Proteomics Analysis of Photo-Thermo-Sensitive Male Sterility Wheat Line BNS during Its Thermosensitive Period

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Abstract

Photo-thermo sensitive male sterile (PTMS) line is one of the important materials in utilizing heterosis in crops. Wheat line BNS (Bainong sterility) is an important nuclear-controlling PTMS line and suitable for growing and seed production in Huang Huai wheat zone in China. It has genetic stability with male sterility when sowing in autumn and male fertility when sowing in spring. Their thermosensitive periods were between stamen and pistil differentiation stage and anther connective stage and they could be regarded key periods for fertility conversion in BNS. To determine the molecular mechanisms of fertility conversion at thermosensitive period, we investigated characters of seed setting, anther and pollen grain of fertile and sterile BNS plants and compared young spike proteome patterns at their thermosensitive periods between the two BNS plants. The results showed that sterile plants had lower seed setting rate and pollen number, small pollen grain and lower pollen vitality than fertile plants. Out of protein spots reproducibly detected and analyzed on two dimensional electrophoresis gels, 76 spots showed significant changes in at least one BNS plant and 36 spots were identified by MALDI-TOF MS. The results showed that proteins involved in multiple biochemical pathways were differentially expressed at thermosensitive period between the two plants, including energy metabolism, stress response, signal transduction and regulation, protein process, amino acid and fatty acid metabolism, nucleic acid metabolism etc. Some of these proteins are reported to be involved in the abortion of anther or pollen grains in MS plants, such as energy metabolism and anti oxidative stress, and some were found to be novel proteins involved in the fertility conversion, such as phytohormones regulation. These results indicated that proteins related with anther or pollen development had expressed differently between the two BNS plants before anther development and phytohormones and signal transduction might be involved in the regulation of fertility conversion at thermosensitive period. Our studies have provided new insight to reveal the molecular mechanisms of fertility conversion at thermosensitive period in PTMS wheat.

Keywords: male sterility, plant hormones, proteomics, *Triticum aestivum*, theomosensitivy

1. Introduction

Male sterility (MS) has been wildly found for the production of hybrid seed in crop plants. Several CMS (cytoplasmic MS) systems have been reported in many crops and have been extensively utilized for hybrid seed production, such as in rich (Li & Yuan, 2000; Virmani, 2003) and rapeseed (Fan et al., 1986; Röbbelen, 1991). The utilization of heterosis in common wheat was made possible by the establishment of the nuclear-cytoplasm interactive male sterility in the three-line system and by the use of chemical hybridizing agents (CHA) (Zhao et al., 1999). However, wheat heterosis has not been utilized extensively in worldwide. The three-line system has a narrow origin and low degree of restoration, and the use of CHA is very limited for its cost and potential residue

problems. Photo-thermo sensitive male sterile (PGMS) lines are utilized as an alternative way of exploiting heterosis in wheat. The PGMS system is advantageous for its broad restoring ability, easy maintenance and multiplication and it is considered to be more efficient than CMS system in hybrid wheat production (Jordaan, 1996; Virmani & Ilyas-Ahmed, 2001).

BNS (Bainong sterility) line is a new type of ecological male sterile wheat line with PGMS character. BNS was obtained from BNY-S, a temperature sensitive nature mutant from wheat *va*. Bainong, and it was improved by continuous backcrossing with a wheat *va*. Lankao (Zhou et al., 2010). Both Bainong and Lankao were released for cultivation in HuangHuai wheat zone, which is the main wheat growing area and the wheat production in this area accounts for more than 60% of wheat production in China (Liu et al., 2011; Zhou et al., 2007). The fertility of BNS was easy to be restored by a few normal wheat varieties in this area (Li et al., 2009). BNS might be a potential, important PGMS line for wheat heterosis utilization in this area.

BNS is a genic PTMS line and its fertility and sterility are stable in different growing years. BNS has the characteristics of high sterility when it was sowed in early or medium autumn and fertility when sowed in later autumn or spring (Li et al., 2009).

As a PTMS line, the temperature played a leading role in its fertility alteration. Our previous studies proved that the temperature sensitive period was between stamen and pistil differentiation stage and anther connective stage, and this period could be regarded as critical for its fertility conversion (Wang et al., 2011). Based on continuous growing years' results, it could be concluded that the complete sterility could be obtained when air temperature goes below 11.4 °C at this fertility conversion period, and hybrid seed could be produced by crossing with restoration lines. BNS showed complete fertility when air temperature is more than 15.9 °C at the fertility conversion period, and fertile seed could be produced by self crossing (Wang et al., 2011). In addition, our research also showed that the male fertility in BNS was controlled by two major genes plus polygene, and a certain amount of cytoplasmic effects was preliminarily found. The two major genes have more effects on the fertility, and their additive effects are much more than their dominant effects (Zhang et al., 2013).

Up to now, many researches on mechanisms of male sterility have focus on the developments of anther or pollen grain. In order to understand the molecular mechanism underlying the key period of fertility conversion in male sterility, we investigated the proteome pattern differences of young spikes at fertility conversion period between fertile plants and sterile plants of BNS. This research will be helpful for elucidation of proteins regulation in conversion of fertility and sterility, and for utilization of this PTMS line in wheat heterosis.

2. Materials and Methods

2.1 Experimental Materials and Cultivation

PTMS winter wheat line BNS was used in this study. Materials were planted at the farm of Agronomy College of Shandong Agricultural University, in Taian City (36°11′N, 117°8′E and 135 m a.s.l), in Eastern China, during 2011-2012 growing season. Sowings occurred at two different dates, autumn (October 5, 2011) and spring (March 10, 2012). Seeds were planted in 3 replicates with plot of 2.0 m × 7 m (14 m^2) in this study. Two hundred seedlings were planted per square meter at equal spacing. All cultural practices, such as irrigation, fertilization, and the controls of weed, insect and disease were standard and uniform according to the local farmer cultivation practices.

