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Full Length Research Paper

Proteomic analysis of cold stress responses in tobacco seedlings

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Cold stress is one of the major abiotic stresses limiting the productivity and the geographical distribution of many important crops. To gain a better understanding of cold stress responses in tobacco (*Nicotiana tabacum*), we carried out a comparative proteomic analysis. Five-week-old tobacco seedlings were treated at 4°C for 4 h. Cold treatment resulted in stress phenotypes of smoothing and shallowing leaves and increased relative electrolyte leakage. The expression changes of total proteins in tobacco leaves were examined using two-dimensional electrophoresis. Quantitative image analysis revealed a total of 101 protein spots that changed their intensities significantly, 21 protein spots were down-regulated, eight were up-regulated after the cold treatment, 50 protein spots only expressed in the control sample, while 22 protein spots were only present in the cold treatment sample. Mass spectrometry analysis allowed the identification of 73 differentially expressed proteins, including well known and novel cold-responsive proteins. The identified proteins are involved in several processes such as photosynthesis, protein processing, redox homeostasis, ribonucleic acid (RNA) processing, signal transduction, translation, cell division/cycle, and metabolisms of carbon and energy. Several types of proteins showed enhanced degradation during chilling stress, especially the photosynthetic proteins. Gene expression analysis of 25 different proteins by reverse transcriptase- polymerase chain reaction (RT-PCR) showed that the messenger RNA (mRNA) levels of 18 genes correlated well with the protein levels. In conclusion, our study provides new insights into cold stress responses in tobacco and needs to be further studied in future.

Key words: Proteomics, cold stress, tobacco (*Nicotiana tabacum*).

INTRODUCTION

A wide array of environmental stresses seriously affects plant growth and crop yields. Among the various abiotic stresses, cold (chilling or low temperature) is a major stress that limits the geographical distribution and the productivity of many important crops such as tobacco and rice. Plants make use of several strategies to resist low expression. The hypothesis that cold-responsive proteins temperature, one of which is regulation of gene are likely to be involved in cold tolerance has resulted in great efforts to study the gene expression profile during cold stress (Thomashow, 2001). Microarray analysis was frequently carried out to examine the global gene expression profile, revealing that many genes are induced or repressed in response to cold (Chen et al., 2002; Fowler and Thomashow, 2002). Therefore, the identification of novel genes, which determine their expression patterns in response to cold stress and understanding their functions in chilling stress adaptation, will provide the basis for engineering effective strategies to improve stress tolerance (Cushman and Bohnert, 2000).

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Numerous physiological and molecular changes occur during cold response, and many studies investigating plant response to cold stress in rice and *Arabidopsis* have been reported over the past years (Chinnusamy et al., 2007; Cook et al., 2004; Doherty et al., 2009; Dong et al., 2006; Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Lee et al., 2001, 2005; Ma et al., 2009; Rabbani et al., 2003; Thomashow, 1999; Vogel et al., 2005; Yun et al., 2010; Zhu et al., 2004, 2007). However, the level of messenger ribonucleic acid (mRNA) does not always correlate well with the level of protein mainly due to post-transcriptional regulation (Yan et al., 2006). Furthermore, the primary effects of low temperature on proteins that give rise to changes of gene expression or post-translational regulation remain unclear. Proteomic studies have identified cold-responsive proteins in several plant species, including rice and other plants (Bae et al., 2003; Cui et al., 2005; Degand et al., 2009; Gao et al., 2009; Hashimoto and Komatsu, 2007; Imin et al., 2004; Lee et al., 2007, 2009; Wang et al., 2009; Wienkoop et al., 2008; Yan et al., 2006). These results provide very vital information for understanding the cold stress-responsive pathway in plant. However, to date there have been very few reports of the analysis of the response of tobacco leaves to cold stress. Proteomic analysis of the tobacco leaves is an important step toward characterization of differentially expressed proteins and the illustration of the mechanisms underlying the cold stress condition.

Tobacco (*Nicotiana tabacum*) is a native of tropical and subtropical America, but it is now commercially cultivated worldwide. Moreover, it is sensitive to cold; hence the molecular mechanism of cold response has been of great concern. In this present paper, a proteomic approach was used to study responses to cold stress in tobacco. Two-dimensional gel electrophoresis (2-DE) was used to separate the cold stress-responsive proteins in the leaves of tobacco seedlings. 73 differentially expressed proteins were subsequently identified and analyzed by mass spectrometry (MS), including many novel cold-responsive proteins.

MATERIALS AND METHODS

Plant growth conditions and cold stress treatments

Tobacco (*N. tabacum* L. cv. K326) seedlings were grown in soil under fluorescent light (700 ± 50 μmole/m^2/s, 14 h light/10 h dark) at 25°C and 60 to 80% relative humidity in a growth chamber. Five-week-old seedlings were treated at 4°C for 0 and 4 h, respectively. Some leaves were collected for detecting the relative electrolyte leakage, while others were frozen in liquid nitrogen and then stored at -80°C for protein and RNA extraction.

Relative electrolyte leakage assay

The leaves were cut into 1-cm segments and washed three times with ultrapure water. The segments were placed in tubes containing5 ml of ultrapure water and incubated at 28°C. Two hours later, the electrical conductivity of the bathing solution (L_t) was measured. Then the tubes were incubated at 100°C for 20 min and subsequently at 28°C for 1 h, and then the electrical conductivity (L_0) was measured again. The relative electrolyte leakage was calculated by the formula $L_t / L_0 \times 100\%$. Five replicates were performed for each sample.

Protein extraction

Tobacco leaves were ground in liquid nitrogen and suspended in ice-cold 10% w/v tricarboxylic acid (TCA) in acetone containing 0.07% w/v dithiothreitol (DTT), incubated at -20°C for 2 h and centrifuged for 20 min at 35,000 × g. The pellets were resuspended in 0.07% w/v DTT in acetone, incubated at -20°C for 2 h and centrifuged for 15 min at 12,000 × g. This step was repeated three times and the pellets were lyophilized. The resulting powder was solubilized in lysis buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT) followed by centrifugation for 20 min at 12,000 × g. The proteins in the supernatant were precipitated by adding four volumes of ice-cold acetone, incubated at -20°C for at least 4 h and centrifuged for 20 min at 12,000 × g. The pellets were dissolved in rehydration buffer (8 M urea, 2 M thiourea, 30 mM DTT, 4% w/v CHAPS, 1% NP-40, 5% v/v pH 3 to 10 immobilized pH gradient (IPG) buffer). Protein concentration was determined by the Bradford assay (Sangon, Shanghai, China) using bovine serum albumin as standard.

