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Anther proteomic characterization in temperature sensitive Bainong male sterile wheat

Y.-Y. LI[1], Y. LI[1,2,3], Q.-G. FU[1], H.-H. SUN[1], and Z.-G. RU[1]*

Henan Institute of Science and Technology, Xinxiang, Henan453003, P.R. China[1]
Institute of Plant Physiology and Ecology, SIBS, CAS, Shanghai, P.R. China[2]
Shanghai Yeslab Biotechnology, Shanghai, P.R. China[3]

Abstract

Male sterile line is a useful material for hybridization, but its sterility mechanism, especially proteomic profile, is still not entirely clear. In wheat (*Triticum aestivum* L.), a male sterile Bainong (BNS) genotype whose sterility could be manipulated by temperature was recently selected. We focused on the proteomic profile change of anthers from the male sterile line (SL) and its conversional line (CL). Two-dimensional gel electrophoresis and MALDI-TOF-MS technologies were utilized for proteomic profiles analysis. Differently abundant protein spots (over 2-fold, $P < 0.05$) were selected for identification analysis. Compared to CL, 24 up-regulated and 23 down-regulated proteins were identified in SL. Protein metabolism-related proteins, which included a ubiquitin-conjugating enzyme E2 (23 kDa) and an ATP-dependent Clp protease proteolytic subunit, were over-accumulated in SL anthers. Alcohol dehydrogenase ADH1A, fructose-bisphosphate aldolase, chloroplast fructose-bisphosphate aldolase, and NADP-dependent malic enzyme were notably down-regulated in SL anthers. Up-regulated prohibitin protein Wph and a translationally-controlled tumor protein homolog, and down-regulated histone acetyltransferases HAT1 and HAT2, and DNA directed RNA polymerase subunit α were identified in SL anthers. These dramatically changed proteins may play a crucial role in abnormal anther development and pollen abortion in BNS.

Additional key words: cytoplasmic male sterility, protein degradation, proteome, *Triticum aestivum.*

Introduction

Male sterility widely exists in the plant kingdom and attention has been paid especially in the main crops such as maize, rice, and wheat when it has been employed in hybrid production. The mechanisms of male sterility (nuclear male sterility, cytoplasmic male sterility, and nuclear-cytoplasmic male sterility) were summarized in detail by Kaul (1988). Both cytoplasmic- and nuclear-encoded fertility restorer (*Rf*) genes are involved in gene-cytoplasmic male sterility (CMS). More genes, such as chimaeric mitochondrial gene in cytoplasmic male sterile rice (*ORFH79)*, cyclooxygenase 2 (*cox2*), and chimaeric open reading frame from cytoplasmic male-sterile wheat (*orf256*) were found to be involved in induction of CMS (Song and Hedgcoth 1994, Ducos *et al.* 2001, Wang *et al*. 2013). A mitochondrial gene with a unique chimaeric sequence which codes a 13 kDa polypeptide (URF13) in mitochondria from the T male-sterile cytoplasm (cms-T) maize may play an important role in male sterility (Forde *et al.* 1978, Dewey *et al.* 1987). In genic male sterility (GMS), nuclear genes are the main functional factors (Chaudhury 1993). In *Arabidopsis*, genes of *apetala-3*, *pistillata*, and *antherless* affect floral morphology and arise male sterility. *CUT1* is another kind of genes which are involved in plant cuticular wax synthesis and its mutation results in male sterility for a waxless pollen (Millar *et al.* 1999).

Texas (T) cytoplasmic male sterility has been widely used in 1960s for its highly stable character under

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Abbreviations: BNS - Bainong male sterility; CL - conversional line; GDH - glutamate dehydrogenase; GMS - genic male sterility; ROS - reactive oxygen species; SHMT - serine hydroxymethyltransferase; sHSPs - small heat shock proteins; SL - sterile line; TCTP - translationally-controlled tumor protein homolog.
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* Corresponding author; e-mail: ruzhengang@163.com

different conditions. Transgenic male sterile plants with a chimaeric ribonuclease gene have been developed (Mariani *et al*. 1990,1991).

The chimaeric ribonuclease gene of *barnase* (extracellular RNase from *Bacillus amyloliquefaciens*) and a tapetum-specific promoter (*TA29*) are expressed in anthers and destroy the tapetal cell layer causing male sterility (Mariani *et al.* 1990). A gene of extracelluar invertase encoding isoenzyme Nin88 shows a spatial and temporal expression in anthers and its repression in an antisense construct leads to male sterility through blocking pollen development (Goetz *et al.* 2001). These results provide a new way for utilization of male sterility in agriculture.

