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Review

MicroRNA-mediated regulation of flower development in grasses

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Flower structure in grasses is very unique. There are no petals or sepals like in eudicots but instead flowers develop bract-like structures – palea and lemma. Reproductive organs are enclosed by round lodicule that not only protects reproductive organs but also plays an important role during flower opening. The first genetic model for floral organ development was proposed 25 years ago and it was based on the research on model eudicots. Since then, studies have been carried out to answer the question whether this model could be applicable in the case of monocots. Genes from all classes found in eudicots have been also identified in genomes of such monocots like rice, maize or barley. What's more, it seems that miRNA-mediated regulation of floral organ genes that was observed in the case of *Arabidopsis thaliana* also takes place in monocots. MiRNA172, miRNA159, miRNA171 and miRNA396 regulate expression of floral organ identity genes in barley, rice and maize, affecting various features of the flower structure, ranging from formation of lemma and palea to the development of reproductive organs. A model of floral development in grasses and its genetic regulation is not yet fully characterized. Further studies on both, the model eudicots and grasses, are needed to unravel this topic. This review provides general overview of genetic model of flower organ identity specification in monocots and it's miRNA-mediated regulation.

Key words: miRNA, grasses, flower development, ABCDE model

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FLOWER STRUCTURE IN GRASSES

The spikelet is the basic unit of the grass inflorescence. It is formed directly on the main axis of the inflorescence or on the branches. The pattern of spikelet formation differs depending on the species. The flower in grasses is located inside the spikelet. The grass flower is composed of two bract-like structures, lemma and palea forming most of the outer whorl. The second whorl is occupied by rounded organ-lodicule which covers stamen and carpel, not only protecting these reproductive structures but also ensuring access to them by swelling during flower opening (Fig. 1). The number of flowers formed in a spikelet is variable depending on the species (Clifford 1987, Bommert *et al*., 2005).

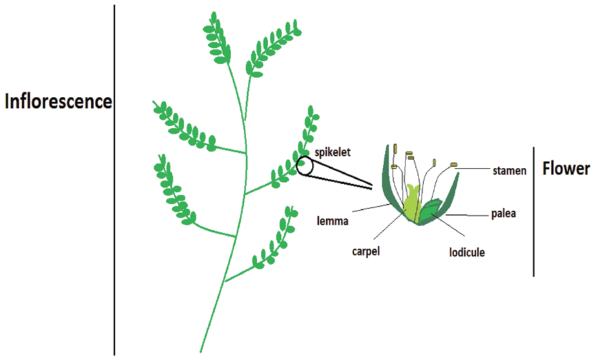

Figure 1. Flower structure in grasses based on the example of rice.

GENETIC REGULATION OF FLOWER DEVELOPMENT-THE ABCDE MODEL

The original ABC flower development model was proposed in 1991 based on the analysis of the phenotypes of floral homeotic mutants with the significant disturbances in floral organ development, whorls in particular. The study was carried out in model eudicots, such as *Arabidopsis thaliana* and *Antirrhinum majus* (Coen & Meyerowitz, 1991). Angiosperm flowers usually have four whorls. According to the original model, in the first whorl only the A genes (*APETALA1*, *AP1*; *APETALA2*, *AP2*) are expressed in addition to the standard leaf developmental genes, and this results in sepal development. In the second whorl, both the A and B genes (*APETALA3*, *AP3*; *PISTILLATA*, *PI*) are functional, and this results in petal production. In the third whorl, the B and C genes (*AGAMOUS*, *AG*) are expressed which promotes stamen production, and in the last whorl only the C class genes are active and therefore the development of carpels occurs (Coen & Meyerowitz, 1991). Later, the D (*STK*) and E (*SEPALLATA1/2/3/4*, *SEP1/2/3/4*, *SHATTERPROOF1*, *SHP1*, and *SHP2*) genes were added to the ABC model. The D-class genes are responsible for ovule specification. What is more, based on phylo-

