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Zm401, a Short-Open Reading-Frame mRNA or Noncoding RNA, Is Essential for Tapetum and Microspore Development and Can Regulate the Floret Formation in Maize

Jinxia Ma,[1] Bingxue Yan,[1] Yanying Qu,[2] Fangfang Qin,[1] Yantao Yang,[1] Xiujing Hao,[1] Jingjuan Yu,[1] Qian Zhao,[1] Dengyun Zhu,[1] and Guangming Ao[1*]

[1]*State key Laboratories of Agrobiotechnology, China Agricultural University, Beijing 100094, China*

[2]*College of Agronomy, Xinjiang Agricultural University, Urumqi 830052, China*

ABSTRACT

In flowering plants, pollen formation depends on the differentiation and interaction of two cell types in the anther: the reproductive cells, called microsporocytes, and somatic cells that form the tapetum. Previously, we cloned a pollen specific gene, *zm401*, from a cDNA library generated from the mature pollen of *Zea mays*. Expression of partial cDNA of *zm401* in maize and ectopic expression of *zm401* in tobacco suggested it may play a role in anther development. Here we present the expression and functional characterization of this pollen specific gene in maize. *Zm401* is expressed primarily in the anthers (tapetal cells as well as microspores) in a developmentally regulated manner. That is, it is expressed from floret forming stage, increasing in concentration up to mature pollen. Knockdown of *zm401* significantly affected the expression of *ZmMADS2*, *MZm3-3*, and *ZmC5*, critical genes for pollen development; led to aberrant development of the microspore and tapetum, and finally male-sterility. *Zm401* possesses highly conserved sequences and evolutionary conserved stable RNA secondary structure in monocotyledon. These data show that *zm401* could be one of the key growth regulators in anther development, and functions as a short-open reading-frame mRNA (sORF mRNA) and/or noncoding RNA (ncRNA). J. Cell. Biochem. 105: 136–146, 2008.

KEY WORDS: *zm401*; ANTHER; DEVELOPMENT; MAIZE; TAPETUM; MICROSPORE

Pollen development is a cooperative process requiring multiple interactions between the sporophytic plant and the developing male gametophyte. The sporophytic microsporocytes undergo meiosis to give rise to a tetrad of four microspores within each anther locule. The callose microsporocyte and intersporal walls are degraded to release the microspores when meiosis is complete and each microspore develops into a pollen grain. Tapetum, which encloses the sporogenous tissue within the anther locule and secretes a β-glucanase to release the microspore tetrads from their callose wall, plays a critical role in the nutrition of microspores, in the regulation of sporogenesis and in pollen wall development [Scott et al., 2004]. The interaction between the sporocytes and sporophytic tissues is important for spore formation [Yang and Sundaresan, 2000]. The formation of microspores relies on the interaction of microsporocyte with several types of somatic anther wall cells including the tapetal cells. Mutations affecting tapetal development lead to abortion of microgametogenesis and final sterility [Wilson et al., 2001; Kapoor et al., 2002]. Several sporophytic mutants that affect the development of tapetal cells have been reported [Wilson et al., 2001; Ito and Shinozaki, 2002]. Pollen has a consistent and simple cell lineage which is relatively easy to follow compared with the lineages that derive most other plant structures, so pollen offers an excellent system to study developmental processes.

In eukaryotes, diverse sORF-mRNAs are induced at specific stages of development. sORF-mRNAs are unusual mRNAs containing only short-open reading frames (<100 amino acid) that accumulate in the cytoplasm (in many cases abundantly) where they may be translated into oligopeptides [MacIntosh et al., 2001]. Their mechanism of action may involve the sORF-encoded oligopeptides and/or the RNA itself. For certain, some sORF-mRNAs are conserved at nucleotide

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*Correspondence to: Guangming Ao, State key Laboratories of Agrobiotechnology, China Agricultural University, Beijing 100094, China. E-mail: guangmingao@yahoo.com

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level but not at the amino acid level suggesting that the RNA itself can play an important role in the function of the gene, and they are referred to ncRNAs [Furini et al., 1997; Erdmann et al., 2001; MacIntosh et al., 2001]. NcRNA is mRNA that is transcribed from DNA but is not translated into protein. Since the sequencing and annotation of the genomes and transcriptomes of several eukaryotes, the importance of ncRNA that produce functional RNAs instead of encoding proteins seems to be more prevalent than previously thought. ncRNAs have been shown to participate in diverse processes, such as organization of embryo cytoplasm, mRNA translation or stability and protein secretion or silencing [Kelley and Kuroda, 2000; Joyce, 2002; Kiss, 2002]. These data led to the accumulating evidence that ncRNA constitute an emerging class of genes.

Pollen specific genes, and ncRNA/sORF-mRNAs genes have been studied extensively [Campalans et al., 2004; Scott et al., 2004]. However, in most cases it remains unclear what roles these genes play in anther development, and very few genes identified function as ncRNA or sORF mRNAs [Campalans et al., 2004]. Previously, we obtained a cDNA fragment from a *Zea mays* mature pollen library, and then its full length was generated, and named it *zm401* [Dai et al., 2004]. There are no significant ORFs in the *zm401* gene by sequence analysis and no detectable translation products have been found for these transcripts by in vitro translation; expression of partial cDNA of *zm401* in maize and ectopic expression of full length *zm401* in tobacco suggested it may play roles in anther development [Ma et al., 2005; Dai et al., 2007]. However, the information about *zm401* expression and its actual function have not been described. Here, we report the expression pattern and function of the *zm401*, a sORF mRNA or ncRNA, which has an essential role in tapetum and microspore development. The possible mechanism of interaction between *zm401* and other growth regulators in anther development is also discussed.

MATERIALS AND METHODS

PLASMID CONSTRUCTION

The chimeric constructs are shown in Figure S1. Construction of the sense *zm401* plasmid was generated as follow: *zm401* was cloned into the *Kpn*I site of the pROK219 plasmid [Messing, 1983] containing a 35S promoter. Then the *Pst*I–*Bam*HI fragment of a pollen-specific promoter ZM13 (cloned by PCR based on the sequence) [Hanson et al., 1989] was inserted into the *Bam*HI and *Pst*I sites of this vector. Finally, the fragment of the 2.0 kb *hpt* cassette containing the hygromycin resistance gene was transferred into the *Hin*dIII site of the plasmid as the selection marker gene. The constructed plasmid was named pBIZM1.2. To construct the RNAi plasmid, the reverse complement *zm401* was cloned into the *Kpn*I site of pROK219 plasmid. The GUS fragment of pBI121 was then inserted into the *Bam*HI site and another fragment *zm401* reverse complement was introduced into the *Sac*I site of the plasmid. Finally, the fragment containing 2.0 kb *hpt* cassette was transferred into the *Hin*dIII site of the plasmid as the selection marker gene. The constructed plasmid was named pBI1.2ASH.