A wheat Bainong male sterile (BNS) line was newly found and it is temperature sensitive (Li *et al.* 2009, Zhou *et al.* 2010). Pollen abortion in the line takes place under 10 °C before pollen meiosis, and pollen normally develops when a temperature is over 13 °C. Convert conditions between male sterile and male fertile could be manipulated by a sowing time. It has been reported that BNS pollen abortion takes place during the bi-nucleic pollen stage (Su *et al.* 2011) which is critical for microspore abortion in BNS and its proteomic profile is less known. In this study, we focused on proteomic differences between a male sterile line (SL) and a conversional line (SL) with the aim to contribute to understanding the mechanism of pollen abortion in BNS.

Materials and methods

Plants cultivation and anther collection: A wheat (*Triticum aestivum* L.) BNS line was planted at the experimental station of the Henan Institution of Science and Technology, Huixian, China, during 1st October to 18th November 2012. This time was divided into seven periods with eight days for each period. The ears of SL were collected from 11th to 19th April 2013 and of CL from 1th to 6th May 2013. Anthers from plants which were sown on 1st October and 9th October displayed a male sterile feature. The anthers were collected at 7:00, and a distance between the flag leaf and the second leaf from the top was 6 - 15 cm. By microscopy assay, the length of the anthers in the uni-nucleic period was 2 mm. Three pieces of the anthers from the first flower in the upper part of spikelets were peeled off on ice. Then the materials were frozen in liquid nitrogen for 1 min and then stored in a freezer at -70 °C. In order to ensure sample sterility correctly, the line from which the samples were collected was labelled in the field, and after 20 d of pollination, a seed set rate were checked. Those anthers collected from the line whose self-seed set rate was zero were selected for protein separation.

The plants sowed after 10th November were CL. In CL, samples were collected at the same developmental period and by the same methods. Those anthers from the plants whose self-seed set rate was over 30 - 70 % were selected for next protein analysis.

Protein extraction: Total proteins from anthers were extracted by the trichloroacetic acid (TCA) method (Damerval *et al.* 1986, Link and LaBaer 2011). Each sample of 2 g was grinded under liquid nitrogen with 10 % (m/v) polyvinylpyrrolidone (PVP) for 15 min and transferred to a 50-cm^3 centrifuge tube with 12 cm^3 of 10 % (m/v) TCA and 12 cm^3 of 30 mM dithiothreitol (DTT), followed by keeping it in a freezer at -20 °C for 2 h. Samples were then centrifuged at 20 000 *g* and 4 °C for 30 min and three volumes of pure acetone and 10 mM DTT dissolved in distilled water were added to precipitates. These mixtures were vortexed and put into a freezer at -20 °C for 0.5 h. This procedure was repeated twice until the supernatant was colorless. After centrifugation at 20 000 *g* and 4 °C for 30 min, three volumes of acetone and 10 mM DTT dissolved in distilled water were added to precipitates. After the mixture was vortexed and kept at -20 °C for 0.5 h, the samples were centrifuged at 20 000 *g* and 4 °C for 30 min. The precipitates were drained in a vacuum freeze dryer and dissolved with 2M thiourea and a *NP40* protein lysate, followed by adding 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM Na_2EDTA, and 20 mM DTT. After sonication for 10 min, the samples were centrifuged at 35 000 *g* for 30 min and the supernatants were vacuum dried. Lysate liquid (300 mm^3) which contained 8 M urea, 2 % (m/v) 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 50 mM DTT, and 0.5 % (v/v) *Pharmalyte* (*Amersham Bioscience*, Piscataway, NJ, USA) was added to the dried protein (3.0 mg). After shaking for 10 min, the samples were centrifuged at 20 000 *g* for 15 min and the supernatants were collected and stored at -80 °C. The Richard's method was utilized for protein quantity assay (Simpson 2003).

Protein identification: For the first dimensional electrophoresis, each sample of 120 μg in a solution containing 8 M urea, 2 % (m/v) CHAPS, 65 mM DTT, and 0.5 % (v/v) immobiline pH gradient (IPG) buffer was loaded into *Immobiline Drystrip* gels (18 cm, pH 3 - 10, *GE Healthcare*, Piscataway, NJ, USA) in an *IPG-phor* system (*Amersham Bioscience*). Isoelectric focusing (IEF) was performed under 30 V for 6 h, 60 V for 6 h, 500 V for 1 h, 1 000 V for 1 h, and 8 000 V for 20 h untill it reached 64 000 Vh. After IEF, IPG strips were transferred to an equilibration solution consisting of 50 mM Tris-HCl, 6 M urea, 20 % (v/v) glycerol, and 2 % (m/v) sodium dodecyl sulphate (SDS) containing 130 mM DTT (equilibration buffer I) and 135 mM

iodoacetamide (buffer II), and vortexed with each buffer for 15 min. The experiments were repeated three times for each group. After visualization by silver nitrate staining, the gels were scanned by *Artix Scan 1010 plus* (*Microtek*, Taipei, Taiwan) and analyzed by the *Image Master*^{TM} *2D* platinum software (*GE Healthcare*, Piscataway, NJ, USA). The protein spots were quantified, background substracted, and differentially abundant protein spots were selected using a criterion of at least a 2-fold change at a 0.05 level determined by the Student's *t*-test (*Shanghai Yeslab Biotechnology*, Shanghai, China).

