Communicating across generations: the Bsister Language

Jamila Bernardi[1], Irma Roig-Villanova[2], Adriano Marocco[1] and Raffaella Battaglia[1]

[1] Università Cattolica del Sacro Cuore, Istituto di Agronomia, Genetica e Coltivazioni erbacee, Via Emilia Parmense, 84, Piacenza, Italia.
[2] Università degli Studi di Milano, Dipartimento di BioScienze, Via Celoria 26, Milano, Italia

Correspondence: Raffaella Battaglia, Istituto di Agronomia, Genetica e Coltivazioni erbacee, Via Emilia Parmense, 84 Piacenza, Italia. Tel +39 0523 599208. E-mail: raffaella.battaglia@unicatt.it

Abstract

B_{sister} proteins form a clade of MADS-box transcription factors that originated 300 million years ago, after ferns diverged but before Angiosperms and Gymnosperms lineages did. Thus, B_{sister} proteins have been found in both Gymnosperm and Angiosperm species such as paddy oat (*Gnetum gnemon)*, ginkgo, yew (*Taxus baccata*), rape seed, rice, maize, wheat, petunia, snapdragon, tomato and Arabidopsis. In all these species, they are expressed in female reproductive organs.
In this review we go over the evolution and pattern of expression of the B_{sister} proteins, and we have a glance on their interaction patterns in the form of high order MADS-box complexes in different species. We describe the functions that have been assigned to them according to the analysis of mutants and RNA interference data. We finish this review discussing from a novel point of view the role that B_{sister} proteins might have, also in tetramer combinations with other MADS-box proteins, on the regulation of tissues communication occurring during reproduction. It is known that a cross-talk is essential for a proper ovule and seed development and B_{sister} and their target genes might play key roles in these communication processes.

Key words: B_{sister}, Integument, MADS-box protein complexes, Ovule development, Reproductive cross-talk, Seed development.

Introduction

The evolutionary success of flowering plants likely depends on the novelties of their reproductive structures. On the female part, tissues with different genetic composition closely differentiate before and after the fertilization. During ovule development, maternal sporophytic tissues, namely the ovule integument(s), coordinately develop together with the haploid generation, the embryo-sac. Next, endosperm and embryo, which are the products of fertilization, growth in close proximity to the maternal seed coat which differentiate from the ovule integument. Mutant analysis supports the idea that a molecular cross-talk occurs in different directions before and after the fertilization: from the integument(s) to the internal tissues and vice versa. Disturbs in these communication mechanisms strongly decrease the fitness of the plant (for reviews see Bencivenga et al. 2011; Nowak et al. 2010).

Focusing on the "mother language", the functional characterization of *sporophytic and megagametogenesis defective* mutants (*smd*) allowed the identification of genes that play a role in the sporophytic control of embryo-sac differentiation (Schneitz et al. 1997; Bencivenga et al. 2011). Successively, the description of female sporophytic sterile plants gave important clues on the signals from the maternal layers to the endosperm and embryo during seed formation (Nowak et al. 2010).

In the next paragraphs, we present the role of the MADS-box genes belonging to the B_{sister} clade (Becker et al. 2002). Besides the observation that B_{sister} gene expression profile and protein interaction patterns are highly conserved in distantly related species, the reduced fertility observed in most of the B_{sister} mutant plants is often sporophytically controlled (de Folter et al. 2006; Deng et al. 2012; Chen et al. 2013; Yin & Xue 2012; Yang et al. 2012; Lee et al. 2013; Mizzotti et al. 2012). In our opinion, understanding the molecular pathways regulated by the B_{sister} transcription factors might strongly contribute to unveil the cell-cell communication mechanisms occurring between the maternal layers and the embryo-sac as well as between the seed coat and the products of fertilization.