2-DE analysis

For 2-DE, 500 μg of proteins were loaded onto the preparative gels. For isoelectric focusing (IEF), the protean IEF Cell System (Bio-Rad, Hercules, CA, USA) and pH 3 to 10 non-linear (NL) IPG strips (18 cm, non-linear, Amersham Biosciences) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 14 h in 340 μL rehydration buffer containing protein samples. Focusing was performed in four steps: 500 V for 1 h, 1000 V for 1 h, 1000 to 8000 V for 0.5 h and 8000 V for 5 h. The gel strips were equilibrated in two steps: 15 min in 10 ml equilibration buffer A (50 mM Tris-HCl buffer, pH 6.8, 6 M urea, 30%v/v glycerol, 1% w/v sodium dodecyl sulphate (SDS), 0.2% w/v DTT), and 15 min in 10 ml equilibration buffer B (50 mM tris-HCl buffer, pH 6.8, 6 M urea, 30%v/v glycerol, 1% w/v SDS, 3% w/v iodoacetamide, 0.002% w/v bromophenol blue), successively. Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% gels using the PROTEAN II xi Cell system (Bio-Rad, Hercules, CA, USA). The gels were run at 25 mA per gel for the stacking gel and followed by 50 mA per separating gel. The protein spots were stained with colloidal Coomassie brilliant blue G-250. At least three replicates were performed for each sample.

Gel scanning and image analysis

The gels were scanned using image scanner^{TM} III (GE Healthcare, USA) at a resolution of 400 dots per inch (dpi). Data were analyzed using PDQuest 8.0 software (Bio-Rad, Hercules, CA, USA). After automated detection and matching, manual editing was carried out. Three well separated gels of each sample were used to create "replicate groups." Statistic, quantitative, and qualitative "analysis sets" were created between the control group and treated group. In the statistic sets, the student's *t* test and significance level of 95% were chosen. In the quantitative sets, the upper limit and the lower limit were set to three and 0.33, respectively, and then the Boolean analysis sets were created between the statistic sets and the quantitative or qualitative sets. The spots from the Boolean sets

were compared among three biological replicates. Only spots displaying reproducible change patterns were considered to be differentially expressed proteins.

In-gel digestion

Protein spots were excised from the preparative gels, placed to clean Eppendorf tubes. In-gel digestion was performed as described previously (Chen et al., 2006). After an overnight digestion, the gel pieces were extracted for 10 min in 100 µL 67% acetonitrile containing 5% formic acid with ultrasonication. The supernatants were pooled and lyophilized in a SpeedVac to about 5 µL for MS analysis.

Capillary column liquid chromatography coupled with tandem mass spectrometry (CapLC-MS/MS analysis)

Tryptic peptides prepared as described earlier were analyzed by CapLC-ion trap mass spectrometry (Bruker Daltonics) coupled with an automated Agilent 1200 LC system equipped with an autosampler and a C18 reverse-phase capillary column (PepMap, 180 µm i.d., 15 cm long, LC-packings). Before separation on the reverse-phase capillary column, the sample was pre-concentrated on a C18 precolumn (500 µm i.d., 3.5 cm long, Bruker). When the sample was separated on the C18 PepMap column, the flow rate of eluting solution was 3 µL/min and the column temperature was set to 25°C. For the chromatography, the following solvents were used: solvent A (98% H_2O, 1.9% acetonitrile, and 0.1% formic acid), and solvent B (95% acetonitrile, 4.9% H_2O and 0.1% formic acid). The peptides eluted from the column were online directed into the mass spectrometry. The LC-MS system was controlled using Chemstation B01 (Agilent) and EsquireControl™ 6.1 (Bruker Daltonics) softwares. The nebulizer pressure was 10 psi. Drying gas flow rate was 5 L/min, drying gas temperature was 300°C and capillary voltage was 4000 V. The full MS scan mode was standard enhanced (m/z: 350 to 1600 Da). Peptide ions were detected in MS scan, and seven most abundant in each MS scan were selected for collision-induced dissociation (MS/MS), using data-dependent MS/MS mode over the m/z range of 100 to 2000 Da.

Data analysis and bioinformatics

Raw mass spectrometry data were processed and Mascot compatible generic files (MGF) were created using Data Analysis™ 3.4 software (Bruker Daltonics) with the following parameters: compounds (Auto MS/MS), threshold 10000, number of compounds 300, and retention time windows 1.0 min. Searches were performed using Mascot software 2.0 (Matrixscience, London, U.K.), and the *N. tabacum* and Rice database downloaded from http:// www.expasy.ch/sprot was used for protein identification. Search parameters were set as follows: enzyme, trypsin; allowance of up to one missed cleavage; peptide mass tolerance, 1.2 Da and MS/MS mass tolerance, 0.6 Da; fixed modification, carbamoylmethylation (C); variable modification, oxidation (at Met); auto hits allowed (only significant hits were reported); and results format as peptide summary report. We confirmed candidate proteins according to the probability-based Mascot scores.

Proteins were identified on the basis of distinct peptides whose ions scores exceeded the threshold, $P<0.05$, which indicates identification at the 95% confidence level for these matched peptides. In this study, most candidate proteins with mascot scores above the threshold were identified based on at least two identified peptides, with the peptide score above 15. For proteins identified by only one peptide with Mascot score exceeding the threshold, their MS/MS spectrum was systematically checked manually. For a protein to be confirmed, (i) the masses of all the major peaks (typically more than 7 peaks) in the MS/MS spectrum had to match those of the theoretically calculated fragment ions; (ii) the assignment had to be based on successive four or more b- or y-series ions; and (iii) the molecular weight of the matched protein was in reasonable agreement with the gel migration data (Forner et al., 2006; Zhao et al., 2004). We kept to the principle of using the minimum set of protein sequences to account for all observed peptides.

Reverse transcriptase- polymerase chain reaction (RT-PCR) analysis

Total RNAs for RT-PCR were extracted from samples using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 µg of total RNA which had been treated with the deoxyribonuclease I [DNase I (Promega, Madison, WI, USA)] using 200 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's recommendations. The cDNA was used for amplification using specific primers for tobacco *actin* gene to equalize the concentrations of the cDNA samples. Subsequently, the synthesized cDNA was used to carry out PCR amplifications using the gene specific primers (Table 1). Each experiment was replicated at least three times.

RESULTS

The physiological responses induced by cold stress in tobacco

As a tropical and subtropical plant species, tobacco is sensitive to chilling; hence we treated the five-week-old tobacco seedlings at 4°C for 4 h. After the cold treatment, the leaves of tobacco became smooth and shallow (Figure 1A). To evaluate the adverse effects of cold stress quantitatively, the relative electrolyte leakage assay was performed and results show that the relative electrolyte leakage increased from 12 to about 60% after the cold treatment (Figure 1B). This indicated that the leaves of tobacco were subjected to chilling injury.

2-DE analysis of total proteins in tobacco leaves

To study the changes of protein expression profiles during the cold stress, we carried out 2-DE analysis of the total proteins in tobacco leaves. For each sample, at least triplicate gels were performed, and they showed the high level of reproducibility. The representative gels are shown in Figure 2. Quantitative image analysis revealed a total of 101 protein spots that changed their intensities significantly by more than three-fold. Most protein spots showed qualitative changes. For example, 50 protein spots only expressed in the control sample, while 22 protein spots were only present in the cold treatment sample. Furthermore, 21 protein spots were down-regulated and eight protein spots were up-regulated after the cold treatment (Figure 3). Figure 4 shows examples

Table 1. The gene-specific primers used in this paper.