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Abbreviations: *REP1, RETARDED PALEA1; DPI, DEPRESSED PALEA1; IDS1, INDETERMINATE SPIKELET1; SPW1, SUPERWOMAN1; DL, DROOPING LEAF; TS4, TASSELSEED4;* SPM, spikelet pair meristem; *SID1, SISTER OF INDETERMINATE SPIKELET1, SNB, SUPERNUMERARY BRACT; SCL, SCARECROW-LIKE* genetic studies, the D-class genes originated from the C-class genes as a result of gene duplications that occurred during evolution before divergence of the eudicot and gymnosperms lineages (Kramer *et al.*, 2004). The E-class genes determine the identity of all four whorls of floral organs, since the proteins that are being produced from those genes create functional complexes with the A, B, C and D proteins (Colombo *et al.*, 1995; Pelaz *et al.*, 2000; Favaro *et al.*, 2003; Pinyopich *et al.*, 2003).

Although this model has not been yet fully characterized in grasses, several studies have been conducted. As in eudicots, presence of the A, B, C, D, E-class genes has been confirmed.

In the case of A genes, there are three *AP1-like* genes present in the rice genome (*OsMADS14/RAP1B*, *OsMADS15/RAP1A* and *OsMADS18*) (Litt *et al.*, 2003). Phenotype of the *osmads15/rap1a* mutant shows that this gene is an important factor in palea development in rice (Wang *et al.*, 2010). However, the triple mutant of *OsMADS14, 15* and *OsMADS18* forms normal palea and lemma structures (Kabayashi *et al.*, 2012). It appears that development of palea, at least in rice, is also regulated by the *REP1* (*RETARDED PALEA1*) and *DPI* (*DEPRESSED PALEA1*) genes. Significantly smaller paleas with delayed differentiation were observed in the case of *rep1* mutant and severe deformation in the central region of paleas in the case of *dpi* mutant (Yuan *et al.*, 2009, Jin *et al.*, 2011). In the paper from 2012, *SUPERNUMERARY BRACT* (*SNB*) and *Os INDETERMINATE SPIKELET1* (*OsIDS1*) – two AP2-like genes were identified in rice to be necessary for lodicule development (Nair *et al.*, 2010; Lee *et al.*, 2012,).

In grasses, the B class of floral identity genes is functionally most similar to those present in eudicots, and therefore it is the best characterized group. In rice, there are two genes that are eudicot *PI* orthologues- *MADS2* and *MADS4*. Suppression of *OsMADS2* results in developmental differences in lodicules does not cause changes in stamen but (Prasad *et al.*, 2003). No alteration in lodicule or stamen is induced in a *OsMADS4* gene mutant, but double mutant of *OsMADS2* and *OsMADS4* displays occurrence of palea-like organs instead of lodicule, and carpel-like structures in place of stamens (Yao *et al.*, 2008). Mutants of *MADS16/ SUPERWOMAN1* (*SPW1*) in rice, and *SI1* in maize (homologs of *AP3*), result in transformation of the second whorl lodicules into paleas and lemmas, and conversion of stamens into carpels (Kang *et al.*, 1998). This phenotype is essentially identical to the eudicots' B-class genes' mutant (Ambrose *et al.*, 2000, Nagasawa *et al.*, 2003 Whipple *et al.*, 2004).

