Cereal Research Communications 43(2), pp. 204–212 (2015)
DOI: 10.1556/CRC.43.2015.2.3

The Analysis of *SKP1* Gene Expression in Physiological Male Sterility Induced by Chemical Hybridizing Agent SQ-1 in Wheat (*Triticum aestivum* L.)

M.Y. WANG[1]**, Y.L. SONG[1]**, S.X. ZHANG[2], X.L. ZHAO[1], J.W. WANG[1], N. NIU[1] and G.S. ZHANG[1]*

[1]Key Laboratory of Crop Heterosis of Shaanxi Province / National Yangling Agricultural Biotechnology and Breeding Center / Yangling Branch of State Wheat Improvement Centre / Wheat Breeding Engineering Research Center, Ministry of Education / Northwest A&F University, Yangling, Shaanxi, 712100, PR China
[2]Institute of Crop Science, Ningxia Academy of Agricultural Sciences, Yongning, 750105, Ningxia, PR China

(Received 18 October 2014; Accepted 16 January 2015;
Communicated by A. Goyal)

Physiological male sterility induced by the chemical hybridizing agent (CHA) overcomes problems of maintenance of sterile lines and restorers. However, the mechanism of sterility is unclear. The process of tapetum of CHA-treated 'Xi'nong 2611' at uninucleate, binucleate and trinucleate were compared with control to determine if tapetum varying differently during developmental stages. Tapetal degradation in CHA-treated 'Xi'nong 2611' began at late uninucleate stage, somewhat earlier than control plants. Cytological observations indicated that the gradual degradation of the tapetum in CHA-treated 'Xi'nong 2611' was initiated and terminated earlier than in the control. These findings implied that CHA-induced male sterility was related to abnormally early tapetal degradation. In order to indicate the role of the *SKP1* gene in fertility/sterility in wheat, its expression was assessed in anthers at uninucleate, binucleate and trinucleate stages. *SKP1* expression was reduced in the later developmental stages, and there was an obvious decrease from the uninucleate to trinucleate stages. Higher expression of the *SKP1* gene occurred in 'Xi'nong 2611' compared to CHA-treated 'Xi'nong 2611'. This implied that *SKP1* gene expression was inhibited during the fertility transformation process and was related to transformation from fertility to sterility. Moreover, the results from this study suggest that *SKP1* plays an essential role of conducting fertility in physiological male sterility.

Keywords: wheat, CHA, tapetum, RT-PCR

Introduction

Male sterility is widely used in plants like maize, rice, tobacco oilseed rape, and to a much lower extent, wheat. However, the establishment and maintenance of male sterile lines (CMS) by the traditional method of sterile lines and maintainer lines is very difficult in

* Corresponding author; E-mail: zhanggsh58@aliyun.com
** The first two authors have contributed equally to this article.

wheat (Li et al. 2006). Physiological male sterility induced by chemical hybridizing agents (CHA) overcomes these difficulties and has significant potential as a tool in research and hybrid production. One aspect of the effect of CHAs is their possible interaction with *SKP1,* a core component of SCF type E3 ubiquitin ligases.

The ubiquitin (Ub)/26S proteasome pathway is a key regulatory mechanism controlling protein turnover. It is responsible for selective degradation of most intracellular proteins by ubiquitination (Smalle and Vierstra 2004). Three key enzymes are involved: Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and Ub-protein ligases (E3) (Hershko et al. 1983; Bai et al. 1996; Hellmann and Estelle 2002). Among the E3 families, SCF protein is a major class and its action is well understood (Chai et al. 2011). The F-box protein performs the crucial role of conferring specificity to the complex for appropriate targets (Hershko and Ciechanover 1998; Pickart 2001; Gagne et al. 2002). S-phase kinase-associated protein 1 *(SKP1)* functions as an adaptor protein connects cullin and F-box proteins. *SKP1* plays crucial roles in cell-cycle progression, transcriptional regulation, flower formation, signal transduction, and many other cellular processes (Stone and Callins 2007; Santner and Estelle 2010; Wang and Deng 2011; Hong et al. 2013).

