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Cloning and expression analysis of *mads*3 in maize CMS-C sterile line and its maintainer line

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Abstract: MADS-box genes involve in various developmental processes in plant, and play an important role in the regulation of floral development. In this paper, *mads*3, a member of MADS box gene family was cloned by RT-PCR in maize. Sequence analysis showed that 8 sits single-base substitution mutations in CDS region were found in CMS-C line in comparison to maintainer line, which may result in 5 individual amino acides change. Furthermore, real-time qPCR analysis showed that *mads*3 was significantly down-regulated in CMS-C line at tetrad stage, and was significantly up-regulated at uninucleate stage. These results laid the foundation for further studying the relationship between MADS-box genes and CMS-C in maize.

Key words: maize; CMS-C; anther; *mads*3

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玉米 CMS-C 不育系和保持系 *mads*3 基因克隆与表达分析

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摘 要: MADS-box 基因参与植物多种发育过程,尤其在花发育过程中发挥着重要的调控作用。该研究以玉米不育系 C48-2 及保持系 N48-2 为材料,采用 RT-PCR 克隆 *mads*3 基因。序列分析发现,在不育系 C48-2 中该基因编码区发生单碱基突变,导致其推定的蛋白质中 5 个氨基酸序列发生改变。荧光定量 PCR 显示,相对于保持系,花粉母细胞时期和四分体时期 *mads*3 基因在 C48-2 下调表达,而单核期和双核期上调表达。这些结果为进一步了解玉米 C 型细胞质雄性不育的发生机制提供参考。

关键词: 玉米; CMS-C; 花药; *mads*3 基因

MADS box genes encoding MADS-box transcription factors are very important for plant development, and they are promising candidates for researching crucial regulatory networks in plant (Yuan *et al.*, 2013). MIKC-type MADS domain proteins all share the same structure (Fig. 1), containing a MADS-box (DNA binding), I intervening, K keratin like (protein interaction) and C-terminal (transactivation). MIKC-type genes have been classified into two types: MIKC^{C}-type and MIKC*-type (Henschel *et al.*, 2002). MIKC^{C}-type

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MADS box genes may involve in the regulation of the development of sporophyte (Gramzow *et al.*, 2010; Smaczniak *et al.*, 2012), and MIKC^{C}-type genes play key roles in pollen development of plant. (Verelst *et al.*, 2007; Adamczyk *et al.*, 2009; Yuan *et al.*, 2013). Increasing numbers of MADS box genes were characterized in rice, *Arabidopsis thaliana*, tomato and maize. These study may contribute to understanding of the mechanism of floral architecture (Coen *et al.*, 1991; Nagasawa *et al.*, 2003; Yamaguchi *et al.*, 2006; Dreni *et al.*, 2007; Yao *et al.*, 2008). For instance, *CFO*1, a MIKC*-type MADS box gene, plays a key role in the regulation of floral organ identity in rice (Xianchun *et al.*, 2012). The loss of functional CFO1 generated chimeric floral organs, defective marginal regions of the palea and ectopic organs phenotype.

A large amount of MADS-box genes were identified in maize, most of them were involved in floral development, pollen tube growth, anther dehiscence and pollen maturation (Schreiber *et al.*, 2004; Danilevskaya *et al.*, 2008; Thompson *et al.*, 2009). Some of them are tissue specific expresssed genes, such as *ZmMADS*2 which is expressed restrictively in pollen and roots (Sigrid *et al.*, 2000). And some MADS box genes were regulated by abiotic stress, such as *ZMM*7-*L* in maize (Zhang *et al.*, 2012). The objective of this study is to clone *mads*3, a MADS-box gene in maize, and analyze the expression patterns of *mads*3 gene in anther at different stages. Which will lay the foundation for further understanding the mechanism of CMS-C in maize.

Fig. 1 The structure of MADS-box

1 Materials and methods

1.1 Plant materials

C type CMS line of maize (C48-2) and its maintainer line (N48-2) were planted in the research field of Sichuan Agricultural University. CMS-C line (C48-2) was generated by successive backcrossing with N48-2.