In grasses, the C class genes (similar to *AG* genes in eudicots) are responsible for development of both types of reproductive organs. Based on the phylogenetic approach, there are four *AG-like* genes present in the rice genome and six in maize (*OsMADS58*, *OsMADS21*, *OsMADS13*, *OsMADS3* and *ZmZAG1*, *ZmZMM2/ZmZMM23*, *ZmZAG2/ZmZMM1*, *ZmZMM25*) (Ciaffi *et al.*, 2011). Loss of *OsMADS3* function results in conversion of stamen into lodicule, with no effect on the carpels' development, although *OsMADS58* knock-down shows disturbance in the carpel structures. That suggests that there is no single gene responsible for stamen and carpel specification, in contrast to the so far studied eudicots (Yamagushi *et al.*, 2006). Phenotype similar to the *mads58* mutant was shown in the case of maize *zmzag1* mutant (Mena *et al.*, 1996). Moreover, in rice the *DL* (*DROOPING LEAF*) gene has been identified as a C-class gene taking part in the carpel development. Mutation in this gene results in conversion of carpels into stamens (Yamagushi *et al.*, 2004). *OsMADS13* and *OsMADS21,* and their maize homologs (*ZmZAG2/ZmZMM1* and *ZmZMM25*), have been categorized as the D-class genes originated from C-class genes in the course of major gene duplications. *OsMADS13* is predominantly expressed in the ovule where its expression overlaps with *OsMADS21* (expression pattern is similar to *STK* gene in Arabidopsis) (Kramer *et al.*, 2004). Knock-out mutant of *OsMADS13* displayed a complete female sterility (Dreni *et al.* 2007).

The E class genes also have been characterized in grasses. Ten genes belonging to that category have been identified in maize and seven in rice, and these are homologs of *SEP* and *AGL6* genes in eudicots. Their expression pattern suggests that they all play an important role in the flower meristem determinacy (Theissen *et al.*, 2000). A mutant of one of the *SEP-like* genes (*OsMADS1*) in rice, produces flowers with palea and lemma-like lodicule and abbreviated number of stamen and carpels; a similar phenotype is produced in a *mads6* mutant (Jeon *et al.*, 2000). The complexity of flower development model in grasses can be a result of its much more complexed genome than is present in Arabidopsis (Fig. 2).

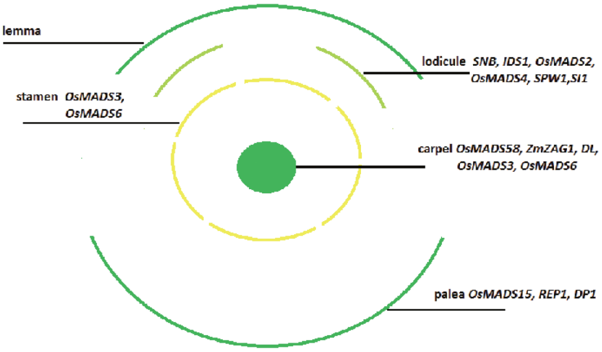

Figure 2. Flower diagram based on the example of rice, with genes corresponding to the development of the flower organs. Due to difficulties with the placement of the classification of individual genes into classes from the ABCDE model in the figure, we provide this classification here: Class A genes – SNB, *IDS1, OsMADS15, REP1, DP1,* Class B genes – *OsMADS2, OsMADS4, SPW1, SI1,* Class C genes – *OsMADS58, ZmZAG1, DL, OsMADS3*, Class E genes – *OsMADS3, OsMADS6.*

MiRNA-MEDIATED REGULATION OF THE FLOWER ORGAN DEVELOPMENT IN GRASSES

MiRNAs are small ribonucleic acid molecules (typically 21 nt in length) which, when incorporated into multi-protein complex RISC (RNA-induced silencing complex), are part of an important regulatory mechanism of gene expression at the transcriptional and post-transcriptional level (Lee *et al.*, 1993; Voinnet *et al.*, 2009; Bartel, 2009; Bielewicz *et al.*, 2012; Bielewicz *et al.*, 2013; Szweykowska-Kulińska *et al.*, 2013). MiRNAs are also a vital element of plant adaptation to changing environmental conditions which was studied in plants responding to the stresses of heat, salinity or excess and deficiency of Cu and Cd (Kruszka *et al.*, 2012; Kruszka *et al.*, 2014; Barciszewska-Pacak *et al.*, 2015). Moreover, the impact of miRNA-mediated regulation on plant growth and development was shown in the case of miRNA393 and an auxin-signaling pathway in *Arabidopsis thaliana* (Windels *et al.*, 2014). Studies conducted so far suggest that the miRNAs' activity is essential in all phases of plant life.

