 2012). Among the genes expressed in ovaries a MADS-box gene similar to the AGL6 of *Zea mays*, *BbrizAGL6*, showed a differential pattern of gene expression in apomictic and sexual plants. This work aims to investigate

the association among *BbrizAGL6* expression and the main events of embryo sac differentiation.

MADS-box genes are transcription factors that are involved in all major aspects of plant development (Bowman et al. 1991; Coen and Meyerowitz 1991) including the differentiation of the cells of the ovule (Matias-Hernandez et al. 2010); for review, see Ng and Yanofsky (2001). MADS-box genes were extensively studied in gymnosperms and angiosperms including *Eucalyptus globulus* (Southerton et al. 1998), *Nicotiana tabacum* (Jang et al. 2002), *Ginkgo biloba* (Jager et al. 2003), *Petunia hybrida* (Vandenbussche et al. 2004), *Alpinia hainanensis* (Song et al. 2010), *Coffea arabica* (Oliveira et al. 2010), *Gnetum gnemon* (Wang et al. 2010), *Prunus serotina* (Liu et al. 2010), *Brassica campestris* (Liu et al. 2012a, b), *Pyrus pyrifolia* (Liu et al. 2012a, b) and *Citrus* (Hou et al. 2013). The MADS-box proteins are characterised by a highly conserved DNA binding domain, the MADS-box, which binds a consensus sequence named CArG box (Riechmann et al. 1996). Modulation of DNA-binding specificity and transcriptional activity is determined by the formation of complexes with other MADS proteins (Theissen and Saedler 2001). For instance, ovule identity is determined by a multimeric complex formed by the C-, D- and E-class MADS-Domain factors, SEEDSTICK (STK), SHATTERPROOF1 (SHP1), SHP2 and SEPALLATA3 (SEP3), respectively (Favaro et al. 2003). Interactions between MADS-box proteins have been demonstrated to be conserved in monocot and eudicot plants (Favaro et al. 2002; de Folter et al. 2005). MADS-box transcription factors were extensively studied in various plant species and were able to form specific homodimers and heterodimers (de Folter et al. 2005). AGL6 from *Arabidopsis* and its homolog in rice, OsMADS6, have shown interaction with SEP-like, SQUAMOSA (SQUA) and AGL6 subfamilies (Moon et al. 1999, de Folter et al. 2005). The SEP subfamily is closely related to the AGAMOUS-LIKE6 (AGL6) and SQUA subfamilies (Favaro et al. 2002; de Folter et al. 2005; Zahn et al. 2005). In *Petunia*, AGL6 was able to interact with proteins that form multimeric complexes determining carpel and ovule identity such as SEP, AG and STK proteins (Rijkema et al. 2009). Recently, it has been shown in *Arabidopsis* that *VERDANDI*, a gene from B3 superfamily, is a direct target for this complex, affecting the identity of antipodal and synergid cells. The evidence indicates that the MADS-box transcription factors are also involved in embryo sac ontogeny (Matias-Hernandez et al. 2010). The involvement of MADS-box genes in the differential development of apomictic plants has not been reported, apart from DEFICIENS in *Hieracium* (Guerin et al. 2000). In this work, we demonstrate that *BbrizAGL6* is differentially expressed in apomictic and sexual *B. brizantha* ovules and is able to interact with those MADS-box proteins that regulate carpel and ovule identity. The involvement of this gene in the regulation of the apomictic pathway 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 (Araújo et al. 2000). Both were cultivated in the field at Embrapa Genetic Resources and Biotechnology (Brasília, DF, Brazil). Ovaries from *B. brizantha* were previously classified in four different stages of development. Stages I and II correspond to megasporogenesis, while stages III and IV correspond to megagametogenesis (Araújo et al. 2000; Rodrigues et al. 2003). Reproductive structures and leaves were collected in the field, whilst roots were collected in the greenhouse.

Amplification of a *Brachiaria AGL6* Homologue

A contig from an EST library of ovaries of *B. brizantha* (Silveira et al. 2012) with 83 % to 95 % similarity to the predicted proteins of ZAG3 and ZAG5 members of AGL6 clade (Mena et al. 1995) was used to design primers AG29F (5'-ATCGATCACCAGCAGGCAGAGAG-3') and AG1052R (5'-CCACGCACACCACAATCACATAG-3') to amplify a *AGL6* homologue. RNA extraction using TRIZOL® (Invitrogen™) and cDNA amplification from a pool of ovaries at the four stages of development were performed as described previously (Rodrigues et al. 2003).