The protein spots were excised to a 96-well plate and fragmented by an ultrasonic system for 5 min. Fragments were unstained with acetonitrile and dried in vacuum for 5 min. The fragments were heated in a water bath with 10 mM DTT and 25 mM NH_4HCO_3 at 56 °C for 1 h. Under the room temperature, 55 mM iodoacetamide and 25 mM NH_4HCO_3 were added and the samples placed in the dark for 45 min. The fragments were washed with 25 mM NH_4HCO_3, 50 % (v/v) acetonitrile and pure acetonitrile, respectively. Trypsin (10 ng mm^{-3}; *Promega*, Madison, WI, USA) diluted with 25 mM NH_4HCO_3 was transferred to well plate on ice for 30 min. These selected protein spots were covered by 25 mM NH_4HCO_3 at 37 °C for one night. Enzymolysis was terminated with 0.1 % (m/v) trifluoroacetic acid .

The tryptic peptides were identified by mass spectrometry *Autoflex II MALDI-TOF/MS* (*Bruker Daltonics*, Billerica, MA, USA) in a positive mode. Peptide mass fingerprint (PMF) maps were analyzed by *Flexanalysis 3.0* with a *SNAP* algorithm and a signal-to-noise ratio 1.5 (*Bruker Daltonics*, Billerica, MA, USA). The mascot search engine from *Matrixscience* (www.matrixscience.com) was utilized, and peptide mass fingerprint data were inputted to search against the *NCBI* non-redundant database (2014/3/22) with theparameter setting of trypsin cleavage, peptide mass tolerance of ± 0.2 Da, and max missed cleavages 1 (Perkins *et al.* 1999).

Quantitative RT-PCR: Wheat anthers were isolated and collected on ice and incubated in 1 cm^3 of *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA). Total RNA of anthers was extracted according to the manufacturer's instruction. Reverse transcription was done with *M-MLV* reverse transcriptase (*Promega*) following the manufacturer's protocol. Each gene specific primer was designed by *Primers 5* (Table 3 Suppl.). Specificities of designed genes primers were assayed by melting curves. Quantitative real time PCR was performed in a 20 mm^3 volume which contained 1 mm^3 of cDNA, 0.5 mm^3 of a primer, 10 mm^3 of *2*SYBR Green*, 5 mm^3 of a Q-PCR enhancer (*RP202*, *Tiangen*, Beijing, China), and 3 mm^3 of distilled water as follows: pre-denaturation at 94 °C for 2 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s. Triplicate samples for each group were selected for quantitative PCR and the procedure was performed in a *Lightcycler 480* (*Roche*, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as reference gene. The gene expression was normalized by *GAPDH*, and differently expressed genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Data analysis: Differently abundant proteins were log-transformed and hierarchically clustered by the centroid linkage method using *Cluster v. 3.0* and visualized with *cluster programs of Java TreeView V1.1.6r2* (Eisen *et al.* 1998, De Hoon *et al.* 2004). Sequences and identification of selected proteins were searched against the database of *AgBase_Community* and *UniProt* using the software of *AgBase*, *Blast2GO*, and *ClueGO* for gene-ontology annotations (Shannon *et al.* 2003, Conesa *et al.* 2005, McCarthy *et al.* 2006, Bindea *et al.* 2009). *Kyoto Encyclopedia of Genes and Genomes* (*KEGG*), which is a database resource for understanding high-level functions and utilities of the biological system, was employed to analyze different abundant proteins (Kanehisa and Goto 2000). Differently accumulated proteins and expressed genes were analysed separately by the *t*-test (*SAS v. 8*).

Results

By two-dimensional gel electrophoresis, 36 over-accumulated protein spots and 31 down-accumulated protein spots were detected in SL compared to CL with expression changes more than 2-fold ($P \leq 0.05$; Fig. 1). With the *MALDI-TOF/MS* assay, 54 protein spots which included 47 unique proteins were successfully identified according to the *MASCOT* method (Tables 1, 2 Suppl., Fig. 2). Protein spots Nos. 65, 67, and 80 were identified as the same protein AF433653_1 (a small Ras-related GTP-binding protein). Protein spots 79, 98, 99, and 154 were identified as the same protein ACO90196 (ascorbate peroxidase). Protein spots 551 and 801 were identified as AEK78081 (a 26S proteasome non-ATPase regulatory subunit). Protein spots 1035 and 1465 were identified as ACO44684 (fructose-bisphosphate aldolase). In total, 47 unique proteins which included 24 up-regulated and 23 down-regulated proteins in SL were hierarchically clustered based on the Pearson correlation and visualized by a tree view tool (Eisen *et al.* 1998, De Hoon *et al.* 2004). All samples were clustered to two groups of SL and CL in the top of the dendrogram, which implies that the data within the groups have high consistency (Fig. 2). Viewed from the left protein tree structure, differently abundant proteins were mainly clustered to two sets of up- and down-regulated proteins (Fig. 2).