miRNA172

MiRNA172 is conserved among higher plants and it acts at the transcriptional and translational level as a regulator of the plant-specific transcription factor and floral organ identity gene *APETALA2* (*AP2*), as well as *AP2-like* genes, including *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) in Arabidopsis (Park *et al*., 2002; Aukerman *et al*., 2003; Schmid *et al*., 2003; Chen, 2004). Also, overexpression of miR172 causes conversion of sepals and petals into carpels and reduction in the stamen number (Chen, 2004).

There have been several studies establishing the role of miRNA172 in maize, rice and barley (Chuck, 2007; Zhu *et al*., 2009; Nair *et al*., 2010).

In barley, suppression of AP2-like mRNA cleavage mediated by miRNA172 results in occurrence of cleistogamus flowering, which means that palea and lemma are closed together during the period of pollen release (Lord, 1981; Nair *et al*., 2010). That situation is caused by the atrophy of lodicule. Under natural conditions, the lodicule swells upon flower opening, pulling the palea and lemma away and ensuring access to carpel and stamens (Bommert *et al*., 2005). Cleistogamy in barley is under control of a single locus-*cleistogamy 1* (*cly1*) (Turuspekov 2004). *Cly1* encodes a transcription factor belonging to the euAP2 family with two AP2 domains. miRNA172 targeted sequence is embedded in the 10th exon of this gene which is common for a number of AP2 genes (Aukerman, 2003; Chen, 2004).

The role of miRNA172 was also established in maize.

Maize is a monoecious plant having tassels and ears which contain male and female reproductive organs. Flowers start as bisexual but then undergo stamen arrest in the ear, and the abortion of pistils in the tassel (Cheng, 1983). The tasselseed4 (*ts4*) mutant in maize displayed feminization of the tassel caused by a lack of pistil abortion (Phipps, 1928) (Fig. 3).

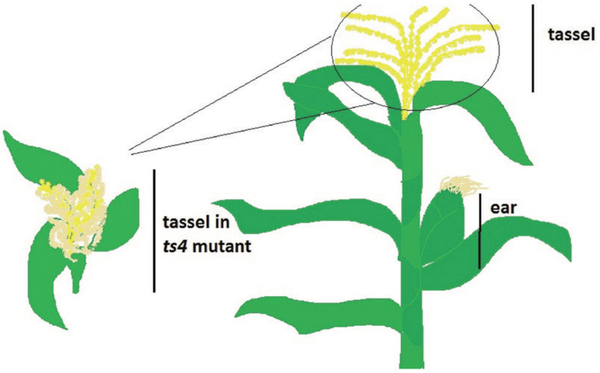

Figure 3. Schematic representation of the wild – type tassel in maize (right) and feminized tassel in the ts4 mutant (left).

TS4 also plays a part in SPM and spikelet meristem determinacy. Inflorescence is initiated from the meristem that under normal conditions produces a regular structure of spikelet pair meristems (SPMs). In case of the *ts4* mutants, the SPMs produced spikelet meristems that display a very irregular pattern. Moreover, it has been established that the *TS4* gene encodes the miRNA172e. Transgenic plants with an insertion within the pri-microRNA transcription unit were produced and the phenotype of these plants was similar to the *ts4* mutant (Chuck *et al*., 2007). MiRNA172e is present in tassel and ears in wild-type plants but undetectable in these structures in the *ts4* mutants (Chuck *et al*., 2007). The zma-miR172e target is the *AP2-like* gene *INDETERMINATE SPIKELET1* (*IDS1*) that is required for spikelet meristem determinacy. An *ids1/ ts4* double mutant had almost completely blocked the defects observed in the *ts4* tassels, supporting the hypothesis that *IDS1* mRNA is a target for miRNA172 (Chuck *et al*., 2007).

