ORIGINAL ARTICLE

Quantitative shotgun proteomic analysis of cold-stressed mature rice anthers

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Abstract Rice crops are vulnerable to low temperatures. During development, the reproductive stage is particularly sensitive to cold exposure, which causes abnormal pollen development and a high degree of male sterility. In this study, shotgun proteomic analysis was used to analyze rice anthers containing pollen grains from a cold-tolerant variety, Dianxi 4. Protein expression was compared between normal anthers and anthers exposed to cold temperatures at the young microspore stage. In total, 3835 non-redundant proteins were identified in the rice anther. Of these, 441 proteins were differentially expressed between normal and cold-treated anthers. Pollen allergens, ATP synthase, actin, profilin, and β-expansin proteins were highly abundant, reflecting anther development, pollen germination, and pollen tube elongation. Starch and sucrose metabolic proteins such as α-amylase precursor and 4-α-glucanotransferase exhibited reduced expression after cold exposure. Among the proteins that exhibited increased expression after cold exposure, C2 domain proteins, and GRPs were identified as candidate signaling factors for mediation of the cold tolerance response. Through high-throughput proteomic analysis we were able to reveal proteomic changes against cold stress and suggest two signaling factors as the candidate genes.

Keywords Cold stress · Early young microspore · Shotgun Proteomics · Rice anther

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Introduction

Plants are affected by various environmental stresses during their life-cycles, such as low temperature, high temperature, high soil salt concentration, and drought. Exposure of plants to low temperatures induces physical and biochemical changes that have particularly serious effects. At the cellular level, low temperature exposure leads to several changes, including alterations to protein and carbohydrate composition and abundance of unsaturated fatty acids that result in physiological symptoms such as wilting, chlorosis, and leaf expansion (Schutzki and Cregg 2007). Low temperature stress experienced during the reproductive phase causes structural and functional abnormalities in reproductive organs that lead to fertilization failure. Rice is vulnerable to low temperatures, particularly during the reproductive stage (Nishiyama 1995). Cytological and histological abnormalities observed in cold-treated rice were more severe in the anthers than in the pistil or other organs of the flower, and fertility was rescued by artificial pollination with healthy pollen collected from non-stressed florets (Satake and Hayase 1970). Exposure to low temperature at the onset of microspore release caused tapetum hypertrophy and disordered microspore development that consequently gave rise to a

high degree of male sterility (Hayase et al. 1969; Nishiyama 1970). The young microspore stage of pollen development, including meiosis, tetrad development, and the first contraction phase, was found to be particularly vulnerable to cold stress (Satake and Hayase 1970).

The molecular changes experienced by developed rice anthers after exposure to cold stress were previously examined using cDNA microarray and proteomic analysis. Microarray analysis of cold-stressed rice anthers in the early microspore stage showed that 160 ESTs were differentially expressed, 38 of which were upregulated and 122 of which were downregulated compared to control anthers. The molecular functions of the differentially expressed genes were related to primary metabolism, proteolysis, signal transduction defense, and secondary metabolism. Approximately 36% of the differentially expressed genes were of unknown function (Yamaguchi et al. 2004). Proteomic analysis of rice anther maturation after cold exposure showed that 70 protein spots were differentially expressed, including several ascorbate peroxidases that probably detoxified activated oxygen species by scavenging hydrogen peroxide generated under cold conditions (Imin et al. 2004). This hypothesis also suggested that programmed cell death and protein degradation might be involved in responses to cold stress during rice anther development.

Abnormal pollen development after cold exposure leads to a high degree of male sterility in rice. However, the very small size of individual pollen grains and technical difficulties in isolating pollen from anthers precludes direct proteomic analysis of pollen. Here, rice anthers containing pollen grains were used for proteomic analysis. Quantitative shotgun proteomic analysis was used to detect changes of protein expression patterns at the trinucleate stage where cold temperature treated during young microspore stage in a cold-tolerant rice variety.

Materials and methods

Plant materials and low temperature treatment

A Chinese low-temperature-tolerant variety of *Oryza sativa*, L., Dianxi 4, was used for proteomic analysis. To confirm the cold tolerance of Dianxi 4, spikelet fertility after low temperature treatment was compared between Dianxi 4 and Mihyang, a low-temperature-susceptible variety, as follows. Dianxi 4 and Mihyang seed were germinated and grown under natural conditions in 15 × 30 cm rows at the Konkuk University experimental field. Individual plants with auricle distances (the distance between the ligule of the flag leaf and the penultimate leaf) of approximately 10 mm were selected and transferred,

along with field soil, to a 15 cm diameter pot. Six rice plants were grown in a growth chamber and treated at 12 °C, with 70% relative humidity, under a 12 h day/12 h night cycle for 4 days. Plants were then transferred to a greenhouse and cultivated until rice grains were fully matured. Control plants were collected and transferred to pots at the same stage as test plants, but remained under natural conditions in the field for 4 days prior to transfer to the greenhouse. Spikelet fertility was assessed using the fully matured spikelet of three replicate rice plants. For Dianxi 4 proteomic analysis, anthers from control and low-temperature-treated plants were harvested from the spikelets of the top three primary branches 1 day prior to anthesis.