2.2 Investigation of Seed Setting, and Characters of Anther and Pollen Grains

Seed setting rates: three spikes (one main and two side tillers per plant) on 10 randomly selected plants in each replication of the two sowing date plants were bagged with butter paper bags before flowering to observe seed setting in bagged spikes. Percent seed setting was calculated as two formulae given below:

Seed set (%) = (Total number of kernels on spikelet base)/(Number of spikelets per spike × 2) × 100 (Chinese seed setting formula) (Song et al., 2005).

Seed set (%) = (Total number of kernels per spike)/(Number of spikelets per spike × 2) × 100 (International seed setting formula) (Zhang et al., 2007).

Characters of anther and pollen grains: 30 anthers of each replicate of the two sowing date plants were analyzed, of which always 10 were taken from the bottom third part, 10 from the middle third part and 10 from the top third part of the spike. Anthers just turning yellow, approximately one day before dehiscence, were removed from the floret with a pincet and measured under a binocular stereomicroscope (16×) with an ocular micrometer. The length and the width of the anther were determined. Pollen grain number pre anther was determined according to Hansen' methods (Hansen & Andersen, 1998). Brifly, the anther was deposited in a vial with 0.5 ml

0.5% aceto carmine, all pollen grains were loosened from the anther by crushing and stirring the anther. The concentration of pollens could be determined using haemocytometer method and the real number of pollen grains per anther could be calculated. At the same time, the diameter of pollen grains were determined under microscopy with an ocular micrometer. The viability of pollen grains was examined under the microscope. Anthers were taken from each spikelet in 5 spikes and squashed with a glass rod on a glass-slide to disperse pollen grains and stained with iodine-potassium iodine solution (2% I-KI). Only those being round, normal sized and dark blue stained were considered fertile (Barnabás & Kovács, 1992).

2.3 Protein Sample Extraction for Proteomics

Proteomics analysis for wheat line BNS during its thermosensitive period were conducted State Key Laboratory of Crop Biology, Shandong Agricultural University. For proteomic analysis, young spikes at the thermosensitive period (between the stage of differentiations of pistil and stamen and the stage of anther connective) were sampled according to the observation under stereomicroscope. Proteins were extracted using a two step trichloroacetic acid/acetone protein extraction protocol with minor modification (Cho et al., 2006). Briefly, a total of 0.3 g of young spikes were ground in liquid nitrogen with mortar and pestle (pre-cooled) into a fine powder. The powder was precipitated with ice-cold acetone containing 10% (w/v) trichloroacetic acid and 0.07% (v/v) 2-mercaptoethanol (acetone-TCA-2-ME) for 2 h at 20 ^{o}C, and then centrifuged at 40,000 g for 25 min at 4 ^{o}C. The pellets were washed with ice-cold acetone containing 0.07% (v/v) 2-ME, for 6 h at 20 ^{o}C and centrifuged again at 4 ^{o}C. Then pellets were lyophilized to powder and were stored at 80 ^{o}C until further use. Six biological replicates were extracted independently for fertile and sterile plants respectively.

2.4 Two-Dimensional Electrophoresis

Isoelectric focusing (IEF) was carried out on immobilized pH gradient (IPG) 24 cm pH 3-10 L strips (Bio-Rad). The running condition was as follows: 500 V for 1 h, followed by 1000 V for 1 h, and finally 8000 V for 14 h. The focused strips were equilibrated for 15 min in 10 ml equilibration solution containing 7 M urea, 30% (w/v) glycerol, 2.5% (w/v) SDS, 1% (w/v) DTT, and 50 mM Tris-HCl buffer, pH 8.8. Separation of proteins in the second dimension was performed by SDS-PAGE in a vertical slab of acrylamide (12% total monomer, with 2.6% cross linker) using a Dodeca Cell (Bio-Rad). For preparative gels 1.8 mg protein were loaded. The protein spots in analytical and preparative gels were visualized by colloidal CBB G-250.

2.5 In-Gel Digestion and MALDI-TOF MS Analysis

The excised protein spots were washed with ultrapure water twice at room temperature, and destained with 100 ml of 100 mM NH_4HCO_3/acetonitrile (50:50, v/v) for 1 h. Gel fragments were dehydrated with 50 ml of acetonitrile for 10 min and dried at room temperature. 10 ml of 20 ng/ml trypsin (Promega) was added to each dried gel fragment and incubated for 45 min at 4 ^{o}C. 10 ml NH_4HCO_3 (50 mM) was added and fragments were incubated at 37 ^{o}C overnight (about 16 h). After digestion of proteins, peptides were desalted with C18 ZipTips (Millipore Corp., Bedford, MA, USA), then spotted on MALDI plates (Bruker Daltonics, Germany) in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA). Finally, peptides were co-crystallized with saturated a-cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% (v/v) acetonitrile containing 1% TFA. To obtain peptide mass fingerprint (PMF) of protein spots, peptide masses were measured using autoflex MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA). External calibration was performed with a peptide calibration standard (Bruker Daltonics, Part No.: 206 195). All obtained PMFs were analyzed with the protein search engine MASCOT (Matrix Science, U.K.) against NCBI's database. Search parameters were set as follows: peptide tolerance (0.2 Da), NCBInr database, Green plants (taxonomy), carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modification). Molecular function of proteins was annotated using the database at http://www.uniprot.org/uniprot (Wen et al., 2010).

2.6 Statistical Analysis

SPSS Version 13.0 (Lead Technologies, Chicago, IL, USA) was used to statistically analyze the results of characters of seed setting, anther and pollen grains, the intensity of protein spots by Student's t-test. Percentage of seed setting was subject to arcsine transformation prior to statistical analysis. Significance was determined at the $p < 0.05$ level.

3. Results

3.1 Characters of Seed Setting, Anther and Pollen from Two Sowing Date Plants

The investigation results of seed setting, characters of anther and pollen from sterile and fertile plants of BNS were shown in Table 1. The seed setting rates of BNS line sowed at autumn and spring were 1.25% and 84.62% (as international calculation method), or 2.96% and 118.67% (as Chinese calculation method), respectively (P <

0.001). The autumn sowing plants were sterile and the spring sowing plants were fertile. The fertility differences of the two plants could be due to the differences of male fertility. The pollen grain number per anther of sterile and sterile plants were 1428.9 and 125.6, respectively (P < 0.001). The vitality of pollen grains from spring sowing plants was much higher than that from autumn sowing plants, and they were 98.9% and 0.32%, respectively (P < 0.001). In addition, effects of sowing dates on BNS fertility also came out on the sizes of anthers and pollen grains. The length and width of anther and the diameter of pollen grains of sterile plants were much less than those of fertile plants (P < 0.01) (Table 1, Figure 1).