The protein sequences of these two sets were

annotated by the *AgBase GOanna*, *Blast2GO*, and *ClueGO* databases. Each listed protein was annotated with a gene ontology term (Table 1 Suppl.). The differently abundant proteins were mainly related to metabolism, nutrition, pollen development, protein cleavage, protein labelling for degradation, cellular components, organelle structure, transcriptional regulation, and stress responses.

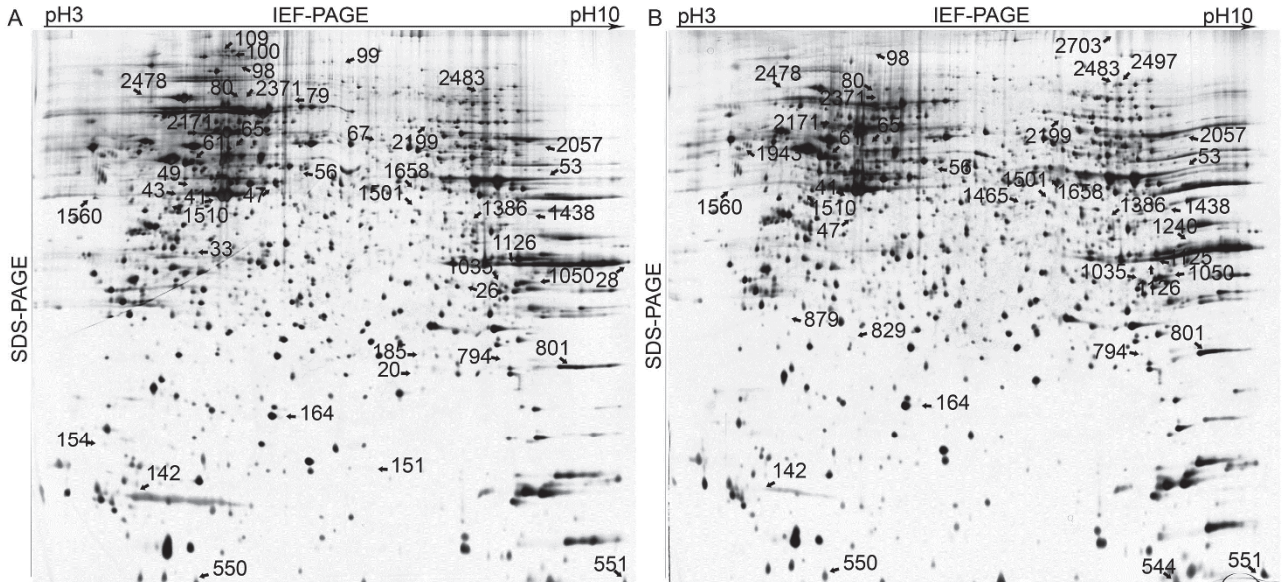

Fig. 1. The 2-D map of anther proteins from Bainong male sterility and conversional lines performed on IPG gel strips followed by vertical SDS-PAGE. Identified proteins are marked with *arrow*s and a code number. *A* - Over-accumulated proteins in the BNS anthers. *B* - Over-accumulated proteins in the conversional line anthers.

The up-regulated proteins in SL included proteins located in plastids, plasma membranes, and mitochondria (Fig. 3*A*). Viewed from the function level, the differently abundant proteins which were involved in the ion binding and oxidoreductase activity accounted for 59 %, and others were mainly involved in enzyme activities, *e.g.*, GTPase activity (Fig. 3*B*), responses to stresses, and others (Fig. 3*C*).

In the down-regulated proteins in SL, we found a protein complex in the cytosol, plastids, mitochondria, and plasma membranes, and each was over 10 % (Fig. 3*D*). The ion binding and oxidoreductase activity consisted of 58 % (Fig. 3*E*). These down-regulated proteins were mainly involved in a response to stress, nitrogen metabolism, and biosynthetic and catabolic processes (Fig. 3*F*).

The database and tool of *Kyoto Encyclopedia of Genes and Genomes* (*KEGG*) was employed to analyze enrichment pathways which these differently accumulated proteins were involved in (Tables 1 and 2 Suppl.). Forty-seven unique proteins were inputted and top 10 were identified according to *KEGG* (Fig. 3*G*). Alcohol dehydrogenase ADH1A (ABL74258.1 and ABL74263.1), a pyruvate dehydrogenase E1 component alpha subunit, fructose-bisphosphate aldolase, and fructose-bisphosphate aldolase of a chloroplast precursor were involved in the glycolysis/ gluconeogenesis pathway (Fig. 3*G*). Four differently accumulated proteins were involved in the metabolism of glycine, serine, and threonine, and in carbon fixation. Alcohol dehydrogenase participates in four metabolism pathways (glycolysis/gluconeo-genesis, glycine, serine, and threonine metabolism, metabolism of cytochrome

Table 1. The transcriptional expression of nine representative protein genes by RT-PCR analysis and their related protein abundances. Means ± SE, $n = 3$.