Rice anther protein extraction

Anthers were placed into 1.5-mL tubes with 2–3 small metal balls and ground with a vortexer to powder in liquid nitrogen. Proteins were extracted by incubating the resultant powder with extraction buffer as 8 M urea/5 mM DTT/1% lithium dodecyl sulfate/100 mM Tris pH 8.5 at room temperature for 30 min. The mixture was then centrifuged at 14,000 g for 15 min, after which the supernatant was retained and filtered through a 0.45 μm membrane filter (Millipore, Billerica, MA, USA). The extracted proteins were precipitated overnight with 20% (v/v) trichloroacetic acid (TCA) and then washed several times with cold acetone until pigments were removed. The extracted proteins were resuspended in resolubilization buffer as 8 M urea/Tris–HCl pH 8.5. Protein concentration was assayed using a 2D-Protein Quant Kit (GE Healthcare, Piscataway, NJ, USA) as described previously (Lee et al. 2007).

1D LDS-PAGE and in-gel digestion

Protein samples (50 μg) were prepared using NuPage® LDS Sample Buffer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and loaded onto 4–12% Bis–Tris acrylamide gradient gels (Invitrogen, Carlsbad, CA, USA). After electrophoresis, gels were fixed in fixing solution (40% deionized water/50% methanol/10% acetic acid) for 10 min with shaking, after which the fixing solution was drained and the gel stained with colloidal Coomassie blue (NOVEX® Colloidal Blue Stain Kit: Invitrogen, Carlsbad, CA, USA). Stained protein lanes were sliced into seven pieces of equal size. Each piece was diced further (approximate size 1 mm²) and transferred to an Eppendorf tube. Destaining buffer [50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (ABC) pH 7.8] was added to remove Coomassie blue and samples were

dehydrated in a SpeedVac. Dehydrated samples were reduced in reduction buffer (10 mM DTT in 25 mM NH$_4$HCO$_3$) at 56 °C for 45 min, then alkylated for 30 min with alkylation buffer (55 mM iodoacetamide in 25 mM NH$_4$HCO$_3$) at room temperature in the dark. Reduced and alkylated samples were digested with trypsin as trypsin 12.5 ng/μL in 50 mM NH$_4$HCO$_3$ overnight at 36 °C. After digestion, tryptic peptides were recovered from the gel by incubation with harvest buffer as 5% formic acid in 50% acetonitrile for 20 min at room temperatures. Supernatants were dried using a SpeedVac, desalted using Pierce® C18 spin columns (Thermo Scientific, Rockford, IL, USA), and finally resuspended in 5% ACN/0.1% formic acid.

LC MS/MS analysis

A nanoflow HPLC instrument (Easy nLC, Thermo Fisher Scientific, San Jose, CA, USA) was coupled on-line to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Columns for analysis (12 cm, 75 μm inner diameter) were packed in-house with Alltima C18-AQ 5 μm resin. Samples were separated using a binary buffer system (Buffer A: 0.1% formic acid; Buffer B: acetonitrile in 0.1% formic acid) with a linear gradient of 3–50% buffer B at a flow rate of 250 nL/min. The total run time for LC MS/MS was 110 min. MS data were acquired using the data-dependent top 8 method, dynamically choosing the most abundant precursor ions from the survey scan (300–2000 Da) for higher-energy collisional dissociation (HCD) fragmentation. The dynamic exclusion duration was 60 s, and the isolation window for precursors was set to 4 m/z. Survey scans were acquired at a resolution of 70,000 at m/z 200 and the resolution for HCD spectra was set to 17,500 at m/z 200.

Analysis of proteomic data

Spectral fragment data from the Q-Exactive analysis were searched against the TIGR Rice Pseudomolecule protein database RELEASE v7.0 (http://rice.plantbiology.msu.edu/annotation_pseudo_current.shtml) using Proteome Discoverer (version 1.3) software (Thermo Fisher Scientific), which provided the p*I* value and molecular weight of each identified protein. Carbamidomethylation of cysteine was used as a fixed modification and oxidation of methionine was set as a variable modification for data searching. Both identifications were filtered using a 1% false discovery rate.

Comparative analysis of relative protein abundance

Output data from Proteome Discoverer (version 1.3) were exported to Microsoft Excel and normalized spectral counts (NSpC) were calculated (Paoletti et al. 2006;

Zybailov et al. 2006; Griffin et al. 2010). The NSpC for each protein k is given by:

$$(\text{NSpC})_k = \frac{\left(\frac{\text{SpC}}{L}\right)_k}{\sum_{i=1}^{n}\left(\frac{\text{SpC}}{L}\right)_i},$$

where the total number of MS/MS spectra matching peptides from protein k (SpC) is divided by the protein length (L), then divided by SpC/L for all N proteins in the experiment.

Bioinformatic analysis of proteomic data

Predicted protein molecular weights and p*I* values were calculated from the rice genome at EMBOSS Pepstats, EMBL-EBI (http://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/) and the EMBOSS Pepstats algorithm (http://emboss.bioinformatics.nl/cgi-bin/emboss/pepstats). Gene ontologies (GO) were retrieved from the TIGR Rice Pseudomolecule protein database Release V7.0. and GO enrichment analysis was performed in agriGO (Du et al. 2010). Differential protein expression was analyzed using Mapman (Thimm et al. 2004).