Spot no./Gene	Gene accession number	Forward primer (5'—3')	Reverse primer (5'—3')	Product size (bp)
D1	CAC84143	TTTCTCGTGTAGCCCGTT	GCAATGCCTGAAGAGTTCTC	521
D6(1)	AAA78277	ATCAACAGGACATCACCCG	CCATACGACCATCACGGAT	623
D6(2)	CAA78703	TTCCAGGCTCCAACAGGTA	GAACTTCTCCATACGACCATCA	584
D7(1)	CAA88841	GTCCCACTTGATGATAACCAG	GGAACCAACTGAAAGACCC	600
D7(2)	AAA18546	CCTATTATTTCTGGCTCTGCTC	GATTCCTCTCCTTTATCAGTCG	541
D9(1)	CAA77361	TGAACAAGTATGGTCGTCCC	CGCAGTAAATCAACAAAGCC	526
D11(1)	CAA36958	CGGAAATGGGAAAGTTACCA	CGATAGCCTGAACAAAGAATCC	579
D11(2)	CAA41187	ACTCTCACCATCTTCCTCTGA	AAAGCCTCTGGGTCATCA	529
D11(3)	CAA36956	CTCTCTCTTCCTCTTCATTTGC	GACCCTTTCCAGTGACGAT	659
D11(4)	ABG73417	TTCCTACCCGACGGTCTCTT	CCAGCAATGACAGTGAGCA	570
D12	AAO62942	CCAGACAGTGGCTAAATCACA	CCAATCGTCCGTTCTTGA	582
D18	AAS79798	TACCATTCCACCAAGAGCG	TTAGGTTCCTCAAGGCTGAAAC	613
U3(2)	CAA77341	ATACCCGTGAGTGAGGCTT	GGCGGTCATACTTCCTTCA	620
CT2(1)	AAD17230	CAGGGCGATTTGATAGACAA	TCACCACTTTGGGCAGAA	609
CT3	BAC55280	TTCACCTACCAGCCGCTTA	TTCACGCTTGCCATTGTC	535
CT4	CAD13178	CGCCCTACTTACACCAACCT	TCACCTTCAGCACCAACCT	621
CT7	AAS92255	CAAACAGGCAGTAACCATCC	GAACGATTAGCATACCTCTCCA	622
CT10	BAD10940	CATCGTGGCGTATTATCTCATC	TGGCTTCATCAGGTTTGG	546
CT14	BAC77634	CCTTCTTCATCTTCTCCCTTC	TCTTGATTGTGGCAGCGT	550
CT15	CAA44214	TCGCTCTCCTTCTCCTTCTA	CTGTTTCTTAGCATCTTCAGCC	455
LT1	ACR56690	GCTGGTAACAAGAAGTTGGTGT	TAGGGTTGGTGACAAGGAGG	536
LT3	AAB71764	TTGGAAGAAGAAGCAGCCC	ACCCACCGACGAATAAAGC	610
LT5	AAA34076	TGTCTCTGCTCAACTCACTCC	TCTCCTCAAGTCACGGTGTG	624
LT7	AAA34075	TCCACCCTTGGCATCTTT	TCAGCAGCCTCCCTAAATG	617
LT9	AAL51055	GCTAACGAATCCTACGAGCA	TTCACCAAACCGTCTCTCC	633
Actin	EU938079	CCACACAGGTGTGATGGTTG	CACGTCGCACTTCATGATCG	706

representing the differentially expressed protein spots in response to the cold stress. These imply that plant cells were able to monitor the cold stress by modulating corresponding protein expression.

Identification of the differentially expressed proteins

The differentially expressed proteins were excised from the preparative gels, in-gel digested by trypsin, and analyzed by CapLC-MS/MS. In total, 73 proteins were successfully identified by the CapLC-MS/MS analysis (Table 2). The results of spot D1 are shown in Figure 5 as an example. Some of the identified proteins were annotated

Figure 1. The physiological responses induced by cold stress in tobacco. Five-week-old seedlings were treated at 4℃ for 4 h. The photographs of the sample were taken at 0 and 24 h treatment time, respectively and the framed regions were enlarged (A). The relative electrolyte leakage is shown in B.

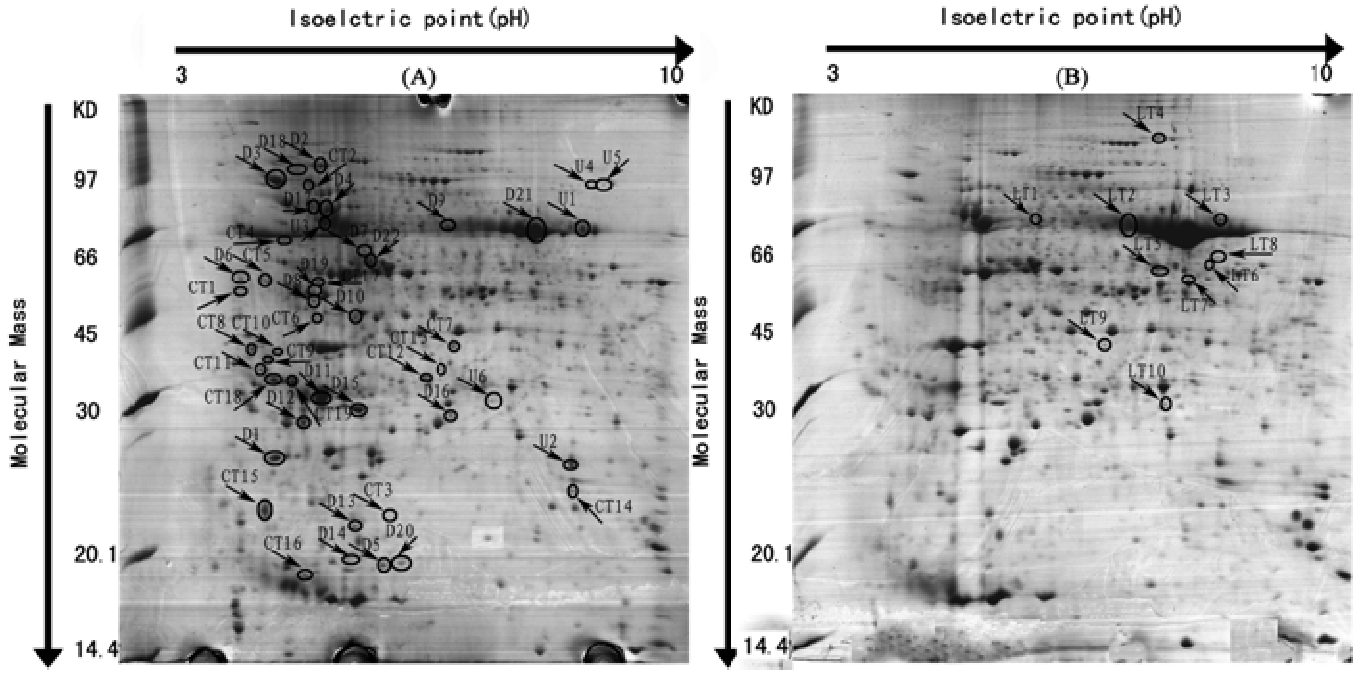

Figure 2. Representative 2-DE gels of leaves proteins from tobacco seedlings. Total proteins were extracted and separated by 2-DE. In IEF, 500 μg of proteins were loaded onto pH 3 to 10 IPG strips (18 cm, non-linear). SDS-PAGE was performed with 12.5% gels. The spots were visualized by colloidal Coomassie brilliant blue G-250 staining. Quantitative image analysis revealed a total of 101 spots that changed their intensities significantly by more than three-fold. (A) 2-DE gel of the control sample treated at 4℃ for 0 h. (B) 2-DE gel of the sample treated at 4℃ for 4 h. 2-DE, Two dimensional gel electrophoresis; IEF, isoelectric focusing; IPG, immobilized pH gradient.

either as unknown and hypothetical proteins or as proteins without specific function in the database. To gain the functional information about these proteins, we searched their homologues with Basic Local Alignment Search Tool program (BLASTP) (www.ncbi.nlm.nih.gov/BLAST/) using their protein sequences as queries. 14 corresponding homologues with the highest homology are shown in Table 3. 13 spots except for spot D8 shared more than 80% identities with homologues at the amino acid level, indicating that they might have the similar function.