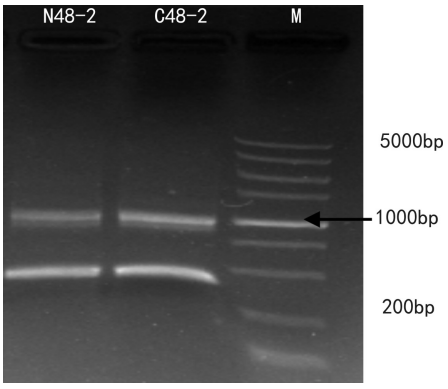

Fig. 2 The RT-PCR amplification of *mads*3

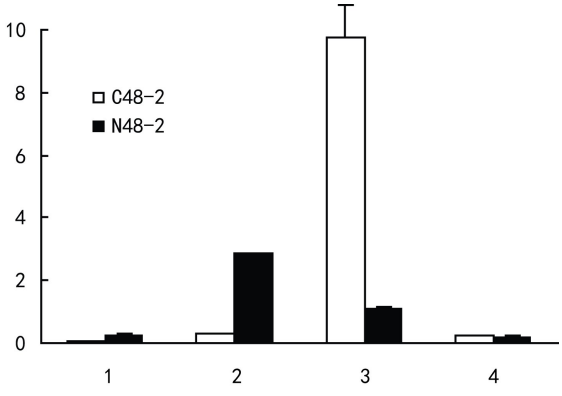

Fig. 3 Expression of *mads*3 revealed by qRT-PCR 1-4: at the pollen mother cells (PMCs) stage, the tetrad stage, the uninucleate stage and the binuclear stage, respectively

The nuclear background of C48-2 is consistent with N48-2. The anthers of maize tassels were collected at the pollen mother cells (PMCs) stage, the tetrad stage, the uninucleate stage and the binuclear stage, respectively.

1.2 RNA extraction and cDNA first strand synthesis

Total RNA of anther was extracted from N48-2 and C48-2 at four stages using Trizol kit according to the manufacturer's instruction. RNA was subjected to reverse transcription using PrimeScript ®RT reagent Kit (TaKaRa). The genomic DNA was completely removed from total RNA before reverse transcription.

1.3 RT-PCR of *mads*3

RT-PCR amplification of *mads*3 was performed with TAR® HS DNA Polymerase (TaKaRa) using specific primer. PCR reaction system (20 μL): cDNA 1 μL, DNTP Mixture 1.6 μL, PCR Forward Primer (10 μmol/L) 0.4 μL, PCR Reverse Primer (10 μmol/L)

A

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C48-2cDNA    TGAACCCCTCCTCACATCCTACCACCTCTTTAAAGCTACC  40
N48-2cDNA    TGAACCCCTCCTCACATCCTACCACCTCTTTAAAGCTACC  40
Consensus    tgaaccctcctcacatcctaccacctctttaaagctacc

C48-2cDNA    CAGCTACCTGCCTGCCTCGCCTCAATCCTCGCCGGCGGCA  80
N48-2cDNA    CAGCTACCTGCCTGCCTCGCCTCAATCCTCGCCGGCGGCC  80
Consensus    cagctacctgcctgcctcgcctcaatcctcgccggcggca

C48-2cDNA    GCAGCAGCATAGACAGGAAGAGAAAGGAAGCAGAGATCGG  120
N48-2cDNA    GCAGCCGCATAGACCGGAAGAGAAACCCCCGTAAAGGTCGG  120
Consensus    gcagcagcatagacaggaagagaaaccaagcaaagatcgg

C48-2cDNA    AGGCAGGCGAAGATGGGGCGCGGCAAGGTGCAGCTGAAGC  160
N48-2cDNA    AGGCAGGCGAAGATGGGGCGCGGCAAGGTGCAGCTGAAGA  160
Consensus    aggcaggcgaagatggggcgcggcaaggtgcagctgaaga

C48-2cDNA    GGATAGAGAACAAGATAAACCGGCAGGTGACCTTCTCCAA  200
N48-2cDNA    GGATAGAGAACAAGATAAACCGGCAGGTGACCTTCTCCAA  200
Consensus    ggatagagaacaagataaaccggcaggtgaccttctccaa

C48-2cDNA    GCGCCGGAACGGGCTGCTGAAGAAGGCGCACGAGATCTCC  240
N48-2cDNA    GCGCCGGAACGGGCTGCTGAAGAAGGCGCACGAGATCTCC  240
Consensus    gcgccggaacgggctgctgaagaaggcgcacgagatctcc

C48-2cDNA    GTCCTCTGCGACGCCGAGGTCGCCGTCATCGTCTTCTCCC  280
N48-2cDNA    GTCCTCTGCGACGCCGAGGTCGCCGTCATCGTCTTCTCCC  280
Consensus    gtcctctgcgacgccgaggtcgccgtcatcgtcttctccc

