

THE EFFECT OF METHYL JASMONATE ON *Zea mays* TASSEL DEVELOPMENT

O EFEITO DE METHYL JASMONATO SOBRE O DESENVOLVIMENTO DO PENDÃO DE *Zea mays*

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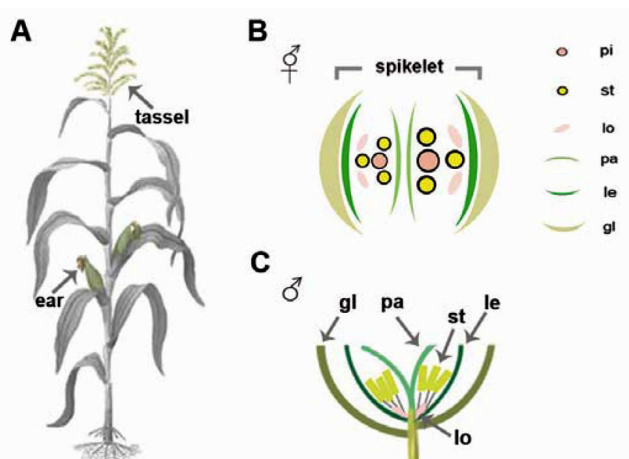
ABSTRACT: Methyl jasmonate (MeJA) is a lipid-derived plant hormone that mediates diverse biological phenomena. Application of MeJA onto rice spikelet could exhibit abnormal floral organ development. Although jasmonic acid (JA) has been proved to be involved in maize tassel sex determination process, the roles of JA and its precursor MeJA in maize tassel development still remain obscure. In this study, we found that tassel development was decelerated by application of 2 mM MeJA. Exogenous MeJA also influenced the number of palea and stamens of tassel spikelets. Exogenous MeJA increased the expression level of some key regulator genes, which may responsible for the phenotypic change in MeJA-treated tassel, and may mediate the crosstalk between MeJA and other hormones.

KEYWORDS: Maize. Methyl jasmonate. Regulator gene. Tassel development. Hormone.

INTRODUCTION

As one of the most important agricultural crops in the world, maize (*Zea mays* L. ssp. *mays*) is an excellent model plant for grass developmental biology research. Maize forms male and female inflorescences on a terminal tassel and on lateral ears, respectively (Supplementary figure 1A, TANAKA et al., 2013). At an early developmental stage, tassel and ear both initiate bisexual spikelets. Each spikelet contains two florets. Each floret

consists of a lemma, a palea, two lodicules, three stamens, and a pistil, and is subtended by two glumes (Supplementary figure 1B). In a so-called sex determination process at a later developmental stage, the pistil in a tassel spikelet and the stamen in an ear spikelet will cease to develop and the plant will ultimately form unisexual spikelets (Supplementary figure 1C). Thus, to form a mature tassel spikelet, a delicate coordination between the establishment of floral organ identity and pistil abortion is required.



Supplementary figure 1. Schematic diagram of maize floral development. (A) Tassel and ear initiate separately in a maize plant. (B) Maize spikelet at an early developmental stage is bisexual. (C) Mature tassel spikelet. Later in development, pistils abort in the floret of tassel spikelet. pi, pistil; st, stamen; lo, lodicule; pa, palea; le, lemma; gl, glume.

Over the past three decades, molecular analyses have led to detailed insights into the genetic regulatory network for Arabidopsis flower morphogenesis (Ó'MAOILÉIDIGH et al., 2014). A classic ABCDE model explains how a series of genes, named class A, B, C, D and E genes, delicately specify the floral organ's identity independently or coordinately (YANOFSKY et al., 1990; COEN; MEYEROWITZ, 1991; HONMA; GOTO, 2001). Among these transcription factor genes, the class C gene *AGAMOUS* (*AG*) is one of the most important regulators. Besides specifying carpel identity, the *AG* gene affects stamen identity in combination with two other class B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (YANOFSKY et al., 1990; COEN; MEYEROWITZ, 1991; PELAZ et al., 2000). Furthermore, *AG* also acts as a meristem gene by suppressing the class A gene *APETALA1* (*API*) in the center of the flower (GUSTAFSON-BROWN et al., 1994) and is itself suppressed by the class B gene *APETALA2* (*AP2*) in the sepal and petal domains (DREWS et al., 1991; YANT et al., 2010). Besides their complicated interactions with each other, some regulator genes, such as *AG* and *API* are reported to mediate in hormone pathways to control floral organ development (Gómez-Mena et al., 2005; ITO et al., 2007; KAUFMANN et al., 2010).