The B_{sister} clade: gene structure and phylogenetic analysis

Within the eukaryotes, MADS-box transcription factors play fundamental roles in developmental control and signal transduction. It is well documented that in higher plants the MADS-box gene family underwent extensive duplications; following these events, neo- and sub-functionalization mechanisms strongly contributed to the evolution of plant form (Theißen, 2000).

Plant MADS-box gene family can be subdivided into two major classes named type I and type II (Alvarez-Buylla et al. 2000). Plant type II MADS-box transcription factors are characterized by a conserved structure, indicated as MIKC-type, comprising a MADS (M), an intervening (I), a keratin-like (K) and a C-terminal (C) domain (Ma et al. 1991; Munster et al. 1997; Theißen et al. 1996). Phylogeny reconstruction of the MADS-box MIKC type genes identified 12 clades known as *AGAMOUS* (*AG*)-, *AGL2*-, *AGL6*-, *AGL12*-, *AGL15*-, *AGL17*-, *DEFICIENS*/*GLOBOSA* (*DEF*/*GLO*)-, *FLOWERING LOCUS C* (*FLC*)-, *GGM13*-(Bsister), *SQUAMOSA* (*SQUA*)-, *StMADS11*-, *TM3*-like

genes. Functional data showed that genes belonging to the same clade have similar expression profiles and highly related functions (Becker et al. 2000).

Undoubtedly, MADS-box gene activity is tightly correlated to the evolution of floral structures; the role of MADS-box genes in the determination of floral organ identity is described by the so-called ABCDE model (Coen and Meyerowitz, 1991; Causier et al. 2010). Accordingly, different classes of gene activity are expressed in overlapping floral domains thus determining organ identity. Class A (*SQUA*-like) genes determine sepal identity, petal formation depends on the activity of class A and B (*DEF*/*GLO*-like) genes; class B and C (*AG*-like) genes are required for stamen differentiation while carpel identity is regulated by C class genes. In some species, member of the *AG* clade have a specific role during ovule formation thus defining the D class (Angenent et al. 1995; Colombo et al. 1995; Pinyopich et al. 2003; Favaro et al. 2003). For almost ten years, functional redundancy masked the existence of E (*SEP1*- or *AGL6*-like) class genes which is indispensable for the differentiation of all floral organs (Pelaz et al. 2000; Ditta et al. 2004).

The founder of the B_{sister} clade is the *Gnetum gnemon GGM13* gene (Becker et al. 2002). Phylogenetic analysis of B_{sister} genes identified in Angiosperms and Gymnosperms suggested that they represent a monophyletic group which is a sister clade of the B (*DEF*/*GLO*) genes. The *DEF*/*GLO* and *GMM13* clades likely originated after the duplication of an ancestral gene in the lineage that led to extant seed plant before the Angiosperm and Gymnosperm lineages diverged 300 Million Years Ago (MYA) (Becker et al. 2002). B_{sister} proteins share with B proteins a shorter I domain compared with other MADS-domain factors, a subterminal PI motif-derived sequence and, in some cases, a PaleoAP3 motif in the C-terminal region (Becker et al. 2002).

Phylogenetic tree showing the common origin of the B and B_{sister} clades as well as the relationship between selected Angiosperm and Gymnosperm B_{sister} genes is represented in Figure 1. Eudicots B_{sister} genes cluster together: a Brassica subclade composed of the four *Brassica napus* paralogous (*BnTT16.1*, *BnTT16.2*, *BnTT16.3* and *BnTT16.4*) and *Arabidopsis* B_{sister}*/TRASPARENT TESTA 16* (*ABS/TT16*) (hereafter indicated as *ABS*) is clearly distinguishable from the other cluster composed of B_{sister} genes identified in three Asterid species, *Antirrhinum majus*, *Petunia hybrida* and *Solanum lycopersicum*. The gene duplication event leading to *ABS* and *GORDITA* (*GOA*) occurred during diversification of the Brassicaceae (Erdmann et al. 2010). The genetic distance between the Arabidopsis B_{sister} genes, *ABS* and *GOA*, is supported by their functional characterization, which suggested that *GOA* evolved under relaxed selection pressure and acquired novel roles compared to *ABS* (Prasad et. al 2010; Prasad & Ambrose 2010, Erdmann et al. 2010). Among the monocots, two branches divide the putative canonical B_{sister} genes orthologous to *ABS* (*ZMM17*, *OsMADS29* and *TaBsis*) and the others originated by duplication of the same ancestor (*OsMADS30*, OsMADS*31* and *ZmMADS31*). Gymnosperm B_{sister} genes, *GGM1* and *GBM10*, originated from the same ancestor of the Angiosperms.