A year later, it has been established that also another gene is regulated by miR172e in maize - *SISTER OF INDETERMINATE SPIKELET1* (*SID1*). No mutant phenotype has been observed in single *sid1* mutants, but *ids1/sid1* double mutants produced fewer tassel branches and generated defects in flower development, proving that miRNA172e targets both *AP2-like* gene mRNAs: *IDS1* and *SID1* (Chuck *et al*., 2008).

The role of miRNA172 has been also studied in the case of rice.

The rice miR172 family contains four members (miR172 a-d) which are predicted to target five *AP2-like* genes: *Os03g60430*, *Os04g55560*, *Os05g03040*, *Os06g43220* and *Os07g13170* (Zhu *et al*., 2009, Lee 2007). One of them – *Os07g13170* (*SNB – SUPERNUMERARY BRACT*) has been shown to be required for determination of floral organ identity. The *snb* mutant displays defects in the floral organ development (Lee, 2007). *SNB*, *Os03g60430*, *Os05g03040* and *Os06g43220* are the putative rice orthologues of maize *SID1*, *IDS1*, *ZmRap2.7* and *GL15*, respectively (Chuck *et al*., 2008). Cleavage of the *Os04g55560* mRNA by the miR172 was detected in a shoot and grain, as well as in the booting panicles, while cleavage of the *Os06g43220* mRNA was only detected in the mixed sample of shoot and grain with a low frequency, and cleavage of the *SNB* mRNA was only detected in the booting panicles. Overexpression of the MIR172b in rice causes abnormalities in the flower organ development, including multiple layers of lemma and palea, twisted lemma and palea, degeneration of either lemma or palea or leaf-like structures replacing them, while in some cases no obvious floral organs were produced (Zhu *et al*., 2009).

These phenotypes are in agreement with results acquired by the *snb* mutants, suggesting that *SNB* and at least one of the other four targets of miR172 were repressed in plants over-expressing miR172b (Zhu *et al*., 2009).

miRNA159

Overexpression of *AtmiR159* in Arabidopsis resulted in various phenotypic changes including anther defects or male sterility, therefore it is believed that miR159 and its target genes are involved in important developmental processes (Achard *et al*., 2004). In Arabidopsis, miRNA159 targets *GAMYB-like* genes (Palatnik *et al*., 2003, Achard *et al*., 2004, Millar *et al*., 2005, Schwab *et al*., 2005). *AtMYB33* and *AtMYB65* belong to the GAMYB-like genes (Stracke *et al*., 2001) and their double mutant displays male sterility and anther defects (Millar *et al*., 2005).

MiRNA159 target sequence was also reported in rice OsGAMYB transcription factors (*OsGAMYBL1* and *OsGAMYBL2*). Also, *OsGAMYBL1* was specifically expressed in flowers and co-expressed with miR159 (Hiroyuki *et al*., 2006). Overexpression of miR159 in rice caused development of deformed flowers. In a mild phenotype, the flowers were sterile and developed either shrunken and whitened anthers, or slightly reduced lemma and palea. In a severe phenotype, the palea and lemma production was distorted and flowers were much smaller than in the wild-type plants. They also did not develop stamen and pistil primordia (Tsuji *et al*., 2006). The knockout mutant of *OsGAMYBL1*, *gamyb-1*, showed similar defects (Kaneko *et al*., 2004).

miRNA171

MiR171 is a member of a well-known family of miRNAs that target the transcription factor *SCL (SCARECROW-LIKE*), belonging to a protein family named GRAS (Bolle, 2004). Those proteins are important in the flowering control and apical meristem development (Lee *et al.* 2008). In Arabidopsis, three *MIR171* genes can be distinguished (a, b and c) and miRNAs produced from them target the *SCL6* genes (Schulze *et al*., 2010; Engstrom *et al*., 2011). Phenotypes of transgenic plants over-expressing miR171c and the *scl6* mutant display similarities, including altered flower structure, which suggests that miR171 can reduce the level of *SCL6* and takes part in these developmental processes (Wang *et al*., 2010).

