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Relationship between male sterility and β-1,3-glucanase activity and callose deposition-related gene expression in wheat (*Triticum aestivum* L.)

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ABSTRACT. In previous studies, we first isolated one different protein β-1,3-glucanase using two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry from normal wheat (*Triticum aestivum* L.) and chemical hybridization agent-induced male sterility (CIMS) wheat. In this experiment, β-1,3-glucanase activity and the expression of a callose deposition-related gene, UDP-glucose phosphorylase (*UGPase*), were determinate in normal, CIMS, and genetic male sterility (GS) wheat. β-1,3-glucanase activity was significantly different between the fertile and sterile lines during callose synthesis and degradation, but there was no difference between CIMS and GS wheat. The *UGPase* gene of callose deposition was highly expressed in the meiophase and sharply

decreased in the tetrad stage. However, the expression of the *UGPase* gene was significantly different between the fertile and sterile lines. These data indicated that β-1,3-glucanase activity and the expression of the *UGPase* gene play important roles in the male sterility of wheat. Consequently, pollen mother cells (PMCs) might degenerate at the early meiosis stage, and differences in *UGPase* gene expression and β-1,3-glucanase activity might eventually result in complete pollen collapse. In addition, the critical period of anther abortion might be the meiosis stage to the tetrad stage rather than what we previously thought, the mononuclear period.

Key words: Wheat; β-1,3-glucanase; UDP-glucose phosphorylase; Male sterility

INTRODUCTION

Callose is widespread in higher plants. It is a component of specialized cell walls or cell wall-associated structures at multiple stages of pollen development (Chen and Kim, 2009). Callose deposition is essential because it can separate developing pollen grains, preventing their underlying walls (exine) from fusing (Nishikawa et al., 2005). Callose is a linear homopolymer of β-1,3-linked glucose residues with some β-1,6-branches. Callose biosynthesis uses UDP-glucose as a substrate. There are 2 chief factors of the effect of callose deposition. First, β-1,3-glucanases catalyze the hydrolysis of β-1,3-glucans. Second, the related gene expression affects callose deposition (Stieglitz, 1977).

In the sterile anthers of *Allium sativum* L., β-1,3-glucanase activity affects male gametophyte development and regulates the degradation of the callose wall (Nishikawa et al., 2005). As meiosis occurs, callose accumulates along the cellular plates, which are formed during cytokinesis, until each individual microspore of the tetrad is attached to a thick callose shell. One study showed that premature expression of an engineered β-1,3-glucanase transgene in the tapetum resulted in partial or total male sterility in tobacco (Hird et al., 1993). If the callose wall is degraded before the completion of meiosis by the premature production of a basic β-1,3-glucanase that has been modified for secretion to the locule, little fertile pollen is produced (Worrall et al., 1992).

In higher plants, the *UGPase* gene is expressed in all tissues, including roots, tubers, leaves, stems, anthers, and young seeds (Park et al., 2010). In addition, *UGPase* is not only involvement in sucrose and starch metabolism but also takes part in cell wall biosynthesis, including callose, pectin, and cellulose biosynthesis (Dong, 2005). Normal callose deposition was disrupted in pollen mother cells of *UGPase*-silenced rice, eventually resulting in complete pollen collapse. This showed that rice *UGPase* was required for callose deposition during pollen mother cell (PMC) meiosis and pollen development (Chen et al., 2007).

In wheat hybrid breeding, cytoplasmic male sterility (CMS) and chemical hybridization agent (CHA)-induced wheat male sterility are the most effective ways to generate sterile wheat. In this experiment, the β-1,3-glucanase activity and *UGPase* gene expression of callose deposition were determined in the anthers of CMS and CHA-induced wheat and corresponding normal wheat (NW).

MATERIAL AND METHODS

Plant growth and material collection

CMS line (S)-1376 (GS) and wheat cultivar 1376 were grown in the experimental field of Northwest A&F University, Yangling, China, on October 8, 2012. In the next April, when wheat developed to the period of female stamen primordia formation to connectivum, 5 kg/hm^2 CHA-SQ-1was sprayed on wheat cultivar 1376 (CIMS), and the control was sprayed with water. GS and its maintainer line of wheat cultivar 1376 and CIMS wheat with the same nuclear background were used in this study. All materials were collected according to the anther development of wheat cultivar 1376. These operations were carried out at 0°-4°C. Anthers were frozen in liquid nitrogen and stored at -80°C until further analysis. We collected the anthers, which can be categorized as the meiophase, tetrad, mononuclear, binuclear, and trinuclear stages.