PLANT TRANSFORMATION

Maize inbred lines Z31 and Q31 were grown under standard greenhouse conditions (25°C by day and 20°C by night, with a supplemented photoperiod of 10 h). Plants from these two lines were cross-pollinated (Z31XQ31). Immature embryos at 9–13 days post-pollination measuring approximately 1–2 mm diameter were excised and calli were induced on N6 media containing 2,4-D (2.0 mg/L) in complete darkness at 27°C. Four hours before bombardment, calli were transferred onto N6 medium containing 0.4 M mannitol. Plasmid DNA was coated onto 1.0 μm gold particles (BioRad) [Wan et al., 1995]. All transformation experiments were conducted with the BioRad Biolistics PDS 1000/He system. Bombardment pressure was 1,350 psi, and the target distance from the launching plate was 7 cm. After bombardment, calli were cultured on N6 medium (containing 2.0 mg/L 2,4-D) for 7 days (dark, 26°C) and then transferred to N6 medium containing 20 mg/L hygromycin for the selection of resistant calli. After 2-month selection, resistant calli were regenerated (light, 26°C on N6 medium without 2,4-D + 5% sucrose). Shooting buds of about 3 cm were cut and further cultured on rooting medium (N6 medium + 2% sucrose). Plantlets of about 5 cm were then transplanted into pots and grown under standard greenhouse conditions.

SOUTHERN BLOT

DNA was extracted from untransformed and hygromycin-resistant plants (according to Junghans and Metzlaff [1990]). Aliquots containing 20 μg of genomic DNA were digested with *Hin*dIII and *Xho*I respectively, analyzed on 0.8% w/v agarose gels and then transferred onto Nylon membranes (Amersham Pharmacia). The membrane was pre-hybridized at 65°C for 3 h in 5 × SSC, 5 × Denhart's solution (0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone), 0.5% SDS and 100 μg/ml salmon sperm DNA. ^{32}P labeled probes were prepared from *hpt* cassette and full-length *zm401* cDNA using a random primed DNA labeling kit (Promega). Hybridization was performed at 65°C overnight (hybridization buffer is same as pre-hybridization buffer). Washing was done at 65°C (according to Sambrook et al. [1989]): 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) 0.1% SDS for 30 min, 1 × SSC 0.1% SDS for 30 min, and 0.1 × SSC 0.1% SDS for 30 min.

NORTHERN BLOT AND QUANTITATIVE REAL-TIME RT-PCR (Q-PCR)

Total RNA from different developmental stages anthers was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Takara). For Northern blot, we generated Northern blot probes by amplifying cDNA fragments of *zm401*, *ZmMADS2*, *MZm3-3*, *ZmC5* and *actin* gene from a cDNA sample of maize anther. Primers are listed in Table S1. We purified the PCR fragment using the PCR purification kit (Qiagen) and radiolabeled them using Random Primers DNA Labeling System (Promega). Fifteen micrograms total RNA were separated in a MOPS/formaldehyde agarose gel, and transferred to a Hybond-N$^+$ nylon membrane (Amersham Biosciences), hybridizations was performed at 65°C overnight in 5 × SSC, 5 × Denhart's solution (0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone), 0.5% SDS and 100 μg/ml salmon sperm DNA. Final washes were as same as in the Southern blot. A fraction enriched with small-sized RNAs and RNA filter

hybridization were performed according to a protocol described by [Park et al., 2002]. Briefly, 1–2 mg total RNA was combined with 50 μl each of 50% PEG8000 and 5 M NaCl, incubated on ice for 2 h, and centrifuged at 15,000*g* for 10 min. After adding 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol to the supernatant, small-sized RNA was spun down at 15,000*g* following incubation at −20°C for 2 h, washed, dried, and resuspended in RNase free water. For RNA filter hybridization, 15 μg small-sized RNA enriched as above was separated on a denaturing 15% polyacrylamide gel containing 8 M urea at 100 V for10 h. RNA was electrophoretically transferred to Zeta-probe GT membranes (BioRad). After electroblotting, RNAs were fixed to the membrane by UV cross-linking and by baking in a vacuum oven at 80°C for 1 h. Membranes were hybridized with ^{32}P-end-labeled oligonucleotide probe at 40°C with Ultrahyb-oligo hybridization buffer (Ambion) and were washed twice at 40°C with 2 × SSC/0.5% SDS. The hybridization signals were visualized by Phosphor Screen, and the quantification of the mRNA amount was performed using ImageQuant5.2 (Molecular Dynamics). Q-PCR was performed with an ABI PRISM 7900 instrument (Applied Biosystems) according to the manufacturer's instructions. Each sample was assayed in a triplicate, and each experiment (i.e., from RNA extraction to quantification) was performed twice independently. The relative expression level of transgene and wild-type maize mRNA was normalized to the amount of *actin* in the same cDNA using the standard curve method described by the manufacturer. We designed primers to cover exon–exon borders in cDNA or span long-intron in DNA, therefore, amplification could not be generated from genomic DNA. Primers are given in Table S2.

IN SITU HYBRIDIZATION

Anthers from different developmental stages cut were incubated at 4°C for 2–4 h with fixative (DEPC-treated PBS containing 4% paraformaldehyde; pH7.5), soaked at 4°C overnight in sucrose solution (DEPC-treated PBS containing 30% sucrose (RNase-free)), and then frozen and stored at −80°C. For in vitro transcription, we cloned *zm401* cDNA into pST19 vector with the Sp6 and T7 polymerases to obtain sense and antisense RNA probes using a DIG labeling Kit (Roche). Anthers were cut into 10 mm thick slices, and in situ hybridization was performed using digoxigenin-labeled RNA probes according to the protocol provided by Roche. The results were detected by immunological detection (BCIP/NBT), and the precipitate was viewed as a blue stain under dark field illumination. Sections were viewed and photodocumented by using the Olimpus light microscope.

HISTOLOGY AND POLLEN VIABILITY TESTS

Anthers from the plants were fixed in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde), infiltrated and embedded in paraffin. Sections at a thickness of 8 μm were cut, heat mounted, stained by safranin-fast green contrast-stain method and photographed. Anthers from the male flowers were squashed in a droplet of I_2-KI stain and observed under a light microscope. Spherical stained pollen grains were considered to be healthy and fertile, and shrunken unstained pollen grains were considered to be sterile.

COMPUTATION

The coding capacity of *zm401* was monitored with the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). RNA secondary structure was predicted using the RNAfold program (Vienna RNA Package [Hofacker et al., 1994]) which is based on dynamic programming algorithms and aims predictions of the structures with minimum free energies as well as at computations of the equilibrium partition functions and base pairing probabilities. We used the free energy of the minimum free energy structure as a measure for secondary structure formation. The lower the free energy, the more stable is the secondary structure. We predicted the possible microRNA products generated from *zm401* using the method [Wang et al., 2004] and the miRscan software [Lim et al., 2003].