Distantly related B_{sister} genes share conserved expression profile

The leitmotif of B_{sister} gene is that they are mainly transcribed in female reproductive organs. This contraposition with the B-class genes, predominantly expressed in male organs, led to the intriguing hypothesis that the B_{sister} genes played a pivotal role in the evolution of reproductive structures in Gymnosperms and Angiosperms (Becker et al. 2002).

Literature information, together with the availability of transcriptome datasets, allows a comprehensive scenario of B_{sister} gene expression in distantly related species. Figure 2 shows B_{sister} gene expression profiles in selected Angiosperms and Gymnosperms. In some cases, detailed expression studies revealed a fine-tuned regulation of B_{sister} gene transcription during ovule formation and early stages of seed development. The *ABS* gene is expressed in the endothelium in mature ovules and young seeds (Mizzotti et al. 2012; de Folter et al. 2006). Dissection of *B. napus* seeds into embryo, endosperm, inner integument and epidermis underlined a predominant transcription of the four *B. napus* B_{sister} genes in the inner integument (Chen et al. 2013). Among the Asterids B_{sister} genes, *FLORAL BINDING PROTEIN 24* (*FBP24*) is transcribed in young ovules and, later in development, the expression is restricted to the endothelium (de Folter et al. 2006). Specific expression in the seed endothelium is reported also for the *DEFICIENS HOMOLOG 21* (*DEFH21*) gene (Becker et al. 2002).

Moving to monocots, the rice *OsMADS29* gene is expressed during ovule differentiation and seed development. Within the seed, *OsMADS29* can be detected in the cells originating from the inner epidermis that are involved in nutrient transfer from the mother plant to the next generation (Yin & Xue 2012; Yang et al. 2012; Lee et al. 2013). Ovule and seed integument expression is reported also for the *ZMM17* and *TaBsis* genes (Becker et al. 2002; Yamada et al. 2009).

B_{sister} gene transcriptional regulation has been studied in *G. gnemon*, G*inkgo biloba* and *T. baccata*; these Gymnosperm species are extremely interesting from an evolutionary point of view since fertilized ovules form a fleshy fruit-like structure (Becker et al. 2002; Lovisetto et al. 2012; Lovisetto et al. 2013). Conservation of B_{sister} gene expression pattern over at least 300 million years is demonstrated by the transcriptional regulation of the *G. gnemon GGM13*, *G. biloba GMB10* and *T. baccata TbBS* genes which are all expressed in female reproductive organs and, more specifically, in the tissue layers surrounding the ovule (Becker et al. 2002; Lovisetto et al. 2013). Furthermore, a derived "fruit" role can be postulated for the *GBM10* gene that is expressed in the outermost seed integument from which the fleshly fruit-like structure originate after the fertilization (Lovisetto et al. 2012; Lovisetto et al. 2013).