C48-2cDNA    CCAAGGGCAAGCTCTACGAGTACGCCTCCGACTCCCGCAT  320
N48-2cDNA    CCAAGGGCAAGCTCTACGAGTACGCCTCCGACTCCCGCAT  320
Consensus    ccaagggcaagctctacgagtacgcctccgactcccgcat

C48-2cDNA    GGACAAAATTCTAGAACGTTATGAGCGATATTCCTATGCT  360
N48-2cDNA    GGACAAAATTCTAGAACGTTATGAGCGATATTCCTATGCT  360
Consensus    ggacaaaattctagaacgttatgagcgatattcctatgct

C48-2cDNA    GAAAAGGCTCTTATTTCAGCTGAATCTGAAAGTGAGGGAA  400
N48-2cDNA    GAAAAGGCTCTTATTTCAGCTGAATCTGAAAGTGAGGGAA  400
Consensus    gaaaaggctcttatttcagctgaatctgaaagtgagggaa

C48-2cDNA    ATTGGTGCCACGAATACAGGAAACTGAAGGCCAAAATTGA  440
N48-2cDNA    ATTGGTGCCACGAATACAGGAAACTGAAGGCCAAAATTGA  440
Consensus    attggtgccacgaatacaggaaactgaaggccaaaattga

C48-2cDNA    GACCATACAAAGATGCCACAAGCACCTGATGGGAGAGGAT  480
N48-2cDNA    GACCATACAAAGATGCCACAAGCACCTGATGGGAGAGGAT  480
Consensus    gaccatacaaagatgccacaagcacctgatgggagaggat

C48-2cDNA    CTGGAGTCTTTGAATCCAAAAGAGCTCCAACAACTAGAGC  520
N48-2cDNA    CTGGAGTCTTTGAATCCAAAAGAGCTCCAACAACTAGAGC  520
Consensus    ctggagtctttgaatccaaaagagctccaacaactagagc

C48-2cDNA    AGCAGCTGGAGAGCTCACTGAAGCACATCAGATCAAGAAA  560
N48-2cDNA    AGCAGCTGGAGAGCTCACTGAAGCACATCAGATCAAGAAA  560
Consensus    agcagctggagagctcactgaagcacatcagatcaagaaa

C48-2cDNA    GAGCCACCTTATGGCCGAGTCAATTTCTGAGCTACAGAAG  600
N48-2cDNA    GAGCCACCTTATGGCCGAGTCAATTTCTGAGCTACAGAAG  600
Consensus    gagccaccttatggccgagtcaatttctgagctacagaag

C48-2cDNA    AAGGAGAGGTCACTGCAGGAGGAGAACAAGGCTCTACAGA  640
N48-2cDNA    AAGGAGAGGTCACTGCAGGAGGAGAACAAGGCTCTACAGA  640
Consensus    aaggagaggtcactgcaggaggagaacaaggctctacaga

C48-2cDNA    AGGAACTTTCAGAGAGGCAGAAGGCGGTCGCTAGCCGGCA  680
N48-2cDNA    AGGAACTTTCAGAGAGGCAGAAGGCGGTCGCTAGCCGGCA  680
Consensus    aggaactttcagagaggcagaaggcggtcgctagccggca

