# ISOLATION AND CHARACTERIZATION OF A POLLEN-SPECIFIC GENE *ZMSTK2_USP* FROM *ZEA MAYS*

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**Abstract.** Microsporogenesis in flowering plants is a complex process involving a range of gene expression. In the present study, a pollen-specific gene *ZmSTK2_USP* (serine threonine kinase) was isolated from *Zea mays*. Mutant *ZmSTK2_USP* showed 56.7% pollen viability capacity, 48.92% germination rates comparing with wild-type. Northern Blot results showed that *ZmSTK2_USP* were specifically expressed in mature pollen. The expression level was highest at late pollen development, followed by bicellular stage. And at early pollen stage, no expression was detected. GUS activity was detected in middle stage, late stage of pollens and germinated pollen. These results indicated that *ZmSTK2_USP* expressed at late pollen development and was pollen-specific.

**Keywords:** *maize, serine/threonine kinase, pollen development, mutant, expression pattern, GUS activity*

## Introduction

The floral organ development of plants needs to cooperate with the expression and regulation of various floral organ-specific genes, including a large number of genes related to anther or pollen development.

In plants, a number of anther- or pollen-specific genes have been isolated and identified. Hanson et al. (1989) reported that *Zmc13* expressed in maize pollen and pollen tube. *OSIPA* promoter conferred pollen-specific expression in both rice and *Arabidopsis*systems (Swapna et al., 2011). *TaPSG719* isolated from wheat were specific for mature pollen, and undetectable in other tissues (Jin and Bian, 2004). *TUA1* expressed in stamens and mature pollen in *Arabidopsis thaliana* development (Carpenter et al., 1992). *SBgLR* promoter in potato was identified as critical for gene specific expression in pollen grains (Zhou et al., 2010).

In the present study, we reported a pollen-specific gene *ZmSTK2_USP* (serine threonine kinase) isolated from *Zea mays*. Mutant *ZmSTK2_USP* pollen viability decreased, causing pollen germination disorder and loss of competitiveness, thus affecting the fertility of maize.

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## Materials and methods

### *Plant materials and growth conditions*

*Table 1* lists all the primers used in this study. *Mu*-induced mutant *ZmSTK2_USP* was donated by Dr. Dooner (Rutgers University, USA) and the insertion information were previously published by Fu et al. (2001). The wild type McC, transgenic lines and mutants were grown in greenhouse with 16 h light /8 h dark cycle.

***Table 1.*** *The list of primers used in the present study*

| Gene | Forward primer | Reverse primer |
|---|---|---|
| **Mu insertion lines**<br>*Mu-F*<br>*ZmSTK2_USPA* | TGTGGTTGTCTTGAACCGCT<br>（S0016） | GCCTCYATTTCGTCGAATCC<br>AAGCACAATGGGCAGGTACA<br>（S2011） |
| **Cloning**<br>ZmSTK2_USP*B* | ATGGGCAGGTACAGCGACG | GTTGTAGTTGGACCTCCATGGA |
| **Control genes**<br>*Actin* | GTTGGGCGTCCTCGTCA | TGGGTCATCTTCTCCCTGTT |
| **Southern Blot Analysis**<br>ZmSTK2_USP*E* | AGACGGGCATGCTGGGGGTGAAGT | ATGGCGGCGACGGGCTGGTGT |
| **GUS for transgenic lines** | GCAACTGGACAAGGCACT | GAGCGTCGCAGAACATTACA |

### *In vitro pollen viability and germination assays*

The pollen of wild type McC and mutant *zmstk2_usp* was collected at 10:00 a.m. every day when the tassel began to loose pollen. After mixing, some of the pollen was quickly brought back to the laboratory within an ice pack.

Proper amount of pollen was put onto clean slides, 2 drops of I2-KI solution were dropped. After treatment with 10 min at 30 constant temperature, the pigmentation of pollen grains were observed under the microscope (ZEISS Axio Scope. A1) and photographed. Mature pollen grains are dark colored, indicating that pollen has strong vitality and shallow coloration indicated that pollen activity is poor or not vigorous. Pollen grains, total no less than 100, were observed under 5 of visual field. Count the number of dark and light colors of pollen grains, and calculate the pollen viability capacity (%). Values are mean with n = 3.

