Plant Cell Rep (2018) 37:293–306
DOI 10.1007/s00299-017-2230-0

ORIGINAL ARTICLE

# GID1 expression is associated with ovule development of sexual and apomictic plants

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Received: 27 June 2017 / Accepted: 18 October 2017 / Published online: 28 October 2017


**Abstract**
***Key message*** ***BbrizGID1*** **is expressed in the nucellus of apomictic** ***Brachiaria brizantha*****, previous to aposporous initial differentiation.** ***AtGID1a*** **overexpression triggers differentiation of** ***Arabidopsis thaliana*** **MMC-like cells, suggesting its involvement in ovule development.**
*Abstract* GIBBERELLIN-INSENSITIVE DWARF1 (GID1) is a gibberellin receptor previously identified in plants and associated with reproductive development, including ovule formation. In this work, we characterized the *Brachiaria brizantha* GID1 gene (*BbrizGID1*). *BbrizGID1* showed up to 92% similarity to GID1-like gibberellin receptors of other plants of the Poaceae family and around 58% to GID1-like gibberellin receptors of *Arabidopsis thaliana*. *BbrizGID1* was more expressed in ovaries at megasporogenesis than in ovaries at megagametogenesis of both sexual and apomictic plants. In ovules, *BbrizGID1* transcripts were detected in the megaspore mother cell (MMC) of sexual and apomictic *B. brizantha*. Only in the apomictic plants, expression was also observed in the surrounding nucellar cells, a region in which aposporous initial cells differentiate to form the aposporic embryo sac. *AtGID1a* ectopic expression in *Arabidopsis* determines the formation of MMC-like cells in the nucellus, close to the MMC, that did not own MMC identity. Our results suggest that GID1 might be involved in the proper differentiation of a single MMC during ovule development and provide valuable information on the role of GID1 in sexual and apomictic reproduction.

**Keywords** Apomixis · *Brachiaria brizantha* · Gibberellin · GID1 · Ovule development · Plant reproduction

## Introduction

Gibberellins are plant hormones directly related to important plant growth and development events including seed

Communicated by Xian Sheng Zhang.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00299-017-2230-0) contains supplementary material, which is available to authorized users.

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germination, flowering and ovule development (Sun 2010; Gupta and Chakrabarty 2013; Gomez et al. 2016; Voegele et al. 2011). The gibberellin receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1) and DELLA proteins, key negative regulators of gibberellins, act on the signal transduction pathway of gibberellin in *Arabidopsis thaliana* and rice (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2005). The binding of gibberellin to the GID1 receptor causes a conformational change in the N-terminal extension of GID1, which inhibits gibberellin action (Sun 2010). The formation of the gibberellin–GID1–DELLA complex results in the rapid degradation of DELLA proteins, releasing the action of gibberellin by destabilizing and degrading DELLA proteins (Davière and Achard 2013; Hartweck and Olszewski 2006; Sun 2010). The GID1 gene was first identified in rice and encodes a protein with similarity to hormone-sensitive lipases (HSL) (Ueguchi-Tanaka et al. 2005). Overexpression analysis of GID1 in rice shows a hypersensitive phenotype to gibberellin, growing taller, containing long light-green leaves and with reduced fertility when compared with the control, while the *gid1* mutant presents insensitivity to gibberellin, suggesting that GID1 is the only gibberellin receptor in rice (Ueguchi-Tanaka et al. 2005). DELLA proteins participate in the correct formation of ovule integument and seed development in *Arabidopsis*, as shown in the *della global* mutant (the quintuple *gaiT6 rgaT2 rgl1-1 rgl2-1 rgl3-1* mutant). Short integuments and no distinction between the inner and outer cell layers of the integuments caused defects in the testa formation of *della global* mutant seeds. Moreover, the number of abortions increased and seed density per fruit was reduced (Gomez et al. 2016).

In angiosperms, ovule development starts with the formation of the ovule primordia from the placental tissue of the ovary. During early ovule development, a single subepidermal cell differentiates from the nucellus, leading to the formation of the megaspore mother cell (MMC). The inner and outer integuments begin to develop as the MMC undergoes meiosis, to give rise to four haploid megaspores. Three of them degenerate and the only one to survive becomes a functional megaspore, usually the one closest to the chalazal region. After three rounds of mitosis and cellularization, an embryo sac (ES) of the Polygonum-type, with three antipodal cells, a diploid central cell, two synergids, and one egg cell is formed, typical of plants of sexual reproduction (Schneitz et al. 1995; Grossniklaus and Schneitz 1998; Yang et al. 2010).

In *Brachiaria*, a genus of forage grass of the Poaceae family, reproduction may be sexual or asexual by apomixis, the latter resulting in progenies that are genetically identical to the mother plants (Carneiro et al. 2006). The apomictic reproduction by apospory predominates in these plants. In apospory, nucellar cells near the MMC differentiate into aposporous initial cells, which will undergo two mitosis events to form unreduced ES of the Panicum-type, with a diploid central cell, two synergids, and one egg cell. The MMC degenerates during meiosis, or complete meiosis resulting in four megaspores, which will degenerate (Araujo et al. 2000; Dusi and Willemse 1999).

In *Brachiaria brizantha* (Syn. *Urochloa brizantha*), a putative GID1-like gene of 606 nucleotides was previously detected by RNA-Seq analysis, as highly expressed in early stages of ovary development. In the model plant *Arabidopsis*, plant hormones were related to a fundamental role in the initiation of the ovule primordia and in the correct determination of the number of ovules (Bartrina et al. 2011; Cucinotta et al. 2014). Three genes were identified in these plants and considered as orthologous to the rice GID1, by a database search and gibberellin receptor activity of their encoded protein: *AtGID1a, AtGID1b* and *AtGID1c* (Nakajima et al. 2006). Functional analysis of double mutant plants *gid1a gid1b* showed reduced fertility; *gid1a gid1c* also reduced fertility and showed fruits with reduced length; *gid1b gid1c* did not show any phenotypic difference from wild-type plants (Gallego-Giraldo et al. 2014; Griffiths et al. 2006). The triple *gid1a gid1b gid1c* mutant showed to be extremely dwarf-sized and was insensitive to gibberellin, resulting in complete infertility. These results suggest that GID1 is the only gibberellin receptor in *Arabidopsis* and that this gene participates in reproductive development (Griffiths et al. 2006). The expression patterns of the three genes of *Arabidopsis* at anthesis, characterized using *pAtGID1::AtGID1-GUS* fusion constructs, showed a GID1a expression signal in all tissues, mainly surrounding the ES; GID1b only appeared in the chalaza region of the ovule and GID1c only in valves, with no expression in ovules (Gallego-Giraldo et al. 2014). Phenotypic traits of the *gid1-1* rice mutant, such as dwarf phenotype and insensitivity to gibberellin, were restored to normal with the overexpression of *AtGID1a, AtGID1b* and *AtGID1c* (Nakajima et al. 2006), showing the conserved nature of GID1 in these species. In *Arabidopsis*, the role of GID1 is associated with seed-set and fruit growth (Gallego-Giraldo et al. 2014); however, the role of GID1 during ovule development is still unclear. Studies related to GID1 might contribute to clarifying the mechanisms underlying the action of gibberellin in ovule development, as well as its participation in the early differential ES development of sexual and apomictic plants.

Our study aimed to evaluate the expression of GID1 during ovary development and, particularly, in the onset of ES of sexual and apomictic plants. The *B. brizantha* gene, named *BbrizGID1*, showed higher expression during ovule development at megasporogenesis. We detected *BbrizGID1* transcripts in the MMC of sexual and apomictic *B. brizantha* and in the surrounding nucellar cells of the apomicts. In an attempt to mimic *BbrizGID1* expression in *Arabidopsis* ovules, we investigated the results of overexpressing

*AtGID1a* under the control of the ovule-specific SEEDSTICK promoter (pSTK) (Kooiker et al. 2005) and the 35S constitutive promoter of Cauliflower mosaic virus (p35S) (Benfey et al. 1990). Morphological characterization of reproductive organs, in particular ovules, in the transformed plants suggested that *AtGID1a* is involved in the differentiation of additional MMC-like cells from nucellar cells near the MMC and in the integument development, suggesting either a delayed megasporogenesis or an earlier growth of integuments in the transgenic plants. Our findings on the GID1 expression during ovule development provide valuable information on the role of GID1 in the ovule development of sexual and apomictic plants.