RESULTS

EXPRESSION PATTERN OF *zm401*: STARTING FROM THE FLORET FORMING STAGE WITH INCREASED CONCENTRATION UP TO MATURE POLLEN; MAINLY IN TAPETAL CELLS AS WELL AS MICROSPORES

The in situ hybridization was performed to study the expression of *zm401* in specific anther tissues and cell types. The florets were sectioned at the floret forming stage; the anthers were sectioned at the premeiosis, tetrad, uninucleate, binucleate and the late binucleate stage according to the aceto-carmine staining for staging. The results showed: *zm401* transcripts could not be detected in the floral meristem and in the anthers before/at the premeiosis stage (Fig. 1A,B); it was first observed at tetrad stage in microspores and tapetal cells (Fig. 1C); The RNA signals became stronger in the anthers at the uninucleate stages (Fig. 1D); and the hybridization signals were distributed both in the nucleus and the cytoplasm of the microspores at this stage (Fig. 1E); after the tapetum degeneration, the hybridization signals could only be observed in microspores at the binucleate and late binucleate stage (Fig. 1F,G); When the pollen grain became mature, *zm401* was expressed predominantly in pollen grains reaching a maximum (Fig. 1H). There were no hybridization signals above background over any other anther regions. Figure 1I shows that only slight background signals were observed in negative control when sense *zm401* RNA was used as a probe for hybridization. The endogenous *zm401* expression levels at floret forming stage and anther different developmental stages were quantified using Real-time Quantitative RT-PCR (Q-PCR). *Actin* was used as an internal control to normalize *zm401* amounts. We did not detect the expression of *zm401* in the root, stem, leaf and female ears, we first detected this gene's expression at floret forming stage in the floral meristem. As shown in Figure 1J, relative to premeiosis stage (set as 1), the transcript amounts were 0.25 at the floret forming stage and slightly increased to 1 at premeiosis stage. While using in situ hybridization, there were no hybridization signals above background observed at these two stages, it could be due to the limitation of this technique. The expression levels of *zm401* increased to 86.96 at uninucleate stage and 95.51 at binucleate stage. The highest transcript amounts (247.15) were present in mature pollen. The results of both in situ hybridization and Q-PCR indicated that the expression of *zm401*

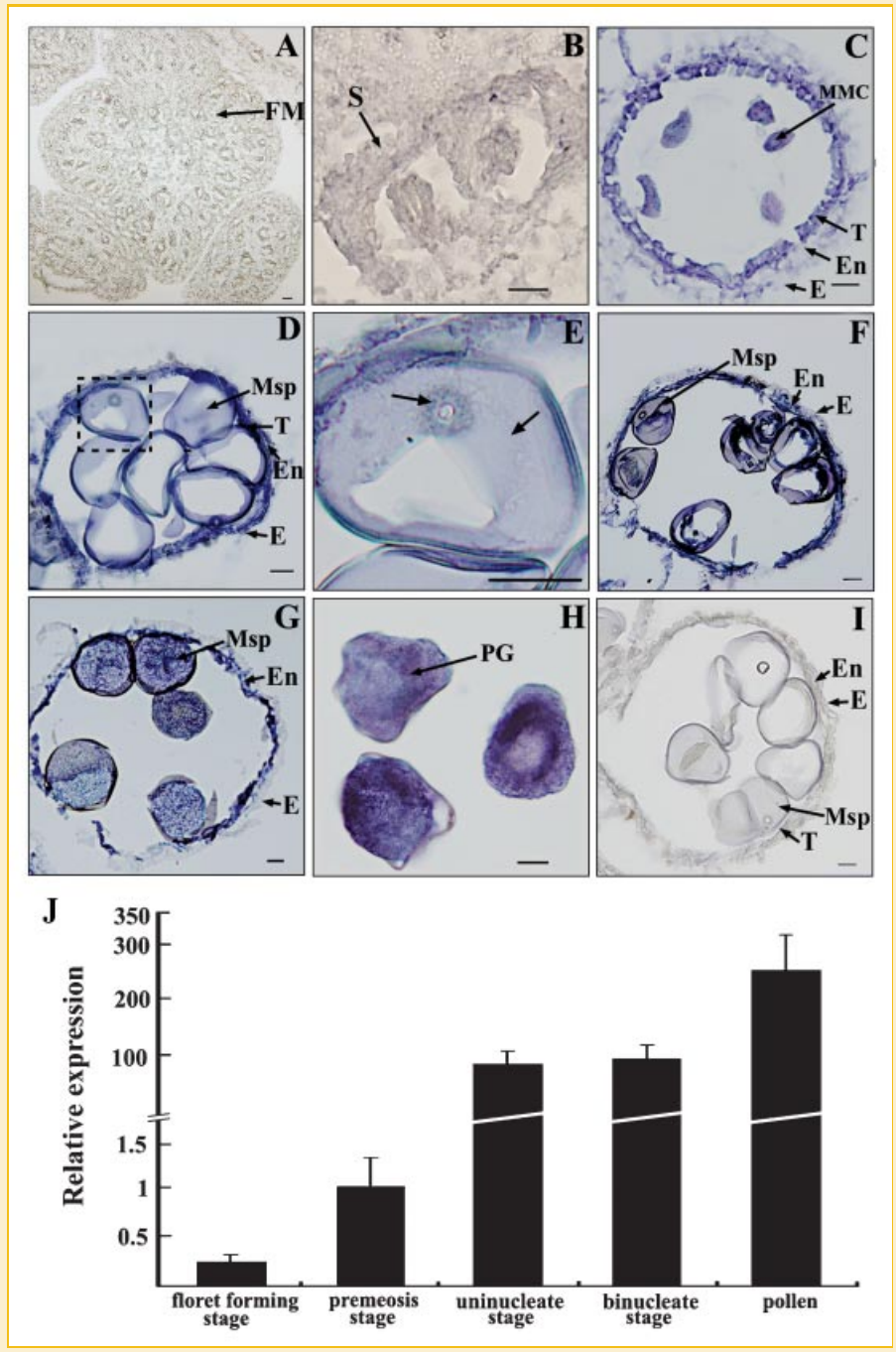

Fig. 1. Expression pattern of *zm401* mRNA. A–I: Expression pattern of *zm401* mRNA in wild-type maize revealed by in situ hybridization. A: No *zm401* mRNA expression was detected in the floral meristem. B: *Zm401* mRNA expression was not detected in anther at premeiosis stage. C: Anther at tetrad stage, positive signals in microspores and tapetum. D: Anther at uninucleate stage, *zm401* mRNA signals were predominant in both tapetal cells and microspores. E: Enlargement of part (in the broken line frame) of D; the arrows indicate that *zm401* mRNA is expressed both in nucleus and cytoplasm. F: Anther at binucleate stage, microspores are positive. G: Anther at late binucleate stage, microspores remain positive. H: Greatly increased expression in pollen grains. I: Control section of an anther at uninucleate stage, which was hybridized with the sense *zm401* RNA probe, showing only the background signals compared with the stronger signals on the other sections hybridized with the antisense *zm401* RNA probe. J: mRNA level in wild-type maize as revealed by Q-PCR. The mRNA level of premeiosis anthers was set to 1. *Actin* was used as an internal control to normalize *zm401* mRNA amounts. Transcript amounts of *zm401* mRNA at different development stages were given, 10–12 different plants of each stage were measured, means of 10–12 plants and s.d. of the means are shown. E, epidermis; En, endothecium; FM, floral meristem; MMC, microspore mother cells; Msp, microspores; PG, pollen grain; S: spikelet; T, tapetum. Bars = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

is associated with the floret development, especially with the development of tapetal cells and microspores.