Histological analysis and phenotypic characterization

Nuclei of wheat anthers at various developmental stages were stained with carbol fuchsin and 0.1% aniline blue to stain the callose walls. Pollen grains of the trinuclear stage anthers were stained with 1% iodine-potassium iodide solution (KI-I_2) (Joseph et al., 2011). Slides were inspected and photographed using an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Scanning electron microscopy (SEM) was performed as described by Dou et al. (2011).

Two-dimensional electrophoresis (2-DE) separation and matrix-assisted laser desorption/ionization time-of-flight-time-of-flight mass spectrometry (MALDI-TOF-TOFMS) analysis

Proteins from anther samples were extracted using the trichloroacetic acid-acetone method with minor modifications (Méchin et al., 2007; Jagadish et al., 2010). Protein concentrations were determined using the Bradford method. 2-DE was used to separate proteins, and the selected protein spots were manually excised from the gel and digested with sequencing-grade trypsin (Sheoran et al., 2005, 2006). MS and tandem MS data were obtained for protein identification using a MALDI-TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA).

Enzyme assay

The β-1,3-glucanase activity was measured reductometrically by monitoring the increase in reducing sugars. The standard assay mixture containing 0.25% (w/v) *Laminaria digitata* laminarin, 50 mM sodium acetate, pH 5.5, and enzyme in a total volume of 50 µL was incubated at 37°C. One unit of enzyme activity was defined as the amount of enzyme required to release 1 mol glucose equivalents per min (Wan et al., 2011).

Total RNA extraction and cDNA synthesis

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified by measuring the absorbance at 260 nm with a Nano Vue ultraviolet/visible

light spectrophotometer (GE Healthcare Life Sciences, Shanghai, China). The purity of RNA was assessed by the 260/280 and 260/230 absorbance ratios, and the integrity was checked with 1% agarose gel electrophoresis. Then, 1 µg RNA was used to synthesize the first-strand cDNA using the PrimeScript RT Reagent Kit (Takara Bio, Tokyo, Japan) according to the manufacturer protocol. The synthesized cDNA was stored at -20°C.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from anthers at different developmental phases in CIMS, GS, and fertile lines. The following specific primers were designed using the primer premier 5.0 software for the full-length cDNA and qRT-PCR analyses: 5'-CGCACCGCCACCTCCCTTCCAGT-3' and 5'-GGACCGCACGGAGGGGATTACAGTT-3' for full-length cDNA, and 5'-GTATTGG CTCTGGTTGAA-3' and 5'-CTGAACAAGAAGTATGGATG-3' qRT-PCR. qRT-PCR was performed on a BIORAD CFX96 real-time system. The 25-mL reaction system contained 1 µL diluted cDNA, 11.25 µL SYBR Green Real-Time PCR Master Mix (TIANGEN, Corp, Beijing, China), and 0.5 µL of each primer. The cycling parameters were as follows: 95°C for 15 min, followed by 39 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A 3-fold serial dilution of cDNA was used to construct a standard curve to determine the PCR efficiency that would be used to convert the quantification cycles (Ctvalues) into the relative quantities (relative gene expression). At the final cycle of amplification, the specificity of PCRs was checked by melting curve analysis (60°-95°C in increments of 0.5°C every 5 s). Each experiment described was repeated independently 3 times. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

Callose deposition was observed under a light microscope to determine the different developmental stages (Figure 1A-H) and then collected. We found that the callose deposition reached its maximum in the tetrad period (Figure 1E). In the mononuclear period, there was trace callose deposition on the edge of the microspore and aperture (Figure 1H).

Fertility investigation

The effect of wheat male sterility was evaluated. The relative male sterility rate in SQ-1-sprayed wheat (CIMS) was almost complete (up to 99.76%), and the seed-setting rate of artificial saturation pollination was high (94.65%) without causing pistil damage. The relative male sterility rate of GS was 99% and the seed-setting rate of artificial saturation pollination was 99%. As shown in Figure 2A, the NW pollen grains, which were stained with KI-I_2, were full of starch; however, the pollen grains of CIMS and GS plants had almost no starch accumulation (Figure 2B-C). By SEM, NW pollen grains had a smooth and particulate exine pattern and nearly round shape (Figure 2D), whereas the pollen of CIMS and GS plants appeared severely malformed (Figure 2E-F).