SKP1 genes have been isolated from many different species. Twenty-one *SKP1*-like genes were identified in Arabidopsis and thirty-one were identified in rice (Farras et al. 2001; Gagne et al. 2002; Risseeuw et al. 2003; Sasaki et al. 2003; Kong et al. 2004, 2007; Chai et al. 2010). Since the crucial role of *SKP1* gene and sterility and no studies had previously demonstrated interactions between *SKP1* gene and physiological male sterility induced by CHAs, therefore, determining the action of *SKP1* gene and elucidating their potential role might reveal the mechanism of male sterility in wheat and the effects of CHAs in disrupting that process.

Materials and Methods

Plant materials

The study was conducted on October 5 of 2010 at Northwest A&F University, Shaanxi. 'Xi'nong 2611', is an early-maturing hard white, semi-dwarf winter cultivar, was planted in the field at a rate to achieve 34,000 plants ha^{-1}. Paired plots of 2.0 m × 2.5 m in three replications were sprayed with CHA, the plants sprayed with water were as the control. Normal crop husbandry measures were taken including three nitrogen fertilizations and pre- and post-emergence herbicide applications. The fungicide treatment was included to control leaf and ear diseases.

CHA spray

SQ-1, is a patented CHA used for hybrid wheat production, was provided by the Key laboratory of Crop Heterosis of Shaanxi, College of Agronomy Northwest A&F University. A hand-pumped back-pack sprayer was used for spraying. The SQ-1 at 5 kg/ha was applied at Feekes growth stage 8.5 (Liu et al. 2003). In order to see the performance of CHA, 2%

iodine-potassium iodide (KI-I_2) was used to test pollen fertility at anthesis. Twenty heads in each plot were bagged prior to anthesis to prevent fertilization by other plants as control.

Plant and light microscropy of anther sections

The uninucleate stage co-incides with when awns are less than 1 cm emerged from the flag leaf sheath; the binucleate stage is when spikes are just completely out of flag leaf sheath; and the trinucleate stage occurs before pollen dissemination. Staged anthers were sampled according to certain external forms of plants we mentioned. Microspores of staged samples were further assured after stained with 5 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) observed with an optical microscope (Nikon ECLIPSE E600, Japan).

Paraffin section for anthers

Spikes at the uninucleate, binucleate and trinucleate stages were fixed in formalin–acetic acid–alcohol (FAA) for 1–3 days at room temperature before being stored in 70% alcohol at 4 °C. Anthers were removed from the fixed spikes, embedded in paraffin, and sliced into 12 μm transverse sections, which were stained with safranin O-fast green prior to observations being made on tapetal development.

DNA and RNA extraction and cDNA synthesis

Total genomic DNA was isolated (Murray and Thompson 1980) from fresh anthers that were assured by both external forms identification and microscope observation. RNA was extracted using TRIzol reagent (Invitrogen, Changsha). Before reverse transcription, total RNA was treated with RNase-free DNase I (Takara, Shanghai) at 37 °C for 30 min to remove DNA contamination. First-strand cDNA was synthesized from total RNA using oligo-(dT) 18 primer and reverse transcriptase (RT) SuperScript (Takara, Dalian). Second strand cDNA was synthesized using 10 U DNA polymerase I (Takara, Dalian) and 3 U RNase H (Takara, Dalian). The resulting double-stranded cDNA was purified by phenol–chloroform extraction and ethanol precipitation, and re-suspended in a final volume of 40 μl ddH_2O (Ba et al. 2014). Approximately one quarter of this volume was placed on an agarose gel. Once confirmed as acceptable with an expected smear between 100 bp and 4,000 bp the cDNA was stored at 20 °C.

SKP1 *gene cloning and primer design for PCR*

In order to obtain the full sequence of the *SKP1* gene from CHA-treated 'Xi'nong 2611', primer sets were designed from the NCBI database (http://www.ncbi.nlm.nih.gov/). PCR was performed with specific primers (forward: 5'-CGCGACTAGAGTTTCCTCGC TAGG-3' and reverse: 5'-ACGATTAAGATTCAGTTTGACAAGT-3') from a binucleate stage anther; 25 μl of the reaction mixture, 0.5 μl of Taq enzyme, 3.75 μl of cDNA, 2.5 μl of forward primer, 2.5 μl of reverse prime and 15.75 μl of ddH_2O. The PCR cycling conditions consisted of 35 cycles of 94 °C for 3 min, at 94 °C for 30 s, and then 55 °C for 30 s, and 72 °C for 10 min. Amplification products were cloned into the Amp/X-gal/IPTG vector at 37 °C, centrifuged at 150 r/min for 15 h and then sequenced. Sequencing results

(conducted by Sunbiotech co, Beijing, China) were compared in the NCBI database (http://www.ncbi.nlm.nih.gov/).