Gene/protein	Group	Protein abundance	mRNA expression
CLP	SL	0.1322 ± 0.0653	5.4051 ± 0.6877
	SC	0.0148 ± 0.0256	1.0089 ± 0.0767
TTTP1	SL	0.0179 ± 0.001	1.1466 ± 0.1029
	SC	0.0043 ± 0.0075	0.7372 ± 0.0156
26SPA	SL	0	0.0113 ± 0.0015
	SC	0.0028 ± 0.0017	0.0156 ± 0.0027
HSP23.6	SL	0.0025 ± 0.001	0.8766 ± 0.1175
	SC	0	1.1460 ± 0.1224
HAT1	SL	0	0.0570 ± 0.0016
	SC	0.0045 ± 0.0009	0.0272 ± 0.001
HAT2	SL	0.0071 ± 0.0063	0.0038 ± 0.0003
	SC	0.0357 ± 0.0075	0.0021 ± 0.0003
RFTN	SL	0.0135 ± 0.0233	1.9740 ± 0.0064
	SC	0.0524 ± 0.0064	0.8502 ± 0.1147
SSBP1	SL	0.0405 ± 0.0404	0.5434 ± 0.0298
	SC	0.1118 ± 0.0045	0.3201 ± 0.029
26SPN	SL	0.0808 ± 0.0532	0.0353 ± 0.0034
	SC	0.2208 ± 0.0456	0.0211 ± 0.003

P_{450}, and fatty acid degradation). The protein putative pyruvate dehydrogenase E1 component alpha subunit was involved in the pathway of glycolysis/ gluconeogenesis, glutathione metabolism, and pentose phosphate and pyruvate metabolism.

Further, anthers were isolated from SL and CL and the expression of related genes were assayed by quantitative real-time PCR. Compared to CL, the mRNA expressions of an ATP-dependent Clp protease proteolytic subunit and of a translationally-controlled tumor protein homolog were up-regulated and their changes were consistent with their proteins abundance (Table 1). In SL, a 26S proteasome ATPase subunit was down-regulated compared to CL (Table 1). This protein displayed a low expression in SL and showed a high consistency with the mRNA expression (Table 1). In the other part, a 26S proteasome non-ATPase regulatory subunit, protein RAFTIN 1A, histone acetyltransferase HAT1, histone acetyltransferase HAT2, a single-stranded nucleic acid binding protein, and small heat shock protein Hsp23.6 showed a poor consistency between their mRNA expression and proteins abundance in SL and CL (Table 1).

Discussion

Male sterile plants fail to produce pollen, which is caused by multiple factors, such as mutation, diseases, and abnormal conditions (Kaul 1988). This processes mainly occur in anthers (Mariani *et al.* 1990, Chaudhury 1993, Clément and Audran 1995, Clément *et al.* 1996). The cytotoxicity, energy deficiency, cell death, and retrograde regulation may be partly accounted for plant male sterility (Chen and Liu 2013). Here we report that the proteomic profile of anther changed between BNS and the conversional line and we tried to understand the mechanism of CMS.

Proteins play an important role in an organ developmental process and their expressions are extremely changed between sterile and fertile anthers. It was reported that the chimaeric ribonuclease gene of *barnase* and *TA29* selectively destruct the tapetal cell layer and cause male sterility in transformed tobacco (Mariani *et al.* 1990). An ATP-dependent Clp protease proteolytic subunit and ubiquitin-conjugating enzyme E2 of 23 kDa were over-accumulated in SL wheat (Table 2 Suppl). RT-PCR results show that the *Clp* mRNA expression was consistent with its protein abundance (Table 1). Clp has the functions of translocation and protein degradation (Weber-Ban *et al.* 1999, Reid *et al.* 2001, Sjogren *et al.* 2006, Bewley *et al.* 2009). Ubiquitin (Ub) is a multifunctional protein and its functions lie mainly in labeling proteins for selective degradation. Under chilling stress, leaf wilting, reactive oxygen species (ROS) accumulation, and membrane damage is lower in sense plants than in antisense plants, which imply that up-regulated Ub is a useful strategy for chilling tolerance (Feng *et al.* 2014). An abounded ubiquitin-conjugating enzyme and Clp protease proteolytic subunit might lead to an excessive degradation of protein in SL and influenced pollen development.