Furthermore, all protein sequences detected and identified were searched against gene ontology tool (www.geneontology.org) and TargetP program (www.cbs-

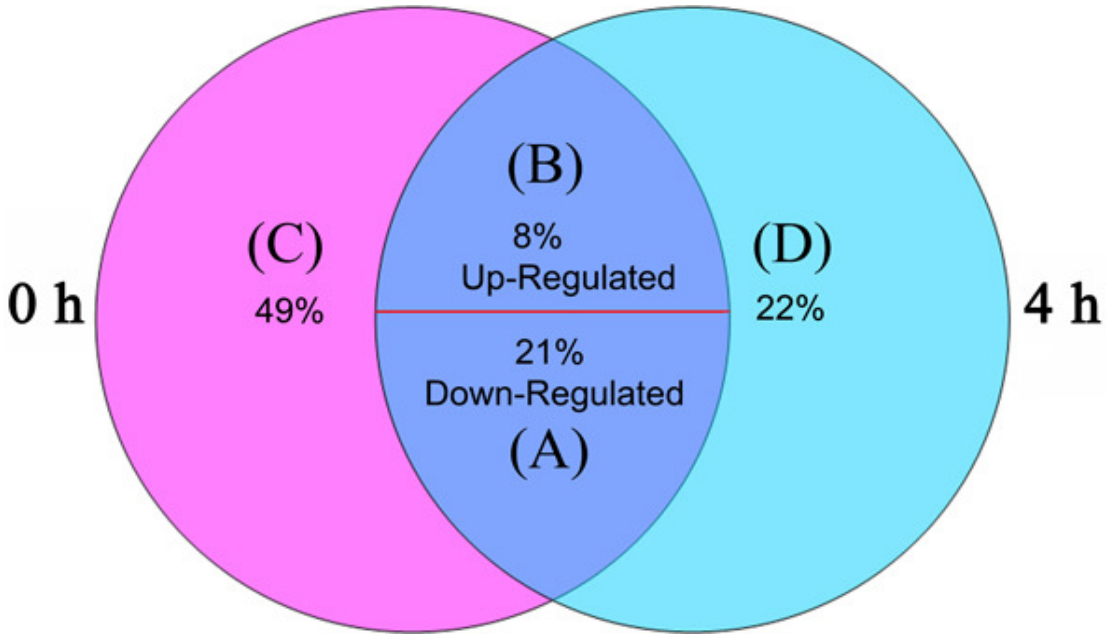

Figure 3. Venn diagram analysis of the 101 differentially expressed protein spots after 4 h of cold stress treatment. 0 h shows control group; 4 h shows cold treatment group; Up-regulated and down-regulated correspond to quantitative difference when samples were treated 4℃ condition.

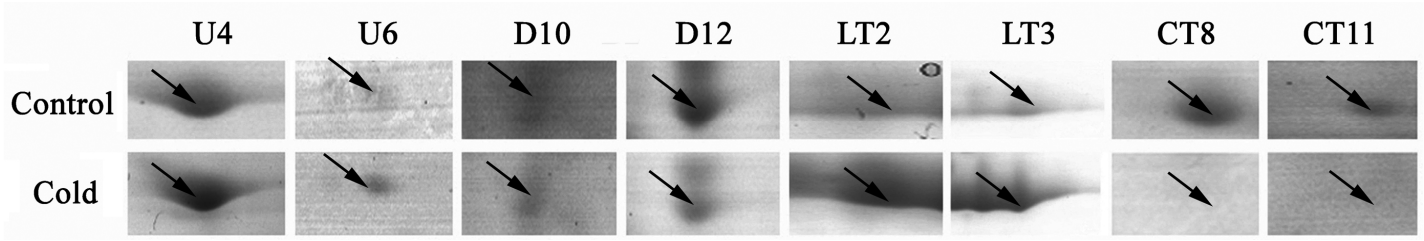

Figure 4. Examples of induction and repression of some protein spots after cold stress treatment. U4, 70 kDa heat shock protein; U6, peptidyl-prolyl cis-trans isomerase; D10, fructose-bisphosphate aldolase; D12, chlorophyll a/b binding protein; LT2, glucose-1-phosphate adenylyltransferase; LT3, catalase; CT8, ribonucleoprotein; CT11, RNA binding protein. RNA, ribonucleic acid.

.dtu.dk/services/TargetP) for functional classification and subcellular localization prediction. These identified proteins were found to be involved in diverse biological processes, including photosynthesis, protein processing, redox homeostasis, RNA processing, signal transduction, cell division/cycle, translation and metabolisms of energy and carbon (Figure 6). The largest functional category was the proteins involved in photosynthesis (24%). Among proteins responsible for photosynthesis, nine protein spots are linked to the ribulose bisphosphate carboxylase/oxygenase, while five protein spots are related to the chlorophyll binding protein (Tables 2 and 3). These results indicate that the photosynthesis pathway was subjective to the cold stress. Using TargetP program, prediction of subcellular localization of identified proteins based on their N-terminal amino acid sequence was performed. The results of prediction show that as much as 41.1% of the differentially expressed proteins are located in the chloroplasts (Table 2). This implied that chloroplasts are one of the organelles inside cells mostly influenced by cold stress.

Gene expression analysis by RT-PCR

To investigate the changes of gene expression at the transcript level, we performed RT-PCR analysis (Figure 7). The gene encoding 25 different proteins from *N. tabacum* in Table 2 were analyzed. For the down-regulated proteins, including only expressed in normal condition, the mRNA levels of 19 genes except for spots D6(2), CT2(1) and CT7 were also down-regulated after the cold treatment (Figure 7). However, for the up-regulated proteins, including only expressed in cold condition, only spots LT1 and LT3 among six different genes were also up-regulated under cold stress at the