Pollen germination experiments of wild type McC and mutant zmstk2_usp were carried out in vitro referring to the methods described in Schreiber and Dresselhaus (2003). A small amount of pollen were immediately and uniformly scattered to the slides coated with the pollen germination medium [medium formula is followed according to Wang et al. (2017), incubated 2 h in a petri dish, with wet filter paper, at constant temperature illumination and observed under the microscope (ZEISS Axio Scope. A1) within 5 of visual field and photographed. The number of germinated and non-germinated pollen grains was counted and the pollen germination rates (%) were calculated. Values are mean with n = 3.

### *Construction of the ZmSTK2_USP expression vector, and maize transformation*

The upstream primer STK2B-F and downstream primer STK2B-R were designed based on the sequence of the *ZmSTK2_USP* gene and listed in *Table 1*. STK2B-F with *BamH*I and STK2B-R with *Hind*Ⅲ cleavage site were employed used to amplifiy coding sequences of *ZmSTK2_USP* using McC pollen cDNA as template. *ZmSTK2_USP* target fragments were introduced into the TA clone plasmid, sequenced to gent positive cloning. The plasmids with positive cloning were digested by *BamH*I and *Hind*Ⅲ. *ZmSTK2_USP* target fragments were cloned into pCAMBIA1301 between cleavage sites of *BamH*I and *Hind*Ⅲ, which an expression vector containing a *ZmSTK2_USP*-GUS fusion gene was constructed.

### *Southern blot analysis*

Leaf DNA of T1 transgenic plants with pCAMBIA1301-GUS was isolated and amplified using primers GUS-F and GUS-R listed in *Table 1*. The DNA probe was labeled using DIG. Genome DNA (40 μg) of positive T1 transgenic plants was digested using Hind III, electrophoresised, then transferred to membrane, hybridized and detected according to DIG High Prime DNA Labeling and Detection Starter Kit Ⅰ (Roche). The colored membrane was recorded by digital camera.

### *Histochemical GUS staining*

For GUS activity analysis, we regularly collected various tissues at different developmental stages (organs) of McC and transgenic $T_2$ generation of *ZmSTK2_USP*-GUS, including immature male flowers, mature male flowers, and mature anthers, three different developmental stages (early stage of pollen development, middle stage of pollen development and late stage of pollen development) and pollen tube.

According to the method described by Jefferson et al. (1987), we put various tissues (organs) into 90% acetone stationary liquid for 20 min in -20 °C refrigerator, PBS buffer rinses 2 times using PBS buffer, each time 10 min, then added GUS staining solution X-Gluc (TritonX-100, 20% methanol), incubated 16 h at 37 °C in dark. After that, the green tissues (organs) was decolorized 2 times using 70%-100%-70% (v/v) ethanol, observed under microscope (ZEISS Axio Scope. A1) and photograph.

## Results and discussion

### *Phenotypic analysis of ZmSTK2_USP mutants*

*Mu*-induced *ZmSTK2_USP*, a non-autonomous 1.4 kb *Mu* element at the beginning of the 5th exon of *ZmSTK2_USP* (*Fig. 1A*), was obtained from Rutgers University, USA (Dooner, unpublished). *ZmSTK2_USP* mutants were detected using the primers listed in *Table 1*.