## Materials and methods

### *BbrizGID1* sequence analysis

To verify the similarity of the *BbrizGID1* sequence previously identified in RNA-Seq libraries of sexual and apomictic ovaries of *B. brizantha*, basic local alignment search tool (BLAST) (Altschul et al. 1990, 1997) was performed against the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) database. BLASTn and BLASTx were used to compare nucleotides and translated sequences, respectively. Sequences showing the most significant nucleotide and amino acid sequence homology were used in multiple sequence alignments with ClustalW2 (Chenna et al. 2003). The cutoff *e* value of $^{>}$ $2e^{-57}$ and identity above 90% was used to define homologs. The Arabidopsis Information Resource database (TAIR, http://arabidopsis.org) was also used for comparative analyses between the BbrizGID1 sequence and the sequences from the *Arabidopsis* model plant. The genomic sequences of *BbrizGID1* from sexual and apomictic genotypes were obtained searching genomic libraries under construction. Restriction map analysis of *BbrizGID1* from sexual and apomictic genomes was performed with Webcutter 2.0.

### Plant material

Two accessions of *B. brizantha* from Embrapa's germplasm collection, grown in the field, were used in this work: BRA 002747, a sexual diploid ($2n = 2x = 18$), and BRA 000591, a facultative apomictic tetraploid ($2n = 4x = 36$) named *B. brizantha* cv. Marandu, with 98% of apospory (Araujo et al. 2007). Ovaries and anthers of both accessions were dissected from flowers of different plants before anthesis (I, II, III and IV), at different stages of development, under a Zeiss Stemi SV11 stereomicroscope. Ovaries at stages I and II contain ovules in megasporogenesis, whereas stages III and IV contain ovules in megagametogenesis (Araujo et al. 2000). The occurrence of nucellar cells and MMC characterizes ovules at stage I, in both sexual and apomictic plants. At stage II, pistils present ovules with degenerated tetrads, and in apomictic plants, aposporous initials can be visualized. At stage III, ovules with coenocytes are observed. Ovaries at stage IV contain mature ES, before anthesis (Araujo et al. 2000; Rodrigues et al. 2003). *Arabidopsis* wild-type (ecotype Columbia) and the *pAtGID1a::AtGID1a-GUS, pAtGIDb::AtGID1b-GUS* and *pAtGID1c::AtGID1c-GUS* transgenic lines (Gallego-Giraldo et al. 2014; Suzuki et al. 2009), provided by Dr. Perez-Amador, were cultivated in a growth room at 22 °C under long-day (16 h light per 8 h dark) conditions.

### Southern hybridization analysis

Genomic DNA was extracted from young leaves of *B. brizantha* using a Dellaporta procedure (Dellaporta et al. 1983) modified by Leblanc et al. (1997). To estimate the copy number of *BbrizGID1*, 30 µg of genomic DNA from each genotype was digested with *Eco*RI, *Hind*III and *Xba*I at 2.5 U/µg of DNA. A 300 bp fragment PCR amplified with the primers GGACAGGGACTGGTACTGGAAG and CTGAGGAAGTCGGCGATCTC for *BbrizGID1* without internal restriction sites for the enzymes used to digest the genomic DNA was used as probe. Labeling the probe with $^{32}$P –αdCTP, hybridization and radioactive detection assays were performed as described (Sambrook and Russell 2001). The hybridized membrane was placed in the cassette containing Imaging Plate (IP) and the image generated on the device Phosphorimager™ FUJIFILM FLA-3000 SERIES, using the Image Reader FLA-3000 V1.8 series programs and image display by Multi Gauge V3.0.

### RNA isolation and RT-qPCR analyses

In sexual and apomictic *B. brizantha* plants, total RNA was extracted in three biological replicates, each constituted of a pool of 250 ovaries, at each of the four stages, using TRIZOL® (Invitrogen) as previously described (Rodrigues et al. 2003). Reverse transcriptase reaction was performed with SuperScript III (Invitrogen) and 2 ug of RNA according to the manufacturer's protocol. RT-qPCRs were done using SYBR Green PCR Master Mix Green (Applied Biosystems). Oligonucleotide pairs for *BbrizGID1* were designed using Primer 3.0 program (Rozen and Skaletsky 2000): forward (TCAAGCTCGTGTACCGCGAG) and reverse (CTGAGG AAGTCGGCGATCTC). The analysis of data was performed by the method ΔΔCt, with *BbrizUBCE* as reference gene, encoding the ubiquitin (forward GGTCTTGCTCTC CATCTGCT) and (reverse CGGGCTGTCGTCTCATAC TT), as previously used (Silveira et al. 2009).

In *Arabidopsis* plants, total RNA was extracted from 1 to 2 inflorescences in three biological replicates of *pSTK::AtGID1a* transgenic plants and *p35s::AtGID1a* transgenic plants, using NucleoZOL® (Macherey–Nagel) according to the manufacturer's instructions. The total RNA was incubated with the Ambion TURBO DNA-free DNase (Invitrogen) and then reverse transcribed using the ImProm-II™ reverse transcription system (Promega). Oligonucleotide pairs were designed using Primer 3.0 program for *AtGID1a* forward (GCTGCGAGCGATGAAGTT) and reverse (GAT TGGCGTTTGCAGTGA), *UBIQUITIN10* forward (CTG TTCACGGAACCCAATTC) and reverse (GGAAAAAGG TCTGACCGACA), and *ACTIN2-8* forward (CTCAGGTAT TGCAGACCGTATGAG) and reverse (CTGGACCTGCTT CATCATACTCTG). *UBIQUITIN10* and *ACTIN2-8* genes were used as references and gene expression analysis was performed using the iQ5 Multicolor real-time PCR detection system (Bio-Rad) with SYBR Green PCR Master Mix (Bio-Rad). The assay was conducted in three technical replicates of each of the three different biological replicates for each sample.

### Expression analysis by in situ hybridization

The RNA probe was synthesized using the DIG RNA labelling kit (Roche) according to the manufacturer's protocol. The same PCR fragment of 300 bp from *BbrizGID1* located in the 3′ region, flanked by positions 1334–1633 of the nucleotide sequence, was used as a probe. The *BbrizGID1* fragment was cloned into pGEM-T Easy Vector System I (Invitrogen) and used as a template for in vitro transcription with SP6 and T7 polymerases, used as sense and antisense probes, respectively. In situ hybridization was performed in semi-thin sections of 3.5 µm of ovaries at megasporogenesis of sexual and apomictic plants. The sample preparation and in situ hybridization were carried out as previously described (Alves et al. 2007; Silveira et al. 2012). Hybridized sections were observed with a Zeiss Axiophot light microscope.

### Construction of vectors and transformation of *Arabidopsis* plants

The full-length *Arabidopsis AtGID1a* gene was cloned using the Gateway system (Invitrogen) and the primers ATGGCT GCGAGCGATGAA and TTAACATTCCGCGTTTAC AAAC. The promoters of the SEEDSTICK gene, pSTK (Kooiker et al. 2005), to drive the expression in ovules, and the 35S constitutive promoter of the Cauliflower mosaic virus (p35S) (Benfey et al. 1990) were used. Plants of *Arabidopsis* ecotype Columbia were transformed with *p35S::AtGID1a* and *pSTK::AtGID1a* constructs by floral dip method (Clough and Bent 1998). The *pSTK::AtGID1a* transformant lines from the ecotype Columbia were selected using BASTA, and *p35S::AtGID1a* transformant lines from the ecotype Columbia were selected using kanamycin. The *pKNU:nlsYFP Arabidopsis* line (Tucker et al. 2012) provided by Dr. Tucker was also transformed with *p35S::AtGID1a* by floral dip method (Clough and Bent 1998). The transformant lines were selected using kanamycin.

### *GUS* expression analysis

The *pAtGID1a::AtGID1a-GUS, pAtGIDb::AtGID1b-GUS* and *pAtGID1c::AtGID1c-GUS* lines (Gallego-Giraldo et al. 2014; Suzuki et al. 2009) were analyzed at different stages of the ovule development. β-Glucuronidase (GUS) assays were performed on inflorescences overnight at 37 °C, as previously described (Liljegren et al. 2000). Samples were incubated in a clearing solution of chloral hydrate:glycerol:water (8:1:2; w/v/v), dissected and observed by DIC microscopy.