Phylogenetic Analysis

Phylogenetic analysis of 27 MADS-box protein sequences (Table S1) was conducted using MEGA 5 (Tamura et al. 2011) with default settings. The K and C terminal regions of MADS-box genes were used for the alignment using program ClustalW (Thompson 1994). The gap opening penalty was ten, and the gap extension penalty was 0.1 for pairwise alignments, and they were ten and 0.2, respectively, for multiple alignments. The Gonnet matrix was selected, and residue-specific and hydrophilic penalties were ON. The gap separation distance was four, and end gap separation was OFF. Phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei 1987). The bootstrap values (%) of 1,000 replicates are shown at the branching points.

Yeast Two-Hybrid Assays

The two-hybrid assays were performed using the Ah109 *Saccharomyces cerevisiae* strain. pBD and pAD vector constructs were selected on yeast synthetic dropout (YSD) media

lacking leucine (Leu) and tryptophan (Trp), respectively. Two-hybrid interactions were assayed on two selective YSD media, the first lacking Leu, Trp and adenine, and the second lacking Leu, Trp and histidine (His) supplemented with 3-aminotriazole at concentration [5 mM]. Genes used for the yeast two-hybrid assays were cloned in the Gateway vector GAL4 system using vectors adapted from pADT7 (Clontech) for activation domain fusions and pGBKT7 (Clontech) for binding domain fusions passing through pDONOR207 (Life Technologies). The cDNA of the genes was amplified by polymerase chain reaction (PCR) with specific primers containing the attB1 and attB2 sequences for homologous recombination. Cloning of AG, SHP1, SHP2 and STK proteins in AD or BD vectors were previously described (Favaro et al. 2003). The SEP3Δ192 cDNA fragment was amplified and cloned according to Brambilla and co-workers (Brambilla et al. 2007). The coding sequences of *AGL6* and *BbrizAGL6* containing the attB1 and attB2 sequences for homologous recombination were amplified using the following primers AtP3094 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGGAAGAGGGGAGAGTG-3') and AtP3095 (5'-GGGGACCCTTTGTACAAGAAAGCTGGGTGTCAAAGAACCACCACTTGGACG-3') for *AGL6* from *Arabidopsis thaliana* and AtP3139 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGGGAGGGGACGGGTC-3') and AtP3140 (5'-GGGACCACCTTTGTACAAGAAAGCTGGGTGTCAAAGAA CCCATCCCAGCATG-3') for *BbrizAGL6* from *B. brizantha*.

RT-qPCR

RNA extraction from *B. brizantha* developing flowers was performed using TRIZOL® (Invitrogen™) as previously described (Rodrigues et al. 2003). RNA from leaves and roots was extracted using LiCL as previously described (Sambrook 2001). For the RNA extraction experiment, four pools of 1,000 ovaries and four pools of 50 anthers of each stage of pistil development were prepared. Ovaries and anther pools were prepared for apomictic and sexual *B. brizantha*. In addition, young leaves, root tissues and a pool of the whole spikelets containing a mixture of the corresponding four pistil developmental stages were isolated from both *B. brizantha* accessions.

Oligonucleotide pairs for *BbrizAGL6*, RT38F1 (5'-TCTGCAAATCGGGTATCCTC-3') and RT38R1 (5'-CCATCCCAGCATGAAGTTG-3') were designed using Primer 3.0 program (Rozen and Skaletsky 2000) with Tm of 60 °C and primer lengths between 19 and 20 bp. The reference gene used was *BbrizUBCE*, an ubiquitin-conjugating enzyme, previously described as the best reference gene for these samples in *B. brizantha* (Silveira et al. 2009). PCR reactions were performed using Syber Green Rox Plus kit (LGCBIO™) to detect dsDNA synthesis according to manufacturer's protocol. Reactions were run

in a Mastercycler Realplex (EppendorfTM) device using the following cycling parameters—95 °C for 15 min; 40 cycles of 95 °C for 15 s, 58 °C for 15 s, 72 °C for 20 s. The dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. No-template controls were included for each primer pair. Each amplicon was then analysed by electrophoresis on 1 % agarose gel to verify the specificity of each amplification reaction. Two biological replicates for each of the samples were used for RT-qPCR analysis, and three technical replicates were analysed for each biological replicate. Relative gene expression was calculated using Q-Gen software (Simon 2003).

In Situ Hybridisation

The RNA probe was synthesised using the DIG RNA labelling kit (Roche) according to the manufacturer's protocol. The same PCR fragment of 120 bp from *BbrizAGL6* amplified for RT-qPCR was cloned into pGEM-T Easy Vector System I (InvitrogenTM life technologies) and used as a template for in vitro transcription with SP6 and T7 polymerases, used as sense and antisense probes, respectively. In situ hybridisation was performed in sections of ovaries, at megasporogenesis and megagametogenesis and of anthers at microsporogenesis of the sexual and the apomictic accessions. In situ hybridisation was performed according to Alves et al. (2007). Sections were observed with a Zeiss Axiophot light microscope.