Identification of β-1,3-glucanase protein

As shown in Figure 3, a spot that had a 2-fold difference between the fertile and sterile lines was identified as β-1,3-glucanase (Figure 3).

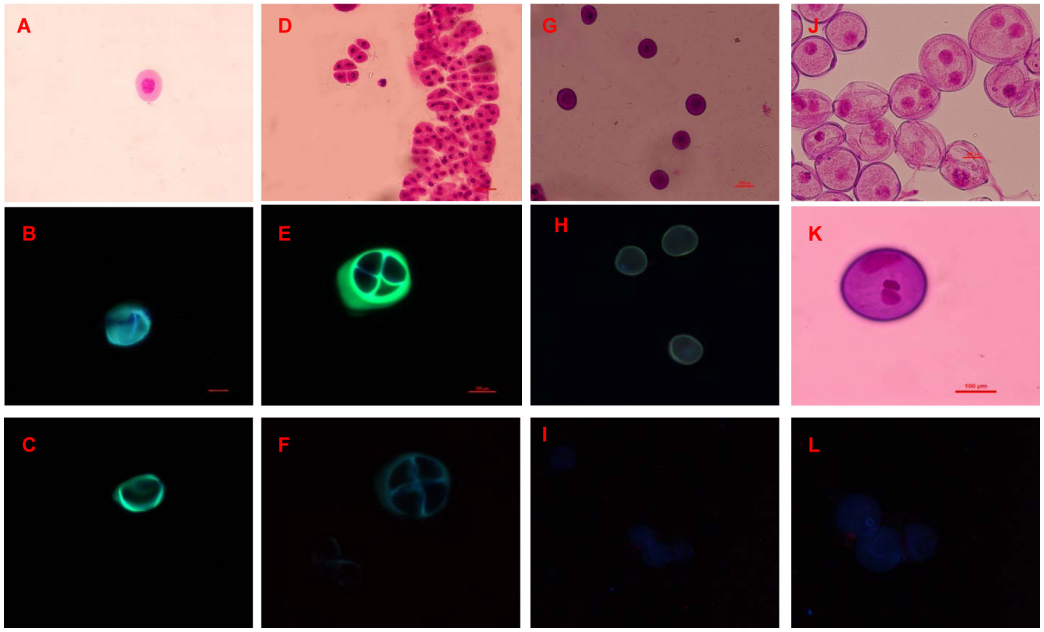

Figure 1. Each stage of microspore and callose deposition: meiophase stage (**A**, **B**, and **C**), tetrad stage (**D**, **E**, and **F**), mononuclear stage (**G**, **H**, and **I**), binuclear stage (**J**), and trinuclear stage (**K** and **L**). Bars = 100 µm. Nuclei of wheat anthers at various developmental stages were stained with carbol fuchsin and 0.1% aniline blue to stain the callose walls.