In some genomes, paralogous B_{sister} genes with not overlapping expression domains can be identified. As already described in the phylogenetic tree, a duplication event occurred within the Brassicaceae and led to the formation of *GOA* and *ABS* in the Arabidopsis genome. Similarly, a monocots specific gene duplication gave rise to B_{sister} paralogous in the rice and maize genomes (*OsMADS29, OsMADS30 and OsMADS31* in rice and *ZMM17* and *ZMM31* in maize). The not overlapping domains

of *ABS* and *GOA* reflects the fact that *GOA* evolved new functions. Besides a wider expression in floral organs with respect to *ABS*, *GOA* is preferentially transcribed in the ovule and seed outer integument (Prasad et al. 2010; Erdmann et al. 2010). Concerning the monocots, *OsMADS30* is expressed throughout all organs of the rice plant while the expression of *OsMADS31* is hardly detectable (Yang et al. 2012). It will be interesting to investigate which evolutionary mechanisms are acting on the function of the rice B_{sister} paralogous genes.

Comparing B_{sister} expression profile in distantly related species confirms the first observation of extremely conserved female organ-specific genes (Becker et al. 2002). A closer look highlights that members of the *GGM13-like genes* clade are active in maternal tissues surrounding the haploid gametophytic generation before fertilization and the endosperm and embryo after the arrival of the spermatic nuclei.

Conserved B_{sister} protein interaction patterns

Protein interaction experiments in yeast and *in planta* (Egea-Cortines et al. 1999; Nougalli Tonaco et al. 2006) indicated that MADS-box floral identity proteins interact to form multimeric complexes also addressed as "floral quartets" (Theißen and Saedler, 2001). These quaternary complexes are supposed to bind two *cis*-regulatory regions termed CArG boxes (for "CC-Arich-GG sequence) (Riechmann et al. 1996; Schwarz-Sommer et al. 1992). While in Angiosperms the class E proteins play an indispensable role in the constitution of floral quartets, it has been recently demonstrated the formation of floral quartet-like complexes through the interaction of Gymnosperm orthologs of class B and C MADS-box proteins (Wang et al. 2010). Diversification of MADS-box proteins function is therefore linked to the formation of different protein complexes; the interacting partners might mainly define the biological role of each complex.

The functional characterization of B_{sister} proteins includes the analysis of their capacity to assemble into MADS-box protein complexes. In Arabidopsis and Petunia, yeast three-hybrid assay (Y3H) demonstrated that ABS can interact with C and D class proteins in presence of the E class factors (Kaufmann et al. 2005; de Folter et al. 2006). Interestingly, in Arabidopsis, the complex between ABS and the class D protein SEEDSTICK (STK) has been recently proved to have a relevant role during fertilization and seed development (Mizzotti et al. 2012). Diverse interaction partners have been described for GOA, compared to its paralogous ABS (Prasad & Ambrose 2010; Erdmann et al. 2010).

In different conifers and *Gnetum* species, the *AGL6*-like genes represent the orthologs of the class E genes (Becker & Theißen 2003; Melzer et al. 2010). Y2H assays and pull-down experiments recently proved heterodimer formation between the GGM13 B_{sister} protein and orthologous of class B (GGM2), class C (GGM3) and AGL6-like (GGM11) proteins (Wang et al. 2010).

The identification of B_{sister} interacting partners represents an important tool to dissect the biological role of these MADS-box factors during plant development and evolution. Indeed, proteins involved in

the formation of higher order complexes often share functional redundancy as the seed phenotype of the Arabidopsis *abs-6 stk-2* double mutant recently demonstrated (Mizzotti et al. 2012).

B_{sister} gene activity contributes to reproductive cross-talk in seed plant

Sequence analysis, expression profile data and protein interaction experiments strongly support the hypothesis that the focus of B_{sister} gene function has been conserved since their appearance 300 MYA; before the separation of the Gymnosperm and Angiosperm lineages but after the diversification of the fern lineage. Clues on the functional roles of B_{sister} genes are recently emerging, the comparison and integration of the results described in different species will contribute to highlight some aspects of the evolutionary mechanisms at the base of seed plant development and evolution.

