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Quantitative proteomic analysis of cold-responsive proteins in *Abelmoschus moschatus*

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1 SUMMARY

***Abelmoschus moschatus* (*A. moschatus*) is an aromatic and medicinal plant, which is susceptible to cold stress. As low temperature is one of the major, abiotic stresses that affect plant growth. A thorough understanding the proteins response to cold stress of *A. moschatus* is essential for engineering cultivars, which have greater resistance to cold stress. To investigate the responses of *A. moschatus* to cold stress, changes in protein expression were analyzed using an iTRAQ (Isobaric Tag for Relative and Absolute Quantification) differential liquid chromatography-tandem mass spectrometry proteomic approach. Twenty-day-old *A. moschatus* seedlings were exposed to cold stress at 4℃, and the total proteins were extracted from leaves for analysis. As a result, a total of 3070 proteins were identified. A subset of 65 proteins was differentially expressed at the 95% confidence interval (ϱ<0.05), with 40 and 25 up- and down- regulated respectively. Functional analysis revealed differential expressed proteins involved in several processes, i.e. signal transduction, RNA processing, translation, protein processing, redox homeostasis, photosynthesis, photorespiration, metabolisms of carbon, nitrogen, sulfur, and energy and so on. In conclusion, our study provides new insights into cold stress responses and prospective genetic improvement in *A. moschatus*.**

2 INTRODUCTION

Abelmoschus moschatus (*A. moschatus,* syn. *Hibiscus abelmoschus* L.) is an aromatic and medicinal plant, which is native to India and planted in tropical and subtropical areas nowadays, including southwest China. Actually, this perennial plant has been applied in Chinese traditional medicine for the treatment of depression and anxiety, antispasmodic activity in the digestive system. *A. moschatus* is also applied externally to treat cramp, poor circulation and aching joints (Du *et al.*, 2008, Liu *et al.*, 2010). Furthermore, the seeds also showed antiplasmodic and aphrodisiac activity (Gul *et al.*, 2011, Maheshwari and Kumar 2009).

Low temperature stress is one of the serious environmental stresses affecting plant growth. *A. moschatus* is a type of is hypersensitive to low temperature, a sudden onset of cold stress may lead it to cease growth or even death. In order to cope with the demand for *A. moschatus*, and meet the challenges of rapid climate change, sustainable *A. moschatus* crops must be developed for cultivation in unfavorable conditions or novel geographical locations. Understanding the molecular mechanism responsive to cold stress provides a basis for engineering improvement of *A. moschatus* to low temperature. To date, there is no information available regarding to cold responsive proteome of *A. moschatus*. Shotgun quantitative proteomics can be used to study global protein expression changes between biological systems. iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) is a shot-gun based technique which allows the concurrent identification and relative quantification of many proteins in different biological samples in a single experiment (Ross *et al*., 2004). To date, the iTRAQ technique has been widely applied to the study of a range of biological samples including bacteria, yeast, plant, animal and so on (Fenselau 2007).

In this study, we comparatively analyzed the protein profiles of *A. moschatus* under cold stress and the control by using iTRAQ labeling coupled with LC/MS/MS. A total of 3070 proteins were identified. Of them, 65 significantly altered proteins were identified (95% confidence interval, $p<0.05$). Further functional categorization revealed that these altered proteins could play essential roles in metabolism, energy pathway, signal transduction and so forth. Such findings will boost our understanding of the molecular mechanism responsive to cold stress and hence provide an approach for engineering improvement of *A. moschatus* to low temperature.

3 MATERIALS AND METHODS

3.1 Plant materials and cold treatment: *A. moschatus* seeds were germinated and grown in polyvinyl chloride pots with soil (20 cm diameter, 50 cm deep) under fluorescent light (600 µmol/m²/s, 16 h light/ 8 h dark) at 25°C and 70% relative humidity in a greenhouse. Twenty day (20 d) after sowing, seedlings were transferred to a cold chamber at 4°C for 72 h for cold stress treatment. The control *A. moschatus* leaves were sampled just before cold treatment (0 h). The samples were frozen in liquid nitrogen, and then stored at -70°C for protein extraction.

3.2 Protein Extraction: The extraction procedure was based on those of Salekdeh *et al.* (2002) with some modifications. Duplicate tissue samples were ground in liquid nitrogen and suspended in 10% w/v TCA in acetone with 0.1% w/v DTT at -20℃ for 2 h, followed by centrifuging at 35,000g (JA-20 108 rotor; Beckman J2-HS) at 4℃ for 30 min. The pellets were washed with ice-cold acetone containing 0.1% DTT, incubated at -20℃ for 1 h and centrifuged again at 4℃. This step was repeated three times and then pellets were lyophilized. The sample powder were then solubilized in lysis buffer (6 M urea, 2% w/v CHAPS, 0.8% w/v Pharmalyte pH 8.5, 1.0% w/v DTT) and the protein concentration was determined by the Bradford assay (Bio-Rad) with BSA as the standard. The proteins were concentrated using 0.5 mL centrifugal filters

(Ultrafree, Millipore, Billerica, MA) and washed with 1.0 mL of water. The supernatant was then collected, and the protein concentration was determined using a protein assay kit according to the manufacturer's protocol (Pierce).