### Morphological analysis of *Arabidopsis* flowers by DIC microscopy

Flowers at different developmental stages were collected, fixed with 9:1 ethanol:acetic acid and cleared overnight using clearing solution. After clearing, ovules were dissected from premature pistils on a slide using a stereomicroscope, mounted with a cover slip, and subsequently observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1). Three different plants of wild-type (ecotype Columbia) and three independent events of each transformant line *p35S::AtGID1a, pSTK::AtGID1a* and the second generation of *pKNU:nlsYFP p35S::AtGID1a* line were analyzed. Three independent events of *pKNU:nlsYFP p35S::AtGID1a* line were observed with a Zeiss Axiophot microscope using a filter with 475/40 nm laser line for excitation. Emissions were detected between 530/40 nm. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

## Results

### *BbrizGID1* encodes a protein with similarity to the GID1 gibberellin receptor

Previous RNA-Seq analysis pointed to a sequence of 606 nucleotides similar to the *GID1-like* gene from different plants. This sequence was present in both genomic libraries and was named *BbrizGID1*. Genomic libraries of sexual and apomictic *B. brizantha* (unpublished results) were screened and a full-length DNA sequence, constituted of 1646 nucleotides (Supplementary Fig. S1) and coding 352 amino acids

(Fig. 1), was found in this analysis. The *BbrizGID1* consists of two exons and one intron, similar to the GID1 structure from rice (Ueguchi-Tanaka et al. 2005). The predicted amino acid sequences of *BbrizGID1* from sexual and apomictic plants have the same number of amino acids, differing in two amino acids at positions 152 and 217. In position 152, an isoleucine (I) found in sexual plants is replaced by a valine (V) in apomicts. In position 217, a lysine (K) from sexual plants is replaced by an arginine (R) in apomicts (Supplementary Fig. S2).

To analyze the similarity of *BbrizGID1* with GID1 sequences from other plants, an NCBI database search was performed. The *BbrizGID1* predicted amino acid sequence showed similarity to the sequences of GID1-like gibberellin receptors of other plants of the Poaceae family (Fig. 1). The most similar sequences were from the following plants (with respective percentage identity): *Setaria italica* (92%), *Sorghum bicolor* (91%), *Saccharum officinarum* (90%), *Zea mays* (87%), *Brachypodium distachyon* (86%), *Oryza sativa Japonica Group* (84%) and *Hordeum vulgare* (83%). The similarity of *BbrizGID1* to the three *Arabidopsis* genes was very close: *AtGID1c* (58%), *AtGID1a* (57%) and *AtGID1b* (55%). Alignment also showed the presence of the consensus sequence of the HSL family, including the conserved HSL motifs HGG and GXSXG in *BbrizGID1* (Fig. 1). The *BbrizGID1* deduced amino acid sequence showed two conserved catalytic centers with the HSL sequence, serine (S) and aspartic acid (D), whereas another catalytic center, histidine (H), was replaced by isoleucine (I) (Supplementary Fig. S3).

### *BbrizGID1* is present in the sexual and apomictic genotypes of *B. brizantha*

In the apomictic genotype of *B. brizantha*, the Southern blot displayed three fragments after *Eco*RI DNA digestion, four after *Hind*III and two after *Xba*I. In the sexual genotype, one fragment hybridized after *Eco*RI DNA digestion, three after *Hind*III and two after *Xba*I (Fig. 2). Restriction map analysis of genomic DNA from both genotypes (Supplementary Fig. S1) showed that there is no site for *Eco*RI and *Xba*I and a site for *Hind*III is found at the intron region, position 444. The probe hybridization site is between positions 1334 and 1633 from the exon region of the nucleotide sequence. Genomic hybridization confirmed that *BbrizGID1* is present in both sexual and apomictic genotypes in a different restriction pattern.

### *BbrizGID1* expression in ovaries of sexual and apomictic plants is higher during megasporogenesis

Analysis of *BbrizGID1* expression profile from the RNA-Seq data of ovaries of sexuals and apomicts suggested higher expression of *BbrizGID1* in ovaries at megasporogenesis stage than in ovaries at megagametogenesis stage (data not shown). To confirm these results, the transcript levels of *BbrizGID1* were compared quantitatively in ovaries of sexual and apomictic plants (Fig. 3), using pistils of *B. brizantha* in four different stages of development as previously described (Araujo et al. 2000; Rodrigues et al. 2003). Ovaries from pistils in stage I, corresponding to the onset of megasporogenesis, showed higher expression of *BbrizGID1*, whereas *BbrizGID1* expression decreased in the later developmental stages. At the 5% significance level, analysis of deviance (ANODEV) test showed that sexual and apomictic reproduction modes do not interfere with *BbrizGID1* expression ($F = 0.903$, GL = 19, GL = 1, $p = 0.353$), but there are significant differences between stages I, II and III of ovule development in both plants ($F = 49.66$, GL = 19, GL = 3, $p = 0.0001$). Comparison of *BbrizGID1* expression in stages III and IV, however, did not show a significant difference.

### *BbrizGID1* is expressed in MMC of sexual and apomictic *B. brizantha* and in nucellar cells of apomicts

In situ hybridization of *BbrizGID1* was performed in the early stages of ovule development of sexual and apomictic plants to understand the cellular localization of *BbrizGID1* within the initial events of ovule development (Fig. 4). The antisense probe of *BbrizGID1* in the early stages of megasporogenesis resulted in high signal of hybridization in MMC of sexual (Fig. 4a, b) and apomictic plants (Fig. 4d, e). Nucellar cells in sexual ovules showed weak signal in comparison to the strong expression of *BbrizGID1* in the nucellar cells of apomicts (Fig. 4d, e). In sexual and apomictic plants, a weak signal was detected in inner and outer integuments (Fig. 4a, d). The sense control probe did not show any hybridization signal in sexual and apomictic ovules, as expected (Fig. 4c, f).

### GID1 proteins show specific spatial expression patterns in *Arabidopsis*

To study the localization of GID1 (a, b, c) proteins during the first stages of ovule development, transgenic *Arabidopsis* lines carrying *pAtGID1::AtGID1-GUS* translational fusions (Gallego-Giraldo et al. 2014; Suzuki et al. 2009) were analyzed (Fig. 5). As visualized in the GUS assay, GID1a is present in the whole pistil during early development, before anthesis (Fig. 5a). The GUS signal was strongly detected in the inner and outer integuments of the ovules, at 2-III stage, in which MMC is in meiosis, and the inner and outer integuments already developing (Schneitz et al. 1995), of *pAtGID1a::AtGID1a-GUS* line. No signal was detected in MMCs and nucellar cells (Fig. 5b). The GUS signal was