In the last ten years, mutant plants affected in B_{sister} activity have been described in Arabidopsis, Petunia, Brassica and rice (Nesi et al. 2002; Kaufmann et al. 2005; Prasad et al. 2010; Prasad & Ambrose 2010; Erdmann et al. 2010; de Folter et al. 2006; Deng et al. 2012; Chen et al. 2013; Yin & Xue 2012; Yang et al. 2012). Intriguingly, the defects due to mutations in B_{sister} genes are sporophytically controlled. The first B_{sister} mutant described is the Arabidopsis *abs* mutant (Nesi et al. 2002). Morphological analysis of the pale *abs* mutant seeds demonstrated that ABS activity correlated with endothelium differentiation where it controls cell structure and pigment accumulation. Endothelial cells in *abs* mutant seeds appeared flatter and irregular in shape compared to wild-type, furthermore, these cells lack proanthocyanidins (PA) accumulation. In *abs* mutant background, seed coat defects are visible immediately after fertilization (Nesi et al. 2002). ABS function in Arabidopsis is partially masked by redundancy with the MADS-box gene *STK* (Mizzotti et al. 2012). The phenotype of the *abs-6 stk-2* double mutant suggested that the MADS-box protein complex composed of ABS and STK is required for ovule and seed formation. Simultaneous lack of ABS and STK activity caused complete absence of endothelium with repercussion on embryo-sac formation, fertilization and seed development. Interestingly, the *abs-6 stk-2* double mutant is female sporophytic sterile as *ABS/abs-6 STK/stk-2* heterozygous plants are fully fertile (Mizzotti et al. 2012). Interestingly, seeds with a reduced amount of endosperm have been described in petunia plants where the two *STK* orthologous (*FLORAL BINDING PROTEIN 7 – FBP7* – and *FBP11*) were down-regulated (Colombo et al. 1997). Genetic analysis showed that this phenotype was sporophytically controlled as the *abs-6 stk-2* phenotype. Taken together, these results suggest that some overlapping functions between the B_{sister} and D-class genes might be conserved in different species.

Target gene identification of the Arabidopsis protein complex composed of ABS and STK will represent a starting point to dissect the molecular cross-talk occurring between the ovule integuments and the developing gametophyte, as well as between the seed coat and the fertilization products.

Plant development in *goa* loss of function mutant definitely demonstrated the fact that *GOA* evolved new regulatory roles not overlapping with *ABS* (Prasad et al. 2010; Prasad & Ambrose 2010; Erdmann et al. 2010). Developmental defects in *goa* mutant plants suggest that *GOA* contributes to

the control of fruit cell expansion and to the differentiation of seed outer integument (Prasad et al. 2010; Prasad & Ambrose 2010; Erdmann et al. 2010). The *goa-1 abs-1* double mutant phenotype showed that *ABS* and *GOA* have additive roles during seed coat development (Prasad et al. 2010).

Four paralogous *ABS* genes (*BnTT16.1*, *BnTT16.2*, *BnTT16.3* and *BnTT16.4*) have been identified in the allotetraploid species *B. napus* (Deng et al. 2012; Chen et al. 2013). Simultaneous down-regulation of the four *BnTT16* genes using an RNA interference (RNAi) approach influences plant size, flowering time, floral morphology, embryo formation, seed development and seed set (Deng et al. 2012). Interestingly, shorter siliques and reduced seed set are visible when the female plants carry the RNAi construct, independent from the male parent genetic background (Deng et al. 2012).

Among the Asterids, functional characterization of a member of the GMM13 clade, the *FBP24* gene, is reported in Petunia (de Folter et al. 2006). *FBP24* loss of function resulted in a maternally-controlled phenotype. Silencing the *FBP24* gene affects endothelium cell identity with repercussion on plant fertility; reciprocal crosses showed a reduced seed set compared to wild-type plants only when the *FBP24* gene is down-regulated in the mother plant. Occasionally, morphological defects are described before fertilization since few ovules lacking the embryo-sac have been identified (de Folter et al. 2006). This phenotype is visible only when the *FBP24* endogenous gene is down-regulated as a consequence of co-suppression, on the contrary transposon insertion within the *FBP24* locus did not cause defects compared to wild-type plants. Taken together, these results suggest functional redundancy with unknown factors which might be silenced in the *fbp24* co-suppressed lines (de Folter et al. 2006).