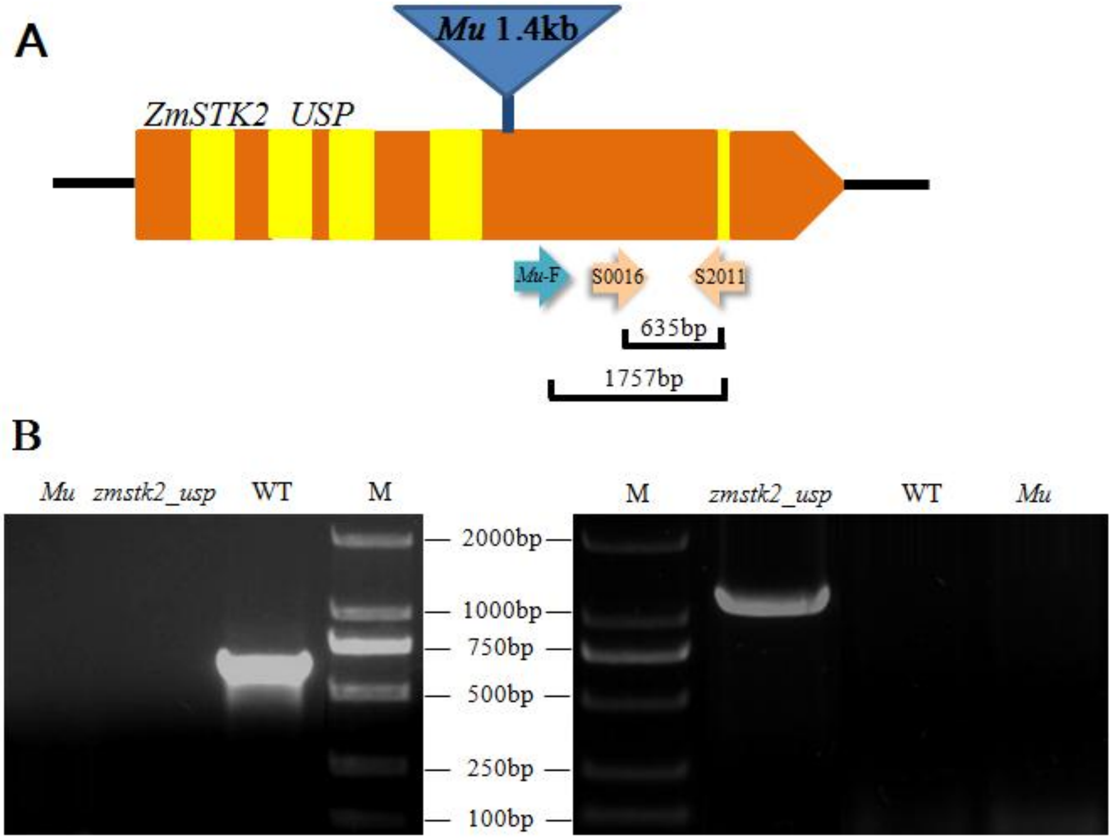

**Figure 1.** *Mu insertion mutation and detection of homozygous mutant. Note: A. Schematic view of the genomic structure of ZmSTK2_USP in maize and sites of Mu insertions; B. RT-PCR test results. M denotes DL2000. Mu was used as a negative control. The orange solid rectangle is the exon; the yellow solid rectangle is the intron; the inverted blue solid triangle is the Mu transposon and its inserted site (the fifth exon origin); the blue arrow is Mu primers and directions; the pink arrows are a pair of primers and directions on ZmSTK2_USP gene; and the black line segment is the length of the RT-PCR product corresponding to the primer combination*

*Fig. 2* shows phenotypic traits of wild-type McC and mutant *ZmSTK2_USP* during the whole period of growth and development. Both wild–type and mutant were semi-compact plant type (*Figs. 2D*, *d* and *E*, *e*) with 12-15 tassel branch (*Figs. 2E* and *e*), purple anther (*Figs. 2F* and *f*) and purple durum grain (*Figs. 2G* and *g*). In our previous study, *ZmSTK2_USP* belonged to receptor-like cytoplasmic kinase (RLCK) and was pollen-specific (Wang et al., 2014; Zhou et al., 2014; Wang et al., 2017). Hence, we observed pollen grains of wild-type plants and mutants. The pollen grains of wild type McC are dyed darkly by $I_2$-KI , very plump and most of them were germinated, while most of mutant pollen grains are partly wrinkled and hollow, dyed brown and not germinated (*Figs. 2A*, *a* and *B*, *b*). The mutant *ZmSTK2_USP* was artificially pollinated with wild type McC plants, and dyed with aniline blue after 6 h, pollen grain germination and pollen tube elongation of *ZmSTK2_USP* were very slow and half of them did not germinated comparing with wild-type (*Figs. 2C* and *c*).