Jasmonic acid (JA) and methyl jasmonate (MeJA) are lipid-derived hormones which have been proved to play a key role in Arabidopsis stamen development (WASTERNAK; HAUSE, 2013). *DEFECTIVE IN ANTHER DEHISCENCE1* (*DADI*) and *DONGLE* (*DGL*) are well-characterized JA-deficient mutants. They have mutations in JA synthesis enzymes and exhibit a delayed anther development phenotype (ISHIGURO et al., 2001; HYUN et al., 2008). Male sterility also occurs in several other JA biosynthesis mutants and a JA perception mutant *coronatine insensitive1* (*coi1*) (XIE et al., 1998; WASTERNAK; HAUSE, 2013). Transcription profiling and genetic analyses indicate that transcription factors, such as *AG*, are involved in JA-dependent stamen development (MANDAOKAR et al., 2006; ITO et al., 2007).

Interestingly, in grass floral morphogenesis the interaction of JA and transcription factors seems not to be limited to stamen development (KIM et al., 2009; CAI et al., 2014). The floral meristem gene *OsMADS1*, working together with the rice *EG1* gene, orthologous to *DGL*, controls floral meristem determinacy and impacts floral organ identity and number via a JA biosynthetic pathway (LI et al., 2009; CAI et al., 2014). Thus, Cai et al (2014) imply

that the role of JA in reproduction has diversified during flowering plant evolution. Because of the different floral features between monocots and dicots and the comparatively few studies in monocotyledonous species (BORTIRI; HAKE, 2007; TANAKA et al., 2013), more work is needed to explain the roles of JA in grass flower morphogenesis.

JA has been proved to mainly affect the pistil abortion process in maize tassel spikelets (ACOSTA et al., 2009; YAN et al., 2012). The *TASSELSEED1* (*TS1*) gene encodes a JA synthesis enzyme (ACOSTA et al., 2009). Maize *ts1* mutants exhibit tasselseed phenotypes, in which pistils of tassel spikelets are not aborted as they normally would be. Compared to the well-documented interaction of JA and transcription factors in Arabidopsis floral organ morphogenesis, their relationship in maize still remains obscure, partly because the functions of most floral regulator genes in maize still await verification (CIAFFI et al., 2011; LI et al., 2014).

To gain some insights into the relationship between JAs and some transcription factors in maize tassel development, we applied exogenous MeJA to the maize tassel at an early stage. By phenotypic observation and expression analysis, we found that application of MeJA altered the number of palea and stamens. We also identified some key transcription factors that can be influenced by MeJA. Our results provide clues for further understanding these genes' functions in tassel spikelet morphogenesis.

MATERIAL AND METHODS

Plant Materials and MeJA Treatment

Plants of the maize cultivar B73 were grown under natural conditions in an experimental field. For the MeJA treatment experiments, we treated tassels of six-leaf stage plants with a solution containing 0.2 mM MeJA (Sigma-Aldrich, MeJA diluted in 1% alcohol solution), 2 mM MeJA, or with 1% alcohol solution as a control. Using plastic pipettes, 2 mL solutions were directly applied onto the tassel inflorescences every 2~3 days until the tassel spikelets had reached maturity.

Phenotypic Characterization and Microscopy

Inflorescence meristems of control plants (treated with 1% alcohol solution) and MeJA-treated plants (<2 mm long) were fixed in 2% glutaraldehyde in phosphate buffer overnight at 4°C, then dehydrated in an ethanol series and subjected to critical point drying. Mounted samples were sputter coated with gold and viewed with an FEI

QUANTA600 (Holland) scanning electron microscope. To perform phenotypic analysis and stereoscopic microscope observation, mature tassel spikelets were dissected and observed under a SteReo Lumar V12 microscope (Zeiss, Germany).