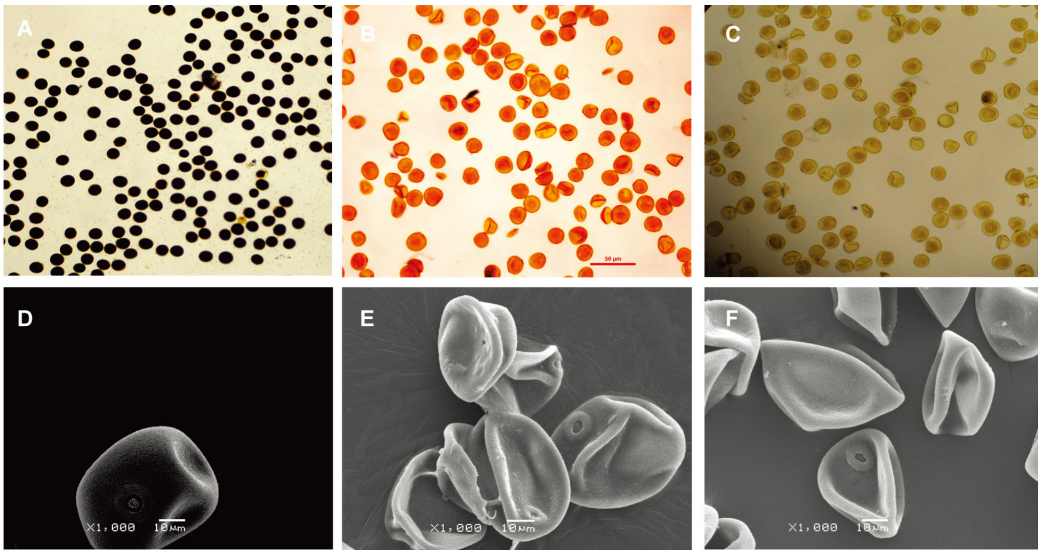

Figure 2. Morphological changes in the anthers of the CIMS and genetic male sterility (GS) lines compared with fertile plants. (**A**) to (**C**) Pollen grains of normal wheat (NW) (**A**), chemical hybridization agent-induced male sterility (CIMS) (**B**), and GS (**C**) plants stained with KI-I_2 solution. (**D**) to (**F**) Scanning electron microscopy analysis of pollen grains from NW (**D**), CIMS (**E**), and GS plants (**F**).

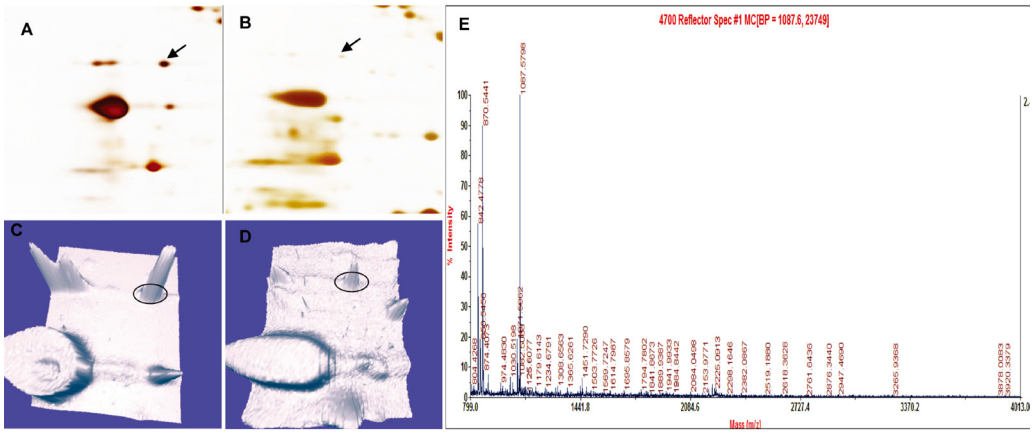

Figure 3. Two-dimensional electrophoresis (2-DE) results of β-1,3-glucanase. Enlarged regions of 2DE gels of NW (**A**) and CIMS (**B**) anthers to identify β-1,3-glucanase, and the corresponding 3-D views (**C** and **D**). (**E**) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the selected spot (β-1,3-glucanase).

The accession number in the National Center for Biotechnology Information database was gi|109150356, the molecular weight was 35 kDa, the isoelectric point was 4.86, the protein score was 225, the protein score confidence interval percentage (CI%) was 100, the total ion score was 147, the total ion CI% was 100, and the peptide count was 11. The average ratio was 1.92. Figure 3E shows the separation profile of this spot.

Determination of the β-1,3-glucanase activity

β-1,3-glucanase activity is significantly lower in CIMS and GS than NW from meiophase to mononuclear stage, but it is significantly higher in the binuclear and trinuclear stages. The level of β-1,3-glucanase activity had a rough trend of "high-low-high-low-low" in the 3 wheat anthers. From the meiosis to tetrad stage, β-1,3-glucanase activity had a sharp decrease, which allowed callose deposition. Then, the activity sharply increased, which indicated callose degradation. In the stages of callose synthesis and degradation, β-1,3-glucanase activity was significantly different between the fertile and sterile lines, but there was no difference between the CIMS and GS lines (Figure 4).

Full-length cDNA of *UGPase*

A pair of specific primers was designed based on the nucleotide sequence of *UGPase* gene from barley (*Hordeum vulgare*) in GenBank (X91347). The full-length cDNA of *UGPase* was cloned by reverse transcription PCR from wheat anther. The length of the cDNA product was 1581 bp, encoding 473 amino acids (Figure 5). Homology analysis for sequences of the *UGPase* gene from wheat anther showed 98 and 96% nucleotide identity with wheat and barley, respectively. This suggests that the gene is the target gene.