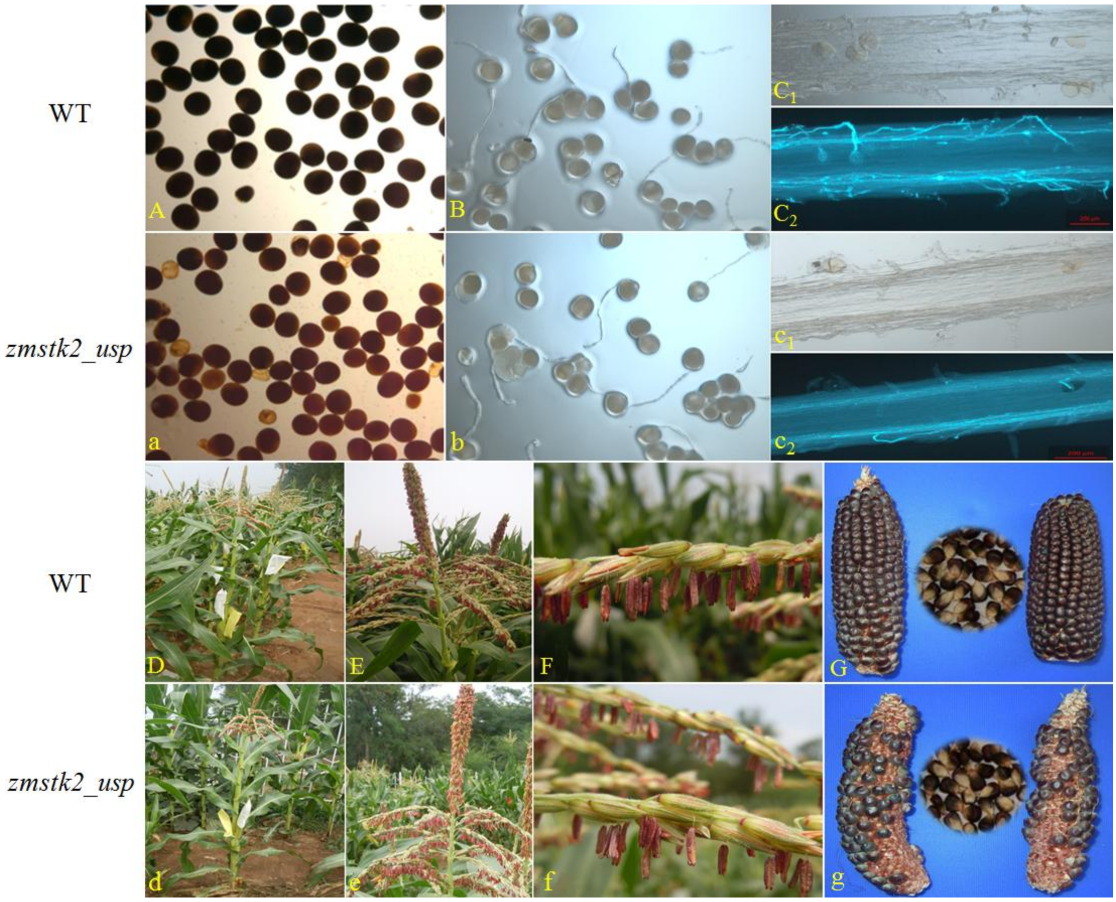

***Figure 2.*** *Phenotypic comparison of wild-type and mutant zmstk2_usp. Note: A and a. Images of pollen viability obtained after staining with $I_2$-KI; B and b. Pollen germination in vitro after 2 h; $C_1$, $C_2$, $c_1$ and $c_2$. In vivo of germinated pollen grains in silks after 6 h; C1 and c1 were observed in bright field; C2 and c2 were observed under fluorescence; D and d. Plant type of the wild type and mutant; E and e Tassel of the wild type and mutant; F and f. Anther of the wild type and mutant; G and g. Grain color and grain type of the wild type and mutant*

*Fig. 3* shows the statistical analysis results of pollen viability capacity, germination rates, filled grains per spike of mutant *ZmSTK2_USP* and wild-type plants. Comparing with wild-type, mutants showed extremely significant difference with 56.7% pollen viability capacity, 48.92% germination rates. The filled grains per spike of mutant *ZmSTK2_USP* were around 40% of wild-type. In summary, *Mu*-induced *ZmSTK2_USP* cause decreased pollen viability, disordered pollen germination, and lower competitiveness, thus affected the fertility of maize.

### *ZmSTK2_USP* expression pattern

To clarify the expression profiles of *ZmSTK2_USP* gene in Maize, we performed Northern Blot and qRT-PCR analysis (*Fig. 4*). Northern Blot results showed that *ZmSTK2_USP* were specifically expressed in mature pollen and no expression was found in other tissues (organs). The expression level was highest at late pollen development, followed by bicellular stage. And at early pollen stage, no expression was detected. The pattern of *ZmSTK2_USP* expression indicates that *ZmSTK2_USP* expressed at late pollen development and was pollen-specific.