3.3 Differential labeling: Total protein (100 mg) was reduced by adding dithiothreitol (DTT) to a final concentration of 10 mM and incubated for 1 h at room temperature. Subsequently, iodoacetamide was added to a final concentration of 40 mM, and the mixture was incubated for 1 h at room temperature in the dark. Then, DTT (10 mM) was added to the mixture to consume any free iodoacetamide by incubating the mixture for 1 h at room temperature in the dark. Proteins were then diluted by 50 mM triethylammonium bicarbonate and 1 mM $CaCl_2$ to reduce the urea concentration to less than 0.6 M and digested with 40 mg of modified trypsin (Promega) at 37℃ overnight. Peptides were labeled with iTRAQ reagent in accordance with the manufacturer's protocol. Extracts from control leaves were labeled with reagent 114 and 116, and the cold treated extracts with reagent 115 and 117. Subsequently, treated and control peptides were combined and further fractionated offline using high-resolution strong catio exchange chromatography (PolySulfoethyl A, 5 µm, 200-Å bead). In total, 40 fractions were collected and combined into 20 final fractions according to the peak area. Each final fraction was lyophilized in a centrifugal speed vacuum concentrator.

3.4 iTRAQ nanoflow LC-MS/MS: The 20 SCX fractions were resuspended in 60 mL 0.1% v/v TFA, 2%v/v ACN and peptides were loaded onto the C18 column with 0.1% v/v formic acid, 2%v/v ACN. Peptides were eluted with three linear gradient steps increasing from 5–95% v/v ACN over 95 min. Mass spectra were acquired on a QStar XL hybrid quadrupole TOF mass spectrometer (Applied Biosystems). The reverse phase nanoLC eluent was subject to positive ion nanoflow electrospray analysis in information dependent acquisition mode. The TOF MS survey scan spectra from m/z 380 to 1600 were acquired for each fraction every 0.5 s, with the three most-intense multiply charged ions (counts 450) sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s in the mass range m/z 100–1600 with a modified Enhance All Q2 transition setting favoring low mass ions so that the reporting iTRAQ tag ion (114, 115, 116 and 117m/z) intensities were enhanced for quantification.

3.5 Database Search and Quantification: GLOBAL PROTEOME SERVER EXPLORER software (Applied Biosystems) was used for submitting data acquired with the MALDI-TOF/TOF mass spectrometer for database searching. The MS/MS data were searched using MASCOT version 2.4.00 (Matrix Science, http://www.matrixscience.com). All searches were performed against a plant protein database. Full scan tolerance was 5 ppm, MS/MS tolerance was 0.9 Da, and up to two missed cleavages were accepted. Fixed modifications were those originating from iTRAQ protocol: iTRAQ-4plex of lysine and N-terminal and methylthio modification of cysteines, whereas oxidation of methionine and iTRAQ-4plex of tyrosine were set as variable modifications. The threshold of significance was set to 0.001, which resulted in a false discovery frequency of less than 0.003 when searched in Mascot against the decoy database of random sequences. The cutoff value for the down-regulated proteins was 0.80-fold and for the up-regulated proteins was 1.20-fold.

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4 RESULTS AND DISCUSSIONS

4.1 iTRAQ quantitatively identified of leaf proteins: Proteins were extracted from leaves of low temperature treated and the control, which grown in normal temperature using a urea/CHAPS extraction buffer. Then the equal amounts of proteins were digested in solution and labeled with iTRAQ reagents. As for labeling, we used iTRAQ reagents 114 and 116 for the controls, 115 and 117 for the cold treated samples. This strategy provided internal technical replicates for these two samples. The iTRAQ labeled peptide samples were commixed and fractionated using SCX column chromatography and the eluted fractions were quantitatively identified by means of LC-MS/MS. The data was searched against NCBI RefSeq database (version 53) using Proteome Discoverer SEQUEST. The experimental design and schematic diagram of this study is shown in Figure 1. A total of 2,638 and 2,815 proteins were identified in the two materials respectively (Fig.1), ranging from metabolism to signal transduction and so on (data not shown). For both technical replicates, the false discovery rate was less than 1.0% (0.90% for experiment 1 and 0.97% for experiment 2). When combined the data from the two samples, the proteome of leaf deduced from the present work is at least 3070 proteins. The rate of overlap was over 85% (Fig. 2).