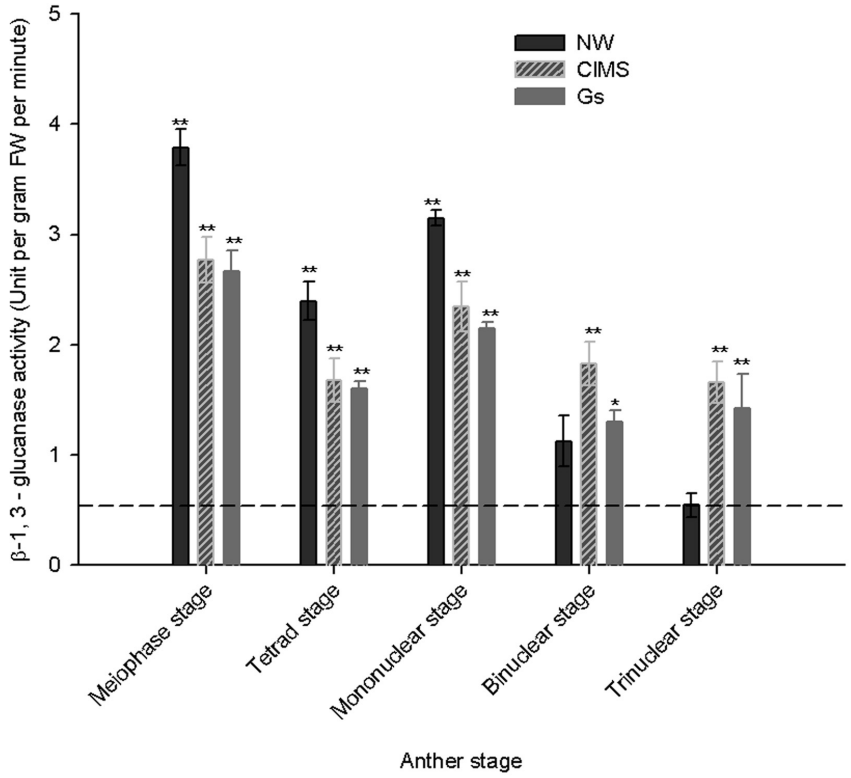

Figure 4. β-1,3-glucanase activity of NW, CIMS, and GS anthers at different stages. FW = formula weight. N = 3. *P < 0.05. **P < 0.01.

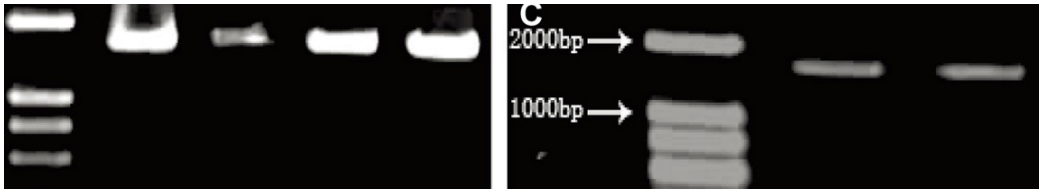

Figure 5. Full-length cDNA of *UGPase*. (**A**) Result of RNA extraction. (**B**) Full-length cDNA of *UGPase* was cloned by reverse transcription polymerase chain reaction of RNA from wheat anthers. (**C**) Result of gel extraction for sequencing.

Result of qRT-PCR of the *UGPase* gene

The standard curve analysis showed that the 18S ribosomal RNA gene (Figure 6A) and *UGPase* (Figure 6B) could be specifically amplified, and the reaction efficiency was higher than 0.99, which showed that the system could be used for fluorescent quantitative PCR amplification. In the fertile line, *UGPase* was highly expressed in meiophase, but it had a sharp decrease in the tetrad stage. Then, the expression remained the same from mononuclear stage to trinuclear stage.