Gene Expression Analysis

For promoter analysis, 2500bp of upstream sequences of some regulator genes were analyzed with PlantCARE database. For quantitative real-time PCR experiments, mature tassels and ears were collected from at least four independent plants. Total RNA extraction and cDNA preparation were performed according to the previous report (ZHENG et al., 2010). Primers were designed with Beacon Designer 7 software and listed in Supplementary Table 1. A master mix of qPCR reaction components was prepared according to the manufacturer's protocol for SYBR Green RealMasterMix (Promega, USA). Thermocycling and fluorescence detection were performed using a Roche LightCycle480 Real-Time PCR machine (Roche, Switzerland). Each experiment was repeated three times. Relative fold changes in gene expression were calculated using the comparative $2^{-\Delta\Delta C_t}$ method with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the endogenous control.

RESULTS

MeJA Treatment Influences the Development of Tassel

To investigate the effects of MeJA on the development of the male inflorescence, we observed the early developmental stage of wild-type and MeJA-treated tassels by scanning electron microscopy. At an early developmental stage, wild-type tassel had a main inflorescence with the spikelet pair starting to initiate and branches differentiating at the base of the main inflorescence (Figure 1A). At this stage, the 2 mM MeJA-treated tassel apparently developed slowly and seemed to just start its branch differentiation (Figure 1B). When the wild-type spikelets were approaching a typical bisexual stage, containing a lower floret (red arrow) and an upper floret as mentioned above (Figure 1C), the MeJA-treated branches lagged far behind those of wild-type and were just at the glume primordia differentiation stage (Figure 1D, star). Based on these observations, we concluded that the application of exogenous MeJA decelerates the developmental pace of the tassel. Since 0.2 mM MeJA treatment had a similar effect on the development of tassel as well, though to a lesser extent (data not shown), we used 2 mM MeJA-treated samples for further research in this study.

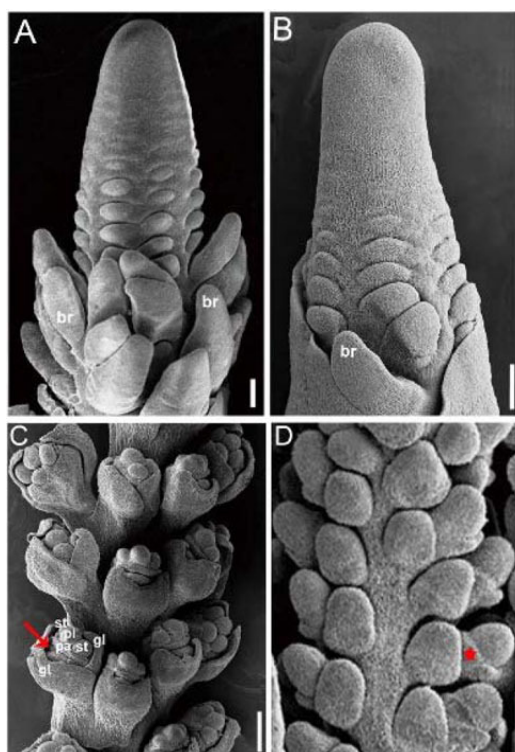


Figure 1. Scanning electron micrographs of wild-type and MeJA-treated tassel. (A) and (B) Male inflorescences (A, WT; B, MeJA-treated plant). (C) and (D) Male branch meristems (C, WT; D, MeJA-treated plant). br, branch; gl, glume; le, lemma; pa, palea; pi, pistil; st, stamen. Bars=100 μ m.

MeJA Treatment Influences the Development of Tassel Floral Organs

To further examine the effect of exogenous MeJA on the development of tassel spikelets, we dissected wild-type and MeJA-treated spikelets and observed their phenotypic features. A mature wild-type tassel spikelet contains two florets subtended by two glumes. Each floret consists of a lemma, a palea, two lodicules and three stamens (Figure 2A).

We found some mature spikelets in MeJA-treated tassels only contained one floret and three stamens (Figure 2B). The size of the spikelet also tended to be smaller than that of wild-type. By analysis of at least 100 spikelets, we found that approximately 8% of the MeJA-treated spikelets changed the number of palea and stamens (Table 1). Taken together, our results demonstrated that the application of MeJA could affect floral organ number of tassel spikelets.



Figure 2. Tassel spikelet morphology of wild-type and MeJA-treated plant. Light microscope images of WT and MeJA-treated spikelet. (A) A WT spikelet. (B) A MeJA-treated spikelet with 3 stamens. gl, glume; le, lemma; pa, palea; st, stamen. Bars=400 μ m.