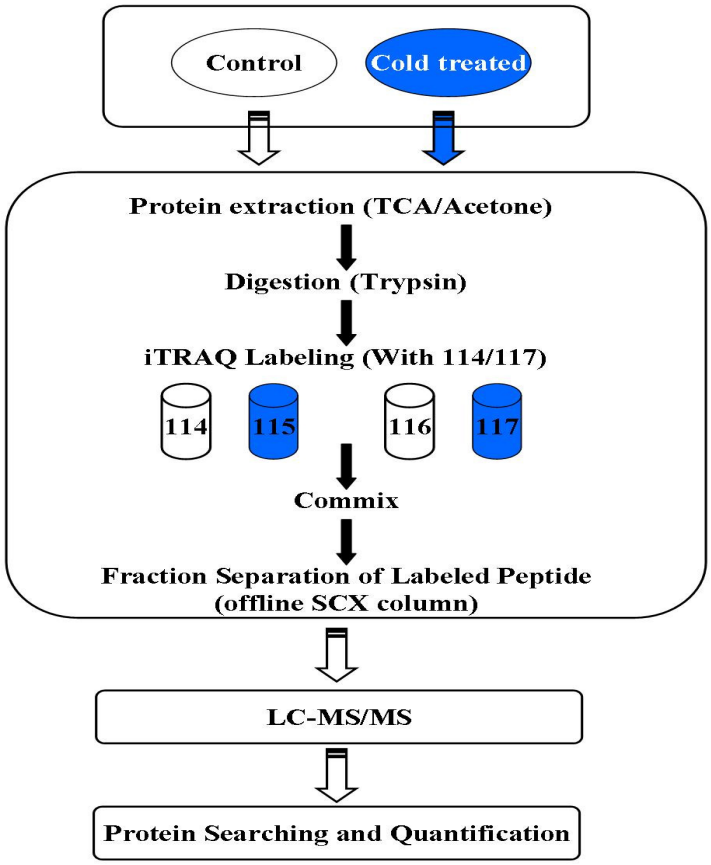

Figure 1: Experimental scheme of the iTRAQ analysis

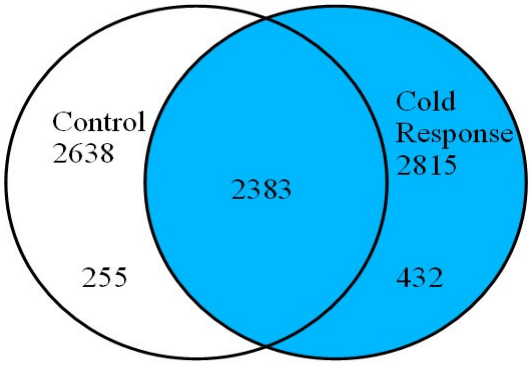

Figure 2: Venn diagram representing the overlap of the identified proteins in the two biological repeats

4.2 Differential expressed proteins and function Classification: The proteins that have at least two corresponding unique peptides were extracted to assess differential expression. Fold changes were determined based on the ratios of the peak areas of iTRAQ reporter ions for the corresponding peptides from the cold-treated and control samples. To evaluate a protein response to cold stress, we used the standards that fold change above 1.2 or below 0.8 for up-regulated or down-regulated respectively, based on a 95% confidence level. Using these standards, a total of 65 differential expressed proteins were further analyzed, with 40 up-regulated and 25 down-regulated (Table 1). These proteins were involved in several processes such as carbohydrate metabolism, energy pathway, transmembrane transport/signal transduction, anti-oxidation, defense response, protein biosynthesis, protein folding and degradation and so on(Fig.3).The biological relevance of these deferential expressed proteins is discussed below

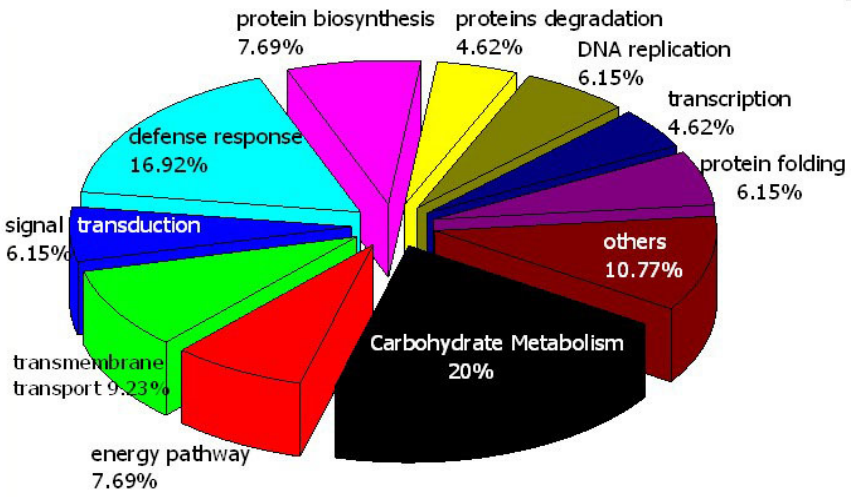

Figure.3: Pie chart showing the various biological processes as a percentage of the 65 differential expressed proteins quantified by iTRAQ.