The 26S proteasome is responsible for a selective protein degradation and removes modified proteins by ubiquitin (Voges *et al.* 1999, Vierstra 2009,). Deficiency of a regulatory particle non-ATPase subunit (RPN5) impairs embryo and seedling development and prevents pollen production by controlling factors involved in cytokine regulation, reducing the activity of proteasomes in a mutant, and participating in protein destruction (Smalle *et al.* 2002, Smalle *et al.* 2003, Ueda *et al.* 2004, Brukhin *et al.* 2005, Book *et al.* 2009). We found that the proteins of the 26S proteasome ATPase subunit and 26S proteasome non-ATPase regulatory subunit were down-regulated in SL compared to CL (Fig. 2). Their unexpected reduction might influence anthers development and pollen production in wheat through abnormal protein degradation under a low temperature. RT-PCR results show that the expression of 26SPA mRNA from SL decreased compared to CL (Table 1). However, the mRNA expression of 26SPN was higher compared to CL, which had a poor consistency with corresponding protein abundances (Table 1). It imply that the transcriptional regulation of 26SPA and 26SPN might be in a different way.

Histone acetyltransferases HAT1 and HAT2 were over-accumulated in CL (Table 1 Suppl.). HAT1 and HAT2 regulate cell proliferation and organ growth. HAT2 is involved in the regulation of the auxin mediated signalling pathway. However, the mRNA expression of *HAT1* and *HAT2* had a poor consistency with their protein abundances, which imply that the proteins might be influenced by a post-transcriptional modification and a translation regulation (Table 1). In contrast, deacetylation related proteins such as prohibitin protein Wph were up-regulated in SL (Fig. 1). Prohibitin is highly conserved evolutionarily and plays an important role in mitochondrial function, proliferation and development. Prohibitin is involved in phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt), in transforming growth factor-β (TGF-β)/signal transducers, and in the Ras-Raf-MEK-Erk pathway (Mishra *et al.* 2010, Chowdhury *et al.* 2013), which imply that prohibitin may perform its multifunctions in anther development. A translationally-controlled tumor protein homolog (TCTP) was also up-regulated in SL (Figs. 1, 2). TCTP is involved in the regulation of the cell cycle and cell growth. Although *TCTP* RNA is highly expressed in the developing and mature phloem in petioles, roots, and apices, in most

cells, the protein is expressed at a lower level and by further research the TCTP is supposed to be involved in translational regulation (Hinojosa-Moya *et al.* 2013) and may play an important role in the development of male sterility.

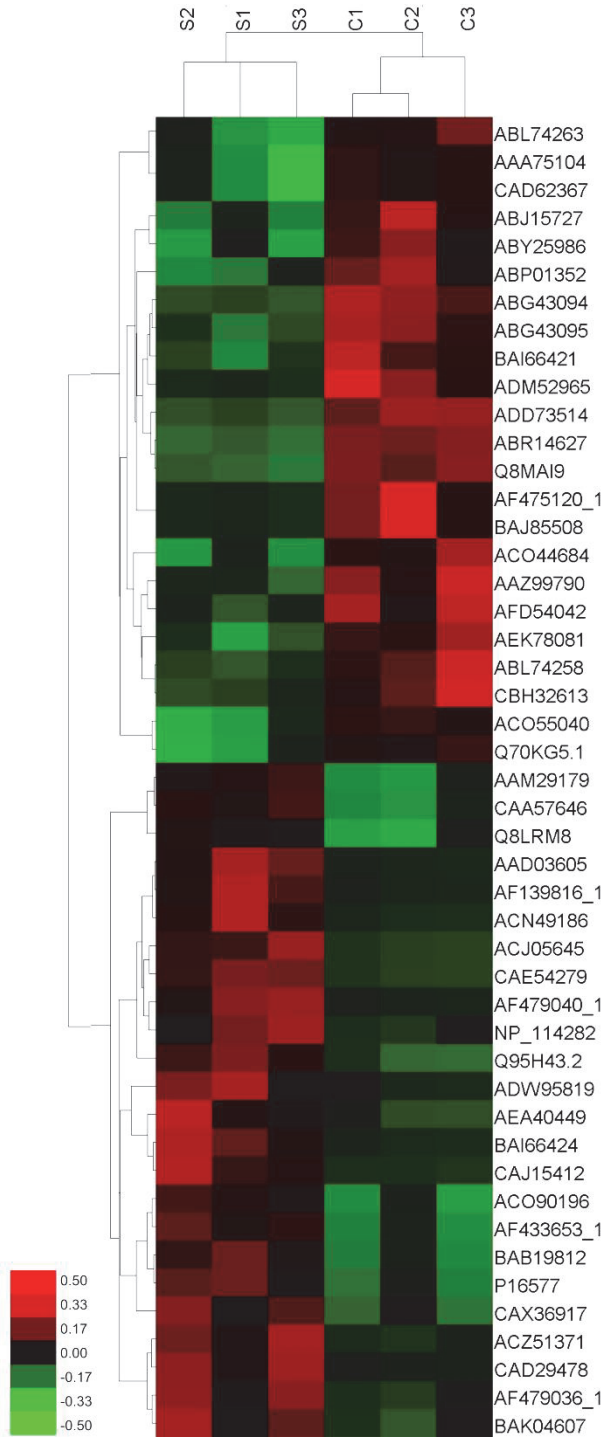

Fig. 2. The heatmap of protein expression from SL and CL by hierarchical clustering analysis. Samples were arranged in columns and identified proteins were hierarchically clustered with a centroid linkage in rows. *Colour blocks* represent differently expressed levels. *Red* - highly accumulated proteins, *green* - low accumulated proteins.