Results and discussion

Cold tolerance in Dianxi 4

The cold-susceptible rice variety Mihyang was used as a control to verify the cold tolerance of Dianxi 4 and the effectiveness of cold treatments. Spikelet fertility under control conditions was $88.39 \pm 2.5\%$ for Dianxi 4 and $94.2 \pm 2.5\%$ for Mihyang. After exposure to low temperature, spikelet fertility decreased to $52.57 \pm 7.9\%$ for Dianxi 4 and $1.5 \pm 0.86\%$ for Mihyang (Fig. 1), indicating that the low temperature treatment was effective. Although Dianxi 4 exhibited lower spikelet fertility after cold treatment than under normal conditions, by comparison with Mihyang, Dianxi 4 was relatively cold-tolerant. Therefore, comparative proteomic analysis between control and cold-treated Dianxi 4 anthers would effectively capture the protein expression response to cold temperature exposure at the early young microspore stage and the effects on anther maturation.

Identification of rice anther proteins

Mature anthers at the trinucleate stage were harvested from cold-treated and control Dianxi 4 rice plants. Proteomes were identified using high-throughput shotgun proteomic analysis at a 0.01 false discovery rate. Three biological replicates were analyzed per treatment. Approximately
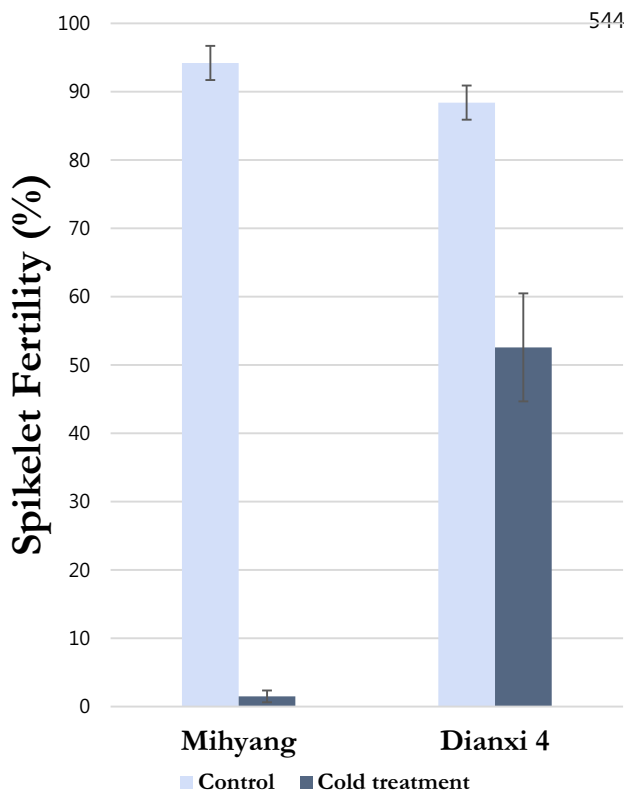
Fig. 1 Comparison of spikelet fertility of Dianxi 4 and Mihyang at normal condition (light blue) and under cold stress (dark blue)

2000 proteins were identified per sample, and 3853 non-redundant proteins were identified in total from the six samples (Supplementary file S1). The analytical incompleteness phenomenon, in which single analytical runs may identify only a fraction of the associated peptides in a highly complex mixture, may explain why all of the proteins were not identified in every sample (Wilkins et al. 2006).

The physiochemical properties of the 3853 proteins were compared with those of the whole predicted rice proteome at TIGR (http://rice.plantbiology.msu.edu/annotation_pseudo_current.shtml). Protein molecular weights spanned a wide range, from 5.28 kDa (LOC_Os04g02670.1: Expressed protein) to 486.00 kDa (LOC_Os09g07300.1: BIG, putative, expressed) (Fig. 2a). Although the proportions of identified proteins < 20 and > 120 kDa were lower than in the predicated rice proteome, the overall molecular weight distribution was similar. The molecular weight range and distribution showed that the shotgun proteomic analysis method used did not exhibit bias in identifying rice anther proteins. The pI values of the identified proteins also covered a wide range, from pH 3.93 (LOC_Os07g41694.1: acidic leucine-rich nuclear phosphoprotein 32-related protein 1, putative, expressed) to pH 12.48 (LOC_Os01g69020.1:

retrotransposon protein, putative, unclassified, expressed) (Fig. 2b). The proportion of proteins with pI values of pH 5–8 was higher for the anther proteins than for the whole predicted rice proteome.

Relative abundance of identified proteins in each sample was estimated using a normalized spectral count method. Several pollen allergens were highly abundant in the matured anther, as well as ATP synthase, UTP-glucose-1-phosphate uridylyltransferase, glyceraldehyde-3-phosphate dehydrogenase, and cupin domain containing proteins (Supplementary table S2, Table 1). The presence of these proteins at high levels was indicative of preparation for pollen tube germination and elongation. Pollen allergens, which were the most abundant proteins, were previously shown to be involved in pollen germination and growth (Songnuan 2013). High levels of carbohydrate metabolism related proteins such as ATP synthase and glyceraldehyde-3-phosphate dehydrogenase are needed for energy supply during tube germination and elongation (Dai et al. 2007). Actin and profilin proteins, as well as expansin precursors, were also highly abundant in mature anthers. Actin is a major component of microfilaments, and is thus important for cell structure, expansion and development, and profilin is a small actin monomer-binding protein (Kovar et al. 2000; McCurdy et al. 2001). β-expansin is involved in loosening the cell wall, which promotes pollen development and pollen tube growth (Kerim et al. 2003). Cupin domain proteins are involved in seed germination and development (Lapik and Kaufman 2003) and have no known role in pollen development. Nevertheless, cupin proteins were highly abundant in mature anthers and might therefore play a hitherto unsuspected role in pollen germination.