Real-time PCR analysis

Real-time PCR was performed with a CFX96 Real-time PCR System (Bio-Rad, Shanghai). The target gene products were amplified with primers (forward: 5'-ACCTGCC AGACTGTTGCT-3' and reverse: 5'-CCGACACCACCTCATAGA-3') designed according to 18S rRNA from wheat (GenBank: AY049040 http://www.ncbi.nlm.nih.gov/nuccore/AY049040). The primers for the target gene were diluted in the SYBR Premix Ex Taq (Takara, Dalian) and 20 µl of each sample was subjected to RT-PCR in three replications. The reaction mix was added to each well at 95 °C for 30 s and 95 °C for 5 s, and then 40 cycles of 60 °C for 30 s and 72 °C for 40 s. Data for *SKP1* gene expression were collected at 60 °C for each cycle. Output data were analyzed with DPS 7.5 according to $F = 10^{\Delta C_{T,T}/A_T - \Delta C_{T,R}/A_R}$ (Zhang et al. 2005).

Results

Light microscopy of anther sections

To be sure that the anthers came from the expected developmental stages from CHA-treated and control plants were stained with 5 µg/mL DAPI and checked microscopically after external forms identification. The observation showed there was no significant difference in staged anthers observations between the CHA-treated and control, but the stages were confirmed as appropriate for DNA extraction.

Pollen fertility assessed on mature pollen stained with 2% KI-I_2

In order to visualize the effect of SQ-1 plants was assessed by 2% KI-I2 staining at anthesis. Pollen from the CHA-treated plants lacked staining (Fig. 1a), whereas pollen from the control was dark blue-black (Fig. 1b). This comparison showed there was no starch in pollen produced by CHA-treated plants and therefore the SQ-1 functioned well on sterility induction.

Degradation of the tapetum

Our results indicated tapetal difference existed between CHA-treated and control at the uninucleate stage. At the late uninucleate stage, enhanced cytoplasmic activity caused a large central vacuole that gradually forced the nuclear to the side of the cell. The process exacerbated the tapetal pre-degradation in CHA-treated plants. Obviously CHA-treated underwent tapetal degradation in advance at late uninucleate (Fig. 2a, 2d) due to less tapetal cells in CHA-treated 'Xi'nong 2611' comparing with the normal tapetum. Total disappearance of tapetum indicated degradation was completed at the binucleate stage in both CHA-treated and control plants (Fig. 2b, 2e). There was no tapetum existed in binucleate and trinucleate (Fig. 2c, 2f). The completion of the process occurred earlier in CHA-treated plants than the control.

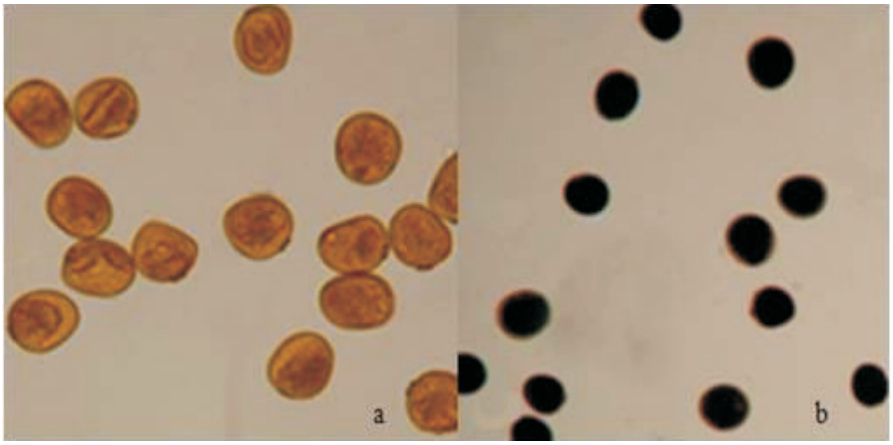

Figure 1. Mature pollen stained with 2% KI-I_2. a, CHA-treated 'Xi'nong 2611'; b, Water-treated 'Xi'nong 2611'