Until now, the only mutant phenotypes correlating with lack of B_{sister} function within the monocots is reported in rice (Yin & Xue 2012; Yang et al. 2012; Lee et al. 2013). The role of *OsMADS29* was recently elucidated using an RNAi construct which specifically silences the endogenous *OsMADS29* gene (Yin & Xue 2012; Yang et al. 2012) and through the characterization of the spontaneous *female sterile* (*fst*) mutation which resulted to map in the *OsMADS29* locus (Lee et al. 2013). In the plant analyzed, *OsMADS29* lack of activity is responsible for sporophytic female sterility. In *fst* homozygous plants, defects are visible before fertilization mainly during ovule integument development (Lee et al. 2013). Following fertilization, both the *fst* mutant and OsMADS29 RNAi plants produced aborted or shrivel seeds mostly lacking endosperm (Yin & Xue 2012; Yang et al. 2012). Detailed morphological analysis showed that the down-regulation of the *OsMADS29* gene correlates with reduced or delayed cell degradation of the maternal tissue named nucellar projection (NP). In wild-type plants, fertilization is followed by programmed cell death (PCD) of the NP tissue; this event is required for the efficient nutrient transfer to both embryo and endosperm. Since *OsMADS29* is not expressed in the endosperm, aborted seeds in mutant plants are likely the consequence of defects during nutrient transportation from the maternal tissues to the next generation (Yin & Xue 2012; Yang et al. 2012). Interestingly, a possible role for the wheat B_{sister} gene (*TaBis*) during seed vascular bundle formation has been postulated (Yamada et al. 2009).

Ectopic expression of B_{sister} genes has been performed in different species (Kaufmann et al. 2005; Prasad et al 2010; Erdmann et al. 2010; de Folter et al. 2006; Lovisetto et al. 2013). While in some cases the phenotype of transgenic plants confirmed the data observed in knock-out plants (35S::GOA plants are smaller than wild-type) in other situation ectopic expression of B_{sister} genes is responsible for pleiotropic defects (Kaufmann et al. 2005; Prasad et al. 2010; Erdmann et al. 2010). Widespread developmental defects as a consequence of ectopic expression of MADS-box transcription factors might likely depends on the unbalanced changes in protein complexes that can be formed when a MADS-box protein is over-produced. Since B_{sister} proteins participate in the formation of quaternary MADS-box protein complexes, the effects of their ectopic expression might not completely reflect the B_{sister} physiological function.

Overall, both monocots and dicots B_{sister} mutant plants indicated that B_{sister} factors play key roles in the differentiation of the ovule sporophytic tissues and maternal-derived seed compartments with consequence on plant fertility. In some case this function is redundantly shared with other MADS-box genes as recently described in Arabidopsis (Mizzotti et al. 2012) while in other species it seems restricted to B_{sister} genes as it can be deduced by the defects observed in the rice *osmads29* mutant plants (Yin & Xue 2012; Yang et al. 2012; Lee et al. 2013)

Concluding remarks and future perspective

In the aim to dissect molecular aspects of reproduction in higher plants, the increasing information regarding B_{sister} gene function in distantly related species represent an important achievement.