Protein expression changes in mature anthers after cold exposure at the early young microspore stage

Relative expression analysis was used to assess the differences in protein expression between control anthers and those exposed to cold stress at the early young microspore stage. Although a total of 3853 proteins were identified from the six anther samples, not all proteins were identified in all samples. Only proteins that were detected in all three biological replicates of either control or treated anthers, with at least two spectral counts per replicate, were used for the comparative analysis: this resulted in 2359 proteins. Of these, 441 proteins exhibited at least twofold differential expression between cold-treated and control anthers, as assessed by t test ($\alpha = 0.05$) (Supplementary table S3). Of these, 371 proteins were overexpressed and 70 proteins were underexpressed in cold-treated anthers compared to the control. GO category enrichment analysis of the 441

Fig. 2 Distribution of molecular weight of extracted proteins (**a**) and distribution of *p*I value of extracted proteins (**b**) from Dianxi4 anther proteins and whole rice genome
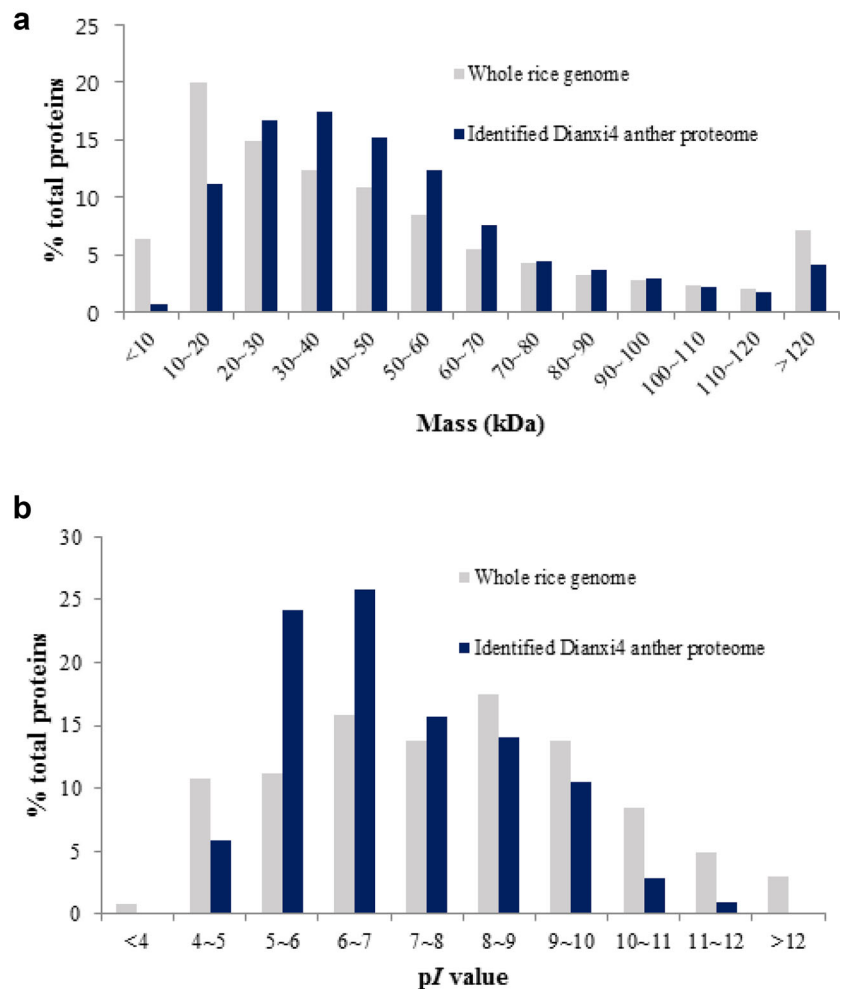
proteins revealed that 31 GO categories were enriched, comprising three biological process, two molecular function, and 26 cellular component GO terms (Table 2). The enriched biological process categories were establishment of localization, localization, and transport. For molecular function, catalytic activity and hydrolase activity were enriched. The 26 enriched cellular component terms encompassed the majority of the proteomic changes that occurred in response to low temperature exposure in the cold-tolerant Dianxi 4 variety. Although spikelet fertility in Dianxi 4 decreased to 52% after exposure to cold treatment at the early microspore stage, fertility remained significantly higher than in the cold-susceptible variety Milyang. Maintenance of cellular functions during pollen development after meiosis is fundamental for proper development. Several of these proteins were upregulated in Dianxi 4 anthers after cold treatment, which may have partially compensated for the effects of cold exposure. We therefore hypothesized that the differentially expressed proteins associated with GO cellular component categories were responsible for cold tolerance in Dianxi 4.