Proteins involved in ATP synthesis and metabolism were repressed in SL (Kaul 1988, Dorion *et al.* 1996, Chen and Liu 2013). The change of sugar supply influence plant reproductive development. The antisense-repression of extracellular invertase Nin88 blocks the early stage of pollen development and induces male sterility in tobacco (Goetz *et al.* 2001). We found that fructose-bisphosphate aldolase (FBA) and the FBA chloroplast precursor were more accumulated in CL compared to SL (Fig. 1). FBA is an important enzyme involved in glycolysis and gluconeogenesis. It was reported that FBA is important in the acquisition of fructose and pyruvate for plant growth, and its expression is negatively regulated by transcriptional regulator HrpG and the transcriptional activator of HrpX cascade (Guo *et al.* 2012). Ribulose-1,5-bisphosphate carboxylase/ oxygenase is an initial carboxylating enzyme and may be a major limiting factor of photosynthesis (Raines 2006). The overexpression of aldolase promotes ribulose-1,5-bisphosphate regeneration and CO_2 fixation (Uematsu *et al.* 2012). Thus, the down-regulation of FBA in SL might influence pollen development (Table 1 Suppl.).

Alcohol dehydrogenase catalyzes the conversion of acetaldehyde to alcohol and its activity is high in pollen and seeds (Dolferus and Jacobs 1984). Alcohol dehydrogenase in maturing and mature pollen grains is denoted ADH-P, which is a modified variant of the major protein ADH1 (Konovalov *et al.* 2003). In this study, ADH1A was down-regulated in SL compared to CL (Figs. 1, 2). The deficiency of ADH1A might repress SL tolerance to a stress.

Our results show that serine hydroxy-methyl-transferase (SHMT) was down-regulated in SL compared to CL (Fig. 1, 2). SHMT, which catalyzes glycine to serine conversion, is involved in photorespiration and salt tolerance in *Arabidopsis* (Somerville and Ogren 1981, Moreno *et al.* 2005). It has been reported that the photorespiratory SHMT activity in *Arabidopsis* requires the accumulation of ferredoxin-dependent glutamate synthase (Jamai *et al.* 2009). The down-regulation of SHMT in SL might depress photorespiration and influence resistance to a low temperature during anther development.

Phytochrome A is supposed to be involved in the regulation of mitochondrial respiration by affecting the expression of succinate dehydrogenase (Jumtee *et al.* 2009, Popov *et al.* 2010, Ganesan *et al.* 2012). We found that phytochrome A type 3 was over-accumulated in SL, which implies that this change might influence photomorphogenesis.

Protein RAFTIN is an anther specifically expressed protein essential for plant pollen development (Wang *et al.* 2003). RAFTIN is targeted to the microspore exine, presented in the pro-orbicule, and accumulated in Ubisch bodies. In our research, RAFTIN was down-regulated in