Table 1. Characters of seed setting rates, anthers and the pollen grains from sterile and fertile plants of BNS line

Plants[*]	Seed setting rate		Size of anther			Pollen grains		
	Internal method	International methods	Length (mm)	Width (mm)	Long × width (mm)	Number per anther	Diameter (um)	Vitality (%)
Sterile plants	1.25±0.8	2.96±0.19	3.5±0.1	0.82±0.12	2.98±0.31	125.6±21.7	67.5±9.3	0.32±0.07
Fetile plants	84.62±7.26	118.67±9.65	4.2±0.3	0.91±0.06	3.95±0.29	1428.9±126.2	71.3±6.9	98.9±7.5

Note. Sterile and fertile plants of BNS were sowed at Oct. 9, 2010 and Feb. 17, 2012 respectively.
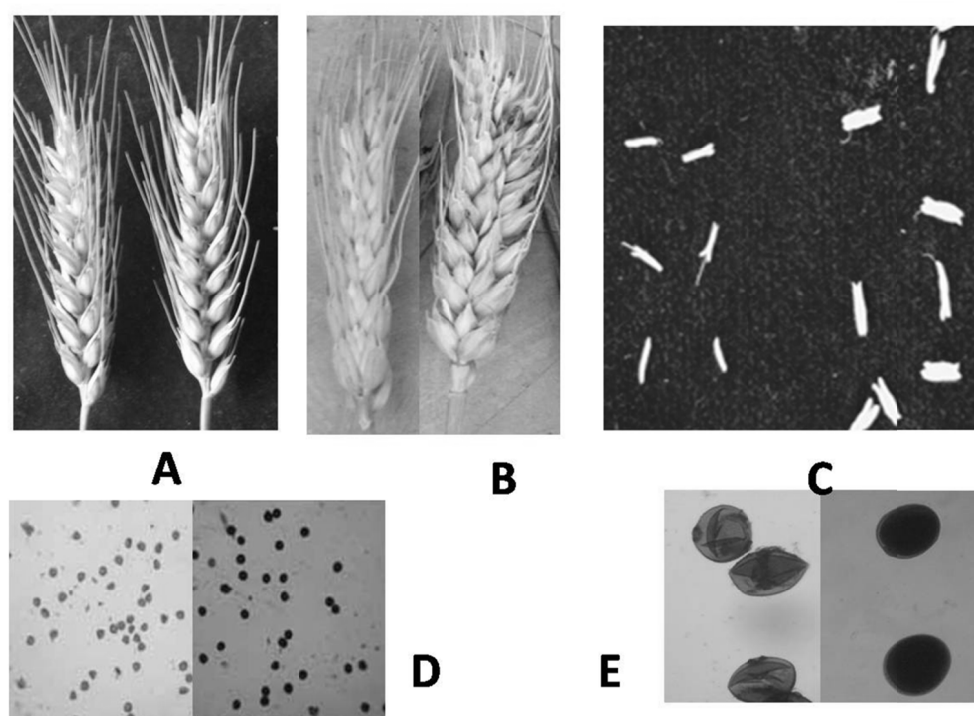

Figure 1. Differences of sterile (sowed at autumn, left) and fertile (sowed at spring, right) plants of BNS. (A) Spikes after jointing stage. Spikes from sterile plants are fluffy, transparent, and glume opening; (B) Mature spikes. Spikes from sterile plants are shriveled, and have few seeds in it; (C) Anthers size. Anthers from sterile plants are smaller than those from fertile, and have little pollen grains in it; (D) Pollen grains. The pollen grains from sterile plants are abortive and those from fertile plants are viable as shown iodine staining experiments; (E) Some pollen grains from sterile plants are irregular shape and those fertile showed normal round

3.2 Protein Expression Differences between Sterile and Fertile Plants of BNS

To investigate differences in protein expression between sterile and fertile plants of BNS line, total soluble proteins in young spikes during thermosensitive period were identified using proteomics techniques. Young spikes of six biological replicates were extracted and separated independently by 2D PAGE. The protein spot numbers and volumes among 2D gel images were reproducibly detected and the protein profiles of the two spikes were compared. Overall, 76 differentially expressed proteins were revealed by 2D PAGE (Figure 2), and

they were identified with high confidence by MALDI-TOF MS. The genomic sequence data analysis of all the differentially expressed proteins identified only 38 proteins from wheat (Table 2). Among these identified proteins, 22 proteins were up-regulated in fertile plants, while 14 were up-regulated in sterile plants. Homologs of some of these proteins were identified in other plant species, and the identification of wheat proteins was low due to the incomplete status of the wheat genome database. According to the functional features described in annotation and literature data, the identified proteins were categorized into several types, including carbohydrate and energy metabolism, stress responses, signaling and regulation, antioxidant and defense pathways, protein synthesis or DNA replication process, amino acid and fatty acid metabolism, nucleic acid metabolism and other unclassified proteins. Detailed information can be found in Table 2.