Analysis of differential protein expression was performed using Mapman analysis (Fig. 3). Expression of the majority of metabolic pathway proteins, particularly those involved in cell division and the cell cycle, increased after cold exposure. Other proteins upregulated in response to cold stress included proteins responsive to abiotic drought or salt stress (LOC_Os04g57890: oligosaccharyl transferase, putative, expressed; and LOC_Os07g05570: ERD4 protein, putative, expressed), miscellaneous proteins (LOC_Os02g13330: bet v I allergen family protein, putative, expressed; and LOC_Os06g02420: ATOZI1, putative, expressed), heat stress proteins (LOC_Os12g27070: heat shock protein DnaJ, putative, expressed; LOC_Os05g26902: chaperone protein DnaJ, putative, expressed; and LOC_Os06g11610: heat shock 22 kDa protein, mitochondrial precursor, putative, expressed), and cold stress proteins (LOC_Os07g39620: C2 domain containing protein, putative, expressed; and LOC_Os02g02870: glycine-rich protein 2, putative, expressed). Expression of proteins involved in redox reactions also increased after cold exposure.

Table 1 List of highly identified proteins in anther of Dianxi4 rice variety at early young microspore stage

Accession	Description	Average of NSpC
LOC_Os06g45180.1 protein	Pollen allergen, putative, expressed	0.018833
LOC_Os09g23899.1 protein	Pollen allergen Cyn d 23, putative, expressed	0.01537
LOC_Os09g23999.1 protein	Pollen allergen Cyn d 23, putative, expressed	0.012543
LOC_Os06g44470.1 protein	Pollen allergen, putative, expressed	0.009966
LOC_Os01g69020.1 protein	Retrotransposon protein, putative, unclassified, expressed	0.009938
LOC_Os04g25160.1 protein	Pollen allergen, putative, expressed	0.009662
LOC_Os04g25190.1 protein	Pollen allergen, putative, expressed	0.009319
LOC_Os04g25150.1 protein	Pollen allergen, putative, expressed	0.008472
LOC_Os06g45230.1 protein	Pollen allergen, putative, expressed	0.007795
LOC_Os08g12520.1 protein	Expressed protein	0.00735
LOC_Os02g03480.1 protein	THION24—plant thionin family protein precursor, expressed	0.006141
LOC_Os09g24020.1 protein	Pollen allergen Cyn d 23, putative, expressed	0.005756
LOC_Os03g01610.1 protein	Expansin precursor, putative, expressed	0.005002
LOC_Os01g63270.1 protein	Alpha-glucan phosphorylase isozyme, putative, expressed	0.004914
LOC_Os04g40950.1 protein	Glyceraldehyde-3-phosphate dehydrogenase, putative, expressed	0.00485
LOC_Os08g03290.1 protein	Glyceraldehyde-3-phosphate dehydrogenase, putative, expressed	0.004802
LOC_Os02g48720.1 protein	Mitochondrial carrier protein, putative, expressed	0.004685
LOC_Os02g38920.1 protein	Glyceraldehyde-3-phosphate dehydrogenase, putative, expressed	0.004656
LOC_Os05g47980.1 protein	ATP synthase, putative, expressed	0.004526
LOC_Os02g02560.1 protein	UTP-glucose-1-phosphate uridylyltransferase, putative, expressed	0.004393
LOC_Os03g17690.1 protein	OsAPx1—cytosolic ascorbate peroxidase encoding gene 1–8, expressed	0.004081
LOC_Os06g45150.1 protein	Pollen allergen, putative, expressed	0.003932
LOC_Os05g02520.1 protein	Cupin domain containing protein, expressed	0.003817
LOC_Os03g50885.1 protein	Actin, putative, expressed	0.003703
LOC_Os03g50290.1 protein	14-3-3 protein, putative, expressed	0.003685
LOC_Os01g49190.1 protein	ATP synthase, putative, expressed	0.003664
LOC_Os06g45160.1 protein	Pollen allergen, putative, expressed	0.003467
LOC_Os07g49400.2 protein	OsAPx2—cytosolic ascorbate peroxidase encoding gene 4, 5, 6, 8, expressed	0.003444
LOC_Os10g17660.1 protein	Profilin domain containing protein, expressed	0.003351
LOC_Os06g09450.6 protein	Sucrose synthase, putative, expressed	0.003167
LOC_Os11g34450.1 protein	14-3-3 protein, putative, expressed	0.003087
LOC_Os12g44350.1 protein	Actin, putative, expressed	0.003045
LOC_Os01g74480.1 protein	Cupin domain containing protein, expressed	0.002854

Differential protein expression between cold stress and normal conditions in a cold-tolerant rice variety

Consideration of prior known functions for proteins upregulated after cold exposure allowed identification of candidate cold-tolerance genes (Table 3). Expression of glycine-rich protein 2 increased 6.86-fold after cold exposure. The glycine-rich protein (GRP) family has a range of functions in plants. All GRPs contain semi-repetitive glycine-rich motifs, and plant GRPs are classified based on the presence of conserved motifs and motif arrangements (Sachetto-Martins et al. 2000; Fusaro et al. 2001; Bocca et al. 2005). GRPs are structurally diverse,

exhibit variable expression and modulation patterns, and are found in a range of subcellular locations, all of which contribute to their diverse functions in plants (Mangeon et al. 2010). GRPs are involved in flower development (Flores Fusaro and Sachetto-Martins 2007) and their expression increases under conditions of abiotic or biotic stress or after abscisic acid (ABA) treatment (Long et al. 2013; Sachetto-Martins et al. 2000; Mangeon et al. 2010). In Arabidopsis, overexpression of AtGRP2, a member of the cold-shock domain protein family, enhanced tolerance to freezing (Kim et al. 2005).