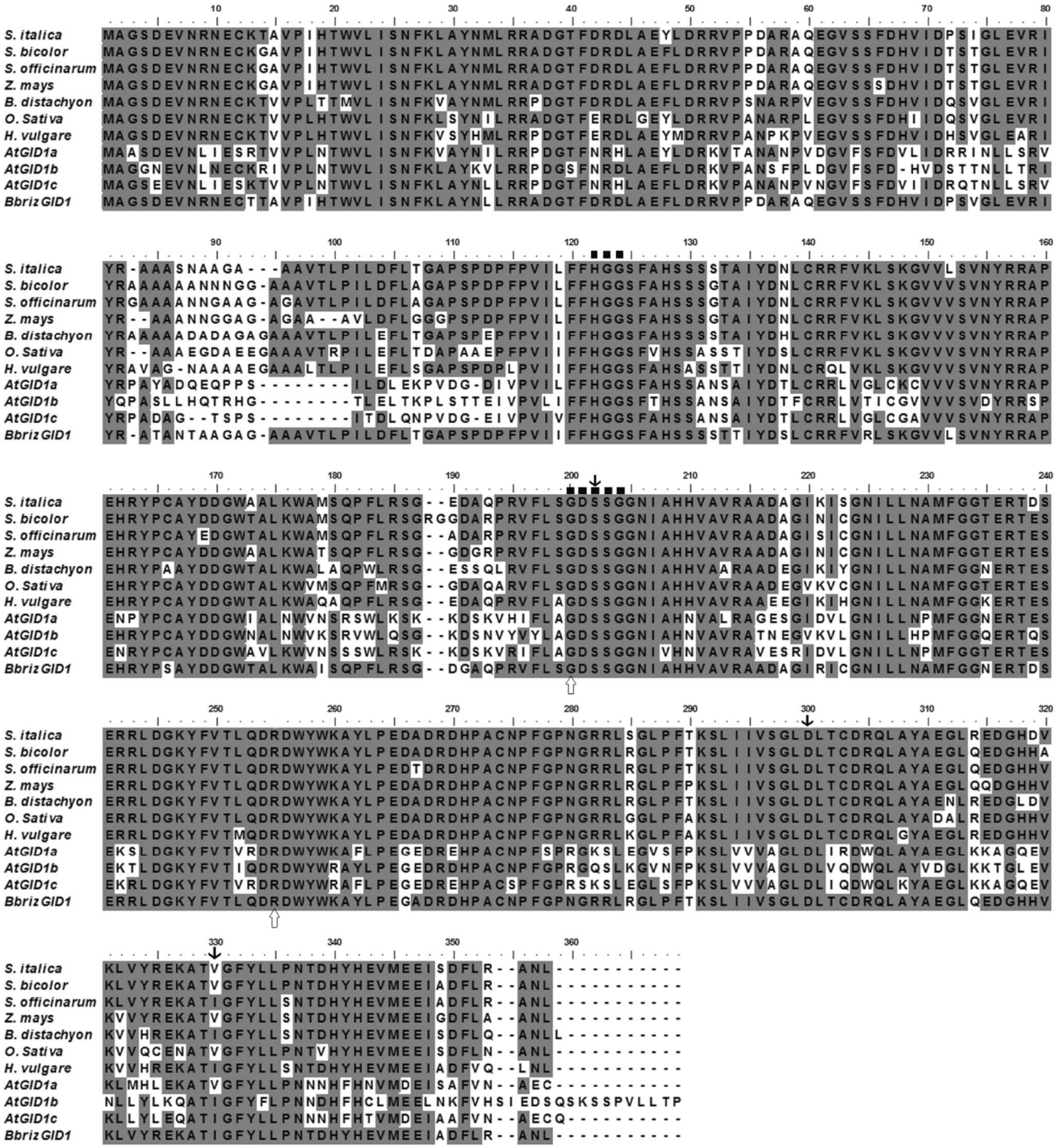


**Fig. 1** Multiple sequence alignment by ClustalW of *BbrizGID1* sequence, from the apomictic plants, showing the *Arabidopsis thaliana* and the most similar sequences found: *Setaria italica* (Accession Number XP_004962116.1), *Sorghum bicolor* (XP_021303311.1), *Saccharum officinarum* (CAP64326.1), *Zea mays* (CAP64327.1), *Brachypodium distachyon* (XP_003568469.1), *Oryza sativa* japonica group (XP_015639961.1), *Hordeum vulgare* (CAO98733.1), *AtGID1a* (At3g05120), *AtGID1b* (At3g63010) and *AtGID1c* (At5g27320). Gray highlight shows the amino acid residue conserved amongst the sequences. Squares represent regions identified as conserved within the hormone-sensitive lipase (HSL) family, HGG and GXSXG motifs. Black arrows show the three catalytic centers, serine (S), aspartic acid (D), and isoleucine (I), which replace histidine (H), characteristic of the HSL family in all the *GID1* sequences of the Poaceae plants. Numbers indicate the position from the start codon. White arrows indicate glycine (G) and arginine (R) residues that are essentials for maintaining the gibberellin binding activity in *OsGID1* in rice (Ueguchi-Tanaka et al. 2005)

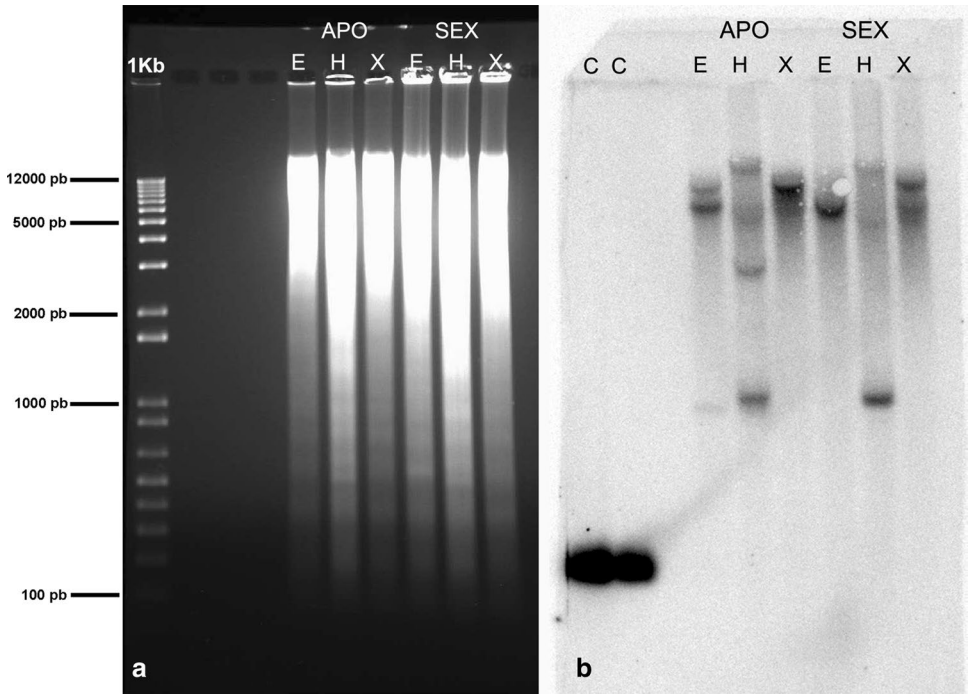


**Fig. 2** Genomic DNA analysis of apomictic (APO) and sexual (SEX) *Brachiaria brizantha* digested with *Eco*RI (E); *Hind*III (H) and *Xba*I (X). **a** Electrophoresis in 0.8% agarose gel in **b** fragments hybridized in Southern blot analysis with a *BbrizGID1* $^{32}$P-labeled probe. In APO three fragments were displayed after *Eco*RI DNA digestion, four after *Hind*III digestion and two after *Xba*I digestion. In SEX, one fragment was hybridized after *Eco*RI digestion, three after *Hind*III and two fragments after *Xba*I digestion. Image was generated on a phosphor imaging plate. C as control, the same fragment used to synthesize the probe