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Table1: iTRAQ quantitative analysis of *A. moschatus* differential expressed proteins under cold stress

NCBI accession no.	Protein name	function	fold change
ADG27841.1	triosephosphate isomerase [*Gossypium hirsutum*]	Carbohydrate Metabolism	10.01
Q6YZX6	Putative aconitate hydratase, cytoplasmic[*Oryza sativa Japonica Group*]	Carbohydrate Metabolism	0.63
ABC73637.1	acetyl-CoA carboxylase carboxyltransferase beta subunit [*Gossypium hirsutum*]	Carbohydrate Metabolism	0.82
BAC21160.1	fructokinase [*Nicotiana tabacum*]	Carbohydrate Metabolism	0.55
AAT40304.1	S-adenosylmethionine synthase [*Medicago sativa*]	Carbohydrate Metabolism	1.69
ADY68848.1	sucrose synthase [*Gossypium hirsutum*]	Carbohydrate Metabolism	0.58
AAQ18140.1	enolase [*Gossypium barbadense*]	Carbohydrate Metabolism	3.53
AAQ77240.1	enolase [*Brassica rapa subsp. campestris*]	Carbohydrate Metabolism	1.49
BAA02729.1	cytoplasmic aldolase [*Oryza sativa Japonica Group*]	Carbohydrate Metabolism	3.68
AAF69802.1	malate dehydrogenase [*Vitis vinifera*]	Carbohydrate Metabolism	8.61
XP_002526594.1	utp-glucose-1-phosphate uridylyltransferase, putative [*Ricinus communis*]	Carbohydrate Metabolism	0.35
ACJ11743.1	pyruvate dehydrogenase alpha subunit [*Gossypium hirsutum*]	Carbohydrate Metabolism	3.11
XP_002530803.1	6-phosphogluconate dehydrogenase, putative [*Ricinus communis*]	Carbohydrate Metabolism	4.59
AFA27410.1	photosystem II CP47 chlorophyll apoprotein, partial [*Tradescantia ohiensis*]	energy pathway	0.22
NP_918587.1	putative 33kDa oxygen evolvingprotein of photosystem II [Oryza sativa Japonica Group]	energy pathway	0.30
YP_001531230.1	photosystem II cytochrome b559 alpha subunit [*Cuscuta obtusiflora*]	energy pathway	0.65
XP_003621695	ATP synthase subunit beta subunit [*Medicago truncatula*]	energy pathway	2.37
NP_051044.1	ATP synthase CF1 alpha subunit [*Arabidopsis thaliana*]	energy pathway	1.67
NP_180726.1	RAB GTPase-like protein A5D [*Arabidopsis thaliana*]	transmembrane transport	0.44
BAA02904.1	ras-related GTP binding protein [*Oryza sativa*]	transmembrane transport	0.35
AAB67994.1	annexin, partial [*Gossypium hirsutum*]	transmembrane transport	2.74
CAA10261.1	annexin P38 [*Capsicum annuum*]	transmembrane transport	2.26
AEN70918.1	lipid transfer protein [*Gossypium turneri*]	transmembrane transport	0.16

AAF07875.1	nitrate transporter [*Oryza sativa*]	transmembrane transport	0.26
NP_176386.2	calcium-dependent protein kinase 19 [*Arabidopsis thaliana*]	signal transduction	1.55
XP_002532559.1	calcium-dependent protein kinase, putative [*Ricinus communis*]	signal transduction	2.46
NP_190150.1	extracellular signal-regulated kinase 1/2 [*Arabidopsis thaliana*]	signal transduction	0.39
ABM67698.1	mitogen-activated protein kinase [*Citrus sinensis*]	signal transduction	0.80
AAZ41971.1	chloroplast Cu/Zn superoxide dismutase [*Gossypium hirsutum*]	anti-oxidation	3.31
XP_003523270.1	PREDICTED: peroxidase 52-like isoform 2 [*Glycine max*]	anti-oxidation	5.66
XP_003545212.1	PREDICTED: peroxidase 4-like [*Glycine max*]	anti-oxidation	2.33
AAP76387.1	class III peroxidase [Gossypium hirsutum]	anti-oxidation	2.33
AAR25792.1	cold-stress inducible protein C17 [*Solanum tuberosum*]	defense response	5.66
AAB53203.1	cold-stress inducible protein [*Solanum tuberosum*]	defense response	4.43
AEE31803.1	high response to osmotic stress 10 [*Arabidopsis thaliana*]	defense response	2.22
ADP30960.1	dehydration-induced 19-like protein [*Gossypium hirsutum*]	defense response	1.61
NP_567770.1	universal stress protein (USP) family protein [*Arabidopsis thaliana*]	defense response	1.59
AED92420.1	universal stress protein (USP) family protein [*Arabidopsis thaliana*]	defense response	2.83
AEE31209.1	Early-responsive to dehydration stress protein (ERD4) [*Arabidopsis thaliana*]	defense response	1.55
NP_568216.1	ribosomal protein L17 family protein [*Arabidopsis thaliana*]	protein biosynthesis	0.49
CAB89353.1	ribsomal protein-like [*Arabidopsis thaliana*]	protein biosynthesis	0.21
NP_039381.1	ribosomal protein S14 [*Oryza sativa Japonica Group*]	protein biosynthesis	0.37
XP_003596000.1	translation initiation factor IF-2 [*Medicago truncatula*]	protein biosynthesis	0.66
XP_002532827.1	mitochondrial translational initiation factor, putative [*Ricinus communis*]	protein biosynthesis	1.88
XP_003543129.1	PREDICTED: heat shock 70 kDa protein, mitochondrial-like [*Glycine max*]	protein folding	1.58
AAD49336.1	low molecular weight heat-shock protein [*Nicotiana tabacum*]	protein folding	3.99
XP_003619696.1	18.2 kDa class I heat shock protein [*Medicago truncatula*]	protein folding	2.86
CAA72092.1	protein disulfide-isomerase precursor [*Nicotiana tabacum*]	protein folding	2.33
XP_002265864.1	PREDICTED: ubiquitin-NEDD8-like protein RUB2 [*Vitis vinifera*]	protein degradation	1.66

