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Analysis of chalcone synthase gene in chemically induced male sterility (CIMS) in wheat (*Triticum aestivum* L.)

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Abstract

Chemically induced male sterility (CIMS) is one of the male sterility system used for hybrid wheat production in China. However, the mechanism of sterility remains unclear. The expression of chalcone synthase (CHS) gene was assessed in anthers to determine its role in fertility/sterility in wheat. The expression levels of CHS gene was evaluated by real-time quantitative polymerase chain reaction (qPCR). qPCR analysis revealed that the expression levels of CHS gene were downregulated in CIMS. The sporopollenin contents in anthers underwent a sharp decrease in 1376-CIMS. Scanning electron microscopy observations revealed that pollen wall formation was significantly altered in 1376-CIMS. This phenomenon implied that CHS gene expression was inhibited during the fertility transformation process and was related to the transformation from fertility to sterility. Moreover, results indicated that CHS plays an essential role in conducting fertility in chemically interfered male sterility.

Key words: Male sterility, chalcone synthase gene, pollen wall, sporopollenin, wheat

Introduction

Sporopollenin is the major constituent of exine of pollen grains. Early studies revealed that the composition of sporopollenin was consistent with that of a series of related polymers derived from long-chain fatty acids as well as modest amounts of oxygenated aromatic rings and phenylpropanoids (Guilford et al. 1988). Several subsequent works implied that phenylalanine is efficiently incorporated into sporopollenin; therefore, phenylpropanoids are genuine components of sporopollenin; flavonoids also eventually become constituents of the pollen wall (Piffanelli et al. 1997; Rittscher and Wiermann 1988). Notably, flavonoids and other phenylpropanoids are precursors for sporopollenin and involved in other pollen structures, such as the pollen coat (Wiermann and Vieth 1983), which are formed later in development. This deduction is supported by the expression patterns of tapetal genes involved in phenylpropanoid biosynthesis, some of which continue to be transcribed well after the completion of exine formation (Shen and Hsu 1992).

More recently, this finding has been confirmed by activity assays and immunocytochemical studies using antibodies raised against two of the key enzymes of the flavonoid pathway, namely, phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). Several studies showed that the gene-encoding PAL and CHS are specifically expressed in the tapetum (Shen and Hsu 1992) and that their down regulation through antisense RNA results in partial or complete male sterility because of abnormal pollen development. Increasing evidence also showed that flavonoids, at least in several plants, play a crucial role in fertility and sexual reproduction. Among the flavonoid biosynthetic genes analyzed in radishes, the expression of CHS was strongly inhibited in the later stages of anther development in cytoplasmic male sterility; the accumulation of putative naringen in derivatives was also inhibited. The perturbation of phenylpropanoid biosynthesis also produces anthers with decreased pollen viability (Elkind et al. 1990). The results are consistent with the hypothesis that CHS expression regulates CMS expression.

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The CHS gene plays a crucial role in the formation of pollen wall. However, no previous studies had demonstrated the relationship between CHS gene and chemically interfered male sterility. Therefore, determining the action of the CHS gene might reveal the mechanism of male sterility in wheat. This study provides a detailed analysis of the expression of this wheat CHS in chemically induced male sterility (CIMS) lines to explore its potential role in CIMS. Notably, the expression of CHS was drastically suppressed prior to the morphological induction of male sterility. This study is the first to show the possible role of flavonoids in pollen development in CIMS.

Materials and methods

Plant materials

A typical CIMS line, Xinong 1376-CIMS induced by CHA SQ-1 ("1376-CIMS"), and its fertile line, Xinong 1376 ("1376"), with common genetic background were grown in experimental fields of Huaibei Normal University in Anhui, China. SQ-1 was sprayed at the rate of 5.0 kg ha^{-1} when the average development was at the 8.5 stage (approximately 175 mm spikes) of the Feekes scale (Parodi and Gaju, 2009).

Microscopic observation

The purity and developmental stages of pollens were checked by staining pollen with 1% acetocarmine (1% carmine in 45% aqueous acetic acid) to establish cytological controls. The male gametophytes mainly aborted at the uninucleate stage (Sheng et al. 2011). The morphology of the anther and pollen were observed using a JSM-6360LV scanning electron microscope. Pollen fertility was tested with iodine-potassium iodide (KI-I2) assays. Pollen grains were collected, stained with a 1% KI-I2 solution, and observed under a fluorescence microscope. Spherical and dark pollen grains were considered healthy and fertile, whereas shrunken and unstained pollen grains were regarded as sterile (Liu et al. 2015).

Determination of the sporopollenin content

The total sporopollenin content was determined using the pyronine B staining method (Sheng et al. 2011). All samples were analyzed three times.