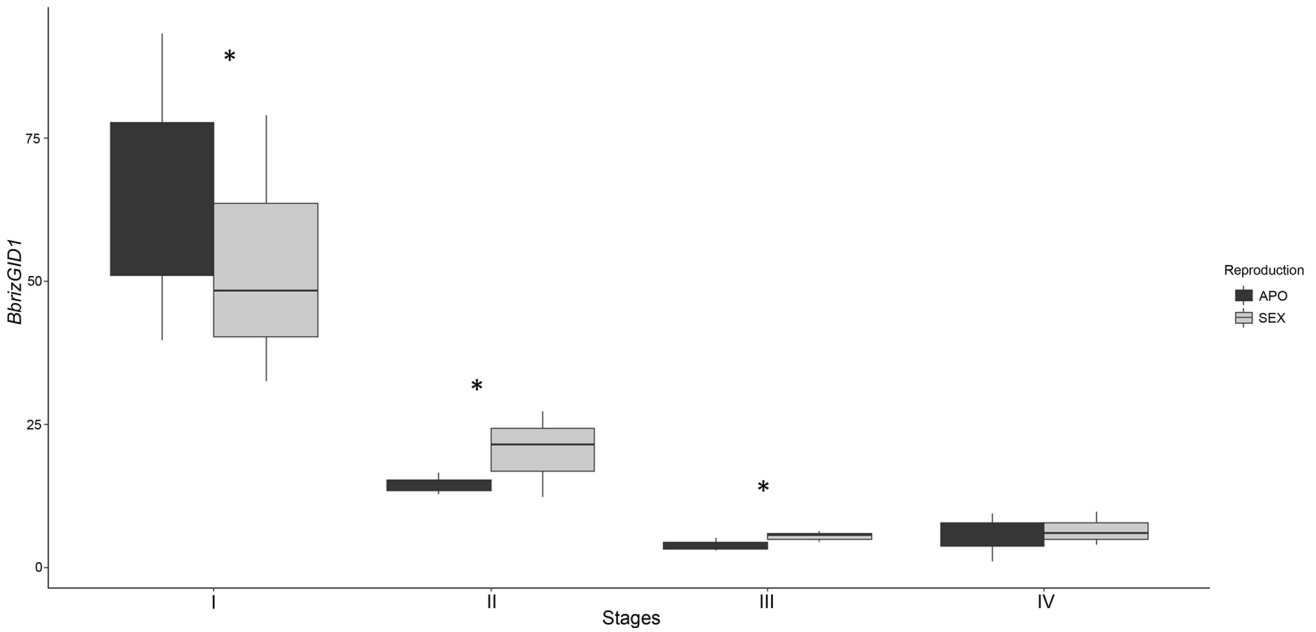


**Fig. 3** Boxplot showing the relative expression levels of the *BbrizGID1* by RT-qPCR in *Brachiaria brizantha*. Analysis in developing ovaries before anthesis at megasporogenesis (stages I, II) and megagametogenesis (stages III and IV) of apomictic (APO) and sexual (SEX) plants. Bars represent standard deviation based on the mean of three biological samples. The mean quartiles, median and ANODEV were calculated in the R Core Team (2016) program. The significant differences between stages of ovary development are marked with asterisks (*$p < 0.05$)

present throughout the mature ovule, including the ES and the integuments (Fig. 5c). In the *pAtGID1b::AtGID1b-GUS* line GUS signal was localized in the inner and outer integuments at 2-III-2-V stage (Schneitz et al. 1995) and no signal was detected in MMCs and nucellar cells (Fig. 5d, e). In mature ovules, at the FG7 stage in which the ES is formed, the GUS signal was localized mainly in the chalaza zone, at the base of the ES (Fig. 5f). *AtGID1c* was expressed

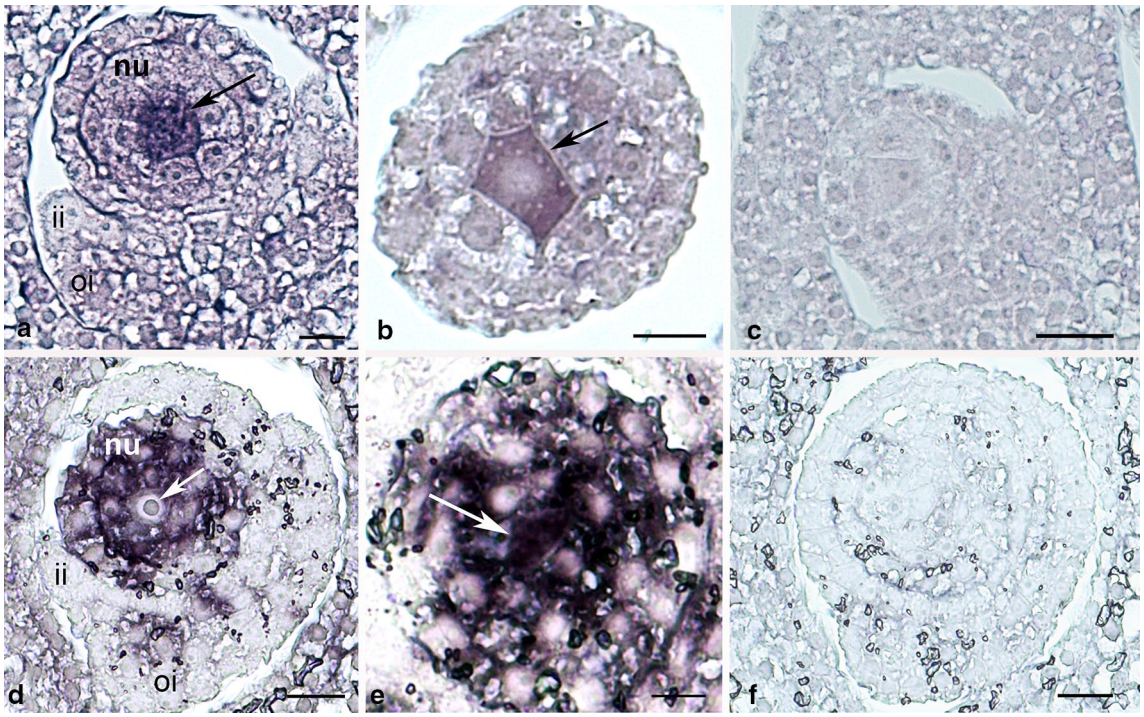


**Fig. 4** Localization of *BbrizGID1* expression by in situ hybridization in semi-thin sections of ovaries of *Brachiaria brizantha* sexual (**a–c**) and apomictic (**d–f**) plants. **a** Ovule with labeling in the megaspore mother cell (arrow) with weaker signal in the nucellar cells (nu), inner (ii) and outer (oi) integuments. **b** Detail of the megaspore mother cell (arrow) with strong signal of hybridization. **c** Control with sense probe showing no signal. **d** Detail of the ovule with labeling in the nucellar cells (nu) and the megaspore mother cell (arrow) with weaker signal in the inner (ii) and outer (oi) integuments. **e** Next section of the anterior ovule showing the cytoplasm of the megaspore mother cell (arrow). **f** Control with sense probe showing no signal. Scale bars **a** 12 µm, **b** 10 µm, **c** 20 µm and **d–f** 10 µm

specifically in the septum, but was completely absent in the ovules at 2-III stage and funiculi of *pAtGID1c::AtGID1c-GUS* line (Fig. 5g, h). *GUS* expression was not observed in ovules.

### Phenotypic characteristics of *Arabidopsis* overexpressed *AtGID1a* lines are distinct from wild-type

To study whether ectopic *AtGID1a* was sufficient to determine the formation of MMC-like cell we have transformed *Arabidopsis* plants with *p35S::AtGID1a* and *pSTK::AtGID1a*. pSTK is specifically active during ovule development starting from stage I (Brambilla et al. 2008). We analyzed three transgenic plants containing *p35S::AtGID1a* and three containing *pSTK::AtGID1a* by RT-qPCR. Results showed that *AtGID1a* expression was higher in the inflorescences of plants from both groups of transgenic lines compared to wild-type plants (Supplementary Fig. S4). The early stages of ovule development of these transgenics were analyzed, especially the finger-like (2-I) stage, in which MMC is formed (Schneitz et al. 1995) and additional MMC-like cells were observed (Fig. 6). The *pSTK::AtGID1a* and *p35S::AtGID1a* plants showed asynchrony in development of the inner and outer integuments as regards megasporogenesis events if compared with wild-type (Fig. 6). These results suggest that ectopic expression of *AtGID1a* could interfere with proper integument development. The additional MMC-like cells of overexpressed *AtGID1a* plants were observed only in ovules at the finger-like stage (2-I). In later stages of development, only one functional megaspore was observed, suggesting that only one MMC completed the meiotic division and entered the gametogenesis process, as twin ES were not observed during the analysis of mature ovules. Results of the phenotype analysis of wild-type *Arabidopsis* and three lines of *pSTK::AtGID1a, p35S::AtGID1a* were evaluated for each plant (Supplementary Table S1). In ovules of wild-type *Arabidopsis*, additional MMC-like cells were also observed but in a reduced number, 17% of the total number of ovules analyzed. In transgenic plants, additional MMC-like cells were observed in 33 and 32% of the total number of ovules analyzed using pSTK or p35S promoter, respectively (Fig. 7). Statistical analysis (t test) showed that there is a significant difference between the number of additional MMC-like cells in the overexpressed *AtGID1a* lines compared to wild-type, independent of the promoter used for *AtGID1a* expression, pSTK ($p = 0.009$) or p35S ($p = 0.013$). In *pSTK::AtGID1a* and