Expression of several heat shock proteins and chaperone proteins such as DnaJ, chaperone proteins, heat shock

Table 2 Enriched GO term of significantly expressed anther proteins in rice under cold stress

GO term	Ontology	Description	Number in input list	Number in BG/Ref	P value	FDR
GO:0006810	P	Transport	64	3278	9.2e−05	0.009
GO:0051234	P	Establishment of localization	64	3278	9.2e−05	0.009
GO:0051179	P	Localization	64	3278	9.2e−05	0.009
GO:0003824	F	Catalytic activity	201	13,508	7.4e−05	0.004
GO:0016787	F	Hydrolase activity	75	4293	0.00063	0.017
GO:0044444	C	Cytoplasmic part	239	10,930	2e−27	3.7e−25
GO:0005737	C	Cytoplasm	250	11,866	8.1e−27	7.4e−25
GO:0044424	C	Intracellular part	260	14,514	2.6e−17	1.6e−15
GO:0005622	C	Intracellular	264	15,144	5e−16	2.3e−14
GO:0044464	C	Cell part	296	19,532	3.8e−10	1.4e−08
GO:0043231	C	Intracellular membrane-bounded organelle	201	11,878	3.1e−09	7.9e−08
GO:0043227	C	Membrane-bounded organelle	201	11,878	3.1e−09	7.9e−08
GO:0005623	C	Cell	320	22,048	3.5e−09	8e−08
GO:0043229	C	Intracellular organelle	205	12,251	4.8e−09	8.7e−08
GO:0043226	C	Organelle	205	12,251	4.8e−09	8.7e−08
GO:0005829	C	Cytosol	79	3345	6.6e−09	1.1e−07
GO:0,005,739	C	Mitochondrion	42	1611	3.4e−06	5.2e−05
GO:0005777	C	Peroxisome	14	301	2.8e−05	0.00,035
GO:0042579	C	Microbody	14	301	2.8e−05	0.00035
GO:0009536	C	Plastid	87	4703	2.9e-05	0.00035
GO:0005783	C	Endoplasmic reticulum	24	768	3.2e−05	0.00037
GO:0005773	C	Vacuole	40	1723	7.5e−05	0.00081
GO:0031975	C	Envelope	7	115	0.00066	0.0057
GO:0031967	C	Organelle envelope	7	115	0.00066	0.0057
GO:0012505	C	Endomembrane system	7	115	0.00066	0.0057
GO:0005635	C	Nuclear envelope	7	115	0.00066	0.0057
GO:0005730	C	Nucleolus	15	495	0.0013	0.011
GO:0044428	C	Nuclear part	20	844	0.0038	0.028
GO:0044446	C	Intracellular organelle part	20	844	0.0038	0.028
GO:0044422	C	Organelle part	20	844	0.0038	0.028
GO:0016020	C	Membrane	121	8187	0.0063	0.044

P biological process, *C* cellular component, *F* molecular function

22 kDa proteins and T-complex proteins increased after cold exposure. Similar increases in expression of these proteins were reported in rice anther after exposure to high temperatures (Kim et al. 2015). This suggested that high and low unfavorable temperatures elicited similar responses in rice anthers. Heat tolerance responses in plants are mediated by increased expression of heat shock proteins and chaperone proteins. These proteins also cooperate with other cellular components to decrease cellular damage in a range of conditions (Wang et al. 2004).

Expression of a C2-domain-containing protein, LOC_Os07g39620.1, which was previously shown to contain a Ca^{2+}-dependent membrane-binding motif, increased 33-fold after cold exposure. Calcium ions play important roles in defense signaling in plant cells (Nalefski

and Falke 1996), and C2-domain-containing proteins, which are targeted to membranes, are associated with tolerance to abiotic and biotic stress (Kim et al. 2003; Yokotani et al. 2009). Overexpression of a single C2 domain protein in Arabidopsis enhanced tolerance to abiotic stresses including high salinity, osmotic stress, dehydration, and oxidative stress, as well as biotic stress caused by *Pseudomonas syringae*.