Table 2. Differentially expressed protein identified by CapLC-MS/MS

Spot no.	Swiss- prot accession no.	Protein name	Species	Location	M_r (kD)	pI	Gravy	Score	C[a] (%)	Sequence[b]
D1	Q8RVF8	Thioredoxin peroxidase	*N. tabacum*	Chloroplast	30.10	8.19	-0.15	242	27	KSYNVLIPDQGIALRG
D2	Q0JBE3	Os04g0538100 protein	*O. sativa*	Secretory pathway	42.11	5.47	-0.2	65	4	KIMTDPFVGSLTFVRI
D3(1)	O03194	Cytochrome b	*N. tabacum*	Mitochondrion membrane	44.52	6.48	0.68	24	1	RLSLLKQ
D3 (2)	A2Y2Y4	Putative uncharacterized protein	*O. sativa*	Chloroplast	69.35	5.25	-0.35	131	6	RNTTLPTSKS
D3 (3)	A2XPB4	Putative uncharacterized protein	*O. sativa*	Cytoplasm	61.45	5.36	0.02	64	3	KTNDSAGDGTTTASVLARE
D4(1)	Q6ZFJ9	Os02g0102900 protein	*O. sativa*	Cytoplasm	64.16	5.77	-0.12	88	4	RQGVVTLEEGKS
D4(2)	B9FR63	Putative uncharacterized protein	*O. sativa*	Cytoplasm	62.84	5.42	-0.12	72	3	RNVVLESKY
D5(1)	Q40490	Cyclin A-like protein	*N. tabacum*	Nucleus	54.69	8.06	-0.19	37	5	KRQAMTANSSLENNNHGKL
D5(2)	Q40491	Cyclin A-like protein	*N. tabacum*	Nucleus	54.53	8.23	-0.2	27	1	KTVSLPRS
D6(1)	Q40460	Ribulose bisphosphate carboxylase/oxygenase activase 1	*N. tabacum*	Chloroplast	48.95	8.44	-0.316	146	6	KGLVQDFSDDQQDITRG
D6(2)	Q40565	Ribulose bisphosphate carboxylase/oxygenase activase 2	*N. tabacum*	Chloroplast	48.54	8.14	-0.331	153	7	KIVDTFPGQSIDFFGALRA
D7(1)	Q42961	Phosphoglycerate kinase	*N. tabacum*	Chloroplast	50.32	8.48	0.148	84	5	KGVTTIIGGGDSVAAVEKV
D7(2)	P68158	Elongation factor Tu	*N. tabacum*	Chloroplast	52.15	6.34	-0.154	71	2	KKYDEIDAAPEERA
D8	Q7G8G4	Os10g0125700 protein	*O. sativa*	Secretory pathway	161.83	8.14	-0.25	43	1	KLLQELSKFQVNRE
D9(1)	P00876	Ribulose bisphosphate carboxylase large chain	*N. tabacum*	---	53.38	6.41	-0.241	111	7	RMSGGDHIHSGTVVGKL
D9(2)	B8ACY2	Putative uncharacterized protein (Fragment)	*O. sativa*	Intracellular	188.12	9.25	-0.42	58	1	RYTGEGESDEAKK
D10	A2ZBX1	Fructose-bisphosphate aldolase	*O. sativa*	---	42.21	6.38	0.19	62	3	RGILAMDESNATCGKR
D11(1)	P27495	Chlorophyll a-b binding protein 40	*N. tabacum*	Chloroplast	28.45	5.48	0.007	103	7	KTVSSGSPWYGPDRV
D11(2)	P27491	Chlorophyll a-b binding protein 7	*N. tabacum*	Chloroplast	28.49	5.27	0.017	101	8	KAKPVSSGSPWYGPDRV
D11(3)	P27496	Chlorophyll a-b binding protein 50	*N. tabacum*	Chloroplast	28.50	5.84	-0.038	47	8	KAKPLSSGSPWYGPDRV
D11(4)	Q0PWS5	Chloroplast pigment-binding protein CP26	*N. tabacum*	Chloroplast membrane	30.49	5.72	0.07	36	6	KTGALLLDGNTLNYFGKN
D12	Q84TM7	Chlorophyll a/b binding protein	*N. tabacum*	Chloroplast membrane	28.78	5.33	-0.04	268	30	KIYPGGAFDPLGLADDPEAFAELKVKE
D13	Q7G8G4	Os10g0125700 protein	*O. sativa*	Secretory pathway	161.83	8.14	-0.25	43	1	KLLQELSKFQVNRE
D14	P00834	ATP synthase epsilon chain	*N. tabacum*	---	14.60	5.18	-0.139	44	11	RIGNNEITVLVNDAEKG
D15	Q7G8G4	Os10g0125700 protein	*O. sativa*	Secretory pathway	161.80	8.14	-0.25	43	1	KLLQELSKFQVNRE
D16	Q7G8G4	Os10g0125700 protein	*O. sativa*	Secretory pathway	161.80	8.14	-0.25	43	1	KLLQELSKFQVNRE
D17(1)	P00876	Ribulose bisphosphate carboxylase large chain	*N. tabacum*	---	53.38	6.41	-0.241	718	38	RELGVPIVMHDYLTGGFTANTSLAHYCRD

Table 2. Contd.

D17(2)	Q6ZFJ9	Os02g0102900 protein	*O. sativa*	Chloroplast	64.17	5.77	-0.12	117	6	KAAVEEGIVVGGGCTLLRL
D18	Q6R0J1	Heat shock protein 90	*N. tabacum*	Cytoplasm	80.36	4.95	-0.58	49	2	KGIVDSEDLPLNISRE
D19	A2ZBX1	Fructose-bisphosphate aldolase	*O. sativa*	---	42.21	6.38	0.19	50	3	RLASIGLENTEANRQ
D20	Q40514	A-type cyclin	*N. tabacum*	Chloroplast	54.73	8.06	-0.2	33	5	KRQAMTSNSSLENNNHGKL
D21	P00876	Ribulose bisphosphate carboxylase large chain	*N. tabacum*	---	53.38	6.41	-0.241	166	8	RELGVPIVMHDYLTGGFTAN TSLAHYCRD
D22	P68158	Elongation factor Tu	*N. tabacum*	Chloroplast	52.15	6.34	-0.154	195	6	KILDEAMAGDNVGLLLRG
U1	B7E588	Serine hydroxymethyltransferase	*O. sativa*	---	46.88	8.75	-0.26	113	7	KNTVPGDVSAMVPGGIRM
U2	A2XZE6	Peptidyl-prolyl cis-trans isomerase	*O. sativa*	Chloroplast	26.75	9.35	-0.12	75	6	RHVVFGQVIEGMDIVKM
U3(1)	P00826	ATP synthase subunit β	*N. tabacum*	---	53.58	5.00	-0.016	272	10	KVALVYGQMNEPPGARM
U3(2)	P00823	ATP synthase subunit α	*N. tabacum*	---	55.48	5.14	-0.036	33	3	KIVNTGTVLQVGDGIARI
U4	Q2QV45	70 kDa heat shock protein	*O. sativa*	Chloroplast	74.27	5.1	-0.27	55	3	KAVVTVPAYFNDSQRT
U5	Q2QV45	70 kDa heat shock protein	*O. sativa*	Chloroplast	74.27	5.1	-0.27	55	3	KAVVTVPAYFNDSQRT
U6	A2XZE6	Peptidyl-prolyl cis-trans isomerase (Fragment)	*O. sativa*	Chloroplast	24.95	4.23	-0.27	120	22	RIVLDGYNAPVTAGNFLDLV ERK
CT1	Q01M83	H0622G10.1 protein	*O. sativa*	Chloroplast	42.74	5.82	-0.15	92	7	RYTGGMVPDVNQIIVKE
CT2(1)	Q9ZP50	FtsH-like protein Pftf	*N. tabacum*	Membrane	74.51	6	-0.1	143	7	RTPGFSGADLANLLNEAAIL AGRR
CT2(2)	A2YG12	Putative uncharacterized protein	*O. sativa*	Membrane	72.61	5.54	-0.08	100	5	RTPGFSGADLANLLNEAAIL AGRR
CT3	Q852S5	Nucleoside diphosphate kinase 2	*N. tabacum*	Chloroplast	25.73	8.46	-0.124	55	6	KLIGATNPLNAEPGTIRG
CT4	Q8VXC9	α-tubulin	*N. tabacum*	Microtubule	50.41	4.85	-0.21	198	9	RAVFVDLEPTVIDEVRT
CT5	Q01M83	H0622G10.1 protein	*O. sativa*	Cytoplasm	42.74	5.82	-0.15	52	4	RLLFEVAPLGFLIEKA
CT6	Q0J878	Os08g0130500 protein	*O. sativa*	Ribosome	34.47	5.37	-0.06	45	4	KGTVEIITPVELIKK
CT7	Q6QND3	Putative pyridoxine biosynthesis protein isoform A	*N. tabacum*	---	33.35	5.93	0.04	247	17	RIAEEAGACAVMALERV
CT8	Q08940	Ribonucleoprotein	*N. tabacum*	Chloroplast	32.35	4.89	-0.2	33	4	RDAFADQPGFMSAKV
CT9	A2XHU1	Putative uncharacterized protein (Fragment)	*O. sativa*	Chloroplast	27.93	4.45	-0.25	65	4	RSYISGYQASKD
CT10	Q5KTN5	14-3-3 c-1 protein	*N. tabacum*	---	29.46	4.77	-0.54	146	12	KAAQDIATTELAPTHPIRL
CT11(1)	Q9T2L8	RNA binding protein (Fragment)	*N. tabacum*	---	3.65	4.32	-0.49	86	39	KELFSEQGNVVDAKV
CT11(2)	Q9T2L7	RNA binding protein (Fragment)	*N. tabacum*	---	3.54	4.51	0.19	78	39	RAGNVEMVEVIYDKL
CT12	P93395	Proteasome subunit β type-6	*N. tabacum*	---	25.23	5.18	-0.124	69	13	RLQTGMIIGGWDKYEGGKI
CT13	B8AMH6	ATP-dependent Clp protease proteolytic subunit	*O. sativa*	Chloroplast	51.85	6.02	-0.25	44	3	KEYGLIDGVIMNPLKA
CT14	Q7XZV3	24K germin-like protein	*N. tabacum*	Apoplast	22.13	5.82	0.53	159	16	KKPSAVTANDFVFSGLATPV KL