In barley, two mature miR171 sequences (hvu-miR171a/b) have been identified (Schreiber *et al*., 2011). There are also nine rice, fourteen maize and four *Brachypodium* miR171 family members. MiRNA171 is mostly expressed in the reproductive tissues. miR171 directs specific cleavage of the *SCL* target mRNAs in rice and barley. Moreover, overexpression of miR171 in rice resulted in a phenotype with altered shoot structure, delayed flowering and spikes' sterility (Curaba *et al*., 2013).

miRNA396

MiRNA396 is a well-conserved miRNA. Recently, involvement of miRNA396 in the control of carpel number and pistil development in *Arabidopsis via* targeting nine *GROWTH REGULATORY FACTORS* (*GRF*) has been established (Liang *et al*., 2014). There are also nine *GRF* genes targeted by miRNA396 in rice, with *GRF6* being the predominant one. Transgenic rice plants overexpressing miR396 displayed malformed flower structures and this effect was correlated with a significant down-regulation of *GRF6* and other members of this family. What's more, this phenotype could be rescued by the expression of *GRF6* carrying changes in the miRNA396 targeted sequence (Liu *et al*., 2014).

SUMMARY

The ABC model of floral organ specification, which was established based on the study of model eudicots, in most part can be applied in the case of grasses as well. Genes in the A class are responsible for the palea development (homologs of eudicot sepals) which was for example shown in the case of the *MADS15* gene in rice (Wang *et al*., 2010). The class B genes are required for the specification of organs in the second whorl which was confirmed in mutants of *MADS16/ SUPERWOMAN1* (*SPW1*) in rice and *SI1* in maize (homologs of *AP3*) (Kang *et al*., 1998; Whipple *et al*., 2004; Ambrose *et al*., 2000; Nagasawa *et al*., 2003). Also, the role of the C-class genes in grasses is similar to those in eudicots. Their involvement in development of reproductive organs was depicted in the loss of function mutant of *MADS3,* and knock-down mutants in the *MADS58* genes in rice (Yamagushi *et al*., 2006).

MiRNAs can regulate flower organ development and flowering time in grasses. In barley, miRNA172 targets a sequence embedded in the *AP2-like* gene responsible for occurrence of the flower cleistogamy (Nair *et al*., 2010). In maize, feminization of the tassel caused by a lack of pistil abortion occurs due to the lack of miRNA172 expression (Chuck, 2007). In rice, miRNA 172 targets the floral organ identity gene (*SNB – SUPERNUMERARY BRACT*), and it's overexpression also results in many problems in the organ development, such as: twisted lemma and palea, degeneration of either lemma or palea, replacement by leaf-like structures or in some cases lack of floral organ production (Phipps *et al*., 1928; Chuck *et al*., 2007; Zhu *et al*., 2009). Also, another miRNA, miR159 regulates the flower organ development in grasses. miR159 targets the *GAMYB-like* gene and overexpression of miR159 in rice causes development of malformed flowers within the leaf sheaths, shrunken and whitened anthers, reduced lemma and male sterility (Hiroyuki *et al*., 2006). Another miRNA that affects flowers in grasses is miRNA171. miRNA171 is found to target the *SCL* gene involved in control of flowering. There are several members of this family found in rice, *Brachypodium* and maize. Overexpression of miR171 in rice resulted in a pleiotropic phenotype with delayed flowering and partially sterile spikes (Curaba *et al*., 2013). Also, miRNA396 regulates the flower development in grasses by targeting the *GRF6* gene which was documented in the case of rice (Liu *et al*., 2014).

A model of floral development in grasses and its genetic regulation is not yet fully characterized. Further studies on both, the model eudicots and grasses, are required to elucidate this mechanism.

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