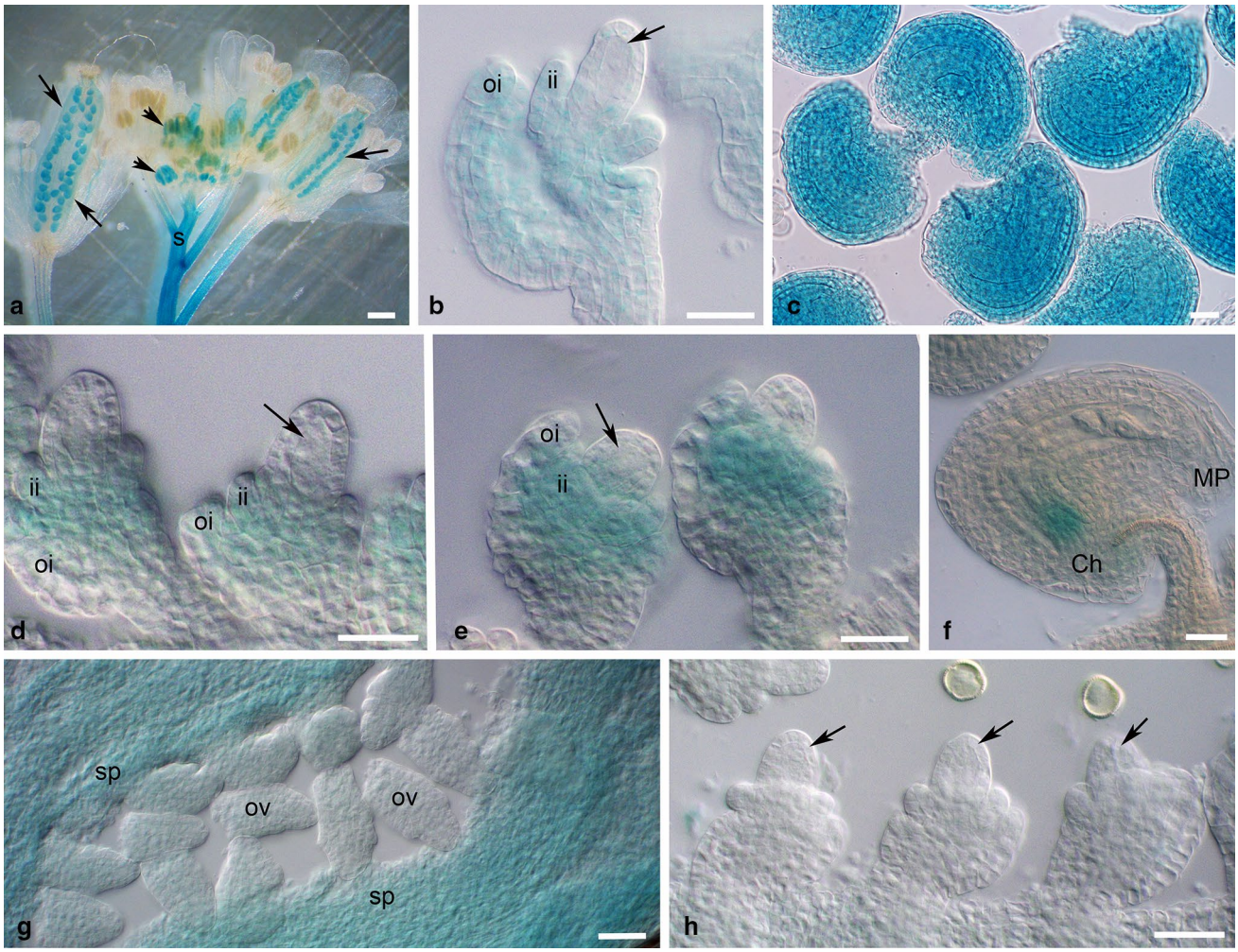


**Fig. 5** Histochemical GUS analysis in inflorescences and ovules of *Arabidopsis thaliana* *pAtGID1::AtGID1–GUS* transgenic lines before anthesis. **a–c** *pAtGID1a::AtGID1a–GUS* line; **d–f** *pAtGID1b::AtGID1b–GUS* line and **g, h** *pAtGID1c::AtGID1c–GUS* line. **a** GUS staining can be observed in stem (S), ovules (arrows) and anthers (arrow heads). **b** At 2-III stage of the ovule development GUS signal was localized in the inner (ii) and the outer (oi) integuments, the expression was not detected in MMC in meiosis (arrow) and nucellar cells. **c** In the mature ovules the GUS signal was localized throughout the embryo sac and the integuments. **d** In the 2-III stage and **e** first stage of gametogenesis, GUS signal was localized in the inner (ii) and outer (oi) integuments of the ovules, the expression was not detected in MMC in meiosis (arrow) and nucellar cells. **f** In the mature embryo sac the GUS signal was localized mainly in the chalaza (Ch) in comparison with the micropyle region (MP). **g** GUS signal was localized specifically in the septum (sp), but was completely absent in the ovules (ov) and funiculi. **h** GUS signal was undetectable in ovules, arrows showing the MMC in meiosis and the nucellar cells. *MMC* megaspore mother cell; Scale bars **a** 1 mm; **b–f** 20 µm and **g, h** 50 µm

*p35S::AtGID1a* plants the number of seeds per silique increased relatively to wild-type. The transgenic plants from both genotypes showed an average of 55 seeds per silique in the 30 siliques counted with 1.5% of aborted seeds, while wild-type plants, under the same environmental conditions, presented an average of 46 seeds per silique in the 32 siliques counted with 2.5% of abortion rate. Statistical analysis (*t* test) showed a significant difference between the number of seeds of the transgenic plants ectopically expressing *AtGID1a*, independent of the promoter, pSTK ($p = 0.0009$) or p35S ($p = 0.001$) in respect of the wild-type plants (Fig. 8, Supplementary Table S1).

To investigate if the additional MMC-like cells had MMC identity, a *pKNU:nlsYFP* line (Tucker et al. 2012) was transformed with *p35S::AtGID1a*. The second generation of transgenic lines overexpressing *AtGID1a* showing 28% of additional MMC-like cells was analyzed (Supplementary Table S1). Plants *pKNU:nlsYFP p35S::AtGID1a* showed only one cell expressing the YFP signal, indicating that one out of the two MMC-like cells acquired the proper MMC identity (Fig. 9).