Table 2. Contd.

CT15	P24929	50S ribosomal protein L12	*N. tabacum*	Chloroplast	19.64	6.34	-0.111	199	34	KLGVTAASFAPAAVAAAPGA AAEAPAVVEEKT
CT16	B2YKT9	Glycine-rich RNA-binding protein	*N. tabacum*	---	15.67	5.22	-0.83	71	9	RDAIEGMNGQDLDGRN
CT17	Q01M83	H0622G10.1 protein	*O. sativa*	Chloroplast	42.74	5.82	-0.15	169	9	RYTGGMVPDVNQIIVKE
CT18(1)	Q9T2L8	RNA binding protein (Fragment)	*N. tabacum*	---	3.65	4.32	-0.49	78	39	KELFSEQGNVVDAKV
CT18(2)	Q9T2L7	RNA binding protein (Fragment)	*N. tabacum*	---	3.54	4.51	0.19	93	39	RAGNVEMVEVIYDKL
CT19	B2YKT9	Glycine-rich RNA-binding protein	*N. tabacum*	---	15.67	5.22	-0.83	77	9	RDAIEGMNGQDLDGRN
LT1	C5J0G6	Enolase	*N. tabacum*	---	48.08	5.4	-0.23	68	4	KAVNNVNSIIGPALIGKD
LT2	Q6AVT2	Glucose-1-phosphate adenylyltransferase	*O. sativa*	Chloroplast	55.79	7	-0.13	68	6	RFPTANDFGSEIIPASAKE
LT3	O24511	Catalase	*N. tabacum*	---	57.23	6.85	-0.57	57	3	KTWPEDILPLQPVGRL
LT4	Q0D9C4	Catalase isozyme B	*O. sativa*	---	57.06	6.47	-0.517	57	2	KTWPEDIIPLQPVGRM
LT5	P09044	Glyceraldehyde-3-phosphate dehydrogenase B	*N. tabacum*	---	47.95	8.83	-0.056	312	14	RVPTPNVSVVDLVVNVAKK
LT6	Q10SA2	cDNA clone:J013118G18	*O. sativa*	---	34.02	4.99	0.09	144	9	KVVAWYDNEWGYSQRV
LT7	P09043	Glyceraldehyde-3-phosphate dehydrogenase A	*N. tabacum*	---	42.12	6.60	-0.031	274	13	KGTMTTTHSYTGDQRL
LT8(1)	Q01IJ6	H0219H12.1 protein	*O. sativa*	Chloroplast	43.03	7.61	-0.02	204	9	KGTMTTTHSYTGDQRL
LT8(2)	A2XC18	Putative uncharacterized protein	*O. sativa*	Chloroplast	47.54	6.22	0.01	131	7	RVPTPNVSVVDLVINTVKT
LT9	Q8W183	Carbonic anhydrase	*N. tabacum*	Chloroplast	34.83	6.19	-0.06	63	8	RYSGVGAAIEYAVLHLKV
LT10	Q8W183	Carbonic anhydrase	*N. tabacum*	Chloroplast	34.83	6.19	-0.06	73	8	RYSGVGAAIEYAVLHLKV

[a]Sequence coverage of matched peptides. [b] The representative sequence of matched peptides. D, down-regulated protein spots after cold treatment; U, up-regulated protein spots after cold treatment; CT, the protein spots only expressed in normal condition; LT, the protein spots only expressed in cold condition. CapLC-MS/MS , Capillary column liquid chromatography coupled with tandem mass spectrometry; pI, isoelectric point;

mRNA levels (Figure 7). In all, seven spots among 25 different proteins showed different expression patterns between protein and mRNA levels. Our results demonstrate that the mRNA levels down-regulated after cold treatment were in accord with protein levels, nevertheless, the mRNA expression levels of up-regulating do not correlate well with protein expression levels in tobacco.

DISCUSSION

Cold stress affects almost all aspects of cellular function in plants and it is, therefore, not surprising that the present comparative proteomics analysis revealed a complex cellular characterization. In this study, tobacco proteome expression was investigated in response to chilling (4℃) stress. The majorities of the identified proteins was involved in photosynthesis and are located in the chloroplasts, indicating that the photosynthesis in tobacco and the organelle of chloroplast are highly distressed by chilling stress. In addition, the present proteomic study identified not only some well known cold-responsive proteins such as enolase, RcbL, RcbA, ascorbate peroxidase and heat shock proteins, but also several novel proteins such as 2-Cys peroxiredoxin, armadillo repeat-containing protein, Lr1-like protein RGA-1 and putative nascent polypeptide-associated complex α chain. These identified proteins can be classified into two groups; the first group consists of regulatory proteins, such as proteins involved in signal transduction, RNA processing, translation, and protein processing; while the second group consists of functional proteins or proteins that probably function in stress tolerance, such as proteins involved in redox homeostasis, photosynthesis, photorespiration, and metabolisms of carbon, nitrogen, sulfur, and energy. Furthermore, our results demonstrate that many proteins might undergo enhanced degradation under chilling stress.