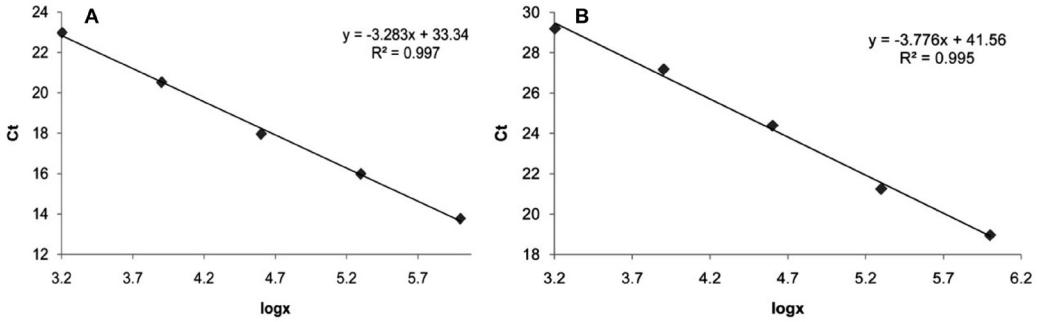

Figure 6. Standard curve graph of *UGPase* and 18S rRNA. (**A**) Standard curve for 18S rRNA. (**B**) Standard curve for *UGPase*.

In CIMS and GS anthers, the *UGPase* gene was also expressed highly in meiosis stage, but it had significantly lower expression than NW anthers ($P < 0.01$). In the tetrad period, the expression level was significantly decreased, and no significant differences were detected in the mononuclear stage. However, *UGPase* gene expression in the binuclear and trinuclear stages of CIMS anthers was markedly increased relative to NW anthers in the trinuclear stage ($P < 0.01$). The expression of *UGPase* was stable in the binuclear and trinuclear stages of GS anthers. The level of *UGPase* gene expression followed a roughly trend of "high-low" in the 3 wheat anthers from meiosis to tetrad stage (Figure 7).

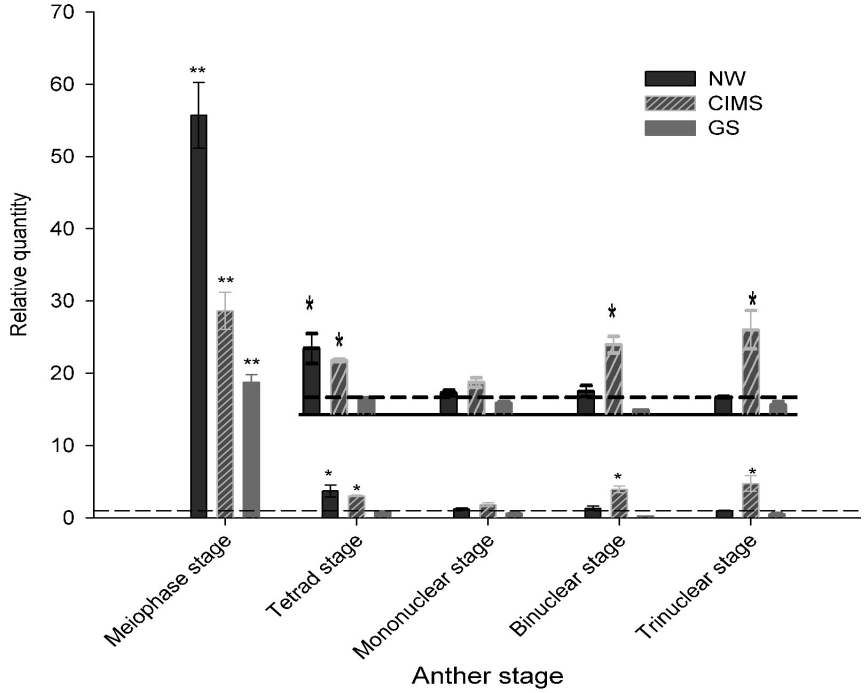

Figure 7. Differences in the relative expression of *UGPase* in NW, CIMS, and GS anthers at different stages. N = 3. *P < 0.05. **P < 0.01.