AEC08206.1	ubiquitin fusion degradation UFD1-like protein [*Arabidopsis thaliana*]	protein degradation	1.44
AEC07154.1	ubiquitin fusion degradation 1 [*Arabidopsis thaliana*]	protein degradation	0.36
CAB79389.1	DNA polymerase III like protein [*Arabidopsis thaliana*]	DNA replication	3.13
AAF19580.1	putative DNA gyrase A subunit [Arabidopsis thaliana]	DNA replication	2.55
AEE28356.1	DNA replication ATP-dependent helicase Dna2 [*Arabidopsis thaliana*]	DNA replication	1.62
BAB18333.1	putative DNA replication complex GINS protein PSF2 [*Oryza sativa Japonica Group*]	DNA replication	0.37
ABX26108.1	myb-like transcription factor 6 [*Gossypium hirsutum*]	transcription	1.88
AEI53160.1	RNA polymerase beta (chloroplast) [*Oryza rufipogon*]	transcription	0.41
NP_196500	RNA polymerase III RPC4 [*Arabidopsis thaliana*]	transcription	0.22
AAZ94181.1	proteinase inhibitor II precursor [*Solanum tuberosum*]	Others	1.66
CAR98204.1	formate dehydrogenase [*Lotus japonicus*]	Others	1.93
BAJ88198.1	predicted protein [*Hordeum vulgare subsp. vulgare*]	Others	1.38
AAK69696.1	putative pectin methylesterase LuPME5 [*Linum usitatissimum*]	Others	0.39
ABD92935.1	alpha tubulin-3D [*Triticum aestivum*]	Others	0.64
XP_003589475.1	Uridine 5'-monophosphate synthase [*Medicago truncatula*]	Others	2.34
AAA33463.1	ferredoxin-dependent glutamate synthase [*Zea mays*]	Others	0.67

4.3 Proteins Involved in Carbohydrate Metabolism: In the present study, 13 proteins relevance to carbohydrate metabolism were identified to differential expressed. In general, the synthesis of carbohydrate would slow down during cold stage. So proteins that participate in anabolic pathways would be expected to down-regulated. Such as the four proteins associated with carbohydrate synthesis, that is, acetyl-CoA carboxylase carboxyltransferase beta subunit (ABC73637.1), fructokinase (BAC21160.1), sucrose synthase (ADY68848.1) and aconitate hydratase (Q6YZX6) were found to be down-regulated with cold-treated. In contrast, the proteins involved in catabolic pathways would be up-regulated, especially for glycolysis. The enzyme catalyzing steps of glycolysis such as triosephosphate isomerase (ADG27841.1), aldolase (BAA02729.1), enolase (AAQ18140.1, AAQ77240.1) and malate dehydrogenase (AAF69802.1)has been fully up-regulated. It should be mentioned that enolase does not occur only in cytoplasm where it functions as a key glycolytic enzyme, but also in plastids, which have their own glycolytic pathway (Andriotis *et al.*, 2010). Furthermore, enolase can be also found in nucleus where it functions as a transcriptional repressor of STZ/ZAT10, a repressor of CBF/DREB1 pathway (Lee *et al.*, 2002). Malate dehydrogenase has been found in plant mitochondria, the cytosol, peroxisomes and glyoxysomes, and its function in glyoxysomes is the β-oxidation of fatty acids (Miller *et al.*, 1998). However, Rinalducci *et al.* (2011) found a malate dehydrogenase was down-regulated. What the role under stress of malate dehydrogenase still remains to confirm.