Results

BbrizAGL6 Shares High Sequence Similarity with *AGL6* Isolated from Other Monocot Species

A sequence of 1,159 bp, similar to the predicted proteins of ZAG3 and ZAG5 members of AGL6 clade, was amplified from cDNA of ovaries of apomictic and sexual *B. brizantha* plants. This sequence, with an identical amino acid predicted sequence in sexual and apomictic plants, was named *BbrizAGL6*.

BbrizAGL6 shows two conserved motifs (I and II) in the C-terminal region, a typical feature of AGL6 clade members (Ohmori et al. 2009) (Fig. 1). The first motif is well conserved in monocots and represents a ten amino acid residue sequence, DCEPTLQIGY; however, in *BbrizAGL6*, glutamic acid is substituted by aspartic acid. The second motif is at the end of the C-terminal region and is composed of a ten amino acid residue sequence, ENNFMLGWVL.

Phylogenetic analyses have also proved that *BbrizAGL6* belongs to the AGL6-like subfamily of proteins and clusters together with the AGL6-like protein of other monocots (Fig. 2).

BbrizAGL6 Interacts with Proteins that Form Ovule and Carpel Identity Complex in *A. thaliana*

To investigate whether *BbrizAGL6* is able to interact with MADS-box proteins related to ovule and carpel identity, a GAL4-based yeast two-hybrid experiment was performed between *BbrizAGL6* and *A. thaliana* MADS-box proteins, those that determine ovule and carpel identity. AG, SHP1 and SHP2 are members of the C-class; the D class STK and SEP3 Δ 192 from E-class were tested. A deleted version of SEP3 was used since the full protein auto-activates the reporter gene (Brambilla et al. 2007). Analyses proved that *BbrizAGL6* homodimerises and heterodimerises with AG, SHP1, SHP2 and STK. However, *BbrizAGL6* interacted with SHP1, SHP2 and STK only when *BbrizAGL6* coding sequence was fused to the activation domain (AD), and SHP1, SHP2 and STK to the binding domain (BD) (Table 1). None of the proteins tested were able to interact with empty vector AD or BD when the transformed yeast was grown in media lacking Trp, Leu and His, supplemented with 5 mM 3-aminotriazole.

BbrizAGL6 Is Sporophytically Expressed in Flowers

The pattern of *BbrizAGL6* expression in apomictic and sexual *B. brizantha* was firstly investigated by RT-qPCR in reproductive and vegetative structures (Fig. 3). Expression of *BbrizAGL6* was restricted to the reproductive tissues; the strongest signal being observed in ovaries. Ovaries from sexual plants showed higher expression compared with those of apomictic plants. The highest *BbrizAGL6* expression was observed at stage II, corresponding to ovules at megasporogenesis (Dusi and Willemse 1999; Araújo et al. 2000). In developing anthers, *BbrizAGL6* expression was lower compared with developing ovules.

In situ hybridisation analysis was performed to investigate in detail the spatial and temporal pattern of *BbrizAGL6* expression. During microsporogenesis, hybridisation signal was stronger in pollen mother cells (PMC) and lower in the tapetum cells (Fig. 4a, d). Transcripts of *BbrizAGL6* were localised in the MMC of ovaries from apomictic and sexual plants (Fig. 4b, c, e, f). In apomictic plants, expression was also present in the nucellus surrounding the MMC (Fig. 4b, c). During megagametogenesis, *BbrizAGL6* expression decreased in the ovaries of apomictic and sexual plants (Fig. 4g, h). Control in situ hybridisation with a *BbrizAGL6* sense probe confirmed that the probe employed is specific since we could not record any signals (Fig. 4i, j).