C48-2cDNA    GCAGCAGCAGCAGCAGGTGCAGTGGGACCAGCAGACACAG  720
N48-2cDNA    GCAGCAGCAGCAGCAGGTGCAGTGGGACCAGCAGACACAG  720
Consensus    gcagcagcagcagcaggtgcagtgggaccagcagacacag
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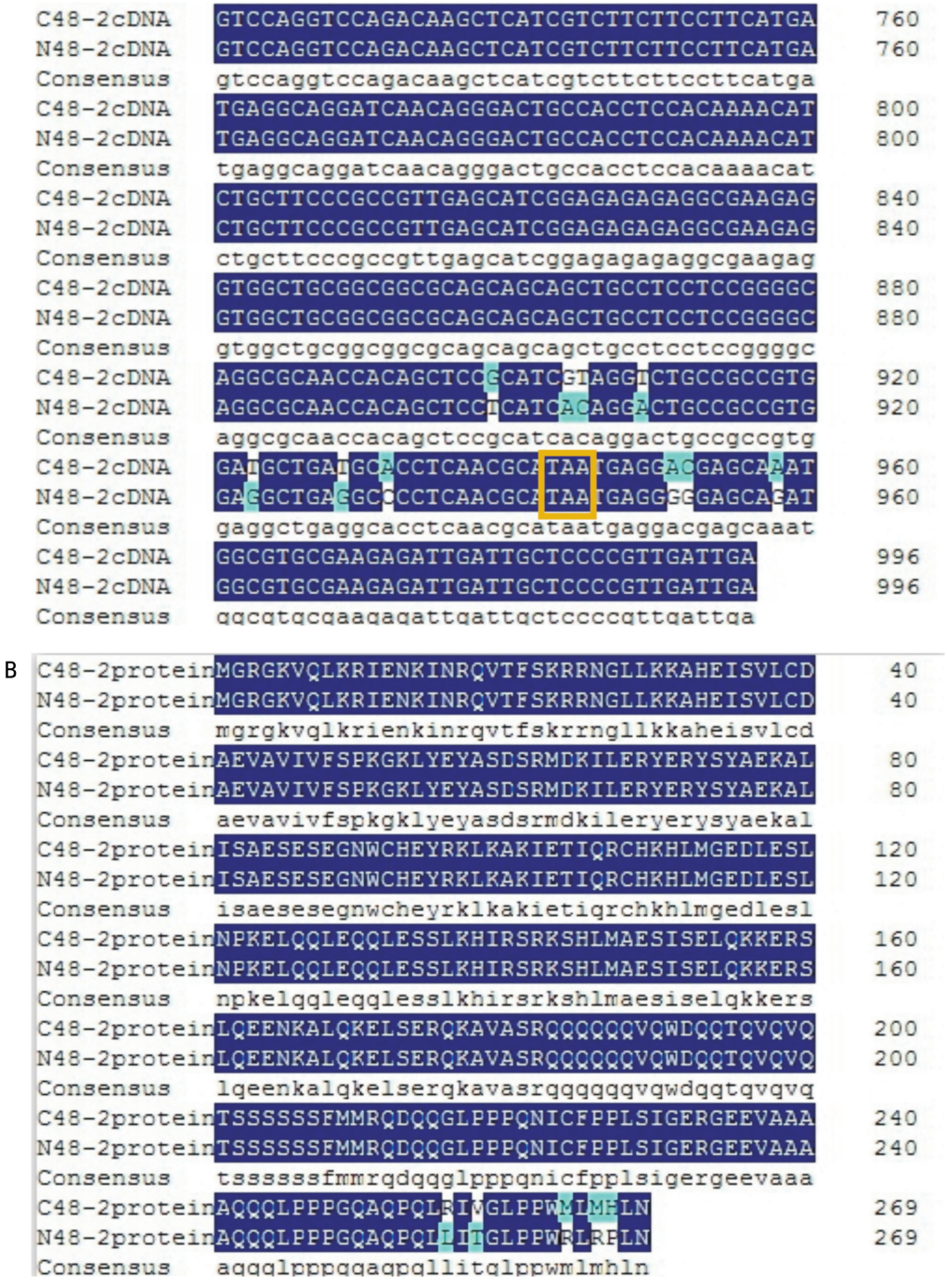

Fig. 4 Alignment of *mads*3 nucleotide (**A**) and amino acid (**B**) sequence between C48-2 and N48-2
Red box is initiation codon, yellow box is termination codon.

0.4 μL, Prime STAR Buffer (Mg^{2+}) 4 μL, Prime STAR HS 0.2 μL, ddH_2O 12.4 μL. PCR reaction condition: Initial denaturation at 95 ℃ 5 min, denaturation at 98 ℃ for 10 s, anneal at 58 ℃ for 5 s, extension at 72 ℃ for 1 min, 35 cycles, re-extension at 72 ℃ for 8 min. The recovered PCR products were connected with pEASY-zero, and then transformed into *E. coli*. Three positive clone were sequenced. The sequence was analyzed by DNAMAN software. Transmembrane helices in proteins was analyzed using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Spatial structure of protein was estimated by SWISS-MODEL homologous modeling (http://swiss-model.expasy.org/) The primer sequences were as fol-

lows：F 5′ CACGCACGCTCATCACACAGAC 3′ and R 5′ CCCTCATTCAACCCCTACACA 3′

1.4 Real-Time qPCR

To detect the expression levels of *mads*3 between C48-2 and N48-2 at different development stages，real-time qPCR was performed using a commercial kit (AccuPower ® 2X Greenstar qPCR Master Mix，Bioneer). Specific primers were designed in the conservative region.18*s* and *β-Actin* were assigned as internal control genes for real time qPCR. The protocol of reaction was as follows：denaturation program at 95 ℃ for 10 min，95 ℃ for 5 s，58 ℃ for 20 s，72 ℃ for 10 s with a single fluorescence measurement，44 cycles. All reactions were performed in triplicate. The primer sequences were as follows：