UTP-glucose-1-phosphate uridylyltransferase (UTP-GPUT, XP_002526594.1) decreased to 0.35 fold to the control. UTP-GPUT not only participates in sucrose/starch pathways, but also directly or indirectly, in the biosynthesis of cell wall polysaccharides and in the synthesis of the carbohydrate moiety of glycolipids and glycoproteins(Kleczkowski *et al.*, 2004). 6-phosphogluconate dehydrogenase (6PGD) is one of the key enzymes of the important plant metabolic pathway, i.e. the pentose phosphate pathway. 6PGD (XP_002530803.1) was observed 4.59 fold up-regulated during cold stress. Komatsu *et al.* (2009) reported that 6PGD was up-regulated in rice leaf sheaths under slat and cold stress. Pyruvate dehydrogenase (ACJ11743.1, up-regulated in present study) is the first component enzyme of pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex contributes to transforming pyruvate into acetyl-CoA by a process called pyruvate decarboxylation. Acetyl-CoA may then be used in the citric acid cycle to carry out cellular respiration, so pyruvate dehydrogenase contributes to linking the glycolysis metabolic pathway to the citric acid cycle and releasing energy via NADH(Ogasawara *et al.*, 2007). S-adenosylmethionine synthase (SAMS) catalyzes the biosynthesis of S-adenosyl-Lmethionine (SAM) from L-methionine and ATP. SAM is a universal methyl group donor in several transmethylation reactions (Farrar and Jarrett, 2009). Amme *et al.* (2006), Cui *et al.* (2005) and Yan *et al.* (2006) have reported enhanced expression of SAMS under stress,. We found the SAMS (AAT40304.1) was 1.69 fold up-regulated in the present study, which was similar to previously reported.

4.4 Affect transmembrane transport and signal transduction: Cellular membranes are fluid structures, and cold temperatures can reduce their fluidity, causing increased rigidity. Plant cells can

sense cold stress through low-temperature-induced changes in membrane fluidity, protein conformation and metabolite concentration. The *Arabidopsis* fad2 mutant defective in oleate desaturase exhibits membrane rigidification and activation of diacylglycerol kinase at higher temperatures (18℃) as compared with the wild type (14℃)(Vaultier *et al.* 2006). The rigid membrane would affect materials transmembrane transport and signal transduction. Lipid transfer protein (AEN70918.1) and nitrate transport protein (AAF07875.1) were found dramatically declined compared to the control. Opposite to this, Wu *et al.* (2004) observed lipid transfer protein was up-regulated in bromegrass under cold stress. The proteins related to vesicular transport (NP_180726.1 and BAA02904.1) were also found down regulated.-Annexins are a family of structurally related, calcium-dependent phospholipid binding proteins which have been proposed to be involved in diverse cellular functions, including phospholipase A2 inhibition, adhesion, exocytosis, and interaction with cytoskeletal proteins. Annexin was considered as a stress induced protein both in plants and in animals (Kovacs *et al.*, 1998, Rhee *et al.*, 2000). In this study, two annexins (AAB67994.1 and CAA10261.1) were identified over 2 fold up-regulated. In cold stress, membrane would increase Ca2+ concentration in the cytosol by ligand-activated Ca^{2+} channels. In addition, subsequently, calcium signal amplification and phospholipid signaling might be involved in cold-stress signaling (Vergnolle *et al.*, 2005, Williams *et al.*, 2005;Komatsu *et al.*, 2007; Chinnusamy *et al.*, 2010,). Two calcium-dependent protein kinases (NP_176386.2 and XP_002532559.1) were detected up-regulated. Besides their effect on calcium signaling, cold stress may also have impact on MAPK signaling pathway (Teige *et al.*, 2004; Ji *et al.*, 2012). Two kinases (NP_190150.1 and ABM67698.1) involved in MAPK signaling pathway were found obviously declined in the present study.

4.5 Protein folding and degradation: Extreme temperature can also raise a potential risk of protein misfolding and resulting in nonfunctional proteins. Misfolded proteins accumulate in cell during cold conditions and plants may manage two strategies to cope up with this situation, one to refold them and the other to remove them (Gao *et al.*, 2009). Accordingly, disulfide-isomerase and heat shock proteins may need to undo the disulfide bond and then refold the misfolded proteins with proper formation (Ding *et al.*, 2008). We observed one disulfide-isomerase(CAA72092.1) and three heat shock proteins (XP_003543129.1, AAD49336.1 and XP_003619696.1) were among the up-regulated proteins. The similar results were reported in different plants under abiotic stresses (Bae *et al.*, 2003; Kawamura and Uemura, 2003; Renaut *et al.*, 2004;Jiang *et al.*, 2009,). And also the ubiquitin play an important role in post-translational regulation by degradation the nonfunctional proteins (Lee *et al.*, 2001; Dong *et al.*, 2006). We found one disulfide-isomerase(CAA72092.1) and three heat shock proteins (XP_003543129.1, AAD49336.1 and XP_003619696.1) were among the up-regulated proteins. Surprisingly, as for the ubiquitins, the exhibit inconsistence, two (XP_002265864.1 and AEC08206.1) of the three were up-regulated and the other one (AEC07154.1) was down-regulated. What was the reason lead to this incongruence remains to be further determined.

