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iTRAQ-facilitated proteomic profiling of anthers from a photosensitive male sterile mutant and wild-type cotton (*Gossypium hirsutum* L.)

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ABSTRACT

Male sterility is a common phenomenon in flowering plants, and it has been successfully developed in several crops by taking advantage of heterosis. Cotton (*Gossypium hirsutum* L.) is an important economic crop, used mainly for the production of textile fiber. Using a space mutation breeding technique, a novel photosensitive genetic male sterile mutant CCRI9106 was isolated from the wild-type upland cotton cultivar CCRI040029. To use CCRI9106 in cotton hybrid breeding, it is of great importance to study the molecular mechanisms of its male sterility. Here, histological and iTRAQ-facilitated proteomic analyses of anthers were performed to explore male sterility mechanisms of the mutant. Scanning and transmission electron microscopy of the anthers showed that the development of pollen wall in CCRI9106 was severely defective with a lack of exine formation. At the protein level, 6121 high-confidence proteins were identified and 325 of them showed differential expression patterns between mutant and wild-type anthers. The proteins up- or down-regulated in MT anthers were mainly involved in exine formation, protein degradation, calcium ion binding,etc. These findings provide valuable information on the proteins involved in anther and pollen development, and contribute to elucidate the mechanism of male sterility in upland cotton.

1. Introduction

Upland cotton (*Gossypium hirsutum* L.), an important economic crop, is used mainly for producing textile fiber. The F_1 progeny of cotton performs strong heterosis in boll number, boll weight, and seed cotton yield. The hybrid seeds are widely produced in India and China [1]. However, the current cotton hybrid seeds are mainly produced through hand emasculation and pollination, which requires a large labor force and cost. As an alternative, male sterile lines are used as the female parent in the hybrid breeding system to simplify the breeding procedure [2].

Male sterility is a widespread phenomenon, which mostly results from failure to produce viable pollen grains, described in over 150 flowering plant species [3]. Pollen development is an elaborated process involving specific events ranging from initial cell differentiation in the floral meristem to pollen formation, maturation and release during anther dehiscence [4]. In the young anther, groups of archesporial cells divide periclinally to form an outer, primary parietal layer and an inner sporogenous layer. The primary parietal layer undergoes periclinal and anticlinal divisions to give rise to several concentric layers that differentiate into the endothecium, middle layer(s) and the innermost tapetum [5]. The tapetum is in direct contact with the developing gametophytes and plays an essential role in pollen maturation, serving as a nutritive tissue for microspore maturation by providing metabolites, nutrients, sporopollenin precursors, pollen wall synthesis, and pollen coat deposition [6,7]. In most studies, male sterility has been found to be related to tapetum abnormalities. A number of *Arabidopsis* genes have been identified that act after tapetum specification, which is required for normal tapetum function and the mutants are defective in microspore development, including *DYSFUNCTIONAL TAPETUM* (*DYT1*) [8], *ABORTED MICROSPORE* (*AMS*) [9,10], and *MALE STERILITY 1* (*MS1*) [11].

Photoperiod-sensitive male sterility (PSMS) is a special type of male sterile line in which the pollen fertility is controlled by day length. Taking advantage of PSMS lines could greatly simplify breeding and seed production procedures, and easily match the restorer lines in hybrid breeding system [2,12]. The mechanism of PSMS has been well illustrated in rice using PSMS mutants. Nongken58S (NK58S), the first spontaneous PSMS mutant found from a *japonica* cultivar Nongken58 (NK58) in 1973, is completely sterile under long-day (>13.75 h) conditions, but converts to partial or complete male fertility under short-day

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(<13.75 h) conditions [13]. Its fertility was regulated by a long-day-specific male-fertility-associated RNA (LDMAR), which is required for normal pollen development in plants grown under long-day conditions. A point mutation occurred in the mutant and altered the secondary structure of the LDMAR. This change brought about reduced transcription of LDMAR specifically under long-day conditions, resulting in premature programmed cell death (PCD) in developing anthers, thus causing PSMS [14,15]. Interestingly, this mutation resulted in thermosensitive male sterility (TSMS) phenotype in *indica* cultivar Peiai64S (PA64S), suggesting that male fertility is controlled by cross-talk between the genetic networks and environmental conditions [15]. Ulike NK58S, the *carbon starved anther* (*csa*) mutant displays male sterility under short-day conditions but fertility under long-day conditions [2]. *CSA*, an R2R3 MYB transcription factor, took part in regulating sugar partitioning from leaves to anthers to promote pollen maturation by directly regulating the expression of *OsMST8*[16]. The *csa* mutation dramatically reduced the *OsMST8* expression in anthers and the amount of sugar accumulation in the anther under short-day conditions, leading to male sterility. However, the *OsMST8* expression level and sugar accumulation level were recovered to near normal levels under LD conditions, as was the fertility [2]. The fine understanding of PSMS mechanisms will help overcome the problems in the current hybrid rice systems and promote the use of rice heterosis [17].