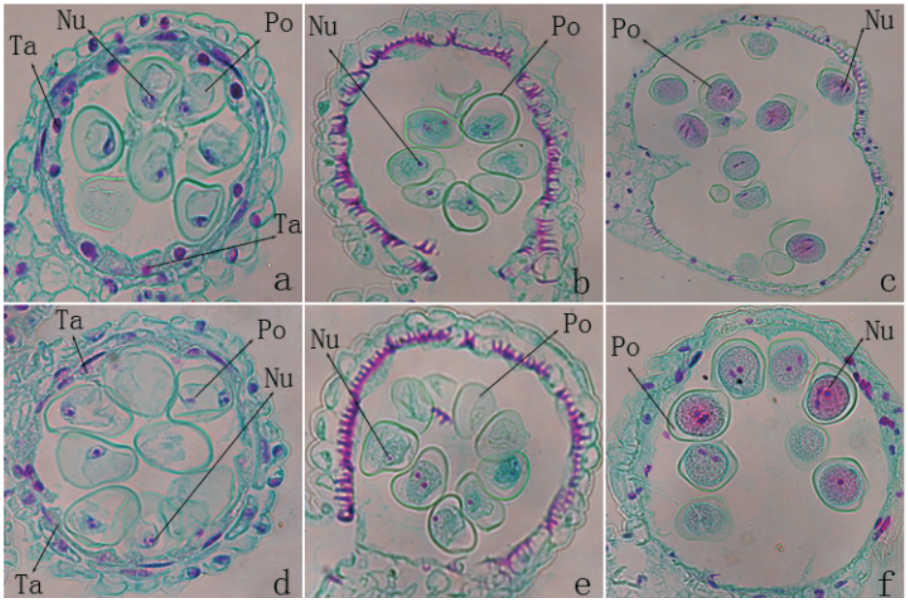

Figure 2. Cross-sections of anthers from male fertile and sterile 'Xi'nong 2611' plants. a–c, Normal tapetal development; d–f, abnormal tapetal development in CHA-treated plants; a and d, uninucleate stage; b and e, binucleate stage; c and f, trinucleate stage. Arrows with Ta: Tapetum; Nu: Nuclear; Po: Pollen

RNA purification and cDNA amplification

RNA purification was confirmed by two clear bands of 28S and 18S. cDNA amplification was conducted after dispersed bands were observed in 2% agarose gel electrophoresis separation detection. A full-length cDNA of the *SKP1* gene was identified in the NCBI database. As expected there was no difference between CHA-treated and control plants. The identified gene was 852 bp, and contained a 527 bp open reading frame (ORF) encoding a peptide of 175 amino acids with an isoeletric point of 4.18. The coding region was the same as published, but some differences in the non-coding region. A difference at No. 475 base occurred as a C in the control, but a G in CHA-treated plant. Besides that, bases were completely the same. The *SKP1* protein was confirmed as non-specific binding sites existed due to base difference according to NCBI database. It also confirmed that the cloned gene was *SKP1*.

Expression of the *SKP1* gene at the three anther developmental stages in CHA-treated and control showed that the *SKP1* gene expression level was lower in later developmental stages, and there was an obvious decrease in the process from uninucleate to trinucleate stage. Higher expression of the *SKP1* gene was observed in control, with lower and even no expression in CHA-treated plants indicating that *SKP1* expression was inhibited during the process of transformation from fertility to sterility (Fig. 3).