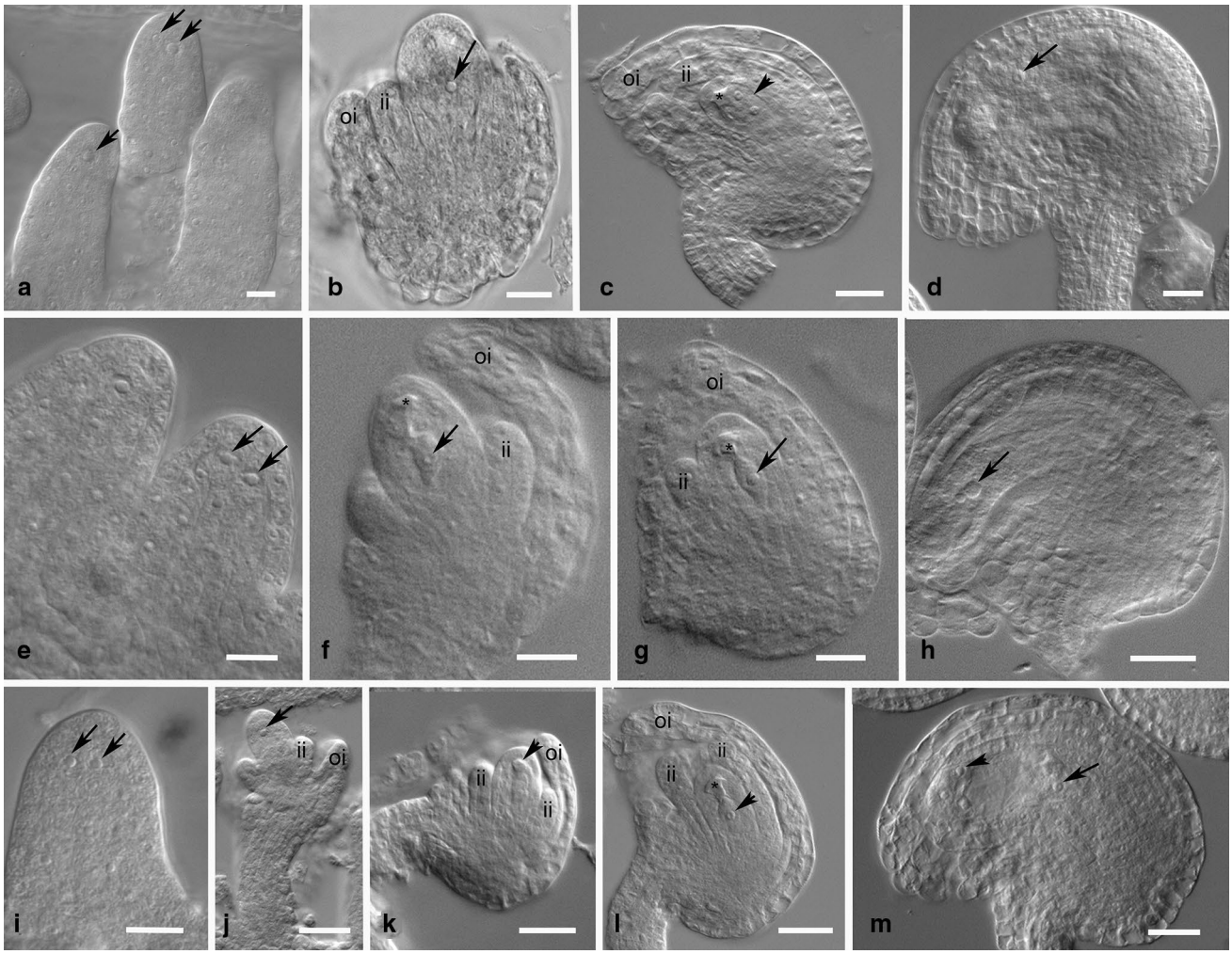


**Fig. 6** Ovules of wild-type and *AtGID1a* overexpression lines of *Arabidopsis thaliana*. **a–d** Wild-type showing normal female gametophyte development. **a** Finger-like stage (2-I) showing MMC-like cells (arrows). **b** FG1 stage displaying MMC (arrow) integument development. **c** FG3 with degenerated megaspores and FG. **d** FG6 stage, arrow indicates the central cell. **e–h** *p35S::AtGID1a* line. **e** Finger-like stage (2-I), showing MMC-like cells (arrows). **f, g** FG1 stage showing well-developed integuments. **h** FG6 stage, arrow indicates the central cell. **i–n** *pSTK::AtGID1a* line. **i** Finger-like stage (2-I), viewing MMC-like cells (arrows). **j** showing MMC (arrow) and inner (ii) and outer (oi) integuments already growing. **k** Tetrad (arrowhead) and growing inner (ii) and outer (oi) integuments. **l** FG1 stage showing functional megaspore (arrowhead), degenerated megaspores and well-developed integuments **m** FG6 stage with antipodal cells (arrow) and three nuclei at the micropylar pole (arrowhead). *ii* Inner integuments, *oi* outer integuments, *MMC* megaspore mother cell, *asterisk* degenerated megaspores, *FG* female gametophyte. Scale bars **a** 20 µm; **b** 10 µm; **c** 20 µm; **d** 40 µm; **e** 20 µm; **f, g** 50 µm; **h** 100 µm; **i** 50 µm; **j–l** 10 µm and **m** 20 µm

## Discussion

GID1 acts in the activation of gibberellin via degradation of DELLA proteins (Davière and Achard 2013; Ueguchi-Tanaka et al. 2007). The detection of GID1 in the transcriptome database of ovaries of sexual and apomictic *B. brizantha* led us to investigate a putative role of this gene in the differential ovule development of sexual and apomictic plants. Alignment of the *BbrizGID1* sequence in databases showed high similarity with sequences from other species of the Poaceae family and the conserved motifs, HGG and GXSXG, from the HSL family. It was suggested that three conserved amino acids of the HSL family form the catalytic triad center: serine (S), aspartic acid (D) and histidine (H) (Osterlund 2001). In all the *GID1* sequences of the Poaceae plants, in which similarity to *BbrizGID1* was detected, replacements of residues from this HSL catalytic center were verified. In rice, a replacement of the H by valine (V) did not affect the binding activity with gibberellin, although an absence of enzymatic activity was detected (Ueguchi-Tanaka et al. 2005). In *B. brizantha*, this same H of the catalytic triad is replaced by isoleucine (I) but S and D are conserved. The residues glicina (G) e arginina (R) are essential to maintain binding between the receptor and the bioactive gibberellin in rice

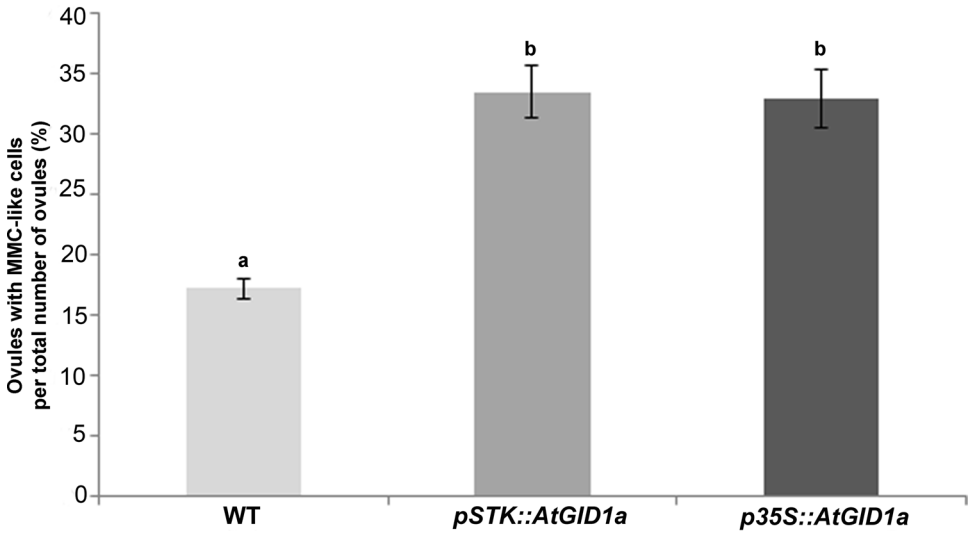


**Fig. 7** Comparison among the number of ovules with additional MMC-like cells of wild-type and transgenic lines *pSTK::AtGID1a* and *p35S::AtGID1a* of *Arabidopsis thaliana*. Different letters indicate statistically significant differences between the number of additional MMC-like cells of transgenic plants and wild-type, independent of the promoter used for *AtGID1a* overexpression, pSTK (p = 0.009) or p35S ($p = 0.013$). *WT* wild-type, *N* number of ovules analyzed, *MMC* megaspore mother cell

(Nakajima et al. 2006; Ueguchi-Tanaka et al. 2005). Comparing the *GID1* sequence of *B. brizantha* with *Arabidopsis* revealed around 60% similarity of the *BbrizGID1* sequence with the three genes *AtGID1a, AtGID1b* and *AtGID1c*. In *Arabidopsis*, analysis of mutant combinations of the three genes showed an overlap of function among them (Griffiths et al. 2006). Complementation with each *AtGID1* gene rescued the dwarf phenotype of the rice gibberellin-insensitive mutant, showing conservation of function among them (Nakajima et al. 2006). *BbrizGID1* is present in the sexual and apomictic genotypes of *B. brizantha*. The restriction pattern of DNA from these plants, observed after *Hind*III digestion and genomic hybridization with *BbrizGID1* probe, suggested a possible different number of copies of the gene in sexual and apomictic plants.

Quantitative analysis of *BbrizGID1* expression showed the highest expression level in ovaries at early megasporogenesis, decreasing in the subsequent stages, independent of the reproductive mode of the plants. At this stage the formation of the MMC in sexual and apomictic genotypes was previously observed, but aposporous initial cells were still not detected in apomicts (Araujo et al. 2000). Analysis of these results suggested that *BbrizGID1* might participate in the early development of ovaries of sexual and apomictic *B. brizantha*, when growing integuments and the differentiation of MMC from nucellar cells occur. Localization of transcripts of *BbrizGID1* in ovules of *B. brizantha* from both reproductive modes revealed that MMC is expressing this gene, while in the surrounding nucellar cells the expression occurs only in the apomicts. These results suggested association of *BbrizGID1* expression not only with the development of MMC but also with the nucellar cells, previous to the differentiation of aposporous initials.