Table 2. Up regulated proteins displayed and identified on gels after 2-DE followed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis in sterile and fertile plants of BNS

Spot	Protein description	Experimenta Mr (kDa)/pIb	Theoretical Mr (kDa)/pI	NCBI accession no.	Protein function	Mascot score	No. of matche peptides	Protein source	Sequence coverage (%)
Up regulated in sterile plants									
14	Cupin protein	45.7/6.02	56.9/6.05	ACG25229.1	Signal transduction	132.7	8	*Zea mays*	25
7	ATP synthase subunit d, mitochondrial-like	16.5/4.82	20.4/5.33	XP_003563178	Energy metabolism	89.5	14	*Brachypodium distachyon*	32
10	Cytosolic Ascorbate peroxidase	29.6/5.92	27.2/5.28	ACG41151.1	Cell detoxification		21		41
12	Cytosolic Ascorbate Peroxidase	23.7/6.42	27.2/5.28	ACG41151.1	Cell detoxification	98.7	17	*Zea mays*	27
8	s-adenosylmethionine synthetase 1	62.7/5.86	43.1/5.50	XP_002312296.1	Gene expression	104.25	9	*Populus trichocarpa*	35
18	Hypothetical protein	27.6/6.05	28.9/7.72	NP_001142197.1	Unclear functional	98.5	12	*Zea mays*	41
2	Oxidase (IM1) mRNA	24.5/3.89	26.7/4.88	AF274001	Metabolism	103.5	15	*Triticum aestivum*	28
20	Triticin precursor	70.2/7.45	57.0/9.37	S62630	Storage protein		19	*Triticum aestivum*	15
22	Superoxide dismutase precursor	28.4/7.36	22.7/8.52	FJ890987	Cell detoxification	98.7	9	*Triticum aestivum*	22
11	NBS-LRR type RGA	24.8/5.69	19.5/5.74	AAZ99787	Disease resistant	78.9	11	*Triticum aestivum*	19
4	Rice homologue of Tat binding protein	63.7/5.12	49.6/5.91	BAA04615	Transcription factor	83.6	13	*Oryza sativa*	27
5	Nitrate reductase apoenzyme	70.2/5.36	101.5/6.11	CAA33817	Electron transport chain	121.5	22	*Oryza sativa*	32
17	KNOX class homeodomain protein	35.8/6.67	33.3/5.21	AAU10751	Unclear function	99.8	14	*Oryza sativa*	41
15	A ABA-responsive, ABR1	48.2/6.34	38.0/6.29	CA524559	Signal transduction	89.6	13	*Capsicum annuum*	21
21	wrab17, LEA/RAB-related COR protein	37.2/7.42	20.6/4.70	AAF68628.1	Signal transduction	98.4	7	*Triticum aestivum*	26
Up regulated in fertile plants									
	14-3-3		28.9/4.73	P48347	Signal transduction	88	8	*Arabidopsis thaliana*	23
F9	Annexin D4	39.7/6.42	36.2/6.88	NP_181409.1	Signal transduction	61	7	*Arabidopsis thaliana*	15
F4	Mitochondrial ATP synthase precu	30.2/5.56	57.8/5.16	AY614716_1	Energy metabolism	131.4	10	*Triticum aestivum*	34
F18	Mitochondrial ATP synthase precursor	34.2/6.23	57.8/5.16	AY614716_1	Energy metabolism	80.19	12	*Triticum aestivum*	28
F19	V-type proton ATPase subunit B1	58.9/6.72	54.1/4.98	P11574	Energy metabolism	78.9	13	*Arabidopsis thaliana*	47
F17	Starch synthase isoform IV	46.2/7.52	103.1/5.87	AAK97773.1	Biosynthetic enzymes, metabolism enzymes	84.3	17	*Triticum aestivum*	24
F10	Putative peptidyl-prolyl cis-trans isomerase family protein	28.4/6.89	23.6/9.58	AFW69420.1	Protein progress, protein folding		18	*Zea mays*	17

F6	DNA ligase OS = Rubrobacter or Polydeoxyribonucleotide synthase [NAD(+)]	82.3/5.82	77.0/5.49	Q1AZ75	DNA replication	77.6	22	*Rubrobacter xylanophilus*	28
F11	Histone H3	27.8/7.64	18.5/10.86	ACG25088.1	DNA replication	89.5	19	*Zea mays*	35
F13	Ubiquinol-cytochrome c reductase complex protein	24.5/7.62	14.7/9.78	NP_001170375.1	Energy metabolism	110.3	10	*Zea mays*	32
F8	TPA: acidic ribosomal protein P2a-3	19.6/5.82	11.5/4.20	DAA35449.1	Protein progress		12	*Zea mays*	29
F12	Chaperonin isoform 1	19.8/7.32	10.5/7.97	NP_001131801.1	Chaperones		8	*Zea mays*	21
F1	ADP-ribosylation factor	24.7/4.48	23.0/5.36	NP_001169258	Intracellular traffic	127.3	9	*Zea mays*	32
F	Retrotransposon protein				DNA replication	89.2	16		
F16	Chi 3 mRNA for chitinase 3, complete cds	34.5/7.62	33.5/6.89	AB029936	Anti stress	106.8	9	*Triticum aestivum*	27
F14	Pyruvate decarboxylase	51.2/7.47	61.1/5.86	AB040742	Glucose metabolism	84.6	17	*Oryza sativa*	38
F7	Isopentenyl pyrophosphate isomerase	27.6/6.12	21.1/5.41	ACF07858	Synthetic proteins	93.5	18	*Triticum aestivum*	40
F15	Cysteine proteinase	36.5/7.18	40.8/6.80	BAE96008	Degradation protein	91.6	21	*Triticum aestivum*	25
F3	Chilling inducible protein	35.7/5.46	43.4/5.84	CAA90866	Cold related	86.7	7	*Oryza sativa*	27
F5	RicMT	18.4/4.82	7.6/4.58	BAG88445	Unclear function	93.6	15	*Oryza sativa*	19
F2	Indole-3-glycerol phosphate lyase	38.5/5.34		AAS45241	Signal transduction	89.4	8	*Hordeum lechleri*	