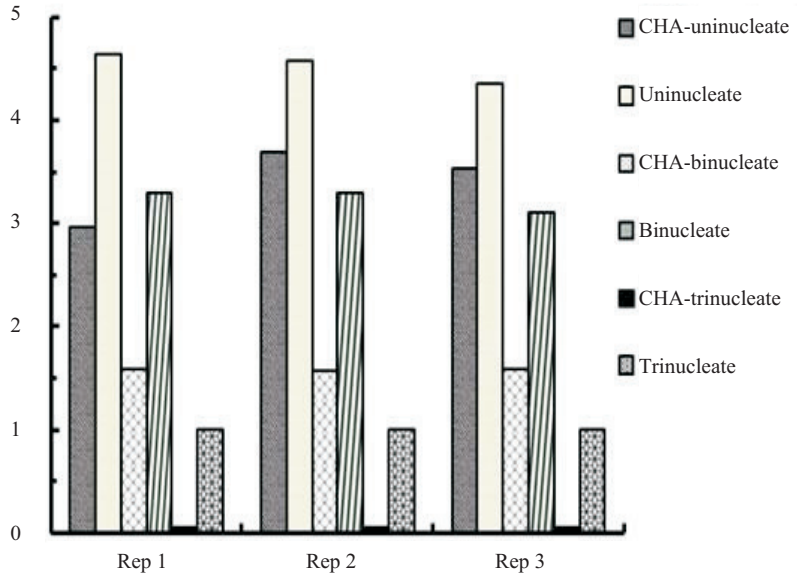

Figure 3. Relative expression of *SKP1* gene in CHA-treated and control 'Xi'nong 2611'

Discussion

Previous studies proved that CHA acts on a key developmental pathway leading to pollen sterility (Aarts et al. 1997; Taylor et al. 1998). We used the CHA SQ-1, which was a key aspect of this study to induce male sterility in wheat cultivar 'Xi'nong 2611'. Because of its high performance in sterility induction SQ-1 has been used in hybrid wheat production and hybrid cultivars have been released in China. It induces high levels of male sterility without bad effect on wheat plants. The rate of male sterility induced by SQ-1 is 95% to 100%, and the hybrid seed setting rate is over 85% (Cheng et al. 2004).

Tapetal cells in the anther wall surrounding the developing pollen provide essential nutrients. The development and degradation of the tapetum is associated with pollen growth. Tapetal disruption in the developmental pathway is a key factor leading to pollen sterility (Aarts et al. 1997; Taylor et al. 1998).

In this study, we confirmed that the gradual degradation of the tapetum in CHA-treated 'Xi'nong 2611' was initiated earlier than in the control. Cytological observations implied that CHA-induced male sterility was related to abnormally early tapetal degradation. The reason for tapetum pre-degradation is not known. Previous studies similarly reported the tapetum of un-functional anther induced by SQ-1 occurs at late uninucleate stage till early binucleate stage (Sheng et al. 2011). These results further proved that CHA-induced physiological male sterility was related to early degradation of the tapetum.

SKP1 plays crucial roles in cell-cycle progression, transcriptional regulation, flower formation, signal transduction, and many other cellular processes (Stone and Callins 2007; Santner and Estelle 2010; Wang and Deng 2011; Hong et al. 2013). Takahashi et al. (2004) found that *Arabidopsis SKP1* genes *(ASK1)* was expressed in pollen. Drouaud et al. (2000) showed that *ASK1* played an important role in male and female gamete development. The *ASK1* gene is required for the separation of homologous chromosomes during male meiosis I and for male fertility (Yang et al. 1999). *ASK1* and *ASK2* are expressed preferentially in dividing cells, whereas nine *ASKs* are expressed in male gametophytes post-meiotically, indicating that they may have a role in male gametophyte development (Marrocco et al. 2003; Zhao et al. 2003a, 2003b). *SKP1*-related proteins control signal transduction for specific degradation of target proteins and formation of complexes with other proteins (Takahashi et al. 2004). The latest research showed that alteration of polyubiquitinated proteins is associated with male sterility in wheat and the differentially expressed polyubiquitinated proteins are mainly related to carbohydrate and energy metabolism, photosynthesis, protein metabolism, transcription, cytoskeleton-associated proteins, and microspore development (Liu et al. 2014). We therefore hypothesised that the *SKP1* gene is related to fertility. However, due to the limited information about *SKP* genes in wheat, there is limited information on fertility in wheat as well. It was important to study the behavior of tapetal cells and *SKP1* gene expression patterns in order to gain clues on the mechanism of male sterility. In order to better understand the relationship of *SKP1* gene and fertility, we examined *SKP1* expression in anthers at the uninuleate, binucleate and trinucleate stages. *SKP1* gene expression studies showed that decreased, or possibly no expression, occurred during the developmental process in CHA-treated 'Xi'nong

2611' compared to the control. This indicated that the *SKP1* gene was down-regulated in conjunction with tapetal degeneration during the period of transformation from fertility to sterility. Moreover, it plays an essential role of conducting fertility transformation in physiological male sterility. However, our results did not clarify whether reduced expression of *SKP1* is the cause of sterility. Further work on *SKP1* gene silencing or *SKP1* gene knockout mutation is needed to better understand the expression of the *SKP1* gene and hence its role in production of normal pollen fertility.