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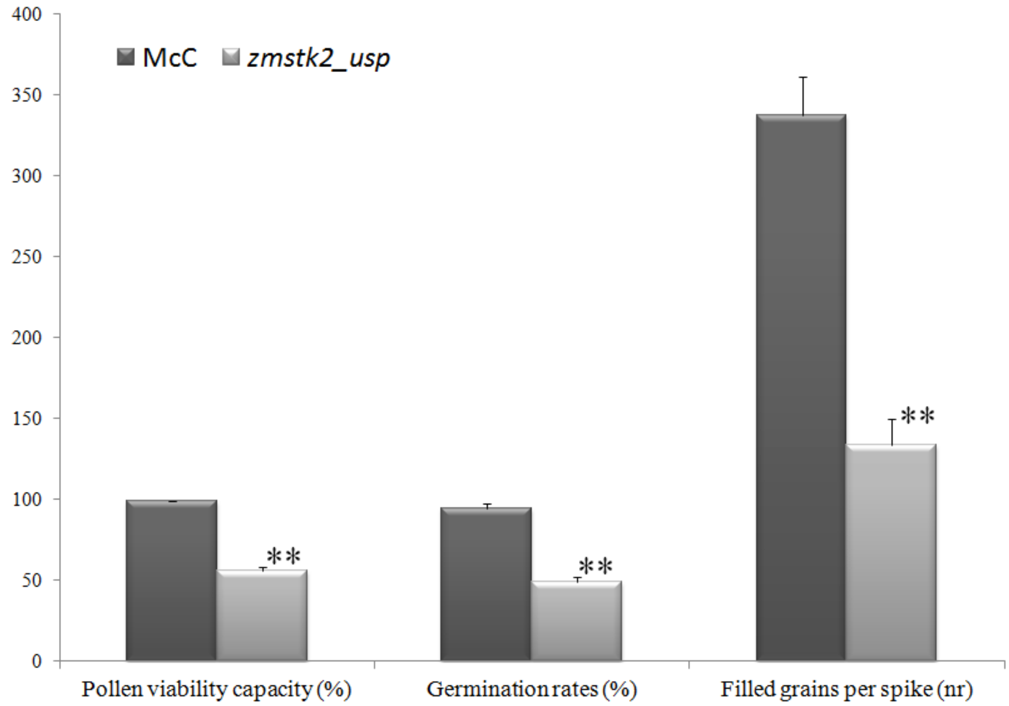

***Figure 3.*** *Analysis of pollen viability capacity (%), germination rates (%), filled grains per spike (nr). Note: ** showed extremely significant difference. The error bars represent standard deviations of the means. Data mean ± SD (n = 3)*

### *Molecular biology detection of transgenic lines*

Using the method of Agrobacterium-mediated transformation of the maize germinating embryo (Wang et al., 2017), four positive plants were conformed by PCR and Southern blot (*Fig. 4*).

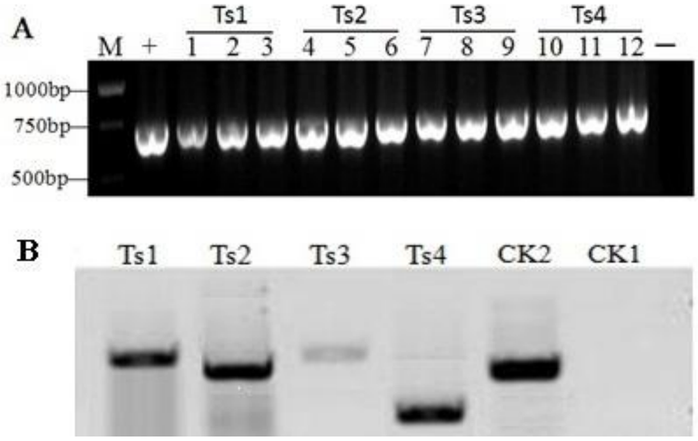

***Figure 4.*** *Molecular biology detection of transgenic lines. Note: A. PCR tests of ZmSTK2_USP-GUS in $T_0$ transgenic lines; B. Southern blotting analysis of transgenic lines ($T_0$). M are DL5000 and DL2000, respectively; + denotes transforming plasmids as positive control; — denotes wild-type McC as negative control. Ts1, Ts2, Ts3 and Ts4 are successfully transformed plants with ZmSTK2_USP-GUS. CK1 is the inbred line McC of non-genetically modified maize. CK2 is the GUS gene*

Among the four positive transgenic lines with *ZmSTK2_USP*-GUS, only $T_2$ seeds of Ts2 and Ts4 were obtained. To validate the tissue specificity of *ZmSTK2_USP*, using non-genetically modified maize McC as control, $T_2$ seeds of Ts2 and Ts4 expressing the GUS fusions, and non-genetically modified seeds (McC) were planted in a greenhouse, and the *ZmSTK2_USP* activity was examined by GUS staining at different developmental stages, then observed, photographed, and recorded under stereomicroscope. Ts2 and Ts4 showed no expression of GUS gene in immature male flower and early stage of pollens, while GUS activity was detected in middle stage, late stage of pollens and germinated pollen (*Fig. 5*). These results indicated that *ZmSTK2_USP* expressed at late stage of pollen development.