Note. A.Spots signed in S Arabian numbers and F Arabian numbers represent up- regulated protein spots in sterile and fertile plants of BNS, respectively; B. Molecular weights (MW) and iso-electric points (pI) were calculated as an average value (n = 6); C. MOWSE score: statistical probability of true positive identification of predicted proteins calculated via MS-fit software. Maximum allowed Mr deviation of experimental and predicted peptide fragments: 50 ppm. Allowed missed cleavage: 1; D. Peptides matched (n): number of peptides matching predicted protein sequences; E. Sequence coverage (%): percentage of predicted protein sequence covered by matched peptides.
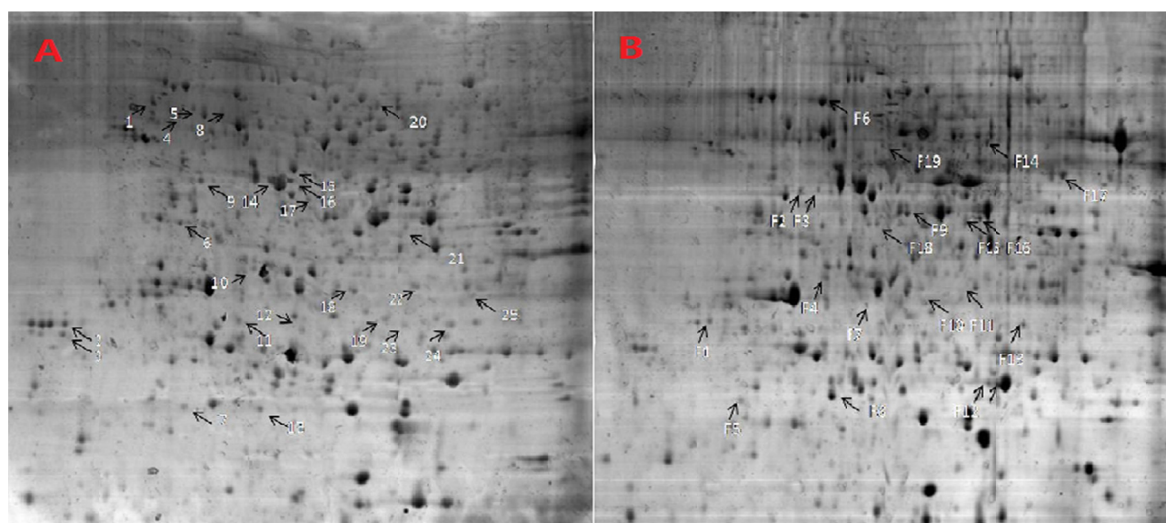

Figure 2. Comparison of the protein patterns of sterile (A), lfertile (B) plants of BNS wheat at fertility conversion phage. Proteins from the whole young spike were extracted and separated on a pH 3-10 linear 24 cm IPG strip followed by 12.0% SDS-PAGE. The spots were visualized with CBB staining and detected by ImageMasterTM 2-D 6.0 software. The numbered spots are those differentially expressed between kinds of plants, they were subjected to MALDI-TOF followed by database searching. Matched peptides are listed in Table 2. Upexpressed proteins in large or small kernels are designated by arrows and numbered in respective 2-D images

4. Discussion

We analyzed the differences of fertility characters between of autumn sowing and spring sowing plants of PTMS winter line BNS. The results indicated that BNS was highly sterile when planted in early autumn in Taian and its seed setting rate recovered to more than 80%, and self-pollinated to normal levels when sowing in spring. This indicated that it could be used as both maintainer and sterile lines in a two-line hybrid system. The present study showed that the sterility of BNS line sowed in autumn might be due to the reduction of pollen number per anther or the decrease of pollen viability. This is consistent with previous studies in other species. In peach, a mutant with male sterility had smaller anthers and lesser number of pollen grains than fertile parental variety (Sreevalli et al., 2003). In eggplant, both the number of pollen per anther and pollen viability in the BC_5 plants with male sterility were lower than those in their fertile nucleus donor (Shiro & Yoshida, 2002). However, the reduction of pollen number per anther and the decrease of pollen viability are influenced by different mechanisms. The pollen number might be related with the development of meiosis of microspore mother cell, or the daparture of pollen grains from tapetum and the realeasement from anther, and the pollen viability might be related with normal development of pollen grains after meiosis of microspore mother cell. Lee et al. (2008) reported that no pollen grain is typically visible during anthesis in a male sterile radish, but a small number of pollen grains could be observed stuck together in the dehiscing anthers in another male sterile radish. Gothandam et al. (2007) showed that pollen viability and grain production decreased significantly when treated at a low temperature in a rice MS line, and the male sterility is due to functional loss of the tapetum which is the most sensitive to low-temperature stress.

The present research showed that, the external appearances had no obvious change at their theomosensitive periods between sterile and fertile plants of BNS, but the expression of a variety of proteins were different significantly between them as shown by proteomics (Table 2, Figure 2). Vijayalakshmi and Bangarusamy (2007) have reported that high temperature induced male sterility in a thermosensitive genie male sterile rice line; and compared with fertile plants, the male sterile plants were associated with accumulation of total phenolics and proline and reductions of soluble proteins at the critical stage of thermosensitivity. Our research indicated that there might be multiple biochemical mechanisms involved in the fertility conversion at thermosensitive period. Among these proteins, proteins related with energy metabolism, antioxidant stress and hormone regulation could be particularly prominent.

Proteins related with energy metabolism and regulation included 4 proteins. ATP synthase subunit d (mitochondrial-like) (spot 7) was up-regulated in sterile plants, and mitochondrial ATP synthase precursor (spot F18), V-type proton ATPase subunit B1 (spot F19) and ubiquinol-cytochrome c reductase complex protein (spot F13) were up regulated in fertile plants.

ATP synthase is an important enzyme that provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP). ATPases are a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion. This dephosphorylation reaction releases energy and this process is widely used in all known forms of life. Mitochondrion, the site of both the tricarboxylic acid cycle and oxidative phosphorylation pathway, plays a crucial role in energy and carbon metabolism in eukaryotic cells (Hatefi, 1985). Up to now, 12 mitochondrion DNA regions associated with CMS have been identified, and most of them are involved in the genes encoding F_0F_1-ATPase subunits (Hanson, 2004). In higher plants, the demand for ATP is highly increased during pollen development (Sabar et al., 2003), and decreased mitochondrial ATP synthesis may be a causal factor in disruption of pollen or microspore development (Yang et al., 2009). These previous researchers have reported that reduction of the proteins associated with energy production and lesser ATP equivalents detected in CMS anther and that indicated that the low level of energy production played an important role in inducing CMS. Oue research indicated that the differences of sterile and fertile plants on energy production has taken place as early as thermosensitive period in BNS, and there is no visible anther in the young spike at this period. The ubiquinol cytochrome c reductase is a key subunit of the cytochrome bc1 complex (complex III) of the mitochondrial respiratory chain (Vedel et al., 1999). The complex shows characteristics associated with a Q-cycle mechanism of redox-driven proton translocation, including two pathways for reduction of b cytochromes by quinols and oxidant-induced reduction of b cytochromes in the presence of antimycin (Berry et al., 1991). But to our knowledge no proof shows that it is related with MS in plants.