DISCUSSION

Male sterility has great application potential in wheat heterosis, but there has not yet been any study to characterize β-1,3-glucanase activity and callose deposition-related gene expression in male sterile wheat. In anthers, β-1,3-glucanases are expressed just before microspores are released, and they function in the degradation of the callose that surrounds the microspore tetrad and contribute to the release of the microspores as pollen grains (Tsuchiya et al., 1995). We demonstrated that the β-1,3-glucanase activity of wheat anthers was different in CIMS and GS compared with NW plants. The CIMS anthers treated with CHA-SQ-1 and GS anthers had lower β-1,3-glucanase activity than NW anthers before the binuclear stage, which suggested that normal callose deposition might be affected in sterile plants. Various reports highlighted the importance of the correct timing of callose activity for the development of fertile pollen. The reduction or absence of callose in PMCs of the *Arabidopsis* mutants, which show early degeneration of PMCs and tapetal cells, suggests that wild-type gene products are required for the normal formation of callose and are perhaps needed for other aspects of sporogenous tissue development (Dawson et al., 1993). β-1,3-glucanase activity had a sharp decrease, and callose deposition reached its maximum. These findings were in agreement with the callose staining of the tetrad stage of anthers. Then, the activities sharply increased, which indicated callose degradation; however, the lower activity in the CIMS and GS wheat than in NW may be a result of premature or delayed callose degradation. In *Petunia*, the rise and degree of callose activity in CMS anthers a short time before the disappearance of callose from the PMC walls is similar to the rise and degree of callose activity a short time before the release of microspores from tetrads in NW anthers (Frankel et al., 1969). However, in another sterile genotype, no callose activity was detected at the end of tetrad stage, and the callose walls remained intact until a very late stage, which suggested that the timing of callose activity is critical for the normal development of the male microspores and that faulty timing may result in male sterility (Izhar and Frankel, 1971). In this experiment, the different callose activity indicated that faulty timing of callose degradation might result in male sterility.

From the mononuclear stage to the trinuclear stage, β-1,3-glucanase activity declined in NW, but there was no obvious decline in the CIMS and GS lines. In the binuclear and trinuclear stage, the β-1,3-glucanase activity of the CIMS and GS lines was significantly higher than that of NW. This phenomenon also shows that enzyme activity may affect the normal development of pollen grains.

UGPase catalyzes the reversible production of glucose-1-phosphate and UTP to UDP-glucose and pyrophosphate. *UGPase* transcripts are present at high levels in florets before flowering, suggesting that it plays a special role in rice flower development (Chen et al., 2007). Woo et al. (2008) found that the suppression of *UGPase* by the introduction of a *UGPase*1-RNAi construct in wild-type plants nearly eliminated seed setting because of the male defect, with developmental retardation similar to the ms-h mutant phenotype, whereas the overexpression of *UGPase*1 in ms-h mutant plants restored male fertility, and the transformants produced T1 seeds that segregated into normal and chalky endosperms. They suggested that *UGPase* plays a key role in pollen development as well as seed carbohydrate metabolism (Woo et al., 2008). In our experiment, the expression level was significantly lower in the tetrad stage than in meiophase in the 3 wheat types. However, the expression level was significantly different in the meiophase of NW and CIMS and GS, which showed that callose deposition might be abnormal in the CIMS and GS lines. There was no significant difference in *UGPase*

expression in the mononuclear stage of the 3 types of wheat, but the *UGPase* gene expression in the binuclear and trinuclear stage of CIMS wheat was markedly increased relative to the expression in the trinuclear stage of NW ($P < 0.01$), which corresponded with the β-1,3-glucanase activity. The expression of *UGPase* was stable in the binuclear and trinuclear stages of GS wheat. The level of *UGPase* gene expression followed a rough trend of "high-low" in the 3 wheat anthers from meiosis to the tetrad stage. In the meiophase, the high level of *UGPase* gene expression provides favorable conditions for callose deposition in NW anthers, and the β-1,3-glucanase activity was also high. In CIMS and GS anthers, *UGPase* gene expression and β-1,3-glucanase activity were significantly lower than in NW anthers. Consequently, the PMCs began to degenerate at the early meiosis stage, and the different *UGPase* gene expression and β-1,3-glucanase activity eventually resulted in complete pollen collapse. In addition, the critical period of anther abortion might be during the meiosis stage to the tetrad stage, rather than the mononuclear period as previously thought.

CONCLUSIONS

In this study, β-1,3-glucanase activity and the expression of the callose deposition-related gene *UGPase* were determined in NW, CIMS wheat, and GS wheat. β-1,3-glucanase activity was significantly different between the fertile and sterile lines during the stages of callose synthesis and degradation, but there was no difference between the CIMS and GS lines. The *UGPase* gene, which is required for callose deposition, was highly expressed in meiophase and sharply decreased in the tetrad stage. However, the expression of the *UGPase* gene was significantly different between the fertile and sterile lines. These data indicated that β-1,3-glucanase activity and the expression of the *UGPase* gene play important roles in male sterility of wheat. Consequently, the PMCs might begin to degenerate during the early meiosis stage, and the different of *UGPase* gene expression and β-1,3-glucanase activity eventually result in complete pollen collapse. Moreover, the critical period of anther abortion might be during the meiosis stage to the tetrad stage, rather than the mononuclear period as previously though.