Discussion

Knowledge about molecular pathways involved in different types of reproduction in flowering plants is still elusive. MADS-box genes are much conserved among living organisms

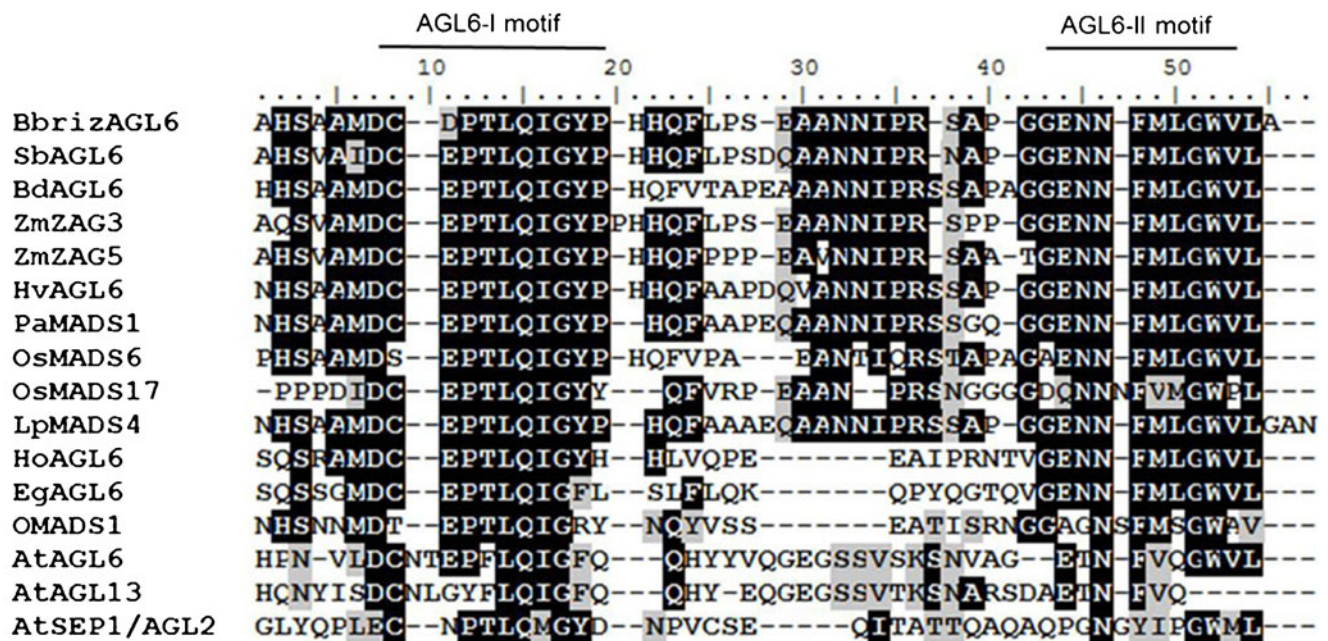


Fig. 1 Alignment of C-terminal region of AGL6-like MADS-box proteins. The C-terminal regions of 15 AGL6-like MADS-box proteins from various monocot plant species and from *Arabidopsis* were used (see Supplemental Table 1) *Arabidopsis* SEP-like protein (at SEP1/AGL2)

sequence was inserted in the alignment for the identification and comparison of the motifs. The positions of two conserved motifs are indicated above the sequences. Identical and similar amino acids are shaded black and grey, respectively. Analyses were done using ClustalW program

(Purugganan et al. 1995). They are widely studied in plants, contributing to the explanation of various aspects related to floral organ formation. This work sheds light on the involvement of MADS-box genes in apomictic and sexual reproduction, since they are poorly described in apomictic plants.

MADS-box transcription factors classified as MIKC are exclusive to plants and are composed of a MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domain (Theissen et al. 1996). The MADS-domain is responsible for DNA binding. K domain mediates interactions, specified by I-domain, between other MIKC-type proteins (Davies and Schwarz-Sommer 1994). The C-terminal domain is the most divergent among different MADS-box genes but displays conserved motifs among members of the same clade (Davies and Schwarz-Sommer 1994), determining their divergent biological functions and possibly forming a specific network of interactions (Egea-Cortines et al. 1999; Honma and Goto 2001). *BbrizAGL6* shows two motifs that are similar to those previously described in different species, especially among monocots (Zahn et al. 2005) and which resemble SEP proteins. This region is known to be essential for protein–protein interaction and transcriptional activation (Honma and Goto 2001). The AGL6-I and AGL6-II motifs were described in rice as transcriptional activation domains, and the AGL6-II has stronger activity than the AGL6-I one (Ohmori et al. 2009). Replacing the glutamic

acid, found in the other monocot sequences, with aspartic acid, in the first motif of *BbrizAGL6*, might not be relevant as both are polar and negative amino acids. The *BbrizAGL6* second motif is an acidic-rich domain and is identical to the AGL6-II motif identified in maize. The negative charge in this motif is critical for the structure of activation domain (Ohmori et al. 2009).