The fine-tuned regulation that MADS-box transcription factors play during plant development is strongly dependent on the formation of interchangeable protein complexes which act as functional units. In this perspective, the identification of protein complexes comprising B_{sister} factors might therefore represent an important step to highlight the biological roles *GGM13-like genes*. Furthermore, the possibility to identify target genes of MADS-box protein complexes comprising the B_{sister} proteins will represent the chance to dissect part of the molecular components involved in tissues communication during ovule and seed formation in higher plants. Promisingly, the Gene Ontology (GO) classification of genes differentially expressed in *fst* rice mutant compared to wild type plants comprises auxin efflux and polarity, hormone regulation, signal transduction, sugar metabolism and apoptosis related genes (Lee et al. 2013). Target genes of the B_{sister} factors likely belong to these functional classes.

Excitingly, we are now at the beginning of understanding the general principles of tissue communication during ovule and seed formation, these aspects are important not only for basic research but also in the perspective to manipulate seed quality for food source.

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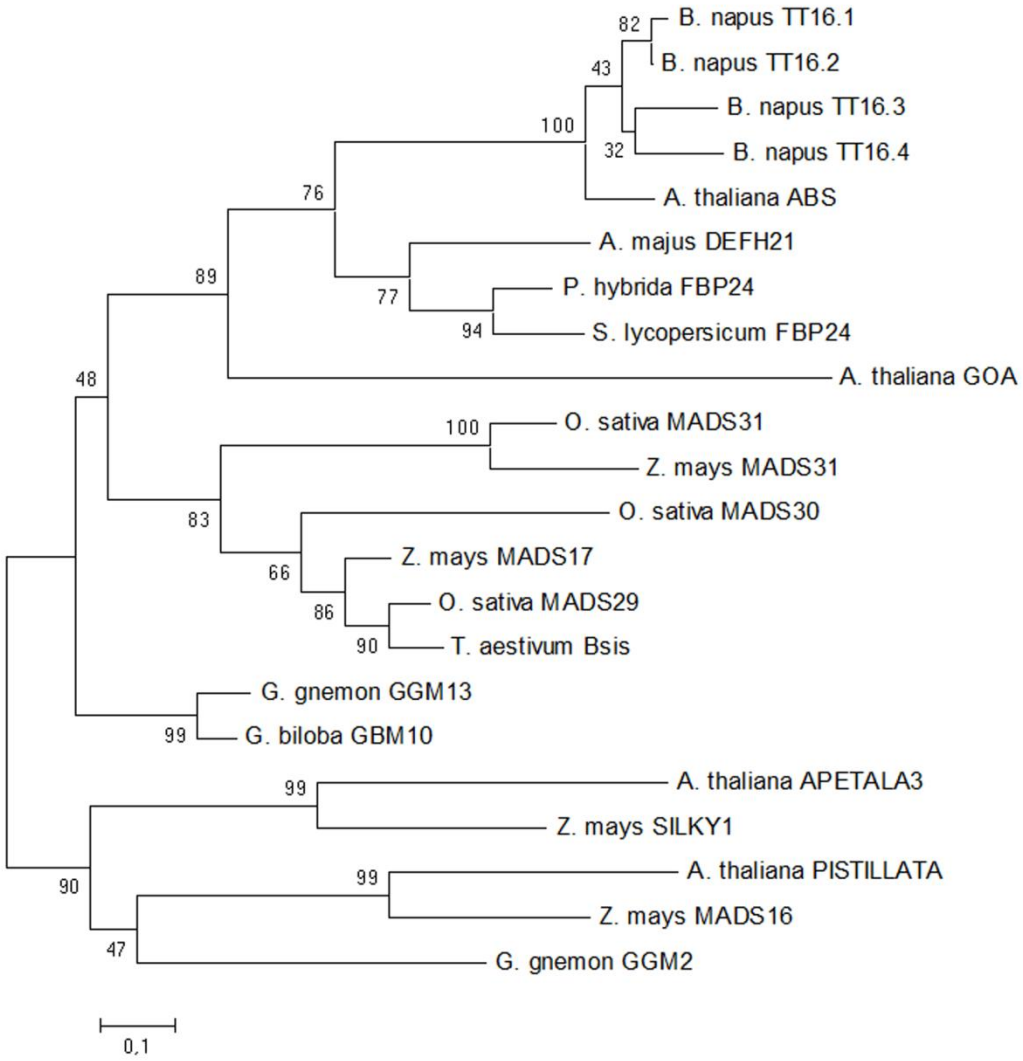