Superoxide dismutase precusor (SOD) (spot 22) and cytosolic ascorbate Peroxidase (spots 10, 12) were found up regulated in sterile plants. Superoxide dismutases are enzymes that catalyze the dismutation of superoxide (O^{2-}) into oxygen and hydrogen peroxide. This is an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide dismutases up regulated in sterile lines might be related with the elimination of activated oxygens in male sterile plants. In rice, compared with the maintainer line, fertile line Yuetai B, a significant

decrease of SOD activity was detected in the CMS line, Yuetai A, which resulted in an increase in the reactive oxygen species (ROS) content (Wang et al., 2013). Ascorbate peroxidases (APX) are enzymes that detoxify peroxides such as hydrogen peroxide using ascorbate as a substrate. Programmed cell death during microgenesis in a Honglian CMS line of rice is correlated with oxidative stress in mitochondria. This pollen disruption was correlated with excess production of reactive oxygen species and down-regulation of the activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) in mitochondria (Li et al., 2004).

Proteins related with hormone synthesis and regulation including 5 proteins. Cupin protein (spot 14), wrab17 (a LEA/RAB-related COR protein) (spot 21) and ABR1 (a ABA-responsive protein) (spot 15) were up-regulated in sterile plants, indole-3-glycerol phosphate lyase and Annexin D4 were up-regulated in fertile plants.

The cupin proteinbelongs to cupin superfamily, which comprises both enzymatic and non-enzymatic members, although it notably contains the non-enzymatic seed storage proteins (Dunwell et al., 2001). In seed, the cupin protein mainly competes with ABA in the activated a-subunit of GTP-binding proteins in the regulation of its germination (Santner & Estelle, 2009; Lapik & Kaufman, 2003). Once ABA interacts with GTP-binding protein receptors, other proteins related to the signal cascade are needed for signal transduction to take place. Wrab17 is a dehydrin-like protein which was shown to be highly homologous (84% amino acid identity) to ES2A, a barley GA3 (gibberellic acid)-responsive protein (Pandey et al., 2009). ABA-RESPONSIVE1 (ABR1), which is highly induced by infection with avirulent *Xanthomonas campestris* and it is also highly induced by treatment with ABA. The pepper ABR1, which localizes to the nucleus, negatively regulates ABA signaling in an SA-dependent manner to resist pathogen attack (Tsuda et al., 2000). Spot F2 had the highest homology to indole-3-glycerol phosphate lyase (IGL2). IGL2 is involved in auxin-mediated signal transduction. The interaction of auxin with its cellular receptors for IGL2 triggers a cascade of events resulting in response including the modification of cell wall components such as lipids, and in altering the orientation of cell wall polysaccharides (Choi & Hwang, 2011). Annexin D4 (spot F9) plays important roles in osmotic stress and ABA signaling in a Ca^{2+}-dependent manner (Macdonald, 1997).

The previous research has proved that almost all the hormones are related with male fertility in plants. Flower tissue-specific auxin reduction is the primary cause of high temperature injury, which leads to the abortion of pollen development in barley and Arabidopsis (Lee et al., 2004). The tapetum-specific expression of a mutated ethylene receptor gene is a potential strategy for inducing male sterility in transgenic tobacco plants (Sakata, 2010). In *B. napus* ABA content in flower buds was higher in fertile plants than that in male sterile plants, and the expression of KIN1, a ABA reactive protein, also reduced in sterile flower (Zhu et al., 2010). Gibberellin-induced gene expression associated with cytoplasmic male sterility in sunflower (Duca et al., 2008).

Our present results indicated that complex plant hormone regulations might exist during the thermosensitive period. Plant hormones might have important roles in the fertility conversion. However, as for what specific hormones mainly involved and what action modes of hormone in fertility conversion are needed to be further studied.

In addition, there are many other proteins expressed differently between the two lines at thermosensitive period in BNS (Figure 2; Table 2). The proteins up-regulated in sterile line included that s-adenosylmethionine synthetase 1, Oxidase (IM1), triticin precursor, NBS-LRR type RGA, rice homologue of Tat binding protein, nitrate reductase apoenzyme, KNOX class homeodomain protein and hypothetical protein. The proteins up regulated in fertile plants included starch synthase isoform IV, putative peptidyl-prolyl cis-trans isomerase family protein, DNA ligase, histone H3, TPA (acidic ribosomal protein P2a-3), chaperonin isoform 1, ADP-ribosylation factor, retrotransposon protein, chitinase 3, pyruvate decarboxylase, isopentenyl pyrophosphate isomerase, cysteine proteinase, chilling inducible protein, RicMT. These proteins might have some roles in the fertility conversion, and they are also worthy of further research.

In conclusion, sterile plants of BNS had lower seed setting rate, lower pollen number, small pollen grain and lower pollen vitality than fertile plants. Proteins involved in multiple pathways were differentially expressed in response to fertility differences at thermosensitive period. Proteins related with pollen abortion had expressed differently at thermosentive period and plant hormones might be involved in the regulation of fertility conversion at this period.

References

Cobb, A. H., & Reade, J. P. H. (2010). *Herbicides and Plant Physiology* (2th ed.). Wiley-Blackwell.

Barnabás, B., & Kovács, G. (1992). *In vitro* pollen maturation and successful seed production in detached spikelet cultures in wheat (*Triticum aestivum* L.). *Sexual Plant Reproduction, 5*(4), 286-291. https://doi.org/10.1007/BF00197380

Berry, E. A., Huang, L. S., & DeRose, V. J. (1991). Ubiquinol-cytochrome c oxidoreductase of higher plants. Isolation and characterization of the bc1 complex from potato tuber mitochondria. *Journal of Biological Chemistry, 266*(14), 9064-9077.