Phylogenetic analysis indicated that the predicted amino acid sequence of *BbrizAGL6* is grouped together with AGL6 from other monocot species and is close to the SEP group, which is highly conserved among various plant species (Reinheimer and Kellogg 2009). *BbrizAGL6* can be included in the same clade formed by ZAG3 and ZAG5 from maize, OsMADS6 from rice and SbAGL6 from sorghum. AGL6-like genes from grasses were shown to be expressed in paleas, lodicules, ovules and floral meristems and show a SEPALLATA-like function (Reinheimer and Kellogg 2009, Li et al. 2011). It was demonstrated that AGL6 has a role in the formation of floral organs in monocots and eudicots (Viaene et al. 2010).

Studies in various plants indicated AGL6's ability to form multimeric complexes with MADS-box transcription factors related to ovule and carpel identity, similarly to SEP (Moon et al. 1999, de Folter et al. 2005). SHP1 and SHP2, related to the shattering of dehiscent fruits (Liljegren et al. 2000), act redundantly with STK in *Arabidopsis* ovules (Pinyopich et al. 2003) being grouped in an AG monophyletic group (Parenicova et al. 2003). *BbrizAGL6* interaction with

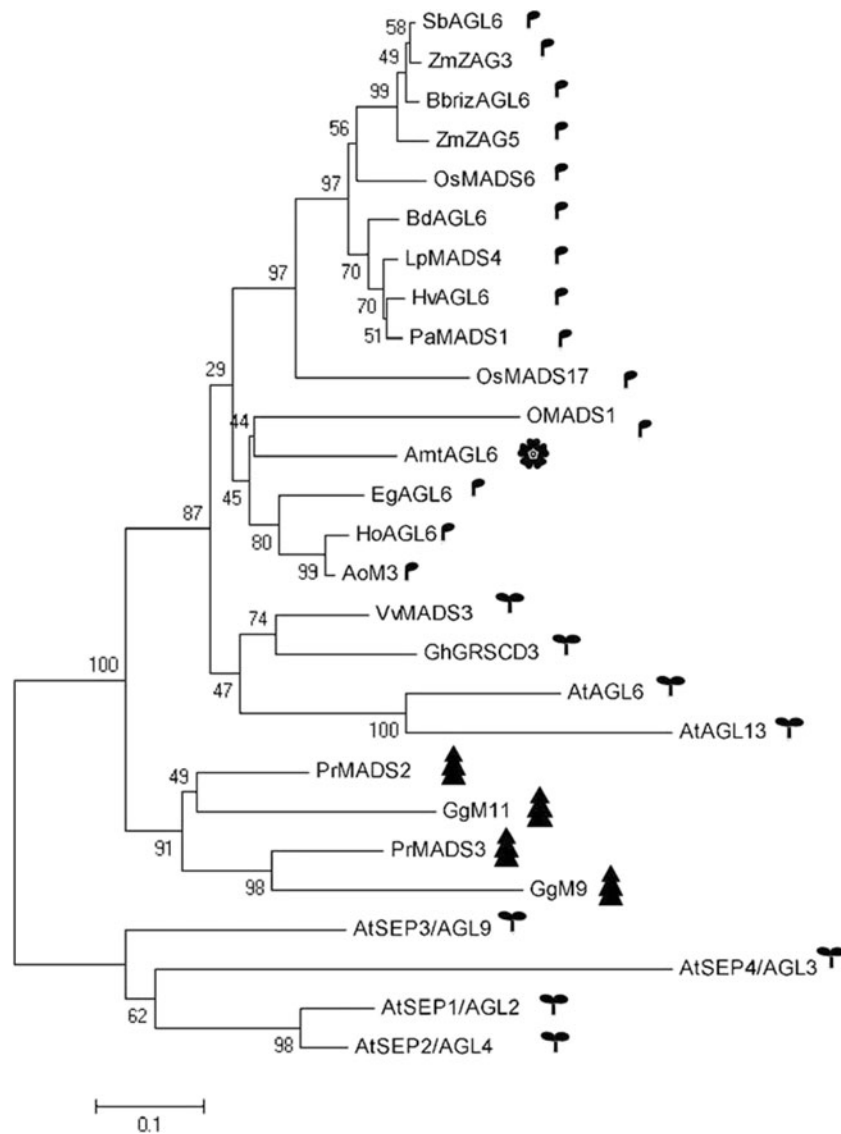


Fig. 2 Phylogenetic tree of MADS-box proteins constructed by the neighbour-joining method using 27 MADS-box protein sequences (see Supplementary Table S1). The bootstrap values (%) of 1,000 replicates





are shown at the branching points.     represent eudicot, monocot, magnoliid and gymnosperm, respectively

Table 1 Yeast two-hybrid study using the GAL4 system for testing interactions of *BbrizAGL6* with other MADS-box proteins: AG, SHP1, SHP2, STK, AGL6 and SEP3Δ