Figure 1. Phylogenetic analysis of class-B and Bsister genes. Nucleotide sequences were translated into proteins and aligned with the muscle algorithm and a Maximum Likelihood tree was constructed. The number at the nodes are bootstrap values after 1000 replicates. Sequence accession numbers are the following: *Brassica napus*: *BnTT16.1* (EU192029), *BnTT16.2* (EE192029), *BnTT16.3* (HM449990), *BnTT16.4* (HM449989), *Arabidopsis thaliana*: *AtABS* (AJ318098), *AtGOA* (AY141243), *AtAP3* (M86357), *AtPI* (D30807); *Antrirrhinum majus*: *AmDEFH21*(AJ307056), *Petunia hybrida*: *PhFBP24* (AF335242), *PhMADS2* (X69947), *Solanum lycopersicum*: *SlFBP24* (XM_004249803), *Oriza sativa*: *OsMADS29* (AK109522), *OsMADS30* (AY174093), *OsMADS31* (AY177698), *Triticum aestivum*: *TaBsis* (AM502893), *Zea mays*: *ZmMADS16* (NM_001111666), *ZmMADS17* (Q8VWM8), *ZmMADS31* (GRMZM2G137387; maizesequence.org), *ZmSILKY1* (AF181479), *Gnetum gnemon*: *GGM13* (AJ132219), *GGM2* (AJ132208), *Ginkgo biloba*: *GBM10* (AB029472).

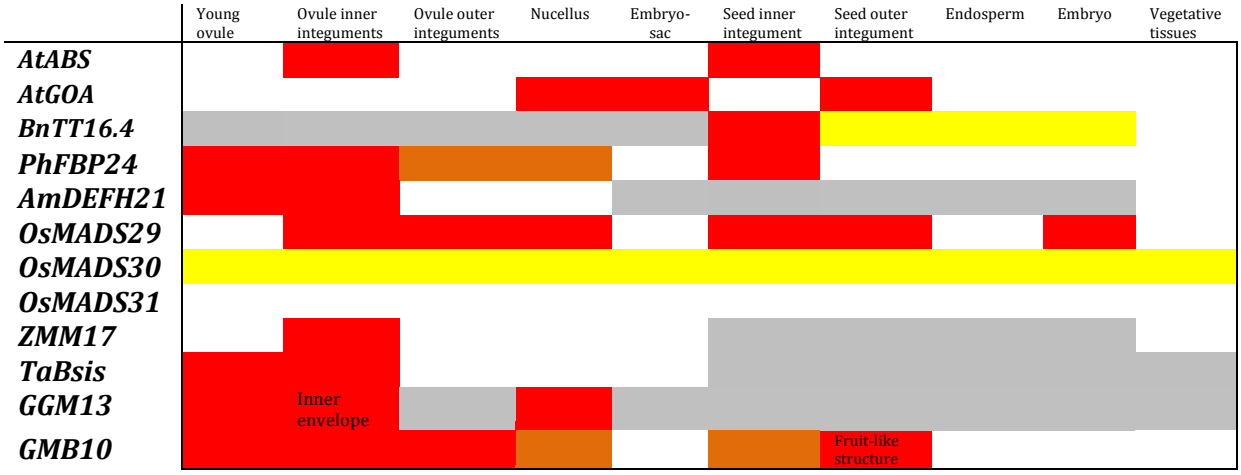

Figure 2. Schematic representation of B_{sister} gene expression levels during ovule and seed formation in selected species. Red: high expression. Orange: medium expression. Yellow: weak expression. White: no expression. Grey: Not known