THE EXPRESSION OF *zm401* WAS SIGNIFICANTLY REDUCED IN TRANSGENIC PLANTS

To investigate the function of *zm401*, we generated full-length of *zm401* (1.2 kb) sense construct and RNAi construct transgenic maize plants. Immature embryos (3,415) were bombarded with either the *zm401* sense or RNAi constructs containing *zm401* cDNA. Transformation efficiency of the two independent experiments was about 1%. Southern blot showed that the transgenic plants contained one to several copies of the transgene. Stable inheritance of *zm401* was investigated in the progeny (T1) of T0 transgenic plants. Some T0 plants displayed identical transgene integration patterns, indicating that they originated from the same transgenic integration event. Therefore, we selected one of these same transgenic integration pattern plants. In total, 245 T1 plants generating from 7 different sense transgenic lines and 9 different RNAi transgenic lines were analyzed, 115 plants showed the stable integration of *zm401*. This indicated that the transgenes displayed stable inheritance in maize plants.

We compared the expression levels of *zm401* in wild-type, sense and RNAi transgenic plants (T0/T1) respectively; 7–10 different plants of each type were analyzed. We originally generated sense transgenic maize plants to up-regulate *zm401* expression; and RNAi transgenic maize plants to down-regulate *zm401* expression. However, the transcript levels of *zm401* were depressed in both of these transgenic plants. The Northern blot and Q-PCR results showed that the transcript levels of *zm401* in transgenic plant were reduced to about 10% (10.8% in sense plants; 9.9% in RNAi plants) relative to the transcript levels of wild-type anthers at uninucleate stage (Fig. 2). We also compared the expression levels of *zm401* in the wild-type and in transgenic anthers at binucleate stage, and similar results were obtained (data not shown). We can detect *zm401* expression in wild-type plants at floret forming stage (the level is very low), while we cannot detect the expression of *zm401* in transgenic plants at this stage by Q-PCR, it could be due to the depression of *zm401*. We further detected short RNAs to confirm the silencing induced using Northern blot. Short RNAs was detected only in transgenic plants and not in the wild-type ones (Fig. S2). All these results showed that the expression of *zm401* in transgenic plants was significantly reduced.

TRANSGENIC MAIZE FLORET AND ANTHER DISPLAYED PLEIOTROPIC PHENOTYPES

Deviation from wild-type phenotypes was exclusively observed in plants expressing the transgene. We selected the same transgenic plants which were used in expression analysis to observe any phenotypic changes (at floret forming stage, we selected plants which have same transgenic integration patterns (T0) or belong to the same lines (T1) of the plants used in expression analysis, since the male organs of the plants were already destroyed). Interestingly, the sense and RNAi transgenic plants showed similar phenotypes (any phenotypic deviation were seen in both transgenic plants). There was no visible alteration in the transgenic plants during the vegetative stage, but abnormal floret and anther

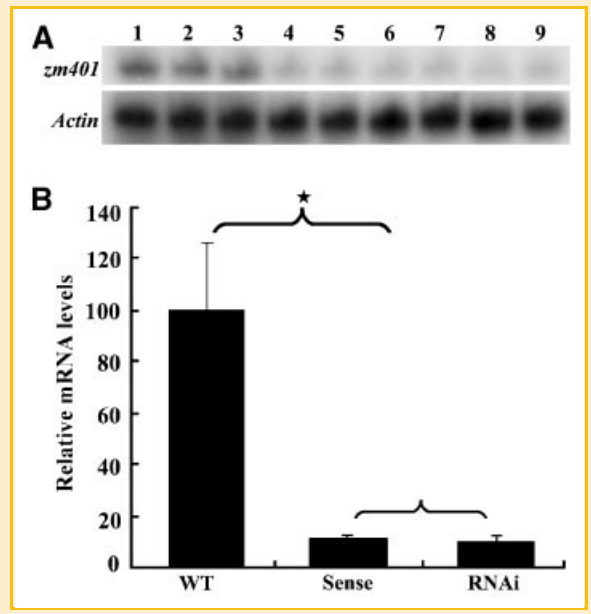

Fig. 2. Relative quantification of *zm401* mRNA levels in wild-type and transgenic anthers at the uninucleate stage. A: Northern blot analyses. Lanes 1–3: Transcripts of the wild-type maize anthers; lanes 4–6: transcripts of the sense construction transgenic maize anthers; lanes 7–9: transcripts of the RNAi construction transgenic maize anthers. *Actin* was used as control to normalize *zm401* mRNA amounts. The hybridization signals were visualized by Phosphor Screen, and the quantification of the transcript level was performed using ImageQuant5.2 (Molecular Dynamics). B: Q-PCR analysis. *Actin* was used as an internal control to normalize *zm401* mRNA amounts. Relative mRNA level in anthers of wild-type was set as 100. Sense and RNAi transcript levels of anthers at uninucleate stage are shown. WT: wild-type plants; sense: sense construction transgenic plants; RNAi: RNAi construction transgenic plants. Seven to ten different wild-type/sense/RNAi transgenic plants were analyzed, means of 7–10 plants and s.d. of the mean are shown; asterisk: $P < 0.05$.

development, which were observed in both of the sense and RNAi construct transgenic plants in T0 and T1 generations. As shown in Figure 3: (1) there was a difference in floret number per spikelet between the wild-type and transgenic plants: transgenic maize spikelets had only one floret with three anthers (Fig. 3B), whereas in wild-type spikelets, two florets developed with three anthers each, for a total of six anthers per spikelet (Fig. 3A); (2) some transgenic anthers ceased to develop and perished at early development stage (Fig. 3C); (3) transgenic anther surface was brown, the anthers were not fully developed and appeared shriveled, and shorter than wild-type (Fig. 3D); (4) very few transgenic tassels were fully developed, which have no floret opening and the tassels remained green for several weeks without occurrence of anthesis, while wild-type tassels reached maturity with floret opening and occurrence of anthesis at the same stage (Fig. 3E).