To make full use of heterosis in cotton, great efforts must be made in seeking suitable male sterile lines and studying the male sterility mechanisms. A novel PSMS cotton mutant CCRI9106 (MT) with a virescent marker was isolated from CCRI040029 (WT) by space mutation breeding technology, and appeared to be male sterile under long-day conditions and fertile under short-day conditions [18]. Although transcriptional profiling analyses of MT and WT anthers have elucidated that the ubiquitin–proteasome system is induced in MT uninucleate pollen (UNP) under long-day conditions and causes protein degradation in MT pollen, how these genes can be regulated still remains largely unknown [18]. Proteins are the final products of genes and are more directly related to cellular metabolisms and biological processes. Moreover, various post-transcriptional regulatory mechanisms can cause differences in the protein abundance levels predicted by their transcript abundances [19]. In recent years, a number of proteomic analyses have been conducted in various male sterile mutants, such as rice [20], tomato [21], rapeseed [22], wolfberry [23], *Zea mays*[24] and pummelo [25]. These works yielded a large amount of proteins defined as contributors to male sterility, including proteasome subunits, ATP synthase subunits, ribonucleoproteins, and flavonoids. Therefore, proteomic analyses of male sterile mutants will greatly facilitate the understanding of male sterility in cotton.

Two-dimensional electrophoresis (2-DE) is a standard method for proteome studies and has been widely applied to detection of protein expression patterns in cotton fiber [26,27], seed [28], leaf [29], and anther [30]. In our previous work, 2-DE was employed to analyze the variation of anther proteomes between CCRI9106 and CCRI040029, and 56 proteins showed differential expression profiles [31]. Although this work advanced our understanding of the molecular defects in CCRI9106 anther, it was far from clearly elucidating the mechanisms of its male sterility. Recently, isobaric tags for relative and absolute quantitation (iTRAQ) has been proved to be an efficient approach to directly quantify and compare the protein expression levels of samples with high accuracy [32] and is successfully used for in proteomic analysis in cotton fiber [33] and somatic embryo [34].

Here, we first applied the iTRAQ technology to cotton anthers to identify the candidate proteins that are directly related with the male sterility of CCRI9106 through analyzing the anther proteomes of CCRI9106 and CCRI040029 at three key developmental stages. Our results revealed a global shift of protein expression patterns corresponding to the pollen development process, including exine formation, protein degradation, and calcium ion binding. Their possible biological functions and potential effects on male sterility are discussed with the aim to determine their roles in pollen development and male sterility in cotton.

2. Materials and methods

2.1. Plant growth and anther collection

Two *G. hirsutum* L. genotypes, a PGMS mutant CCRI9106 and its WT line, CCRI040029, were used in this study. CCRI040029 was an elite upland variety bred in our lab, and the mutant line, CCRI9106, was created by space mutation in 2010 [18]. Our previous genetic analysis showed that the sterility was caused by the mutation of a single recessive allele in CCRI9106 [31]. Therefore, the genetic background of CCRI9106 and CCRI040029 are very similar, with the exception of male fertility site. They were grown in an agronomic field in Anyang (Henan, China) from April to October and in Sanya (Hainan, China) from October to early April. Thirty rows (8 m in length × 0.8 m in width) were prepared for each genotype, and every 10 rows formed one replicate.

As in our previous study [18,31], during the anthesis period, flower buds of different lengths were observed to identify the pollen developmental stages and then were sampled for anther collection every other day. To test the pollen fertility, anthers were stained with Alexander's solution [35]. They were then photographed using an Olympus DP72 light microscope. Additionally, anthers from both MT and WT at different development stages were collected for further analysis. The collected anthers were immediately fixed for cross-section, scanning electron microscopy (SEM) or transmission electron microscopy (TEM) analyses or frozen in liquid nitrogen and stored at −80 °C until proteins and mRNA extractions were performed.

2.2. Cross-section, SEM and TEM

For cross-sections, anthers were fixed in formalin-aceto-alcohol (FAA) and dehydrated in an ethanol series (from 30% to 100%). The samples were then embedded in paraffin. Longitudinal sections were cut using a Leica RM2265 (Solms, Germany) ultramicrotome, stained with toluidine blue and photographed using the Olympus DP72 (Osaka, Japan) light microscope.

For SEM, anthers were infiltrated with 2.5% (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.2), dehydrated in a graded series of ethanol (from 30% to 100%), treated in acetone for 15 min, and transferred to isoamyl acetate for 20 min. The samples were then dried with a CO_2 critical-point drying system (HITACHI HCP-2, Japan). Subsequently, pollen grains were coated with gold:palladium and imaged using a scanning electron microscopy (HITACHI S-530, Japan).

For TEM, anthers were firstly fixed in 3% glutaraldehyde and 4% paraformaldehyde, and then post-fixed with 1% OsO_4. Samples were dehydrated in an ethanol series (from 30% to 100%), and treated in acetone, and infiltrated with Spurr's resin (Electron Microscopy Sciences). Sections of 90 nm were cut, counterstained with saturated uranyl acetate and 2.5% lead citrate, and visualized using a New Bio-TEM H-7500 transmission electron microscope (HITACHI, Japan).

2.3. Protein extraction and quantification

For protein extraction, TCA–acetone (trichloroacetic acid) method [36] was selected, performed according to Pang et al. with minor modifications [26]. In brief, ~1.5 g of frozen anther was ground with 10% polyvinyl polypyrrolidone (w/w) in liquid nitrogen using a mortar and pestle. The resulting fine powder was mixed with 10% (w/v) TCA in cold acetone containing 0.07% (w/v) 2-mercaptoethanol for at least 2 h and subsequently centrifuged at 12,000 *g* for 1 h at 4 °C. The pellet was washed first with cold acetone containing 0.07% (w/v) 2-mercaptoethanol and then with 80% cold acetone and finally was suspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, 2% EDTA-free protease-inhibitor). The supernatant was