Real-time quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). Before reverse transcription, total RNA was treated with RNase-free DNase I (Takara) at 37^{o}C for 30 min to eliminate genomic DNA contamination. First-strand cDNA was synthesized from total RNA using oligo (dT)18 primer and reverse transcriptase (RT) SuperScript (Takara) according to the specifications of the manufacturer. Oligo (dT) primer and random 6 mers were used as primers for cDNA synthesis and were supplied with the kit. The housekeeping gene (18S rRNA gene) was used as an internal control to normalize for variation in the amount of cDNA template. Quantitative RT-PCR was performed to detect the expression level of the CHS gene. The total reaction volume of 25 µL contains 12.5 µL Brilliant II SYBR® Green qPCR Master Mix, 1.0 µL of 10 mM forward primer, 1.0 µL of 10 mM reverse primer, 2.0 µL cDNA templates, and 8.5 µL nuclease-free PCR-grade water. The RT-PCR program adopted was as follows: 95^{o}C for 10 min and 35 cycles of 95^{o}C for 30 s, 55^{o}C for 60 s, and 72°C for 30 s. The primers were designed by using the software Primer-Premier 5.0. The primer pair sequences were as follows: CHS forward: 5′-CCTGAACAAGGAACGGAT-3′; CHS reverse: 5′-GACGCTATGGAGGACAAC-3′; CHSL forward: 5′-TCGTCCTTATGACCTCGTT-3′; CHSL reverse: 5′-CTATGCGGCTTTCAATCTT-3′; 18S rRNA forward: 5′-CGTCCCTGCCCTTTGTACAC-3′; 18S rRNA reverse: 5′-AACACTTCACCGGA CCATT-CA-3′. The relative value of the expression level of each gene was calculated by comparing the cycle thresholds of the target genes with that of the housekeeping gene (18S rRNA gene) using a previously described method (Yuan et al. 2006). The results were presented as the means ± SD of three biological replicates.

Results and discussion

The purity and developmental stages of pollens were checked by staining the pollen with 1% acetocarmine to establish cytological controls and photographed under a fluorescence microscope (Axiovert 200M, Germany). Anthers at different developmental stages were collected from the corresponding young spikes and used in experiments (Ba et al. 2014b).

Identification of pollen sterility

Pollen fertility was assessed on mature pollen stained with 2% KI-I2. Plants were assessed by 2% KI-I2 staining at anthesis to visualize the effect of SQ-1. Pollens from the CHA-treated plants were not stained, whereas pollen from the control stained dark blue-black. This comparison indicated that no starch was found in pollen in 1376-CIMS, and thus, SQ-1

functioned well on sterility induction. At this stage, pollen grains in 1376 were full of starch (Fig. 1a). By contrast, the microspores were abnormally developed with deformity, but were still visible at this stage. Poor contents in the pollen grains were also observed; in particular, no starches were observed in 1376-CIMS (Fig. 1b).

Ultrastructure observation of the pollen wall

Previously, the pollen sizes of 1376-CIMS were observed to be smaller than those of 1376. The phenotypicits phenotype were analyzed further by examining the pollen surface structure using scanning electron microscopy. At the mature pollen stage, the pollen grains of 1376 had a smooth and particulate exine patterning (Fig. 1c), whereas the pollen surface of 1376-CIMS appeared severely deformed and shrunken (Fig. 1d).

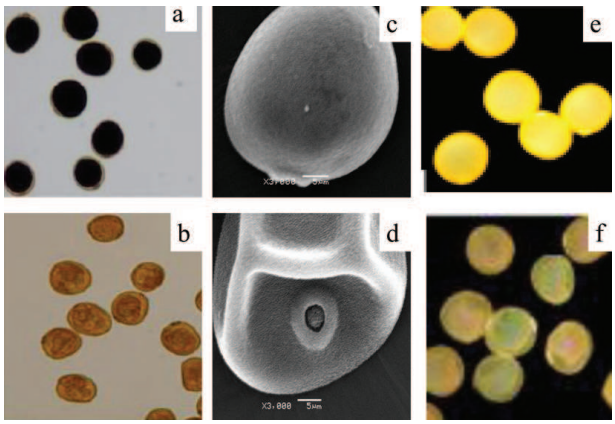

Fig. 1. Morphological analysis of anther and pollen at mature pollen stages in 1376 and 1376-CIMS. a, c, and e Pollen grains from 1376; b, d, and f pollen grains from 1376-CIMS. a and b Pollen grains from 1376 and 1376-CIMS stained with 1% I2-KI solution. c and d Morphology of the pollen. e and f Relative optical density of pollens. CIMS, chemically induced male sterility