To further investigate the phenotypic differences between wild-type and transgenic anthers, anthers were sectioned at several development stages in wild-type, sense and RNAi transgenic plants. In wild-type anthers, it was visible that tapetum lined the anther locule, microspores bound together as tetrads in the pollen sac (Fig. 4A); while in the transgenic anthers, the tapetal cells

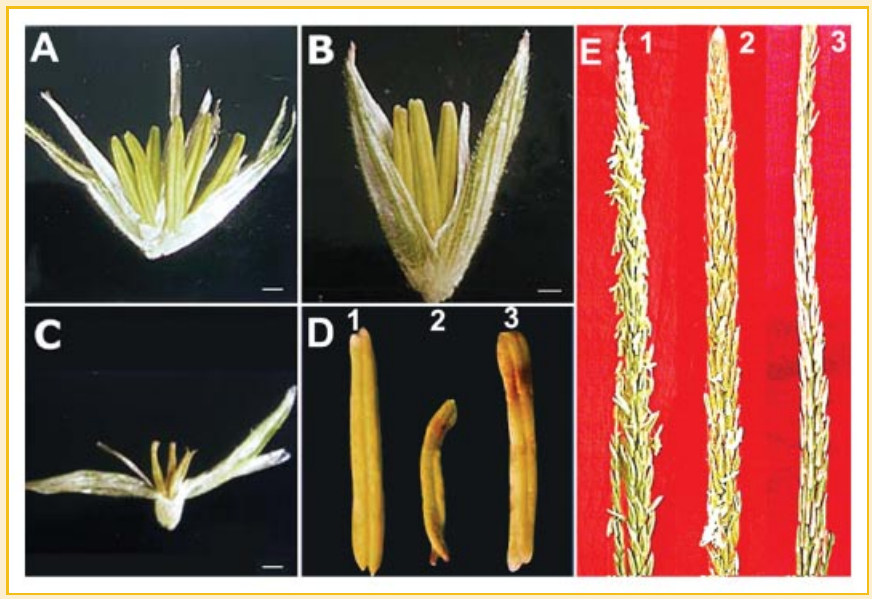

Fig. 3. Transgenic maize expressing *zm401* sense and RNAi transcripts displayed a variety of phenotypes. A: A spikelet from a wild-type plant containing two florets and producing three anthers each, for a total of six anthers per spikelet. B: A spikelet from a sense/RNAi transgenic plant containing only one floret with three anthers. C: A spikelet from a sense/RNAi transgenic plant showing arrested anthers at an early development stage. D: Anthers from sense/RNAi transgenic plants compared with wild-type plants. D1: wild-type anther; D2, D3: transgenic anthers. E: Tassels from sense/RNAi transgenic plants compared with wild-type plants. E1: Tassel of a wild-type plant at anthesis; E2, E3: tassels of sense/RNAi transgenic plants at the last stage of development, showing fully developed tassels but no opening of male florets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

abnormally expanded, occupying a large space of the sac; the microspores were distorted in shape; the tetrads could not be observed at this stage (Fig. 4B,C). No differences were found in outer epidermis, endothecium and middle layer between the wild-type and the transgenic plants at tetrad stage. At uninucleate stage, the tapetum of wild-type anther began to degrade and the microspores became round and was filled with cytoplasm (Fig. 4D); while in the transgenic plants, the tapetum was denser than wild-types, and the microspores were distorted and had no cytoplasm (Fig. 4E,F). The other anther wall layers of transgenic anthers were same to those of wild-type anthers. At the binucleate stage, tapetal cell layers of the wild-type plants had completely degraded and microspores had full cytoplasm (Fig. 4G); while tapetum of transgenic anthers were still incompletely disintegrated, and the microspores had no cytoplasm (Fig. 4H,I). These results indicated that anther development was perturbed in transgenic maize.

The degrees of sterility were also analyzed. Anthers from the male flowers were stained with I_2-KI. Spherical stained pollen grains were considered to be healthy and fertile, and shrunken unstained pollen grains were considered to be sterile. A few hundreds pollen from different anthers of transgenic lines and wild-type plants were analyzed. About 98% of the wild-type pollen reached maturity; while only about 8% of the transgenic pollen reached maturity (about 2% of the pollen was arrested during the normal anther development). This indicated that 90% of the pollen of the transgenic plants was sterile (Table S3). At the same time, even the fully developed pollen in transgenic plants, neither germinated in vitro nor led to progeny kernels after selfing or outcrossing to Z31 wild-type plants.

When the female ears of these transgenic plants used as female parents were crossed with wild-type, nearly all ovules produced viable seeds similar to the wild-type, indicating that the transformation does not affect female fertility (data not shown).

All transgenic plants in T0 and T1 generations showed parts or all phenotypes described above except for a few transgenic plants, these plants showed the same phenotype as the wild-type plants. We detected the expression of *zm401* in these transgenic plants, and found that expressions of *zm401* were not depressed. This phenomenon could be the result of transgenic silencing since the exogenous *zm401* can be integrated into different regions of genome.

THE EXPRESSION LEVEL OF THREE CRITICAL GENES IN ANTHER DEVELOPMENT SIGNIFICANTLY CHANGED IN TRANSGENIC PLANTS

To investigate the potential interaction between *zm401* and the genes involved in anther development (all the data above suggested that *zm401* has crucial role in anther development), we selected three crucial genes in maize anther development: *MZm3-3*, which is expressed specifically during male gametogenesis and at high levels in the tapetum [Lauga et al., 2000]; *ZmMADS2*, expressed during pollen development at all stages [Heuer et al., 2000; Schreiber et al., 2004] and *ZmC5*, which is expressed specifically during late pollen development [Wakeley et al., 1998]. We compared the expression of these genes between the wild-type and the transgenic plants. Seven to ten different plants from T0 and T1 wild-type and transgenic maize were analyzed. As revealed by Northern blot and Q-PCR; *MZm3-3* transcription levels in transgenic anthers was increased about fivefold at uninucleate stage; *ZmMADS2* and *ZmC5* transcription levels in transgenic anthers were both decreased to about 10% (10.6% and10.3% respectively) relative to the transcription levels in the wild-type plants at the binucleate stage (Fig. 5).