18*s*：F 5′ CTGAGAAACGGCTACCACA 3′ and R5′ TCTGAGAAGGAAGTATTGGCTATGC 3′

β-Actin：F 5′ GTCCCTCACCCTCCCAAAAG 3′ and R 5′ GCTGCCTCAACACCTCAACCC 3′

*mads*3：F 5′ GAAGCACATCAGATCAAGAAAGA 3′and R 5′GAAGCAGATGTTTTGTGGAGGT 3′

2 Results and discussion

2.1 Sequences analyses of *mads*3

The *mads*3 in anther at uninucleate stage was evaluated. Two transcripts were cloned and sequencing (Fig. 2)，the first is 997 bp，the second is 350 bp. Sequences analyses of the first transcript showed that 8 sits single base substitution mutations in CDS region were found in C48-2 compared to N48-2，which may result in 5 individual amino acides change (Fig. 4). The physical and chemical parameters of its products (MADS3) might be different from the normal MADS3 in N48-2 based on primary protein structure prediction. the space conformational of C48-2 putative protein is slightly different form N48-2 (Fig. 5). However，the predicted transmembrane domains and conservative structure domain of MADS3 in C48-2 was the same with N48-2. The function of altered MADS3 remains to be seen and further explored by reverse genetics approach. In addition，the second transcript was identified as a novel transcript of *mads*3 by means of sequences sequence alignment，which underwent alternative splicing. The sequence of novel transcript in C48-2 is the same with in N48-2.

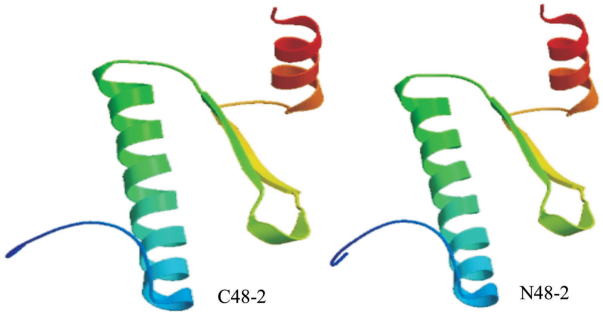

Fig. 5　Tertiary structure prediction of first transcript in *mads*3

The AG homologs MADS3，a C-class MADS box gene in rice，regulate floral organ identity during early flower development. OSMADS3 was necessary for stamen specification in rice，developmental defects were observed if the function *mads*3 was impaired or lost (Yamaguchi *et al.*，2006). *MADS*3 mutant displayed defective anther walls，aborted microspores，and complete male sterility (Hu *et al.*，2011). Interestingly，a specific amplified band was found in CMS-C line (Fig. 2). The specific fragment in CMS-C line was identified as a nonspecific amplification by means of sequence analysis. Why the nonspecific segment only present in CMS-C line was ambiguous for us. One more special product of *mads*1 was found in rice CMS Zhenshan 97A compared to its maintainer line Zhenshan 97B at the same developmental stage(Yuan *et al.*，2000). It illustrated that the expression of MADS-box gene in rice CMS line was different from its maintainer line.

2.2 The expression profiling of *mads*3

The expression patterns of *mads*3 at the pollen mother cells (PMCs) stage，the tetrad stage，the uninucleate stage and the binuclear stage were determined (Fig. 3). It was up-regulated at the uninucleate stage and the binuclear stage，while was down regulated at the pollen mother cells (PMCs) stage and the tetrad stage in the CMS-C line in comparison to maintainer line. Except binuclear stage，the differences of *mads*3 expression level between CMS-C line and maintainer line are remarkable. Especially，the expression level of

CMS-C line at uninucleate stage was nearly tenfold as compared with maintainer line. This phenomenon just was good fit with pollen abortion stage in C48-2. Therefore, the relationship between *mads*3 and anther pollen abortion is amusing, and this results laid a foundation for further research.

MADS3 is a key transcriptional regulator for male reproductive development in rice, which is highly expressed in the tapetum and microspores during late anther development (Hu *et al*., 2011). *ZMM*4, a MADS-box gene in maize, was significantly up-regulated in shoot apices after the floral transition, and over-expression of *ZMM*4 have changed the flowering time in transgenic maize (Danilevskaya *et al*., 2008). Cytoplasmic male sterility was caused by nuclear genes which was mediated by retrograde signaling emitted from mitochondria (Yang *et al*., 2007). The immense amounts of studies have demonstrated that pollen abortion is responsible for cytoplasmic nuclear incompatibility. As a consequence, the expression of MADS-box genes would be regulated by a detrimental interaction between nuclear and mitochondria.