Acknowledgements

This research was sponsored by the National High Technology Research and Development Program of China (No. 2011AA10A106), the Key Scientific and Technological Innovation Special Projects of Shaanxi Program 13115 (No. 2014KTZB02-01-02 and 2011KTZB02-01-01). We thank to Dr. R.A. McIntosh for the manuscript revision.

References

Aarts, M.G., Hodge, R., Kalantidis, K., Florack, D., Wilson, Z.A., Mulligan, B.J., Stiekema, W.J., Scott, R., Pereira, A. 1997. The Arabidopsis MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. The Plant Journal **12**:615–623.

Ba, Q.S., Zhang, G.S., Wang, J.S., Niu, N., Ma, S.C., Wang, J.W. 2014. Gene expression and DNA methylation alterations in chemically induced male sterility anthers in wheat (*Triticum aestivum* L.). Acta Physiol. Plant. **36**:503–512.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., Elledge, S.J. 1996. *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86**:263–274.

Chai, L.J., Biswas, M.K., Ge, X.X., Deng, X.X. 2010. Isolation, characterization, and expression analysis of an *SKP1*-like gene from 'Shatian' pummelo (*Citrus grandis* Osbeck). Plant Mol. Biol. Rep. **28**:569–577.

Chai, L.J., Ge, X.X., Biswas, M.K., Deng, X.X. 2011. Molecular analysis and expression of a floral organ-relative F-box gene isolated from 'Zigui shatian' pummel (Citrus grandis Osbeck). Mol. Biol. Rep. **38**:4429–4436.

Cheng, C.S., Lu, Y.F., Zhang, G.S., Zhang, Z.J., Li, P., Zhang, B.Y. 2004. The synthesis and biological of lamicital acid on chemical hybridizing agent. Pepsiticides **43**:315–317. (In Chinese with English abstract)

Drouaud, J., Marrocco, K., Ridel, C., Pelletier, G., Guerche, P. 2000. A *Brassica napus skp1*-like gene promoter drives GUS expression in *Arabidopsis thaliana* male and female gametophytes. Sex. Plant Reprod. **13**:29–35.

Farras, R., Ferrando, A., Jasik, J., Kleinow, T., Okresz, L., Tiburcio, A., Salchert, K., del Pozo, C., Schell, J., Koncz, C. 2001. SKP1-SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. EMBO J. **20**:2742–2756.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., Vierstra, R.D. 2002. The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis.* Proc. Natl Acad. Sci. USA **99**:11519–11524.

Hellmann, H., Estelle, M. 2002. Plant development: regulation by protein degradation. Science **297**:793–797.

Hershko, A., Heller, H., Elias, S., Ciechanover, A. 1983. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. **258**:8206–8214.

Hershko, A., Ciechanover, A. 1998. The ubiquitin system. Annu. Rev. Biochem. **67**:425–479.

Hong, M.J., Kim, D.Y., Seo, Y.W. 2013. *SKP1*-like-related genes interact with various F-box proteins and may form SCF complexes with Cullin–F-box proteins in wheat. Mol. Biol. Rep. **40**:969–981.

Kong, H.Z., Leebens-Mack, J., Ni, W., dePamphilis, C.W., Ma, H. 2004. Highly heterogeneous rates of evolution in the *SKP1* gene family in plants and animals: Functional and evolutionary implications. Mol. Biol. and Evol. **21**:117–128.

Kong, H.Z., Landherr, L.L., Frohlich, M.W., Leebens-Mack, J., Ma, H., dePamphilis, C.W. 2007. Patterns of gene duplication in the plant *SKP1* gene family in angiosperms: Evidence for multiple mechanisms of rapid gene birth. Plant J. **50**:873–885.

Li, C.J., Liang, Y., Chen, C.B., Li, J.H., Xu, Y.Y., Xu, Z.H., Ma, H., Chong, K. 2006. Cloning and expression analysis of *TSK1*, a wheat *SKP1* homologue, and functional comparison with *Arabidopsis ASK1* in male meiosis and auxin signaling. Functional Plant Biol. **33**:381–390.