Expression of putative peroxidase precursor proteins LOC_Os03g02920.1, LOC_Os11g10460.1, LOC_Os02g14180.1, and LOC_Os01g16450.1 increased 27.68-, 22.73-, 15.65-, and 2.56-fold, respectively, after cold exposure. These proteins enhance resistance to abiotic and biotic by catalyzing reactions with reactive oxygen species (ROS) such as H_2O_2. Salicylic acid and
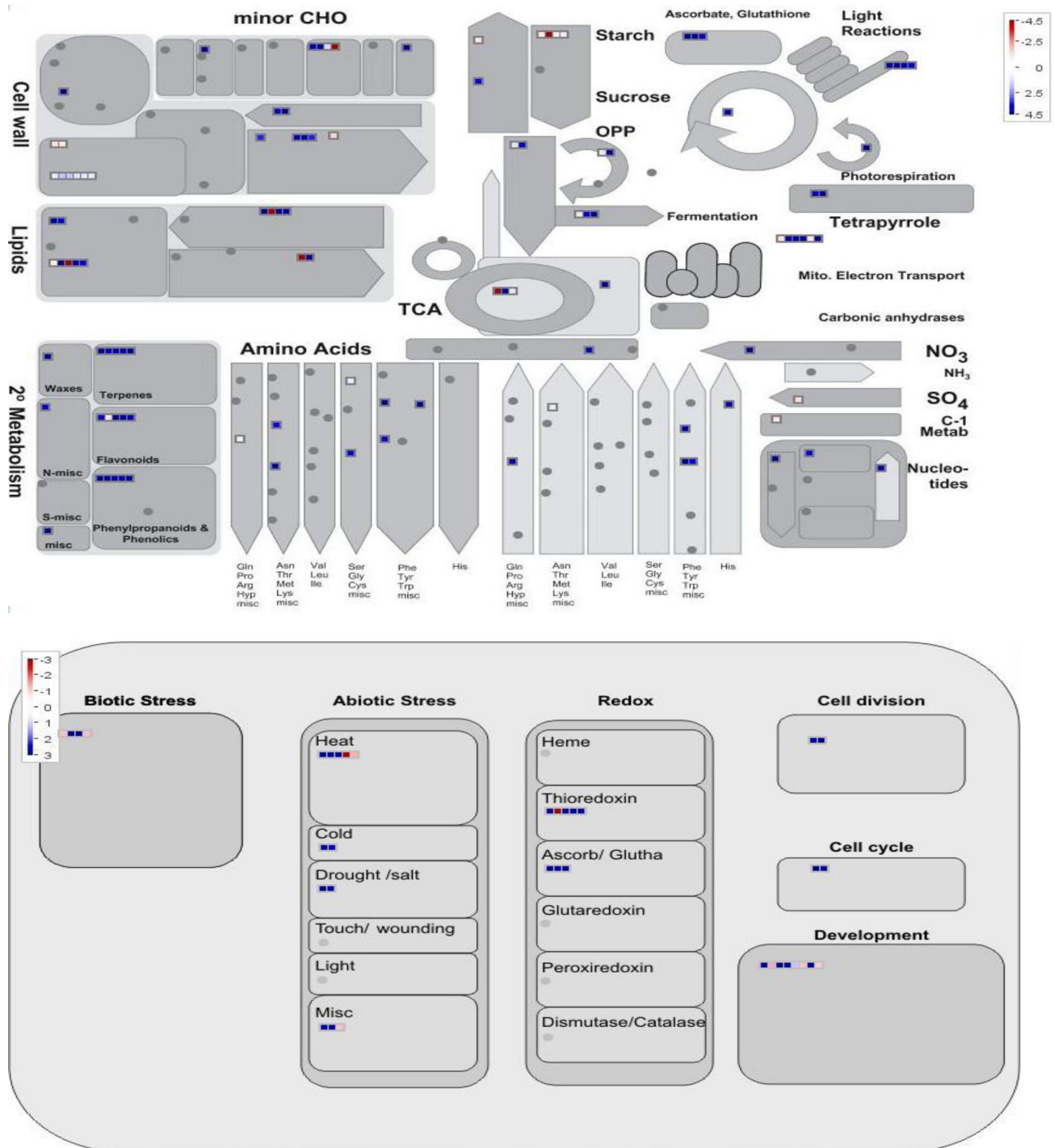
Fig. 3 Mapman analysis in order to visualize expression pattern of the differentially expressed anther proteins under cold stress at early young microspore stage

nitric oxide produced by these processes are active in plant defenses. Peroxidase proteins participate in signaling and release of ROS in response to various stresses (Mittler 2002). Together, these results indicate that several signaling systems are active under cold stress conditions (Dat et al. 2000; Zhu 2016). Peroxidases are also involved in stress-induced phenylpropanoid metabolism (Dixon and Paiva 1995). Phenylpropanoid metabolism increased under cold stress conditions and abundance of the resultant phenolic compounds increased (Leyva et al. 1995). Phenolic compounds were oxidized by peroxidase, and these enzyme activities

Table 3 List of twofold of upregulated and down-regulated proteins under cold stress