3 Conclusion

RNA sequence analysis of *mads*3 showed that 8 sits single base difference in CDS region were detected in CMS-C line in comparison to maintainer line. qRT-PCR analysis showed that *mads*3 was significantly up-regulated in maize CMS-C line at uninucleate stage. The differenced expression of *mads*3 gene during anther development may contributes to understanding of the mechanism of CMS-C in maize.

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References:

Adamczyk BJ, Fernandez DE, *et al*. 2009. MIKC* MADS domain heterodimers are required for pollen maturation and tube growth in *Arabidopsis*[J]. *Plant Physiol*, **149**(4): 1 713－1 723

Coen ES, Meyerowitz EM. 1991. The war of the whorls: genetic interactions controlling flower development[J]. *Nature*, **353**(6 339): 31－37

Dreni L, ColomboL, Kater MM, *et al*. 2007. The D-lineage MADS-box gene OsMADS13 controls ovule identity in rice[J]. *Plant J*, **52**(4): 690－699

Gramzow L, Theissen G, *et al*. 2010. A hitchhiker's guide to the MADS world of plants[J]. *Gen Biol*, **11**(6): 214

Henschel K, Münster T, Theissen G, *et al*. 2002. Two ancient classes of MIKC-type MADS-box genes are present in the moss Physcomitrella patens[J]. *Mol Biol Evol*, **19**(6): 801－814

Hu LF, Hu JP, Dabing Zhanga, *et al*. 2011. Rice MADS3 Regulates ROS homeostasis during late anther development[J]. *The Plant Cell*, **23**(2): 515－533

Medard Ng, Martin F, Yanofsky, *et al*. 2000. Function and evolution of the plant MADS-box gene family[J]. *Nat Rev Gen*, **2**(3): 186－195

Olga N, Evgueni V, Ananiev Michael, *et al*. 2008. Involvement of the MADS-box gene ZMM4 in floral induction and inflorescence development in Maize[J]. *Plant Physiol*, **147**(4): 2 054－2 069

Schreiber DN, Bantin J, Dresselhaus T, *et al*. 2004. The MADS-box transcription factor ZmMADS2 is required for anther and pollen maturation in maize and accumulates in apoptotic bodies during anther dehiscence[J]. *Plant Physiol*, **134**(3): 1 069－79

Sigrid Heuer, Horst Lörz, Thomas Dresselhaus, *et al*. 2000. The MADS box gene ZmMADS2 is specifically expressed in maize pollen and during maize pollen tube growth[J]. *Sex Plant Reprod*, **13**(2): 21－27

Smaczniak C, Angenent GC, Kaufmann K, *et al*. 2012. Developmental and evolutionary diversity of plant MADS-domain factors: Insights from recent studies[J]. *Development*, **139**(7): 3 081－3 098

Verelst W, Saedler H, Münster T, *et al*. 2007. MADS-complexes regulate transcriptome dynamics during pollen maturation[J]. *Gen Biol*, **8**(11): 249

Yamaguchi T, An GH, Hirano HY, *et al*. 2006. Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in *Oryza sativa*[J]. *Plant Cell*, **18**(1): 15－28

Yao SG, Ohmori S, Kimizu M, *et al*. 2008. Unequal genetic redundancy of rice PISTILLATA orthologs, OsMADS2 and OsMADS4, in lodicule and stamen development[J]. *Plant Cell Physiol*, **49**(5): 853－857

Yang JH, Zhang MF. Mechanism of cytoplasmic male-sterility modulated by mitochondrial retrograde regulation in higher plants[J]. *Acta Agron Sin*, **29**(10): 1173－11181

Yuan ZQ, Qian M, Yang JS, *et al*. 2000. cDNA cloning and analysis of two MADS -box genes in rice[J]. *Progr Nat Sci*, **10**(2): 129－134

Yuan L, Günter Theißen, Zheng MG, *et al*. 2013. Functional conservation of MIKC * -Type MADS box genes in *Arabidopsis* and rice pollen maturation[J]. *The Plant Cell*, **25**(4): 1 288－1 303

Zhang ZB, Wang TY, Li Y, *et al*. 2013. Characterization and expression analysis of six MADS-box genes in maize (*Zea mays*)[J]. *J Plant Physiol*, **169**: 797－806