Table 1. Alteration in organ number of wild-type and MeJA-treated spikelets

Plants	Total ^a	Altered ^b			Percentage of Altered/Total
		3paleae	3stamens	5stamens	
WT	100	0	0	0	0
MeJA-treated WT	178	6	4	4	7.87

* ^aNumber of total spikelets used in each assay. ^bNumber of altered spikelets.

Expression Analysis of Some Regulator Genes

To get a clue about genes that may be related to the floral phenotypic changes triggered by exogenous MeJA, we selected some key regulator genes related to floral development (Table 2). Next, we analyzed the promoter sequences of these selected genes, paying attention to modules that are related to hormones. Among these eight genes, *ZAP1*, *S11*, *ZAG1*, *ZMM2*, and *TS1* had MeJA responsive elements, which may imply they react directly to MeJA application. All of the eight genes, except *ZMM2*, have GA responsive elements. Other hormone-responsive elements also were found in the promoter regions of these genes. The correlation of these hormone elements and the function of these genes needs further experimental test.

Since some selected genes still await functional verification (CIAFFI et al., 2011; LI et

al., 2014; Table 2), we tested their expression patterns in tassel and ear by quantitative real-time PCR. Among eight genes examined in our study, class B gene *S11* was expressed slightly more in tassel than in ear, and putative class A gene *ZAP1*, class C gene *ZAG1*, meristem genes *ZAG3* and *IDS1*, were expressed mainly in ear. The other three genes, putative class C genes *ZMM2* and *ZMM23*, and sex determination gene *TS1* displayed no biased expression pattern between tassels and ears (Figure 3A, 3C). The expression level of all examined genes, except *ZMM2*, were increased by application of MeJA (Figure 3B, 3D). Taking these genes' promoter analysis into consideration, we speculated that *ZAP1*, *S11*, *ZAG1* and *TS1* may directly respond to exogenous MeJA, while other genes that showed increased expression level in MeJA-treated tassels may respond to MeJA via other hormones.

Table 2. Promoter prediction of selected regulator genes

Site name	Function	Gene name							
		ZAP1	SI1	ZAG1	ZMM2	ZMM23	ZAG3	IDS1	TS1
CGTCA-motif	MeJA-responsive element	✓	✓	✓	✓				✓
TGACG-motif	MeJA-responsive element	✓	✓	✓	✓				✓
GARE-motif	Gibberellin-responsive element	✓	✓			✓	✓	✓	✓
P-box	Gibberellin-responsive element		✓	✓		✓			✓
TATC-box	Gibberellin-responsive element					✓		✓	
ABRE	Abscisic acid-responsive element	✓		✓	✓	✓	✓	✓	✓
TGA-element	Auxin-responsive element	✓	✓	✓					✓
TCA element	Salicylic acid-responsive element	✓	✓	✓	✓	✓	✓	✓	