<i>BbrizAGL6</i>	C-class			D-class	E-class		
	AG	SHP1	SHP2	STK	AGL6	SEP3Δ	<i>BbrizAGL6</i>
-W-L-A ^a	+a	+a	+a	+a	-b	+a	-
-W-L-H ^b	+	-	-	-	++	++	+

^a Selective dropout medium lacking Trp, Leu and adenine (-W-L-A)

^b Selective dropout medium lacking Trp, Leu and His (-W-L-H) supplemented with 3-aminotriazole at concentration [5 mM]

++ Interactions clear in both directions, + interactions positive only in one direction, - no growth, *a* interactions could only be tested in one direction due to autoactivation of the GAL4 reporter gene by intrinsic transcription activation domain, *b* interactions could not be tested due to autoactivation of the GAL4 reporter gene by intrinsic transcription activation domain

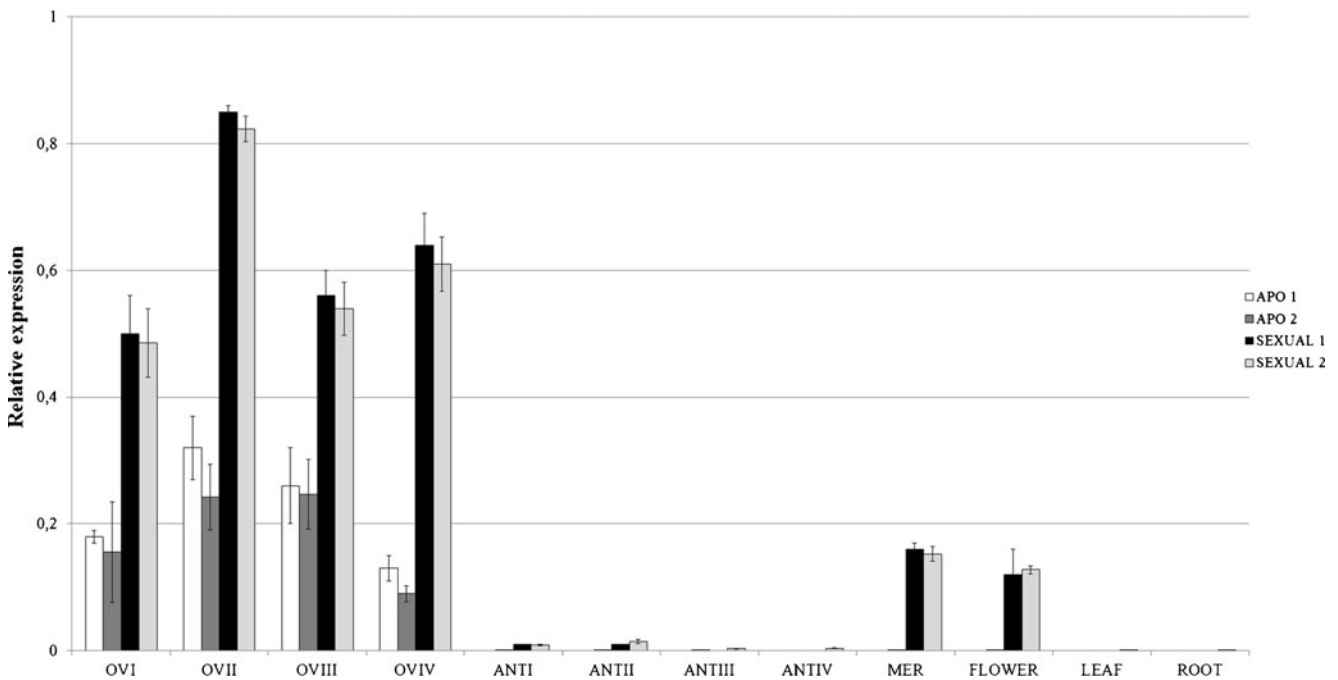


Fig. 3 Expression profile of *BbrizAGL6* by RT-qPCR in ovaries in megasporogenesis (ovI, ovII) and megagametogenesis (ovIII, ovIV); anthers in microsporogenesis (ANTI, ANTII) and microgametogenesis (ANTIII, ANTIV); inflorescence meristem (MER); flower; leaf and root from apomictic and sexual plants. *White and black columns* represent first biological replicate from apomictic (APO 1) and sexual (SEXUAL 1)

plants, respectively. *Dark and light grey columns* represent second biological replicate from apomictic (APO 2) and sexual (SEXUAL 2) plants, respectively. *Bars* represent the standard error of technical variation. *BbrizAGL6* is more expressed in sexual plants than in apomictic plants, especially in ovaries. Higher *BbrizAGL6* expression was detected in sexual ovII; the stage where MMC starts its development

SHP1, SHP2 and STK can be due to its SEP-like function since SEP3 gene was designated as glue in the formation of the multimeric complex in *Arabidopsis* (Immink et al. 2009). This multimeric complex can be related to *Brachiaria* reproduction, since *BbrizAGL6* is modulated in apomictic and sexual plants.