4.6 Affects protein synthesis: The ribosomal proteins, in conjunction with rRNA, make up the ribosomal subunits involved in the cellular process of translation. The translation elongation factor is an essential component for protein synthesis and plays a role in polypeptide elongation. It interacts with

aminoacyl tRNA and transports the codon specific tRNA to the aminoacyl site on the ribosome (ribosomal A-site) during translation elongation step. Three ribosomal proteins were reported to affect under cold stress in previous studies (Rogalski *et al.*, 2008). In the present study, three ribosomal proteins (NP_568216.1, CAB89353.1 and NP_039381.1) and a translation initiation factor (XP_003596000.1) showed decreased abundance when plants were subjected cold condition. This may suggested that much of the plant proteins synthesis apparatus were damaged under cold stress. Interestingly, the translation initiation factor located in mitochondrial (XP_002532827.1) showed nearly 2-fold increased after cold-treated. This may be explained that the plant may need more mitochondrial (to provide energy) to cope with adverse environment. Cui *et al.* (2005) found initiation factor 4A is up-regulated in rice seedling under cold condition.

4.7 Energy pathway: We observed two proteins encoding ATP synthase subunits, one for alpha subunit (NP_051044.1) and the other for beta subunit (XP_003621695) were up-regulated under cold stress. ATP synthase is an important enzyme that provides energy for the cell to use through the synthesis of ATP. This implies that more energy is required for enhancing plant resistance to cold stress. Beside this, three proteins (AFA27410.1, NP_918587.1 and YP_001531230.1) participating in photosynthesis (photosystem II) were detected decrease remarkably. This indicates that cold destroys the plant photosystem II and thus affect its photosynthesis. Chen . (2009) reported photosynthetic antenna protein CP29 was decreased in wheat under environmental stresses. While Andersson *et al.* (2001) found that the CP29 antisense lines have a decreased level of CP24 protein and weakened photosystem II function in *Arabidopsis* under environmental stress. Yan *et al.* (2006) reported that many photosynthetic proteins were partially degraded by chilling stress in rice.

4.8 DNA replication and RNA transcription: Under stress conditions, DNA damages are likely occurred. To cope with these, plant may increase proteins involved in DNA repair / replication. A DNA polymerase III like protein (CAB79389.1) which was an error-prone DNA polymerase involved in DNA repair(Vijeh Motlagh *et al.*, 2006), was found exceed 3-folds up-regulated. Hongsthong *et al.* (2008) also reported a cold induced DNA polymerase III protein in *cyanobacterium* Spirulina. DNA gyrase and DNA replication helicase participate in loosening the helix structures of DNA. Many unusual helix structures may form under stress conditions, and the DNA gyrase and DNA replication helicase are to be expected. We detected a DNA gyrase (AAF19580.1) and a DNA replication helicase (AEE28356.1) increased under cold stress. On the contrary, a putative DNA replication complex protein (BAB18333.1) was determined dramatically decreased compared to the control. This may imply that plant cut down the DNA replication to tackle cold stress, as also documented by Steward *et al.*, (2002). Cold acclimation temperatures induce profound changes in the plant transcriptome. In *Arabidopsis*, cold-regulated genes have been estimated to constitute ~4% to 20% of the genome [review by Chinnusamy *et al.*, 2010]. There are many transcription factors that responsible for gene differential expression under various stress conditions (Haga *et al.*, 2007; Simon *et al.*, 2007;Dubos *et al.*, 2008). In this study, a MYB -like transcription factor 6(ABX26108.1) was found up-regulated after cold treatment. Under stress condition, the transcriptional apparatus are unavoidable be damage (Wang, 2002; Opalka *et al.*,

2003; Yousefi-Nejad *et al.*, 2011). Two RNA polymerase proteins (AEI53160.1 and NP_196500) were observed severely decreased.