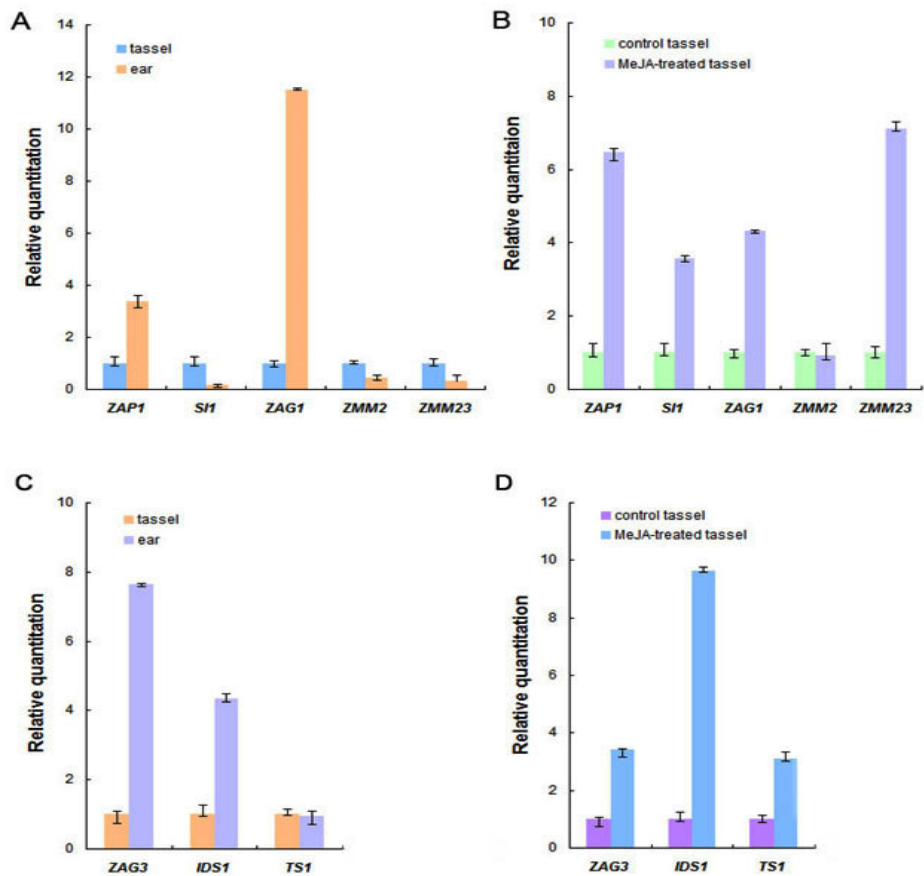


Figure 3. Expression pattern analysis of selected regulator genes via quantitative real-time PCR. (A) Expression pattern of some floral organ identity genes in tassels and ears. (B) Expression pattern of some floral organ identity genes in WT tassels and MeJA-treated tassels. (C) Expression pattern of some meristem genes in tassels and ears. (D) Expression pattern of some meristem genes in WT tassels and MeJA-treated tassels.

DISCUSSION

In this study, we showed that exogenous application of MeJA to maize impacts its tassel development. The effects included a reduced developmental pace of spikelets and alterations in floral organ numbers. Exogenous MeJA also increased the expression level of key developmental floral organ identity genes, meristem genes and tassel sex-determination genes, suggesting that MeJA has a role in maize floral organ development, at least in the development of stamen and palea.

Class C gene function may divergent in maize inflorescence development

Maize contains three genes that are homologous to *AG1*, *ZAG1* and *ZMM2/ZMM23* (ZAHN et al., 2006). *ZAG1* is mainly expressed in carpel, and *ZMM2* is mainly expressed in anthers (SCHMIDT et al., 1993; MENA et al. 1996). Though mutants of *ZMM2/ZMM23* have not yet been identified, it is thought that *ZMM2/ZMM23* probably specify the organ identity of stamens and *ZAG1* specifies floral meristem determination (SCHMIDT et al., 1993; THEISSEN et al., 1995; MENA et al., 1996; LI et al., 2014). Consistent with this hypothesis, our results also showed that *ZAG1* is expressed mainly in the ear, and *ZMM2* and *ZMM23* are expressed slightly higher in the tassel than in ears (Figure 3A). A noticeable expression difference also existed between *ZMM2* and *ZMM23*. *ZMM23*, containing putative GA responsive elements rather than MeJA responsive elements in its promotor, showed a MeJA responsive expression pattern, while *ZMM2* did not. Our results may provide another clue about their divergent role in maize inflorescence development mediated by different hormone pathways.

JA and regulator genes in maize tassel development

JAs have been proved to be involved in stamen development and usually result in male sterility. In our study, application of 2 mM MeJA did not result in male sterility (data not shown). However, alterations in the number of stamens and

palea in MeJA-treated tassels were phenotypically similar to those in MeJA-treated rice plants, which showed an alteration in spikelet organ numbers (KIM et al., 2009). Our study proved that exogenous MeJA has a similar influence in maize floral organ development, at least in stamen and palea development.

indeterminate spikelet1 (ids1) is an *APETALA2 (AP2)*-like gene which is required for the timely conversion of the spikelet meristem into the floral meristem. *IDS1* has also been reported to be a target gene of the *TASSELSEED4* gene. Mutation in the *IDS1* gene will result in a tasselseed phenotype which is similar to the JA-deficient mutant *ts1* (CHUCK et al., 2007, 2008). In our study, the expression level of *IDS1* and *TS1* both increased in MeJA-treated tassels. It will be worthwhile to study whether there exists an interaction between the *ts1* pathway and the *ts4* pathway during the tassel sex determination process.