	Accession	Description	Ratio of cold treatment/control
Upregulated	LOC_Os02g22780.1 protein	T-complex protein, putative, expressed	163.9444
	LOC_Os05g48200.1 protein	Glutamate synthase, chloroplast precursor, putative, expressed	129.8811
	LOC_Os12g21798.1 protein	40S ribosomal protein S3a, putative, expressed	126.8662
	LOC_Os01g53720.2 protein	Expressed protein	126.8662
	LOC_Os10g35720.1 protein	OsGrx_S17—glutaredoxin subgroup II, expressed	90.64269
	LOC_Os01g32800.2 protein	Proteasome subunit, putative, expressed	86.61428
	LOC_Os12g17910.1 protein	T-complex protein, putative, expressed	69.7043
	LOC_Os03g08570.1 protein	Amine oxidase, flavin-containing, domain containing protein, expressed	50.00869
	LOC_Os05g32630.1 protein	Mitochondrial carrier protein, putative, expressed	49.46094
	LOC_Os04g32920.3 protein	Potassium transporter, putative, expressed	43.40457
	LOC_Os02g02530.2 protein	Signal peptide peptidase domain containing protein, expressed	41.83511
	LOC_Os04g57890.1 protein	Oligosaccharyl transferase, putative, expressed	41.80034
	LOC_Os03g29260.2 protein	Elongation factor protein, putative, expressed	38.35577
	LOC_Os10g36490.1 protein	HEAT repeat family protein, putative, expressed	34.54285
	LOC_Os07g39620.1 protein	C2 domain containing protein, putative, expressed	33.03606
	LOC_Os01g40860.1 protein	Aldehyde dehydrogenase, putative, expressed	30.09889
	LOC_Os07g32010.1 protein	UDP-glucuronosyl and UDP-glucosyl transferase domain containing protein, expressed	30.06412
	LOC_Os04g56590.1 protein	ATP/GTP binding protein, putative, expressed	29.61937
	LOC_Os01g51220.1 protein	Transport protein particle component, Bet3, domain containing protein, expressed	28.04992
	LOC_Os05g26902.1 protein	Chaperone protein DnaJ, putative, expressed	27.90396
	LOC_Os03g02920.1 protein	Peroxidase precursor, putative, expressed	27.68158
	LOC_Os01g55040.1 protein	1,3-Beta-glucan synthase component, putative, expressed	26.25121
	LOC_Os11g10460.1 protein	Peroxidase precursor, putative, expressed	22.73021
	LOC_Os01g74610.1 protein	Zinc finger, C3HC4 type domain containing protein, expressed	20.97315
	LOC_Os07g31270.1 protein	Cupin 2, conserved barrel domain protein, putative, expressed	20.97315
	LOC_Os06g11610.1 protein	Heat shock 22 kDa protein, mitochondrial precursor, putative, expressed	20.93838
	LOC_Os02g14180.1 protein	Peroxidase precursor, putative, expressed	15.65344
	LOC_Os12g27070.1 protein	Heat shock protein DnaJ, putative, expressed	12.20887
	LOC_Os02g02870.1 protein	Glycine-rich protein 2, putative, expressed	6.862826
	LOC_Os01g16450.1 protein	Peroxidase precursor, putative, expressed	2.562197
	LOC_Os03g12300.1 protein	HEAT repeat family protein, putative, expressed	0.469713
Down-regulated	LOC_Os02g22780.2 protein	T-complex protein, putative, expressed	0.003571
	LOC_Os01g51754.3 protein	Alpha-amylase precursor, putative, expressed	0.004036
	LOC_Os01g51754.1 protein	Alpha-amylase precursor, putative, expressed	0.481787
	LOC_Os07g43390.1 protein	4-Alpha-glucanotransferase, chloroplast precursor, putative, expressed	0.493793

contributed to enhanced stress resistance (Rivero et al. 2001).

Several proteins exhibited reduced expression levels after cold exposure. These included proteins associated with starch metabolism. Expression levels of two α-amylase precursors, LOC_Os01g51754.1 and LOC_Os01g51754.3, were 0.48- and 0.004-fold lower, respectively, in cold-exposed anthers than in control anthers. The role of α-amylase is to produce smaller oligosaccharides from complex carbohydrates such as starch and cellulose. Expression of another starch metabolism protein, 4-α-glucanotransferase (LOC_Os07g43390), decreased 0.49-fold after cold exposure. The role of 4-α-glucanotransferase in carbohydrate metabolism is to transfer a segment of 1,4-α-D-glucan to other carbohydrates such as glucose or 1,4-α-D-glucan (Takaha et al. 1993).

During rice anther development, large amounts of sugars are mobilized to the anthers to support early development (Castro and Clément 2007). Physiological damage caused by exposure to low temperatures can block sugar mobilization to the young microspore, culminating in abortion of pollen development and sterility (Oliver et al. 2007). ABA was previously shown to inhibit α-amylase synthesis and growth (Khan and Downing 1968), and cold stress resulted in an increase in ABA synthesis (Chinnusamy et al. 2007). Although protein expression changes were examined in a cold-tolerant rice variety, Dianxi 4, in this study, the detected proteomic changes nevertheless reflect the impacts of cold stress. The observed decrease in α-amylase was consistent with ABA responses to cold stress. (Oliver et al. 2007).

Conclusions

Rice variety Dianxi 4 exhibited cold tolerance at the early young microspore stage. High-throughput shotgun proteomic analysis identified 3835 non-redundant proteins in matured rice anther (trinucleate stage) with a wide range of molecular weight and pI values. Pollen allergens, ATP synthase, actin, profilin, and β-expansin proteins were highly abundant in mature anthers, reflecting anther maturation, pollen germination, and pollen tube elongation functions. The proteomes of cold-exposed and control anthers were compared and, in total, 441 differentially expressed proteins were detected. GO enrichment and Mapman analysis of the differentially expressed proteins suggested that the cold tolerance of Dianxi 4 resulted from increased levels of proteins associated with cellular components that limited damage from cold stress. Cold exposure was associated with reduced levels of starch metabolism proteins. Among the proteins that exhibited increased expression after cold exposure, C2 domain proteins and GRPs were identified as candidate signaling factors for mediation of the cold tolerance response. Protein expression changes may not directly contribute to cold tolerance. Nevertheless, understanding protein expression patterns in cold-tolerant rice varieties can contribute to our understanding of tolerance responses and can highlight candidate genes for further study.

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