Liu, H.W., Zhang, G.S., Wang, J.W., Wang, X.L., Fang, Z.W. 2003. Effect of male sterility on different wheat genotype induced by SQ-1. Northwest Univ. Agric. Forestry Nat. Sci. **31**:15–18. (In Chinese with English abstract)

Liu, H.Z., Zhang, G.S., Zhu, W.W., Wu, W.K.K., Ba, Q.S., Zhang, L., Zhang, L.Y., Niu, N., Ma, S.C., Wang, J.W. 2014. Differential proteomic analysis of polyubiquitin-related proteins in chemical hybridization agent-induced wheat (*Triticum aestivum* L.) male sterility. Acta Physiol. Plant. **36**:1473–1489.

Marrocco, K., Lecureuil, A., Nicolas, P., Guerche, P. 2003. The *Arabidopsis SKP1*-like genes present a spectrum of expression profiles. Plant Mol. Biol. **52**:715–727.

Murray, M.G., Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8**:4321–43254.

Pickart, C.M. 2001. Mechanisms underlying ubiquitination. Annu. Rev. Biochem. **70**:503–533.

Risseeuw, E.P., Daskalchuk, T.E., Banks, T.W., Liu, E., Cotelesage, J., Hellmann, H., Estelle, M., Somers, D.E., Crosby, W.L. 2003. Protein interaction analysis of SCF ubiquitin E3 ligase subunits from *Arabidopsis.* Plant J. **34**:753–767.

Santner, A., Estelle, M. 2010. The ubiquitin-proteasome system regulates plant hormone signaling. Plant J. **61**:1029–1040.

Sasaki, A., Itoh, H., Gomi, K., Ueguchi-Tanaka, M., Ishiyama, K. 2003. Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science **299**:1896–1898.

Sheng, Y., Zhang, G.S., Li, Y.X., Zhang, L.Y., Wang, S.P., Zhao, X.L., Wang, L.M., Song, Y.L. 2011. The relationship of anther tapetum, sporopollenin and expression of *RAFTIN1* in physiological male sterile wheat. Scientia Agricultura Sinica **44**:3937–3944. (In Chinese with English abstract)

Smalle, J., Vierstra, R.D. 2004. The ubiquitin 26S proteasome preteolytic pathway. Annu. Rev. Plant Biol. **55**:555–590.

Stone, S.L., Callins, J. 2007. Ubiquitin ligases mediate growth and development by promoting protein death. Curr. Opin. Plant Biol. **10**:624–632.

Takahashi, N., Kuroda, H., Kuromori, T., Hirayama, T., Seki, M., Shinozaki, K., Shimada, H., Matsui, M. 2004. Expression and interaction analysis of *Arabidopsis Skp1*-related genes. Plant Cell Physiol. **45**:83–91.

Taylor, P.E., Glover, J.A., Lavithis, M., Craig, S., Singh, M.B., Knox, R.B., Dennis, E.S., Chaudhury, A.M. 1998. Genetic control of male fertility in *Arabidopsis thaliana.* Structural analyses of postmeiotic developmental mutants. Planta **205**:492–505.

Wang, F., Deng, X.W. 2011. Plant ubiquitin-proteasome pathway and its role in gibberellin signaling. Cell Res. **21**:1286–1294.

Yang, M., Hu, Y., Lodhi, M., McCombie, W.R., Ma, H. 1999. The *Arabidopsis SKP1-LIKE1* gene is essential for male meiosis and may control homologue separation. Proc. Natl Acad. Sci. USA **96**:11416–11421.

Zhang, C.Y., Xu, S.G., Huang, X.X. 2005. A novel and convenient relative quantitative method of fluorescence real time RT-PCR assay based on slope of standard curve. Prog. Biochem. Biophys. **9**:883–887. (In Chinese with English abstract)

Zhao, D., Han, T., Risseeuw, E., Crosby, W.L., Ma, H. 2003a. Conservation and divergence of *ASK1* and *ASK2* gene functions during male meiosis in *Arabidopsis thaliana.* Plant Mol. Biol. **53**:163–173.

Zhao, D., Ni, W., Feng, B., Han, T., Petrasek, M.G., Ma, H. 2003b. Members of the *Arabidopsis-SKP1*-like gene family exhibit a variety of expression patterns and may play diverse roles in *Arabidopsis.* Plant Physiol. **133**:203–217.