4.9 Anti-oxidative and Stress-Responsive Proteins: Electrons leaked from electron transport chain, together with the higher solubility of oxygen, hydrogen peroxide and super oxide ions can be easily produced at low temperature. The higher concentration hydrogen peroxide and super oxide ions have an undesirable effect on the structure and activity of membranes and cytoplasmic proteins. To handle this problem, proteins such as superoxide dismutase (AAZ41971.1), peroxidase (XP_003523270.1, XP_003545212.1 and AAP76387.1) were observed over expressed in cells to clear harmful superoxide ions. Dismutases catalyse the conversion of superoxide radicals to molecular oxygen. Their function is to destroy radicals that are normally produced within cells and are toxic to biological systems (Bannister *et al.*,1987). Peroxidase enzymes can degrade hydrogen peroxide by donating electrons to bind to other substrate substances, such as ferrocyanide and ascorbate. As a result, this substance is converted into harmless components, water and oxygen (Horling *et al.*, 2003). In the current investigation, several proteins such as dehydration induced 19 like protein (ADP30960.1 and AEE31209.1), universal stress protein (USP) family protein (NP_567770.1 and AED92420.1), high response to osmotic stress protein (AEE31803.1) and other cold-stress inducible protein (AAR25792.1 and AAB53203.1) were identified as cold stress responsive proteins, which had also partly been previously identified in the proteomic analysis other plants in response to cold stress(Imin *et al.*, 2004; Cui *et al.*, 2005; Amme *et al.*, 2006; Yan *et al.*, 2006). On cold condition, plants suffer dehydration which caused an osmotic stress imbalance in intracellular. Subsequently, In order to adjust the osmotic pressure, proteins response to osmotic stress and dehydration are expected to be increased (Sanchez-Ballesta *et al.*, 2004;Wagstaff *et al.*,2010).

4.10 Other proteins involved in cold stress: Formate dehydrogenase (FDH) is a soluble mitochondrial enzyme capable of oxidizing formate into CO_2 in the presence of NAD^+. FDHs are found in methylotrophic bacteria and yeasts where they provide NADH to the respiratory chain, as well as in plants where their function has not been fully elucidated (Igamberdiev *et al.*, 1999). In higher plants, FDH is localized to the mitochondrial matrix (Colas des Francs-Small *et al.*,1993), but it has recently been reported in the chloroplasts of *Arabidopsis thaliana* leaves (Olson *et al.*, 2000). In the present study, a FDH protein (CAR98204.1) was detected remarkably up-regulated. FDH transcripts (and protein) were also found to be accumulated in potato leaves under cold stress (Suzuki *et al.*, 1998) and in barley roots by iron deficiency stress (Hourton-Cabassa *et al.*, 1998). Tubulin is the building block of microtubules. Microtubules are involved in diverse functions that include cell movement and vesicle transport (Downing and Nogales,1998; Downing and Nogales, 2010). An alpha tubulin-3D (ABD92935.1) was revealed declined expressed under cold stress. Shibasaki *et al.* (2009) reported that after 12 h of cold stress, microtubules were severely disrupted in Arabidopsis. Concomitantly, a putative pectin methylesterase (AAK69696.1), a type of cell wall enzyme, was also found down-regulated. Pectin methylesterases are ubiquitous enzymes that modify the degree of methylesterification of pectins, which are major components of plant cell walls. Such changes in pectin structure are associated with changes in cellular adhesion, plasticity, pH and ionic contents of the cell wall and influence plant

development and stress responses (review by Pelloux *et al.*, 2007). Uridine 5'-monophosphate synthase is an enzyme that catalyzes the formation of uridine monophosphate (UMP), an energy-carrying molecule in many important biosynthetic pathways (Santoso and Thornburg,1998; Krungkrai *et al.*, 2001).Uridine 5'-monophosphate synthase (XP_003589475.1) was observed increased after cold stress in present study. An enzyme participates in nitrogen metabolism, i.e. ferredoxin-dependent glutamate synthase (AAA33463.1) was detected decreased markedly. These indicate that at low temperature, the N utilization efficiency declined. Beside these, a proteinase inhibitor II precursor protein (AAZ94181.1) and a predicted protein (BAJ88198.1) participate in cell cycle were found up regulated. Several biotic and abiotic stresses were proved to be lead to the synthesis of proteinase inhibitors, which regulate the activity of cysteine proteinases(Yang and Yeh, 2005; Huang *et al.*, 2007).Low temperatures would prolong the cell cycle and reduce the rate of cell division in plant such as maize (Francis and Barlow, 1988; Rymen *et al.*, 2007). Cell cycle regulation plays an important role in growth during adverse conditions.

5 CONCLUSIONS

There have no proteomic studies carried out on *A. moschatus* in response to cold to date. We hence investigated the molecular responses to cold stress at the protein level. In the present study, the use of reporter ions in MS/MS scans provides a means to accurately quantify changes in protein abundance. As a result, sixty-five proteins were revealed to be differentially expressed, 40 and 25 of them were up- and down- regulated respectively. These proteins were involved in several processes such as carbohydrate metabolism, energy pathway, transmembrane transport/signal transduction, anti-oxidation, defense response, protein biosynthesis, protein folding and degradation and so on. The identification of novel cold-responsive proteins provides not only new insights into cold stress responses but also a good starting point for further dissection of their functions using genetic and other approaches.

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