By qPCR, we found 7 regulator genes displayed MeJA-responsive expression patterns, although their roles in MeJA-treated tassel undoubtedly need more detailed study. Among these genes, *ZAP1*, *ZAG1*, *ZAG3* and *IDS1* were mainly expressed in the ear. Considering that all of these four genes contain GA responsive elements in their promoters and GA is reported to interact with JA in Arabidopsis stamen development (PENG, 2009), it will be interesting to explore whether the crosstalk between JA and GA exists in maize tassel development.

ACKNOWLEDGMENTS

We would like to thank Dr. Jinsheng Lai (China Agricultural University) for providing B73 seed. We also thank members of the Key Laboratory of Agricultural Biotechnology of Liaoning Province for their assistance in this study. We also thank Dr. Terence Murphy (University of California, Davis, US) for his critical reading of the manuscript. This work was supported by grant from the National Natural Science Foundation of China (No.31071422).

RESUMO: jasmonate (MeJA) é um derivado lipídico vegetal hormônio que medeia Diversos fenômenos biológicos. Aplicação de MeJA para arroz spikelet poderá apresentar Desenvolvimento anormal DOS órgãos florais. Apesar de Ácido jasmônico (Ja), precursor de MeJA, mostrou ser envolvido no processo de determinação do sexo de milho tassel, OS papéis DOS dois compostos de milho tassel Desenvolvimento ainda permanecem obscuros. No presente estudo, descobrimos que o Desenvolvimento FOI desacelerada pelo pedido do tassel de 2 mm MeJA. MeJA exógeno também influenciou o número de palea e estames de tassel spikelets. MeJA aumentou o nível de expressão exógena, um regulador chave genes, o que Pode o responsável PELA alteração fenotípica EM MeJA tratados tassel, e podem mediar a interferência entre MeJA e outras hormonas.

PALAVRAS – CHAVE: Zea mays. Jasmonato de metilo. Gene regulador. Desenvolvimento de borla. Hormona

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ANEXES**Supplementary Table 1.** Primers used in this study

Primers name	Locus	Primers sequence
GDPDH	X07156	F 5`-CCTGCTTCTCATGGATGGTT-3` R 5`-TGGTAGCAGGAAGGGAACA-3`
IDS1	NM_001111434	F 5`-TCCGAGACAGTAGTAGTT-3` R 5`-CAATGCTTGTTGAGTCATAA-3`
SI1	NM_001111481	F 5`-GTTGGTTAGTTCGCTCATG-3` R 5`-CAGTAATTGGGTTTGCAGTAT-3`
TS1	NM_001112509	F 5`-GTTACCATTTAGCCTCTC-3` R 5`-CGACTATAATGAGTGGAA-3`
ZAP1	NM_001111863	F 5`-ATGGCGTCAGCAACTAAG-3` R 5`-TTACAGCAAGTCACGAACC-3`
ZAG1	NM_001111851	F 5`-GCACCAACTATGTCTCCTTAA-3` R 5`-GTATATGACCACCGCCATC-3`
ZAG3	NM_001111862	F 5`-CATCAGATGGATGTCCAAACT-3` R 5`-ATGAAGGTGCCGTTCTTA-3`
ZMM2	X81200	F 5`-CGGTCAGTCCATATCATC-3` R 5`-ATCTATATTAAAGCACACAAATCA-3`
ZMM23	AJ430637	F 5`-CCAACTCGGATGATGAAA-3` R 5`-GTACAGCCACATGACATAT-3`

Supplementary Table 2. Selected regulator genes and their function

Gene name	Function	Expression domain	Arabidopsis homolog	References
ZAP1	Putative class A gene	Lemma, palea, lodicule	AP1	Munster et al., 2002
SI1	class B gene	Anther, lodicule primordium	AP3	Ambrose et al., 2000
ZAG1	class C gene	Carpel, anther	AG	Schmidt et al., 1993
ZMM2	Putative class C gene	Anther	AG	Theissen et al., 1995
ZMM23	Putative class C gene	-	AG	Munster et al., 2002
ZAG3	Meristem gene	Floral meristem, palea, lodicule, carpel	AGL6	Thompson et al., 2009
IDS1	Meristem gene	Spikelet meristem	AP2	Chuck et al., 1998
TS1	Sex-determination gene	Pistil primordium	-	Acosta et al., 2009