Yeast two-hybrid of OsMADS6, a rice AGL6 homolog, did not show any interaction with B and C class of MADS-box. Moreover, it was recently demonstrated that OsMADS6 is a key regulator of transcription levels of B-, C- and E-class genes (Li et al. 2011). However, ZAG3 from maize showed interaction with C class of MADS-box genes (Moon et al. 1999; Thompson et al. 2009), results similar to that observed with *BbrizAGL6*. Interestingly, the rice AGL6-II motif is slightly different from those of *BbrizAGL6* and ZAG3, which are identical. Analysis using yeast two-hybrid demonstrated that *BbrizAGL6* has the same role as AGL6 from *Arabidopsis* and as observed in the literature with maize. AGL6 belongs to MIKC MADS-box type genes. Generally, genes from this group are expressed only in diploid tissues that control various aspects of sporophyte development. They are involved in almost all levels of the regulatory network that controls reproductive development. Some of them are affected by stress treatment regulating flowering time (Gramzow and Theissen 2010).

The expression of *BbrizAGL6* in the ovary during megasporogenesis of sexual *B. brizantha* is consistent with data obtained in other species.

In *Arabidopsis*, rice and maize, AGL6 expression is more abundant in ovule sporophytic tissues, and its expression is restrained to flower organ (Mena et al. 1995; Ohmori et al. 2009; Reinheimer and Kellogg 2009; Koo et al. 2010). In rice, AGL6 mRNA was strongly detected in MMC and microspores (Zhang et al. 2010). The peak of expression of *BbrizAGL6*, observed at stage II ovaries of apomictic and sexual plants, could be related to events that are more recurrent in this stage, such as meiosis. The localisation of expression at this stage was preferentially detected inside MMC and PMC, cells which will enter meiosis. Remarkably, in apomictic plants, a broader expression of *BbrizAGL6* included the nucellar region near the MMC, where AI cells differentiate to form unreduced embryo sacs. These data suggest a deregulation of the spatial expression pattern of AGL6 during sporogenesis of apomictic plants. It is possible that the presence of AGL6 in the nucellar region of apomictic plants enables AI formation; alternatively, AGL6 could mark the differentiation of sporogenous cells, either the MMC in sexual plants, or the AI precursor cells in apomictic plants, suggesting that the expression of *BbrizAGL6* could be involved at sporogenesis, regardless of mode of reproduction.