The network involved in cold stress responses

Tobacco can perceive cold stress signals by

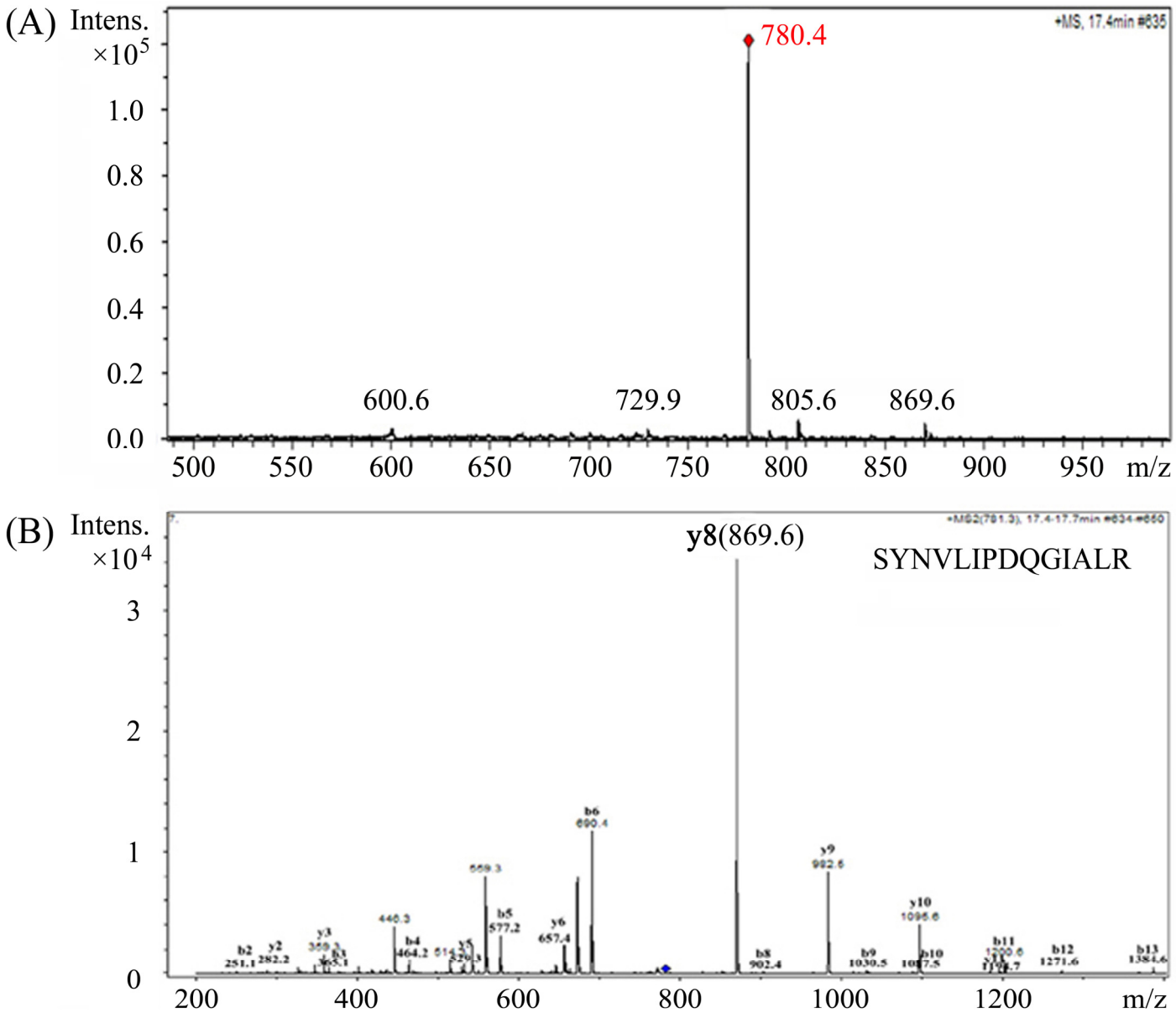

Figure 5. Identification of the protein spot D1 by MS. The protein excised from gels was digested with trypsin, and the resulting peptides were analyzed using the CapLC-ion trap mass spectrometry (Bruker Daltonics) proteomics analyzer. (A) The MS spectra. The ion 780.4 marked with an asterisk was analyzed by MS/MS. (B) MS/MS spectra of ion 780.4. The *y/b* ions and the corresponding peptide sequence are shown. The protein was identified as thioredoxin peroxidase (Swiss-Prot accession number Q8RVF8) after database searching. CapLC-MS/MS, Capillary column liquid chromatography coupled with tandem mass spectrometry

putative sensors and transmit them to the cellular machinery by signal transduction to regulate gene expression. Gene expression can be regulated at transcriptional, post-transcriptional, translational and post-translational levels, resulting in the abundant activities of functional proteins working cooperatively to establish a new cellular home-ostasis under the cold stress condition; proteins involved in these processes were also identified. Acidic ribosomal protein P0, FtsH-like protein Pfrf, proteasome, adenosine triphosphate (ATP)-dependent Clp protease proteolytic subunit, ribo-nucleoprotein, peptidyl-prolyl cis-trans isomerase, heat shock protein, elongation factor, RNA binding protein, germin-like protein, Lr1-like protein RGA-1 and cyclin were proteins involved in protein translation, protein and RNA processing, signal transduction and cell division/cycle. The differential regulation of different components of the regulatory machinery suggests that there is a complicated mechanism controlling regulatory network in response to cold stress in tobacco. Heat shock proteins (spots U4 and U5) and peptidyl-prolyl cis-trans isomerase (spots U2 and U6) act as molecular

Table 3. The homologues of the unknown proteins from BLASTP (www.ncbi.nlm.nih.gov/BLAST/) were used to search the homologues of the unknown proteins in Table 2. The homologues with the highest homology are shown.

Spot	Accession no.[a]	Homologue				
		NCBI accession no[b]	Name	Organism	Ident[c] (%)	E-value (%)
D2	Q0JBE3	P34811	Elongation factor G, chloroplastic	*G. max*	87	0.0
D3 (2)	A2Y2Y4	A4ZYQ0	Chloroplast heat shock protein 70	*P. americanum*	85	0.0
D3 (3)	A2XPB4	B6SXW8	Rubisco large subunit-binding protein subunit α	*Z. mays*	89	0.0
D4(1)	Q6ZFJ9	B6THN2	Rubisco large subunit-binding protein subunit β	*Z. mays*	91	0.0
D4(2)	B9FR63	P21240	Rubisco large subunit-binding protein subunit β, chloroplastic	*A. thaliana*	83	0.0
D8	Q7G8G4	A7U1X3	Lr1-like proteinRGA-1	*T. aestivum*	35	0.0
D9(2)	B8ACY2	B9EZ83	Fructose-bisphosphate aldolase	*O. sativa*	97	0.0
CT2(2)	A2YG12	Q655S1	Cell division protease ftsH homolog 2, chloroplastic	*O. sativa*	100	0.0
CT5	Q01M83	P46285	Sedoheptulose-1,7-bisphosphatase, chloroplastic	*T. aestivum*	93	0.0
CT6	Q0J878	P41095	60S acidic ribosomal protein P0	*O. sativa*	100	1 e-175
CT9	A2XHU1	Q40680	Elongation factor 1-δ 1	*O. sativa*	83	1 e-103
LT6	Q10SA2	B4F8L7	Glyceraldehyde-3-phosphate dehydrogenase B	*Z. mays*	94	1 e-171
LT8(1)	Q01IJ6	P09315	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	*Z. mays*	95	0.0
LT8(2)	A2XC18	B4F8L7	Glyceraldehyde-3-phosphate dehydrogenase B	*Z. mays*	92	0.0

[a]The accession number of the unknown proteins in Table 2.[b] The accession number of the homologues; [c]Identities.

chaperones, and may play a pivotal role in preventing aggregation of the denatured proteins and facilitating the refolding under cold stress.