Cho, K., Torres, N. L., Subramanyam, S., Deepak, S. A., Sardesai, N., Han, O., … Rakwal, R. (2006). Protein extraction/solubilization protocol for monocot and dicot plant gel-based proteomics. *Journal of Plant Biology, 49*(6), 413-420. https://doi.org/10.1007/BF03031120

Choi, D. S., & Hwang, B. K. (2011). Proteomics and functional analyses of pepper abscisic acid-responsive 1 (ABR1), which is involved in cell death and defense signaling. *The Plant Cell, 23*(2), 823-842. http://doi.org/10.1105/tpc.110.082081

Duca, M., Port, A., Orozco-Cardenas, M., & Lovatt, C. (2008). Gibberellin-induced gene expression associated with cytoplasmic male sterility in sunflower. *Biotechnology & Biotechnological Equipment, 22*(2), 691-698. https://doi.org/10.1080/13102818.2008.10817536

Dunwell, J. M., Culham, A., Carter, C. E., Sosa-Aguirre, C. R., & Goodenough, P. W. (2001). Evolution of functional diversity in the cupin superfamily. *Trends in Biochemical Sciences, 26*(12), 740-746. https://doi.org/10.1016/S0968-0004(01)01981-8

Fan, Z., Stefansson, B. R., & Sernyk, J. L. (1986). Maintainers and restorers for three male-sterility—Inducing cytoplasms in rape (*Brassica napus* L.). *Canadian Journal of Plant Science, 66*(2), 229-234. https://doi.org/10.4141/ cjps86-036

Gothandam, K. M., Kim, E. S., & Chung, Y. Y. (2007). Ultrastructural Study of Rice Tapetum under Low-Temperature Stress. *Journal of Plant Biology, 50*(4), 396. https://doi.org/10.1007/BF03030674

Hansen, N. J. P., & Andersen, S. B. (1998). In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). *Euphytica, 102*(1), 101-108. https://doi.org/10.1023/A:1018348 816205

Hanson, M. R., & Bentolila, S. (2004). Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *The Plant Cell, 16*(Suppl. 1), S154-S169. https://doi.org/10.1105/tpc.015966

Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annual Review of Biochemistry, 54*(1), 1015-1069. https://doi.org/10.1146/annurev.bi.54.070185.005055

Jordaan, J. P. (1996). Hybrid wheat: advances and challenges. In M. P. Reynolds, S. Rajaram, & A. McNab (Eds.), *Increasing Yield Potential in Wheat: Breaking the Barriers* (Vol. 66, pp. 66-67). CIMMYT Press, Mexico.

Lapik, Y. R., & Kaufman, L. S. (2003). The Arabidopsis cupin domain protein AtPirin1 interacts with the G protein α-subunit GPA1 and regulates seed germination and early seedling development. *The Plant Cell, 15*(7), 1578-1590. https://doi.org/10.1105/tpc.011890

Lee, S., Lee, E. J., Yang, E. J., Lee, J. E., Park, A. R., Song, W. H., & Park, O. K. (2004). Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. *The Plant Cell, 16*(6), 1378-1391. http://doi.org/10.1105/tpc.021683

Lee, Y. P., Park, S., Lim, C., Kim, H., Lim, H., Ahn, Y., … Kim, S. (2008). Discovery of a novel cytoplasmic male-sterility and its restorer lines in radish (*Raphanus sativus* L.). *Theoretical and Applied Genetics, 117*(6), 905-913. https://doi.org/10.1007/s00122-008-0830-3

Li, J. M., & Yuan, L. P. (2010). Hybrid rice: Genetics, breeding, and seed production. In J. Janick (Ed.), *Plant Breeding Reviews* (Vol. 17, pp. 15-158). John Wiley & Sons, New York. https://doi.org/10.1002/ 9780470650134.ch2

Li, L. J., Ru, Z. G., Gao, Q. R., Jiang, H., Guo, F. Z., Sun, Z., & Wu, S. W. (2009). Analysis on the fertility of wheat male-sterile line BNS and its F_1 hybrid. *Journal of Triticeae Crop, 4*, 008.

Li, L. J., Ru, Z. G., Gao, Q. R., Jiang, H., Guo, F. Z., Sun, Z., & Wu, S. W. (2009). Male sterility and t hermo-photosensitivity characteristics of BNS in wheat. *Scientia Agricultura Sinica, 42*(9), 3019-3027.

Li, S., Wan, C., Kong, J., Zhang, Z., Li, Y., & Zhu, Y. (2004). Programmed cell death during microgenesis in a Honglian CMS line of rice is correlated with oxidative stress in mitochondria. *Functional Plant Biology, 31*(4), 369-376. https://doi.org/10.1071/FP03224

Liu, Y. P., Ru, Z. G., Chen, X. Y., Li, H., Li, Y. J., Zhao, A. J., & Su, Z. Q. (2011). Study on Fertility Alteration of Wheat Male Sterile Line BNS in Shijiazhuang. *Journal of Hebei Agricultural Sciences, 6*, 017.

Macdonald, H. (1997). Auxin perception and signal transduction. *Physiologia Plantarum, 100*(3), 423-430. http://doi.org/10.1111/j.1399-3054.1997.tb03046.x

Pandey, S., Nelson, D. C., & Assmann, S. M. (2009). Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. *Cell, 136*(1), 136-148. https://doi.org/10.1016/j.cell.2008.12.026

Röbbelen, G. (1991). Citation at the occasion of presenting the GCIRC Superior Scientist Award to FU Tingdong. *Proceedings of 8th International Rapeseed Congress*. Sasktoon, Canada.