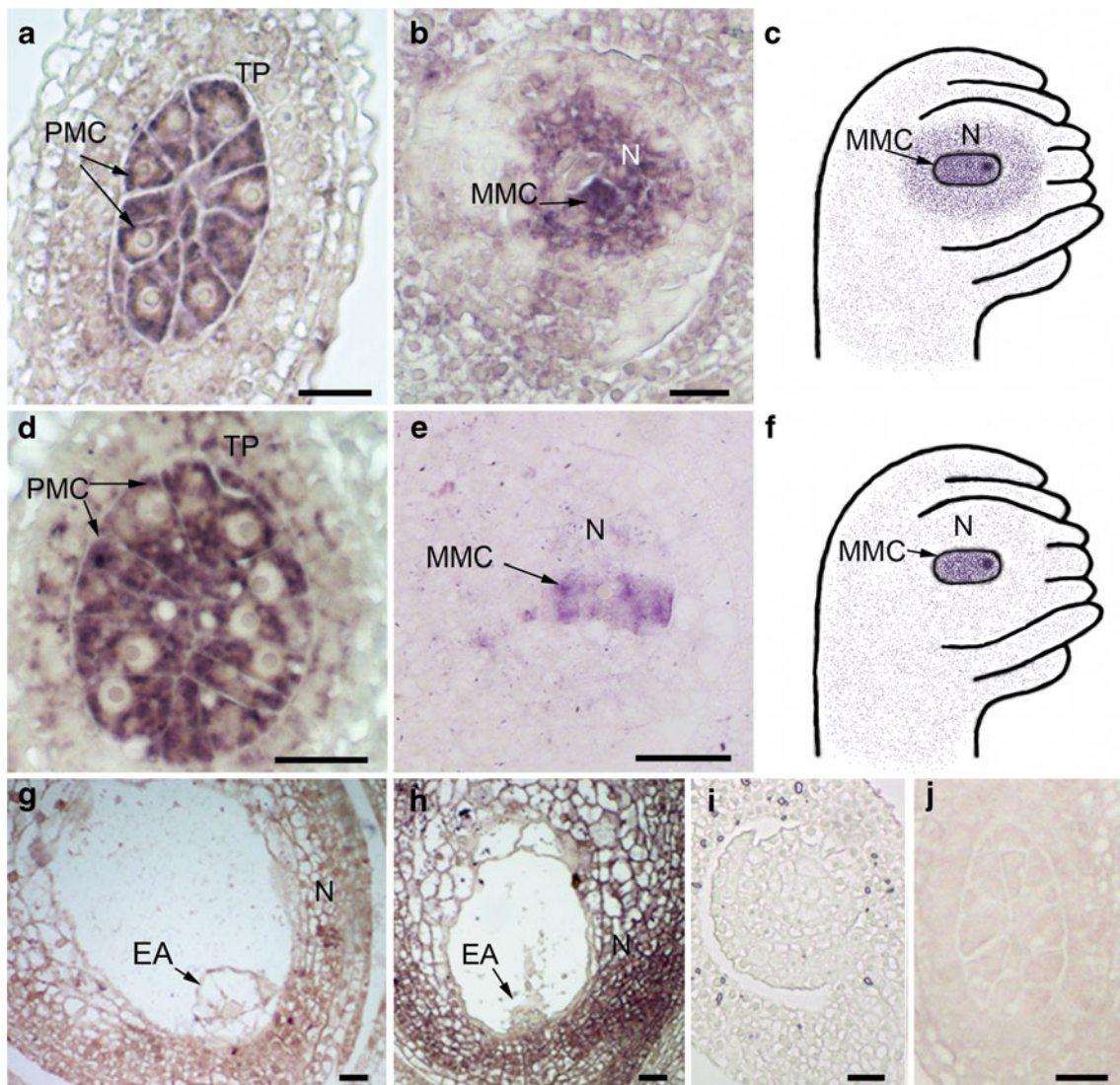


Fig. 4 Detection of *BbrizAGL6* transcripts by in situ hybridisation in semi-thin sections of anthers (**a, d**) and ovaries (**b, e, g, h**) of apomictic (**a, b, g**) and of sexual (**d, e, h**) *B. brizantha*. Schematic representation of in situ hybridisation at megasporogenesis of apomictic (**c**) and sexual (**f**)

plants. Hybridisation of sense probe in ovary (**i**) and anther (**j**) of apomictic *B. brizantha*. EA, egg apparatus; MMC, megaspore mother cell; N, nucellus; PMC, pollen mother cell; TP, tapetum. Bar=20 µm

The presence of *AGL6* in male sporogenous cells of sexual and apomictic *B. brizantha*, also observed in rice (Zhang et al. 2010), suggests that the latter is most likely the case.

Based on in situ analysis showing that *AGL6* is not present in gametophytic cells in rice (Zhang et al. 2010) and *B. brizantha* sexual and apomictic plants (this paper), this could be a conserved element in *AGL6* regulation, regardless of mode of reproduction. Therefore, *AGL6* appears to be required not only for sexual reproduction but also for the apomictic process. This corroborates the hypothesis that apomixis is a consequence of deregulation of the sexual pathway (Koltunow and Grossniklaus 2003; Rodriguez-Leal and Vielle-Calzada 2012).

We have successfully cloned and characterised a MADS-box gene, *BbrizAGL6*, from *B. brizantha* plants. The phylogenetic

association with MADS-box genes of other monocots was shown. In ovaries, *BbrizAGL6* was expressed only in the MMC of sexual plants while in apomictic plants it was expressed more broadly in the MMC and surrounding cell types. *BbrizAGL6* can be a useful marker for the characterisation and study of apomixis in *Brachiaria*.

Acknowledgements The authors acknowledge Ana L.M. Lacerda and Andrea D. Koehler for technical help in RNA extraction and Flávia S. Ferreira for dissecting ovaries. This work was supported by the National Council for Scientific and Technological Development-CNPq (490749/2008-9-VTCC) and the Brazilian Agricultural Research Corporation-Embrapa (0209020010.000- VTCC). This work is part of LAG's PhD thesis from Pós-Graduação em Biologia Molecular, University of Brasilia-UnB, Brazil, with a partial fellowship from CNPq and Embrapa. CNPq also provided a 1-year fellowship for LAG at Università degli Studi di Milano, Italy.

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