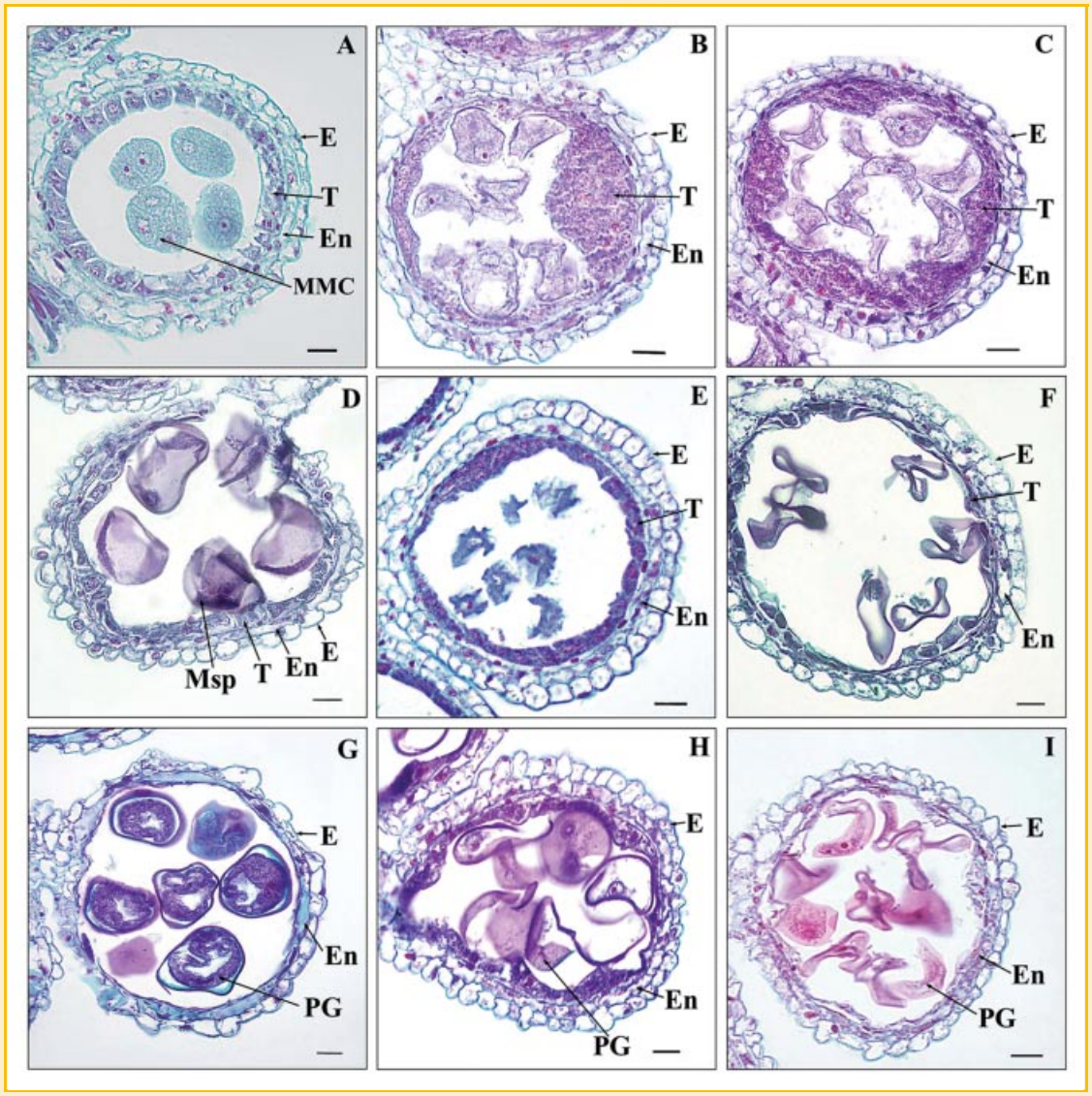

Fig. 4. phenotypes of anther development in transgenic plants compared with the wild-type. A: A wild-type anther at tetrad stage, with the individual microspores separated by callose. B: An anther of transgenic plants with the *zm401* RNAi construct at tetrad stage; the tapetum begins to enlarge and the tetrad cannot be observed. C: An anther of transgenic plants with sense *zm401* cDNA promoted by ZM13 promoter at tetrad stage, showing the enlarged tapetum cells and no tetrad can be observed. D: A wild-type anther at uninucleate stage. The tapetum shows signs of degradation and the microspores become round and had full cytoplasm. E: An anther of transgenic plants with the *zm401* RNAi construct at uninucleate stage, the tapetum is still dense and the microspores are distorted with no cytoplasm. F: An anther of transgenic plants with sense *zm401* cDNA promoted by the ZM13 promoter at uninucleate stage, with the same phenotype as anthers from RNAi construct plants. G: A wild-type anther at binucleate stage. The tapetum has degenerated and microspores are full of cytoplasm. H: An anther from transgenic plants with the *zm401* RNAi construct at binucleate stage, the tapetum are incompletely disintegrated and the microspores have no cytoplasm. I: An anther from transgenic plants with sense *zm401* cDNA promoted by ZM13 promoter at binucleate stage, it has the same phenotype as anthers from RNAi construct plants. E, epidermis; En, endothecium; MMC, microspore mother cells; Msp, microspores; PG, pollen grain; T, tapetum. Bars = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These results indicated that the expression of these genes had significantly changed in transgenic plants.

Zm401 HAS sORFs; IT HAS HIGHLY CONSERVED SEQUENCES AND RNA SECONDARY STRUCTURE IN MONOCOTYLEDON

Previously we found that *zm401* lacks any significant ORF by sequence analysis [Dai et al., 2007]. By using NCBI ORF finder, we found that *zm401* contains multiple starts and stop codons and lacks an extensive ORF. Among these short ORFs that are present in the *zm401* cDNA sequence, the longest spans 269 bp with a potentially functional translation initiation site which would yield a product of 89 amino acids, and can be identified at 791–1,060 bases from the 5′-end. We also examined the predicted secondary structures of *zm401* RNAs by computer analysis using the Vienna RNA package [Hofacker et al., 1994]. The results showed that *zm401* can fold into a stable RNA secondary structure (Fig. S3): about 65% of the sequences (744 bp relative to 1,150 bp) will be paired; the free energy of *zm401* is about −570.08 kcal/mol. The secondary