Sabar, M., Gagliardi, D., Balk, J., & Leaver, C. J. (2003). ORFB is a subunit of F1 FO-ATP synthase: Insight into the basis of cytoplasmic male sterility in sunflower. *EMBO Reports, 4*(4), 381-386. https://doi.org/10.1038/sj.embor.embor800

Sakata, T., Oshino, T., Miura, S., Tomabechi, M., Tsunaga, Y., Higashitani, N., ... Higashitani, A. (2010). Auxins reverse plant male sterility caused by high temperatures. *Proceedings of the National Academy of Sciences, 107*(19), 8569-8574. http://doi.org/10.1073/pnas.1000869107

Santner, A., & Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. *Nature, 459*(7250), 1071-1078. https://doi.org/10.1038/nature08122

Shiro, I., & Yoshida, S. (2002). Characteristics of the Cytoplasmic Male Sterility in the Eggplant (*Solanum melongena* L.) Carrying the Cytoplasm of S. violaceum Ort. *Bulletin Faculty Agricultural of Saga Univerity, 87*, 87-93.

Song, X. Y., He, B. R., Ma, L. J., Hu, Y. G., & Li, H. B. (2005). Genetic study of thermo-sensitive male sterility on thermo-sensitive male sterile wheat line A3314. *Scientia Agricultura Sinica, 38*, 1095-1099.

Sreevalli, Y., Baskaran, K., & Kulkarni, R. N. (2003). Inheritance of functional male sterility in the medicinal plant, periwinkle. *The Indian Journal of Genetics & Plant Breeding, 63*, 365-366.

Takada, K., Ishimaru, K., Kamada, H., & Ezura, H. (2006). Anther-specific expression of mutated melon ethylene receptor gene Cm-ERS1/H70A affected tapetum degeneration and pollen grain production in transgenic tobacco plants. *Plant Cell Reports, 25*(9), 936-941. http://doi.org/10.1007/s00299-006-0147-0

Tsuda, K., Tsvetanov, S., Takumi, S., Mori, N., Atanassov, A., & Nakamura, C. (2000). New members of a cold-responsive group-3 Lea/Rab-related Cor gene family from common wheat (*Triticum aestivum* L.). *Genes & Genetic Systems, 75*(4), 179-188. http://doi.org/10.1266/ggs.75.179

Vedel, F., Lalanne, É., Sabar, M., Chétrit, P., & De Paepe, R. (1999). The mitochondrial respiratory chain and ATP synthase complexes: Composition, structure and mutational studies. *Plant Physiology and Biochemistry, 37*(9), 629-643. https://doi.org/10.1016/S0981-9428(00)80093-5

Vijayalakshmi, D., & Bangarusamy, U. (2007). Biochemical mechanisms leading to male sterility in thermosensitive genic male sterile rice. *Indian Journal of Agricultural Research, 41*(2), 112-116.

Virmani, S. S. (2003). Advances in hybrid rice research and development in the tropics. In S. S. Virmani, C. X. Mao, & B. Hardy (Eds.), *Hybrid Rice for Food Security, Poverty Alleviation, and Environmental Protection, Proceedings of the 4th International Symposium on Hybrid Rice* (pp. 7-20). Hanoi, Vietnam.

Virmani, S. S., & Ilyas-Ahmed, M. (2001). Environment-sensitive genic male sterility (EGMS) in crops. *Advances in Agronomy, 72*, 139-195. https://doi.org/10.1016/S0065-2113(01)72013-5

Wang, K., Gao, F., Ji, Y., Liu, Y., Dan, Z., Yang, P., ... Li, S. (2013). ORFH79 impairs mitochondrial function via interaction with a subunit of electron transport chain complex III in Honglian cytoplasmic male sterile rice. *New Phytologist, 198*(2), 408-418. https://doi.org/10.1111/nph.12180

Wang, M. T., Gao, Q. R., Sun, Z. J., Yuan, K., Yu, S., Zhang, B. L., … Ru, Z. G. (2011). Spike differentiation process and the male sterility of BNS in wheat. *Molecular Plant Breeding, 9*, 294-301.

Wen, F. P., Zhang, Z. H., Bai, T., Xu, Q., & Pan, Y. H. (2010). Proteomics reveals the effects of gibberellic acid (GA3) on salt-stressed rice (*Oryza sativa L.*) shoots. *Plant Science, 178*(2), 170-175. https://doi.org/10.1016/j.plantsci.2009.11.006

Yang, J. H., Huai, Y., & Zhang, M. F. (2009). Mitochondrial atpA gene is altered in a new orf220-type cytoplasmic male-sterile line of stem mustard (*Brassica juncea*). *Molecular Biology Reports, 36*(2), 273-280. https://doi.org/10.1007/s11033-007-9176-1

Zhang, B. L., Zhang, W. D., Gao, Q. R., Wang, M. T., Li, N. N., Zhang, Y. Y., … Ru, Z. G. (2013). Genetic Analysis on Male Sterility of Thermo-Photo-Sensitive Male Sterile Line BNS in Wheat. *Scientia Agricultura Sinica, 16*, 1533-1542.

Zhang, L. P., Zhao, C. P., Shan, F. H., Zhang, F. T., & Ye, Z. J. (2007). The mixed genetic analysis of photoperiod-temperature sensitive male sterility of BS210 in wheat. *Acta Agronomica Sinica, 33*, 1553-1557.

Zhao, C. P., Wang, X., Zhang, F. T., Ye, Z. J., & Dai, H. J. (1999). Advances of hybrid wheat and two-line system of photo-thermo sensitivity. *Beijing Agric. Sci., 17*(2), 3-5.

Zhou, M. L., Ru, Z. G., Luo, Y. Q., Luo, P., Li, Q., Guo, X. K., & Zhou, P. (2010). Male fertility transformation of two-line wheat sterile lines BNS. *Journal of Nuclear Agricultural Sciences, 24*(5), 887-894.

Zhou, Y., He, Z, H., Chen, X. M., Wang, D. E., Zhang, Y., & Zhang, G. S. (2007). Genetic Gain of Wheat Breeding for Yield in Northern Winter Wheat Zone over 30 Years. *Europe PMC, 33*, 1530-1535.

Zhu, Y., Dun, X., Zhou, Z., Xia, S., Yi, B., Wen, J., ... Fu, T. (2010). A separation defect of tapetum cells and microspore mother cells results in male sterility in Brassica napus: the role of abscisic acid in early anther development. *Plant Molecular Biology, 72*(1-2), 111-123. http://doi.org/10.1007/s11103-009-9556-0