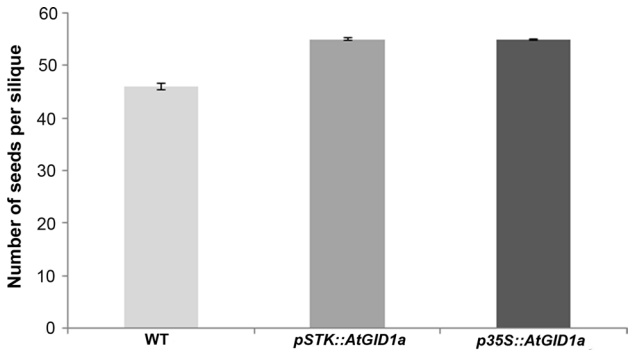


**Fig. 8** Comparison among the number of seeds per silique in *pSTK::AtGID1a* and *p35S::AtGID1a Arabidopsis thaliana* relatively to wild-type (WT) plants. Different letters indicate statistically significant differences between the number of seeds of the wild-type and the transgenic plants ectopically expressing *AtGID1a*, independent of the promoter used, pSTK ($p = 0.0009$) or p35S ($p = 0.001$)

The overexpression of the *AtGID1a* gene in *Arabidopsis* significantly increased the number of additional MMC-like cells of the wild-type Columbia ecotype, suggesting that this gene might be associated with the differentiation of an additional MMC-like cell in the nucellus. Analysis of the second generation of *pKNU:nlsYFP p35S::AtGID1a* suggested that the trait was stably inherited. Different ratios of additional MMC-like cells were previously reported in *Arabidopsis* ecotypes (Rodríguez-Leal et al. 2015). Analysis of *ago9* mutants showed a percentage of abnormally MMC-like cells varying from 37.16 to 47.7%, with identity to gametic cells that do not undergo meiosis, a phenotype that resembles apomictic reproduction by apospory (Olmedo-Monfil et al. 2010). In our work, the identity of MMC was investigated by the expression of YFP marker genes under the control of the KNUCKLES promoter, which drives expression to the MMC (Tucker et al. 2012). *Arabidopsis pKNU:nlsYFP* line, overexpressing *AtGID1a*, showed only one cell with MMC identity in each ovule,

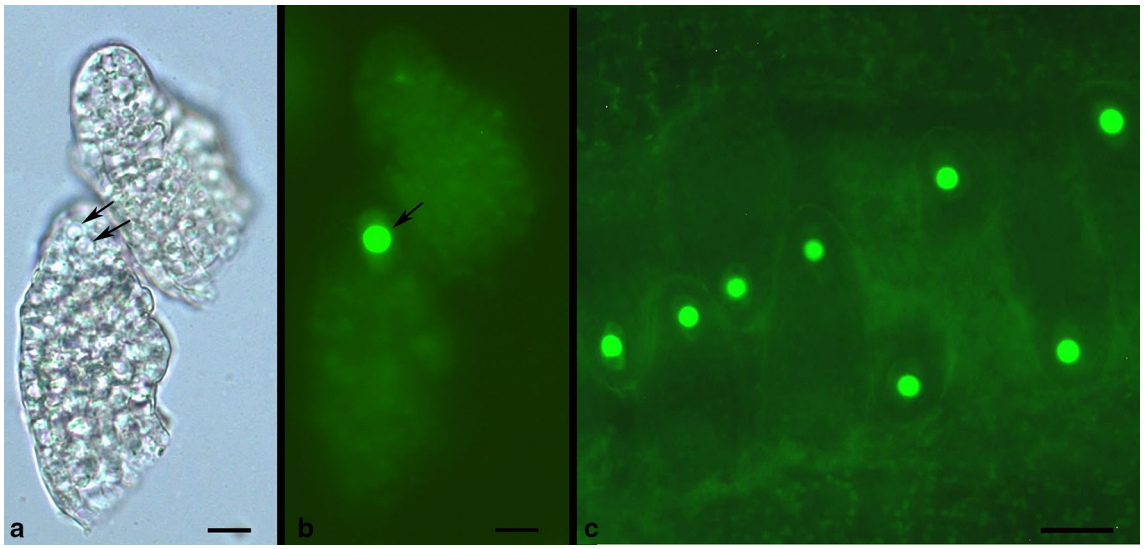


**Fig. 9** *Arabidopsis thaliana* ovules of *pKNU:nlsYFP* line with the MMC YFP-labeled overexpressing *AtGID1a* under the control of p35ScaMV. **a** Ovules at the 2-III stage with two MMC-like cells (arrows) and **b** at the same moment using UV light showed signal of fluorescence only in the MMC (arrow), not in the additional enlarged cells of the overexpressed plants. **c** Ovules finger-like (2-I) stage, showing yellow florescence in the MMC cells. *MMC* megaspore mother cell; scale bars **a**–**c** 10 µm

comparable to what happens in plants of the *pKNU:nlsYFP* line, suggesting the possibility of identity other than with MMC to the MMC-like cells. It is important to note that the KNUCKLES promoter was not previously tested for identity of MMC-like cells.

Gallego-Giraldo et al. (2014) previously verified the differential expression of *AtGID1a, AtGID1b* and *AtGID1c* in ovules, at anthesis, using GUS staining. Expression of *AtGID1a* was detected in all tissues of the ovules, with higher expression observed in the surrounding of the ES, while *AtGID1b* expression was observed mainly in the chalaza zone, at the base of the ES, and *AtGID1c* was undetectable in the ovules. In our study, we detected *AtGID1a, AtGID1b* and *AtGID1c* expression reported by GUS assays during the early stages of ovule development, before anthesis. *AtGID1a* and *AtGID1b* were localized in the inner and outer integuments, not in MMCs and the surrounding nucellar cells. Expression signal of *AtGID1c* was not detected in the ovules, before anthesis. Our results suggested that *AtGID1a* and *AtGID1b* expression is associated with integument development in *Arabidopsis*. The association of the gibberellin and integument development was previously described (Gomez et al. 2016). In plants overexpressing *AtGID1a*, in comparison to the wild-type plants, an asynchrony between integument development and the stage of gametogenesis suggests that the ectopic expression of *AtGID1a* resulted in either a small delay in megasporogenesis or a faster growth of integuments in the transgenic plants. Consistent with the importance of gibberellin in the correct seed development to form seed-filled siliques, overexpressed *AtGID1a* plants were fertile without any sign of ovule or seed abortion and their siliques were seed-filled.

We demonstrated *GID1* expression during megasporogenesis in ovules of sexual and apomictic *B. brizantha*, especially in the MMC. Specifically in ovules of the apomictic plants, *BbrizGID1* is also expressed in the nucellar cells, previously to aposporous initial cell differentiation. In *Arabidopsis*, overexpression of *AtGID1a* triggered the differentiation of MMC-like cells in the nucellar region. These data suggest the involvement of *GID1* in the early events of ovule and ES development.

**Author contribution statement** LGF, DMAD, ASTI, LC and VTCC designed the project; LGF, DMAD, ASTI, ACMMG, MAM and VTCC performed experimental procedures; LGF, DMAD, MAM, LC and VTCC, participated in the research, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

**Acknowledgements** The authors acknowledge Dr. Perez-Amador who provided seeds of *pAtGID1a:AtGID1a-GUS, pAtGID1b:AtGID1b-GUS* and *pAtGID1c:AtGID1c-GUS* transgenic lines and Dr. Tucker who supplied seeds of *pKNU:nlsYFP* line. We also thank the assistance of Simona Masiero, Lilian Florentino and Júlio Rodrigues in the laboratory; Roberto Togawa and Priscila Grynberg in the bioinformatics analysis; and Joseane Padilha in the statistical analyses. This work was supported by the National Council for Scientific and Technological Development-CNPq (449636/2014-3-VTCC) and the Brazilian Agricultural Research Corporation-Embrapa (02140101400.00-VTCC). This work is part of Luciana Gomes Ferreira's PhD thesis from Pós-Graduação em Biologia Molecular, University of Brasilia-UnB,

Brazil, with fellowship from Coordination for the Improvement of Higher Level Personnel—University of Brasília, CAPES-UnB, which also provided a 1-year fellowship at Università degli Studi di Milano, Italy. Foundation for Research Support of the Federal District-FAPDF provided financial assistance for LGF's participation in congresses.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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