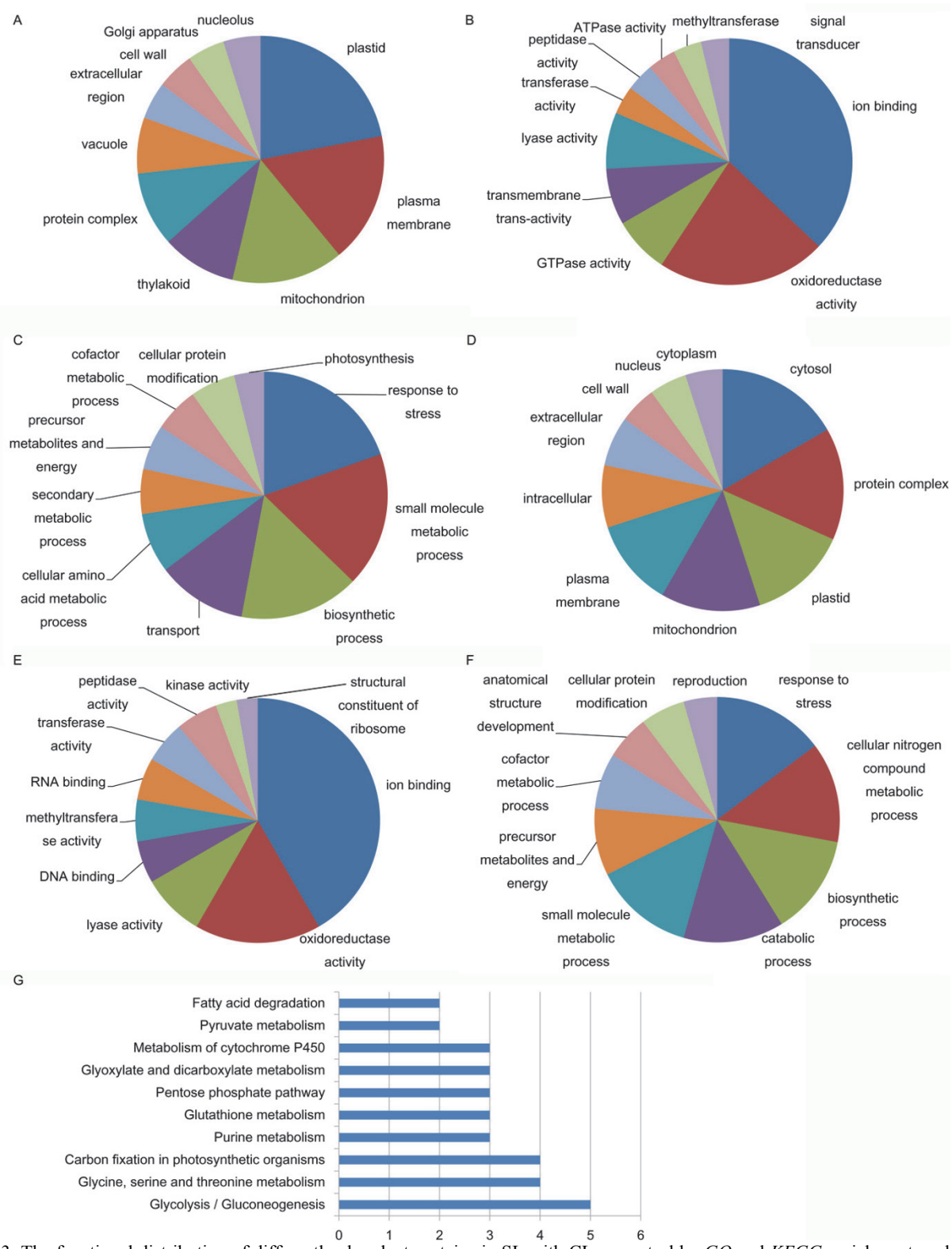

Fig. 3. The functional distribution of differently abundant proteins in SL with CL as control by *GO* and *KEGG* enrichment analysis. *A* - *GO* enrichment of cellular component classification in over-accumulated proteins in SL. *B* - Molecular function classification in ovr-accumulated proteins in SL. *C* - Biological process classification in over-accumulated proteins in SL. *D* - Cellular component classification in over-accumulated proteins in CL. *E* - Molecular function classification in over-accumulated proteins in CL. *F* - Biological process classification in over-accumulated proteins in CL. *G* - Top 10 enrichment metabolism pathways by *KEGG* analysis. The quantity of proteins involved in the pathway is represented by the length of the *horizontal bar*.

SL compared to CL (Figs. 1, 2), which might lead to the functional deficiency of anthers and influence the anther development in SL.

Small heat shock proteins (sHSPs) are a diverse and important protein family. They are molecular chaperones which are involved in preventing misfolding and an irreversible aggregation of target proteins (Waters 2013). Small heat shock protein Hsp23.6 was up-regulated in response to a low temperature in SL anthers (Table 2 Suppl.).

GTP-binding proteins are an important protein family. Under UV-radiation, a kind of a Ras-related small GTP-binding protein is involved in an early response (Fritz and Kaina 1997). We found that the small Ras-related GTP-binding protein was up-regulated in SL. ROS scavenging is an important function in plant cells for eliminating oxidative damage. Ascorbate has multiple functions and plays an important role in antioxidative defense through the ascorbate/glutathione cycle. Ascorbate peroxidase is a key enzyme for H_2O_2 metabolism (Ishikawa and Shigeoka 2008). The over-accumulated ascorbate peroxidase (Figs. 1, 2) and ferritin 1A might reflect the abrupt response to a stress and reduce oxidative damage in SL.

Alanine aminotransferases (AlaATs) are widely distributed in prokaryotes and eukaryotes and catalyse the reversible interconversion of alanine and 2-oxoglutarate to pyruvate and glutamate, respectively. It has been reported that peroxisomal AlaAT has the alanine-glyoxylate transaminase activity for glycine synthesis associated with photorespiration in *Arabidopsis* (Liepman and Olsen 2001). Mitochondrial isoform m-AlaAT catalyzes alanine synthesis, whereas glutamate synthesis using alanine as amino donor was inhibited under hypoxia, followed by the accumulation of alanine as major amino acid instead of asparagine in *Medicago truncatula* seedlings (Ricoult *et al.* 2006). The over-accumulation of AlaATs in SL (Table 2 Suppl.) imply that AlaATs might be involved in development of male sterility.

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