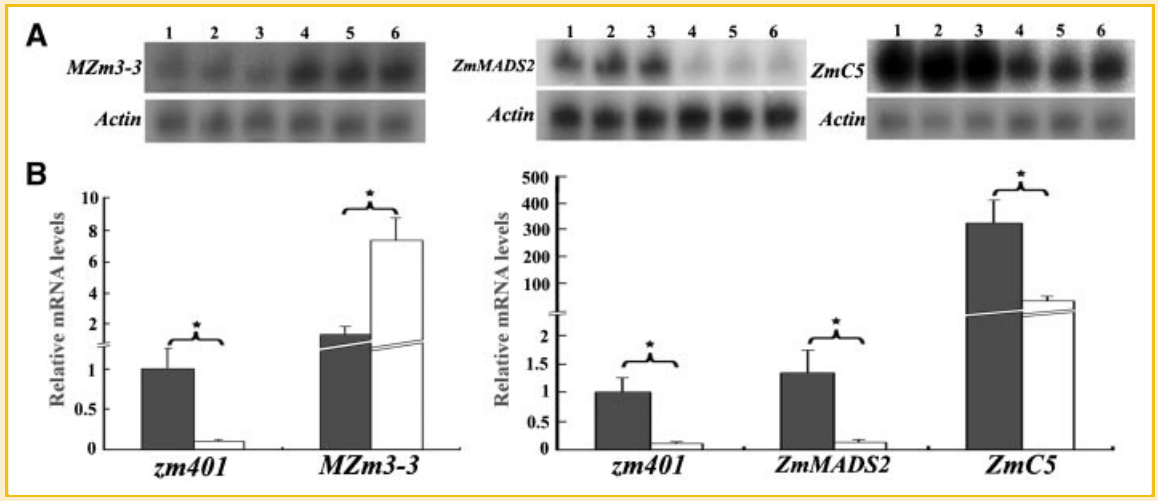

Fig. 5. mRNA level of *ZmC5*, *ZmMADS2* and *MZm3-3* genes changed greatly in *zm401* transgenic plants. A: Northern blot analyses. Lanes 1–3: mRNA transcripts of the wild-type maize anthers, lanes 4–6: mRNA transcripts of the transgenic maize anthers. *MZm3-3* mRNA transcripts in maize anther at uninucleate stage are more highly expressed in transgenic plants than in wild-type ones; *ZmC5* and *ZmMADS2* mRNA transcripts in maize anthers at binucleate stage are expressed at lower levels in transgenic plants than in wild-type plants. *Actin* was used as a control to normalize *zm401* mRNA amounts. The hybridization signals were visualized by Phosphor Screen, and the quantification of the transcript level was performed with ImageQuant5.2 (Molecular Dynamics). B: *MZm3-3*, *ZmMADS2* and *ZmC5* mRNA transcript levels in wild-type and transgenic anthers as revealed by Q-PCR. *Actin* was used as an internal control to normalize *zm401* mRNA amounts. Seven to ten different wild-type/transgenic plants were analyzed, means of 7–10 plants and s.d. of the means are shown; asterisk: $P < 0.05$.

structures are very stable with the free energy measuring $\Delta G = -1$ to -3 kcal/mol per base pair [Pasquali, 2005]. For *zm401*, the ΔG is about -1.53 kcal/mol per base pair ($-570.08/(744/2)$). The predicted structure at 30–35°C is different from the structures at 5–25°C, indicated that temperature may affect the activity of *zm401*. The possible microRNA produced from *zm401* were predicted using the method [Wang et al., 2004] and the miRscan software [Lim et al., 2003], and no microRNA products were found. Northern blot using *zm401* sense RNA transcribed from a full-length *zm401* cDNA as probe also showed that there are no microRNA products produced from *zm401* in maize (Fig. S2).

No homologous DNA sequences were found in NCBI GeneBank database, except for one partial homologous EST (from maize apex tissues, the accession number in GenBank is CF048681). The homologous sequences of *zm401* (765–968) and CF048681 (10–212) have about 86% identity. Using *zm401* cDNA as a probe in Southern blot, no signal was detectable in dicotyledon plant genomes such as Arabidopsis, tobacco or tomato, while signals were seen in monocotyledon plant genomes such as rice, wheat and millet. We obtained the *zm401* homolog from the rice, wheat and millet genome by both PCR and RT-PCR, and the sequences of these genes showed over 99% homology with maize *zm401* cDNA, only few bases are different (unpublished data).

DISCUSSION

Zm401 EXPRESSES FROM THE FLORET FORMING STAGE AND IS REQUIRED FOR FLORET FORMATION AND DEVELOPMENT

The previous study showed that *zm401* was transcribed from the tetrad stage of microspore development using RT-PCR and Northern blot analysis [Dai et al., 2004]. In this study, we found that *zm401* was transcribed from the floret forming stage using Q-PCR. The different results could be due to different methods used. The facts that *zm401* is not expressed in root, stem, leaf and female ear, give the reason why there is no phenotype difference between the wild-type and transgenic plants in these tissues/organs throughout the life of the plants; *zm401* is expressed from the floret forming stage in the floral meristem; there were phenotype and *zm401* expression difference between the wild-type and transgenic plants indicted that *zm401* begins to play a role in the growth and development of male organ from the floret forming stage. We can detected the expression of *zm401* at the floret forming stage in wild-type plants (the expression level is the lowest compared to other developmental stages), while in transgenic plants, we could not detect the expression of *zm401* at the floret forming stage. This could be due to the depression of *zm401* in the transgenic plants. Reduced floret number in transgenic plants could be the result of the depressed expression of *zm401*. As the floret growth and development, the abnormal expression of *zm401* disrupted the floret development and resulted in no opening of the florets in transgenic plants. Similar results were also obtained from our previous study. The ectopic expression of *zm401* in tobacco perturbed the flower development of tobacco while in comparison to control plants, about 80% of the flower buds in transgenic tobacco gradually fell off before flowering, and eventually most rachis became brown [Ma et al., 2005]. It can be concluded that *zm401* could affect the floret formation and development in maize.

Zm401 HAS AN ESSENTIAL ROLE IN TAPETUM AND MICROSPORE DEVELOPMENT IN MAIZE

As revealed by in situ hybridization and Q-PCR, *zm401* is expressed in a developmentally regulated manner in anthers. Normally, the

expression pattern of the gene is consistent with the function of the gene, thus *zm401* should play important roles in tapetal and microspores growth and development.

In order to study the function of *zm401*, we generated full-length of *zm401* cDNA (1.2 kb) transgenic plants: sense construct to over-express *zm401* and RNAi construct to silence *zm401*. Unexpectedly, we found that the expression levels of *zm401* in both sense and RNAi construct transgenic plants were reduced and all the transgenic plants showed similar phenotypes. In transgenic anthers, the tapetum underwent hypertrophy at tetrad stage and the tapetum degraded later than wild-type due to the reduced expression of *zm401*. The tapetum is a critical tissue, mediating between the gametophyte and sporophyte, and this is highlighted across the plant kingdom that tapetal malfunction is regarded as the prime cause of male sterility [Kaul, 1988]. It plays important roles in the nutrition of microspores, in the regulation of sporogenesis and in pollen wall development and disintegrates in the later stages of pollen development, and also, microsporocytes and tapetal cells share many developmental pathways during pollen wall formation [Scott et al., 1991; Goldberg et al., 1993]. In our study, besides the tapetum, the microspores in transgenic anthers were also different from those of wild-type. In transgenic anthers, the tetrads could not be observed and the microspores were distorted with no cytoplasm; while in the wild-type anthers, the tetrads could be observed clearly and the microspores became round and be filled with cytoplasm at the same development stage. A number of genes have been identified that have a specific effect in microspore development. For example, *HEN1* is required for male fertility [Chen et al., 2002]; *SEK1* and *SEK2* are required for microspore formation [Albrecht et al., 2005; Colcombet et al., 2005]. Other results from mutations analyses show that the defects of tapetal cells can affect microspore formation [Wilson et al., 2001; Ito and Shinozaki, 2002]. From this study, we can conclude that poor pollen viability of transgenic plants could be the results of both the lagged degradation of tapetum and developmental defect of microsporocytes.