Sporopollenin content in 1376-CIMS

Sporopollenin is the major constituent in the pollen grains. Sporopollenin formation is necessary to develop viable pollen grains, as recently shown for the *Arabidopsis* ms2 mutation; specifically, a lesion in a tapetal gene involved in exine deposition results in male sterility (Aarts et al. 1997). The abnormalities of the pollen wall in 1376-CIMS indicated possible defects in the synthesis of sporopollenin. The sporopollenin content between the 1376 and the 1376-CIMS anthers at the trinucleate stage were compared to verify the assumption. The data indicated that as compared with the anthers in 1376 pollens, the 1376-CIMS anther possessed significantly altered sporopollenin content (Figs. 1e and 1f). The average optical density of sporopollenin in 1376 pollens was 0.0432. By contrast, the average optical density of sporopollenin decreased to 0.0272 in the 1376-CIMS anther (Fig. 2). Notably, the sporopollenin content in

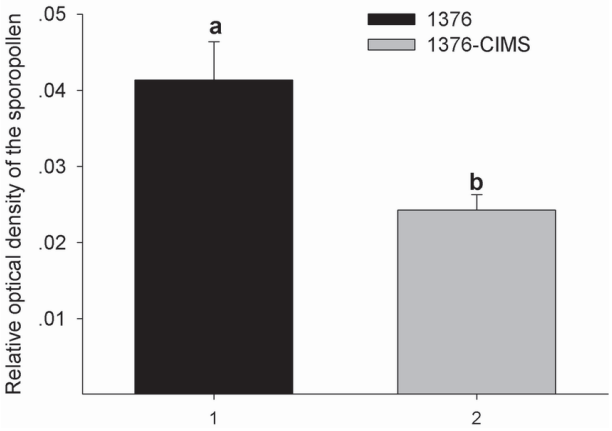

Fig. 2. Sporopollenin histological stain of pollen grains with the pyronine B staining method. CIMS, chemically induced male sterility

1376 pollens increased to 1.6-fold compared with that in 1376-CIMS.

In 1376-CIMS anther, the increase in flavonoids (approximately 2.29-fold compared with 1376) confirms that conversion of flavonoids to sporopollenin in 1376-CIMS anther is significantly blocked. The results implied that the gene involved in the conversion of fatty alcohols is possibly repressed in 1376-CIMS anther.

CHS and CHSL gene expression analysis

During pollen wall development, a good correlation was observed between the expression pattern of anther-specific CHS genes and the predicted male-specific components; therefore, transgenic *Petunia hybrida* plants with suppressed CHS gene expression produces abnormal anthers devoid of flavonoid pigments (Taylor and Jorgensen, 1992). Furthermore, the expression of CHS was strongly inhibited in the later stages of anther development in the male sterility line (Yang et al. 2008; Xie et al. 2016). Thus, CHS enzymes play a critical role in the development of functional pollen. One downregulated gene in 1376-CIMS, CHS (AY286097.1), was specifically expressed

in anthers at the uninucleate stage (Fig. 3) and exhibited substantial homology to CHS (Hihara et al. 1996). Another CHS, CHS-like gene (TaCHSL1), is a novel anther-specific CHS-like gene (EU408770.1) and a gene highly expressed in the wheat tapetum (Wu et al. 2008). This gene was also downregulated in 1376-CIMS (Fig. 3).

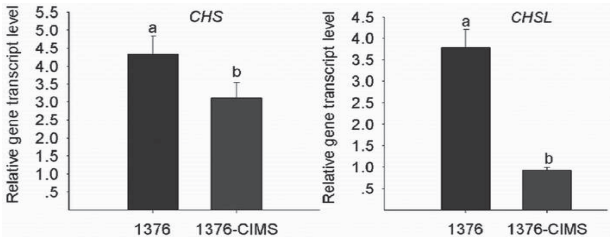

Fig. 3. Relative expression levels of CHS in 1376 and 1376-CIMS anthers. CIMS, chemically induced male sterility

In general, tapetal cells play an essential role in pollen development, thereby producing protein, lipids, and flavonols that are secreted into the pollen sac and form part of exine (Goldberg et al. 1993). Moreover, several genes involved in secondary metabolism, including CHS, are specifically or predominantly expressed in the tapetum (Shen and Hsu, 1992).

The dysfunction of the tapetum in 1376-CIMS (Ba et al. 2014a) is concomitant with the inhibition of anther-specific CHS expression (Fig. 3). As a result, the level of sporopollenin might decrease to below the level required to generate the pollen exine. CHS is one of the main enzymes in the flavonoid biosynthesis pathway. An alteration in CHS expression might affect the accumulation of all classes of these compounds.

In summary, the results of this study lay the foundation for epigenetic studies on CIMS and provide further clues to reveal the molecular mechanism of sterility-to-fertility transition induced by CHA in two-line hybrid wheat.

Authors' contribution

Conceptualization of research (GPL); Designing of the experiments (QSB); Contribution of experimental materials (GSZ, ZLF); Execution of field/lab experiments and data collection (LJZ, GSZ); Analysis of data and interpretation (CC, YXZ); Preparation of manuscript (GPL, QSB).

Declaration

The authors declare no conflict of interest.

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