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REFERENCES

Chen R, Zhao X, Shao Z, Wei Z, et al. (2007). Rice UDP-glucose pyrophosphorylase1 is essential for pollen callose deposition and its cosuppression results in a new type of thermosensitive genic male sterility. *Plant Cell* 19: 847-861.

Chen XY and Kim JY (2009). Callose synthesis in higher plants. *Plant Signal. Behav.* 4: 489-492.

Dawson J, Wilson ZA, Aarts MGM, Braithwaite AF, et al. (1993). Microspore and pollen development in six male-sterile mutants of *Arabidopsis thaliana*. *Can. J. Bot.* 71: 629-638.

Dong X (2005). Functional Investigation of *Arabidopsis* Callose Synthases and the Signal Transduction Pathway. Doctoral thesis, Department of Plant Biology, The Ohio State University, Columbus.

Dou XY, Yang KZ, Zhang Y, Wang W, et al. (2011). WBC27, an adenosine tri-phosphate-binding cassette protein, controls pollen wall formation and patterning in *Arabidopsis*. *J. Integr. Plant Biol.* 53: 74-88.

Frankel R, Izhar S and Nitsan J (1969). Timing of callase activity and cytoplasmic male sterility in *Petunia*. *Biochem. Genet.* 3:451-455.

Hird DL, Worrall D, Hodge R, Smartt S, et al. (1993). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana A6* gene displays similarity to β-1,3-glucanases. *Plant J.* 4: 1023-1033.

Izhar S and Frankel R (1971). Mechanism of male sterility in *Petunia*: the relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. *Theor. Appl. Genet.* 41: 104-108.

Jagadish SVK, Muthurajan R, Oane R, Wheeler TR, et al. (2010). Physiological and proteomic approaches to address heat tolerance during anthesis in rice (*Oryza sativa* L.). *J. Exp. Bot.* 61: 143-156.

Joseph CA, Chen Z, Ma D and Zeng HL (2011). Analysis of short photo-periodic sensitive genic male sterility and molecular mapping of *rpms3*(*t*) gene in rice (*Oryza sativa* L.) using SSR markers. *Genes Genom.* 33:513-519.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402-408.

Méchin V, Damerval C and Zivy M (2007). Total protein extraction with TCA-acetone. *Methods Mol. Biol.* 355: 1-8.

Nishikawa SI, Zinkl GM, Swanson RJ, Maruyama D, et al. (2005). Callose (β-1,3 glucan) is essential for *Arabidopsis* pollen wall patterning, but not tube growth. *BMC Plant Biol.* 5:22.

Park JI, Ishimizu T, Suwabe K, Sudo K, et al. (2010). UDP-glucose pyrophosphorylase is rate limiting in vegetative and reproductive phases in *Arabidopsis thaliana*. *Plant Cell Physiol.* 51:981-996.

Sheoran IS, Olson DJ, Ross AR and Sawhney VK (2005). Proteome analysis of embryo and endosperm from germinating tomato seeds. *Proteomics* 5:3752-3764.

Sheoran IS, Sproule KA, Olson DJH, Ross AR, et al. (2006). Proteome profile and functional classification of proteins in *Arabidopsis thaliana* (Landsberg erecta) mature pollen. *Sex. Plant Reprod.* 19:185-196.

Stieglitz H (1977). Role of β-1,3-glucanase in postmeiotic microspore release. *Dev. Biol.* 57:87-97.

Tsuchiya T, Toriyama K, Yoshikawa M, Ejiri SI, et al. (1995). Tapetum-specific expression of the gene for an endo-β-1,3-glucanase causes male sterility in transgenic tobacco. *Plant Cell Physiol.* 36:487-494.

Wan L, Zha W, Cheng X, Liu C, et al. (2011). A rice β-1,3-glucanase gene *Osg1* is required for callose degradation in pollen development. *Planta* 233:309-323.

Woo MO, Ham TH, Ji HS, Choi MS, et al. (2008). Inactivation of the *UGPase1* gene causes genic male sterility and endosperm chalkiness in rice (*Oryza sativa* L.). *Plant J.* 54:190-204.

Worrall D, Hird DL, Hodge R, Paul W, et al. (1992). Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. *Plant Cell* 4:759-771.