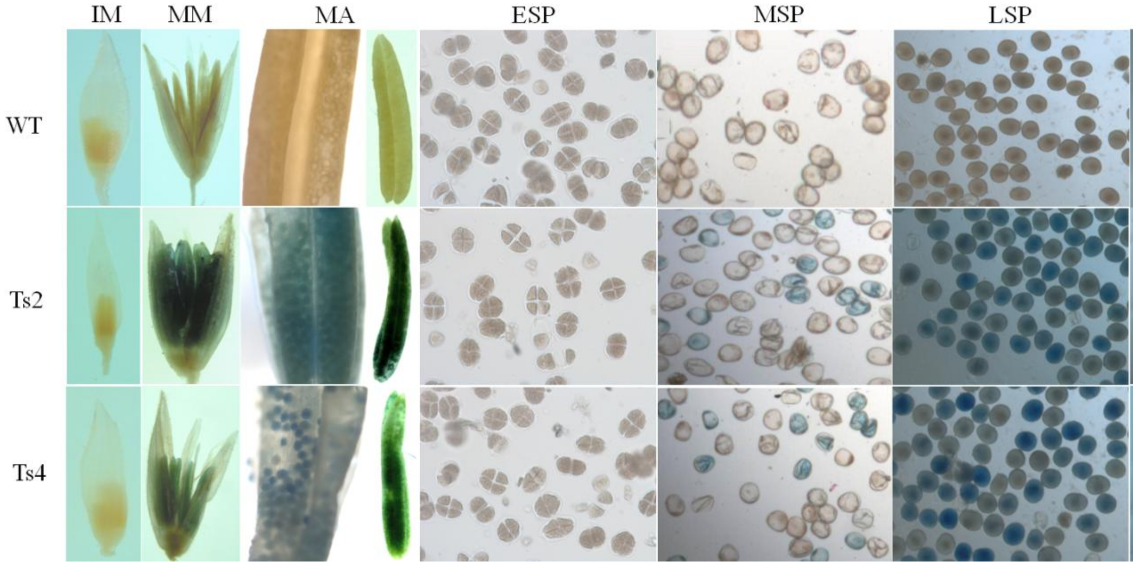

***Figure 5.*** *Histochemical GUS staining of maize transformed with ZmSTK2_USP-GUS. Note: WT is the inbred line McC of non-genetically modified maize; Ts2 and Ts4 are successfully transformed plants with ZmSTK2_USP-GUS; IM: Immature male flower; MM: Mature male flower; MA: Mature anther; ESP: early stage of pollens; MSP: middle stage of pollens; LSP: late stage of pollens*

## Conclusion

The molecular mechanisms involved in pollen development and pollen tube elongation are extremely complex. Besides a range of organ differentiation and tightly controlled changes in cellular physiology and biochemistry, a large number of genes are involved in expression and regulation. Most of these genes are also expressed in other tissues of plants, but there are still 10%-20% genes that are specifically expressed in pollen (Willing et al., 1988). At present, there are relatively few reports about pollen development genes in maize.

In our previous study, ZmSTK2_USP belong to the serine/threonine kinase (STK), containing a Usp domain on N-terminal (Wang et al., 2014; Zhou et al., 2014). In the present study, we cloned *ZmSTK2_USP* gene with specific-expressed at late stage of pollen development. The mutant *ZmSTK2_USP*, with wrinkled and withered pollen grains, showed 56.7% pollen viability capacity, 48.92% germination rates comparing with wild-type, which led to the reduction of seed setting rate. To investigate the specific expression of *ZmSTK2_USP*, Northern blot and qRT-PCR analysis results revealed that *ZmSTK2_USP* expressed at late stage of pollen development. Further, GUS staining of transgenic plants verified that *ZmSTK2_USP* gene has tissue specific expression at mature pollen. All these results are useful for providing theoretical basis for future study on the molecular mechanism of reproductive development in maize.

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