Transgenes are susceptible to silencing in all plant species studied [Jeong et al., 2002]. Silencing results from interactions among multiple copies of transgenes or when additional copies of an endogenous gene are ectopically expressed. Although a number of speculative hypotheses have been put forward and extensively reviewed, the mechanism of homology dependent gene silencing is not yet clearly understood in many respects [Matzke et al., 2001; Henderson and Jacobsen, 2007]. Homology dependent gene silencing appear to involve at least two distinct processes, one operating at the transcriptional level—through repression of transcription, termed transcriptional gene silencing (TGS) and one at post-transcriptional levels—through mRNA degradation, termed post-transcriptional gene silencing (PTGS) [Vaucheret et al., 1998]. The reason of *zm401* expression in sense transgenic plants was reduced may be the result of transgenic silencing: both the transgene and the endogenous gene produce transcripts that are quickly degraded in the cytoplasm, and as a result, little transcripts was produced in affected cells; or via promoter homology resulting in the reduction of transcription. Another reason maybe *zm401* is an essential gene in maize, over-expression might be more harmful than depressed expression, there are regulators to limit it's over-expression. The expression of *zm401* in both sense and RNAi construct transgenic maize were reduced, and the transcript levels were almost the same. It could be the reason why sense and RNAi construct transgenic plants showed the same phenotypes.

Zm401 IS ONE OF THE KEY GROWTH REGULATORS IN ANTHER DEVELOPMENT

Recently, many genes involved in anther development have been identified [Zik and Irish, 2003; Scott et al., 2004], while in maize, only a few pollen specific genes have been studied [Wakeley et al., 1998; Schreiber et al., 2004; Bommert et al., 2005]. Silencing of *zm401* by RNAi and sense transgenic plants, which both reduced expression level of *zm401*, significantly affected (directly or indirectly) the expression of *MZm3-3*, *ZmC5*, and *ZmMADS2*, three crucial genes in anther development. This suggested that *zm401* could be an important member of the complex regulatory network regulating anther development. *MZm3-3* gene is expressed specifically during male gametogenesis and its expression is highly and preferentially detected in the tapetum, from the pollen mother cell to uninucleate stage, it has an important role in tapetal development [Lauga et al., 2000]. In our results, tapetums did not degrade in transgenic anthers and the expression level of *MZm3-3* in transgenic plants was higher than in wild-type at uninucleate stage. It suggested that the increased expression of *MZm3-3* could be one of the reasons of later degradation of the tapetum in transgenic anthers. *ZmC5* is a maize pectin methylesterase (PME)-like gene. The expression of the *ZmC5* gene is very similar to that of the *P. inflate* PPE gene, and other late pollen expressed genes (polygalacturonase and pectate lyase) with putative roles in pectin breakdown. The PME-like gene product from *ZmC5* has a role in pollen maturation and pollen tube elongation [Wakeley et al., 1998]. In our study, the development of microspores was abnormal and the expression level of *ZmC5* in transgenic plants was lower than those of wild-type, suggesting that the lower expression level of *zm401* could affect the expression pattern of the genes that are involved in microspore development, and perturb the microspore development of transgenic maize plants. As for the *ZmMADS2* gene, which is expressed during pollen development all stages, its expression is required for anther and pollen maturation [Heuer et al., 2000; Schreiber et al., 2004]. *ZmMADS2* is a crucial gene in anther development, especially in late pollen development. In our transgenic plants, the expression level of *zm401* was decreased to about 10% compared to that of wild-type plants; meanwhile, the expression level of *ZmMADS2* (as well as *ZmC5*) was also decreased to about 10% in transgenic plants. It suggested that these genes may be in the same regulatory pathway or have very closely relationships in regulating anther development; they compose a complex regulatory network to accurately regulate anther development. The possible interaction of *zm401* and *Zm3-3*, *ZmC5*, *ZmMADS2* is shown in (Fig. S4).

Zm401 IS A NEWLY IDENTIFIED sORF RNA OR ncRNA

The PCR from genomic DNA and RT-PCR from cDNA cloned homologous of *zm401* in some monocotyledon plants, including rice, showed about 99% identical with maize *zm401*, while the blast

result of *zm401* in NCBI using blastn, no homologous DNA sequences were found. The results were very confusing for us. At first, we doubted whether the PCR-cloned homologues of *zm401* sequences were the results of sample contamination. Repeated experiments using new materials in PCR, RT-PCR and sequencing in another lab (only carry out animal molecular biology research) by different person, still gave the same results, excluded the possibility of sample contamination. It is indeed that *zm401* is a newly identified gene, and the sequences of this gene/homologues gene are highly conserved between maize, rice, wheat and millet, although the genomic DNA sequences of rice have been published. It is still possible that the sequences of some small gap sequences have not been identified, which could be a reason why we were unable to find this gene in GenBank, while we were able to amplify it from rice genomic DNA.

Previously, we found that *zm401* lacks any significant ORF by sequence analysis [Dai et al., 2007]. By using NCBI ORF finder, we found that *zm401* lacks an extensive ORF and it contains multiple starts and stop codons. The longest ORF spans 269 bp with a potentially functional translation initiation site, and it would yield a product of 89 amino acids. We proposed that the function of *zm401* could be associated with the secondary structures of RNA transcript or small peptides. This is supported by the following observations: there are no significant ORFs in the *zm401* gene and no detectable translation products have been found for these transcripts by in vitro translation [Dai et al., 2007]; no microRNA of *zm401* were found by both of miRscan software prediction and the Northern blot. There exists a high degree of overall sequence conservation (over 99%) and therefore highly conserved stable RNA secondary structure in maize, rice, wheat and millet. The fact that the *zm401* homologs have retained a high degree of similarity despite the long divergence times among these species suggests that these genes have crucial conserved functions in all these plants. Although *zm401* could not be translated in vitro, it remains possible that *zm401* does encode small peptides, which could not be detected by in vitro translation. Some ncRNA or small polypeptides involved in anther or floral development have been found recently [Butenko et al., 2003; Kidner and Martienssen, 2003; Wen et al., 2004]. *Enod40*, a well-known ncRNA and/or sORF mRNA, colocalize with MtBP1 into cytoplasmic granules, and this mechanism of action involve the *enod40* RNA itself. The sORFs of *enod40* can be translated in vitro, and the *enod40* RNA accumulates in nuclear as well as cytoplasmatic particles in living plant cells [Van de Sande et al., 1996; Rohrig et al., 2002; Campalans et al., 2004]. *Enod40* plays roles as oligopeptide or as ncRNA or both. *Zm401* shares some characteristics with *enod40*. Both have short-opening reading frames, highly conserved sequences and stable RNA secondary structures, and are expressed in both the nucleus and cytoplasm. The function of *zm401* could be associated with secondary structure RNA, or the polypeptides that translated from *zm401*, or both.

In conclusion, we identified a novel ncRNA or sORF gene—*zm401*, which is expressed mainly in tapetal cells and microspores. The silence of *zm401* dramatically affects two processes: stamen growth and anther development. It is an essential gene that plays a pleiotropic role during the development of the male flower, and can be used to generate sterile male plants. Future studies will characterize the molecular nature of *zm401* and clarify the interaction between *zm401* and its target genes.

Sequence data from this article can be found in the GenBank data libraries under accession number: AY911609 (*zm401* cDNA).

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