Many research results suggest that photosynthesis is greatly inhibited by low temperature in various crops (Allen and Ort, 2001), although the mechanism is still not completely understood. In this paper, our proteomic results show that many photosynthetic proteins were partially degraded by cold stress (Table 1). Due to the fact that the photosynthetic components are functionally linked, damage of any components may lead to the overall reduction of photosynthetic activity. Ribulose 1,5-bisphosphate carboxylase/ oxygenase enzyme (Rubisco), fructose-bisphosphate aldolase (FBPase) and phosphor-glycerate kinase (PGK) are involved in photorespiration, which has been suggested to be important for maintaining electron flow to prevent photoinhibition under stress conditions. The degradation of Rubisco, FBPase and PGK will lead to the inhibition of photorespiration. This is probably one of the most important reasons why tobacco is sensitive to cold stress. Chlorophyll a/b binding proteins and chloroplast pigment-binding protein are related to the chlorophyll biosynthesis and chloroplast development. The reduction of these proteins expression will result in reducing the photosynthesis under cold condition. This is perhaps another reason why tobacco is sensitive to cold stress.

In addition, increasing evidence shows that redox homeostasis is a metabolic interface between stress perception and physiological responses (Foyer and Noctor, 2005). Reactive oxygen species (ROS) are readily produced in cold conditions and can act as the signaling molecules for cold responses. Furthermore, they can also cause damage to cellular components. Plants can control the ROS level through accurately mechanisms such as scavenging them by catalase (CAT) (spots LT3 and LT4) and thioredoxin peroxidase (spot D1), which were identified in this paper. Recently, a new function has emerged for the vitamin B6 compounds in cellular antioxidant defense (Havaux et al., 2009), and one putative pyridoxine biosynthesis protein (spot CT7) was identified in this study.

The primary metabolisms, such as metabolisms of carbon, nitrogen and energy, need to be modulated to establish a new homeostasis under cold stress. Glyco-lysis is thought to be the archetype of a universal metabolic pathway of carbon and energy. Glyceraldehyde-3-phosphate

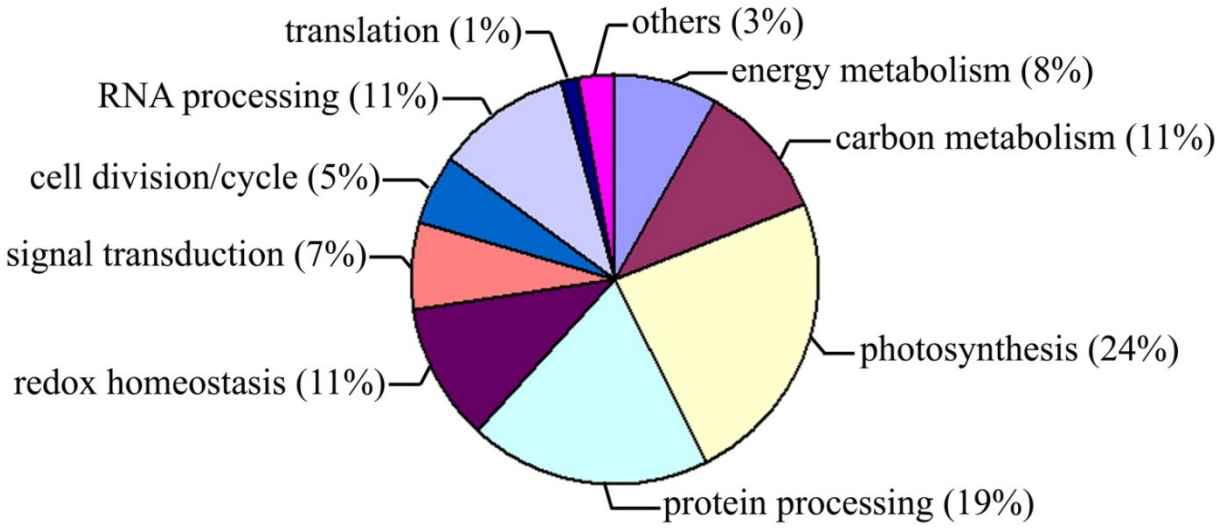

Figure 6. The functional category distribution of the 73 identified proteins.

dehydrogenase (GAPDH) is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. Enolase, also known as phosphopyruvate dehydratase, is a metallo-enzyme responsible for the catalysis of 2-phosphoglycerate (2-PG) to phospho-enolpyruvate (PEP), the ninth and penultimate step of glycolysis (Lee et al., 2002). As expected, cold stress up-regulated expression of GAPDH (spots LT5, LT6, LT7 and LT8) and enolase (spot LT1), two enzymes involved in glycolysis. Their up-regulation might help to produce more energy needed in cold defense processes.

The photosynthetic carbon reduction (Calvin) cycle is the primary pathway for fixation of atmospheric CO_2. This cycle plays a central role in plant metabolism, providing intermediates not only for starch and sucrose biosynthesis, but also for isoprenoid metabolism and shikimic acid biosynthesis. The sedoheptulose-1,7-bisphosphatase (SBPase) (spot CT5) is important in the Calvin cycle for participating in carbon fixation, which functions at a branch point in the Calvin cycle between regeneration of the acceptor molecule ribulose 1,5-bisphosphate (RuBP) and export to starch biosynthesis (Lefebvre et al., 2005); its down-regulation might indicate that the cold stress have damaged the Calvin cycle in tobacco.

Carbonic anhydrase, form a family of enzymes that catalyze the rapid conversion of carbonic acid to bicarbonate and protons, a reaction that occurs rather slowly in the absence of a catalyst (Badger and Price, 1994). In plants, carbonic anhydrase helps to raise the concentration of CO_2 within the chloroplast in order to increase the carboxylation rate of the enzyme Rubisco. Serine hydroxymethyltransferase (SHMT) is an enzyme which plays an important role in cellular one-carbon pathways by catalyzing the reversible, simultaneous conversions of L-serine to glycine (retro-aldol cleavage) and 5,6,7,8-tetrahydrofolate to 5,10-methylenete-trahydrofolate (hydrolysis) (Appaji et al., 2003). Glucose-1-phosphate adenylyltransferase,also called ADP-glucose pyrophosphorylase(ADP-GPPase), is an enzyme that catalyzes α-D-glucose 1-phosphate and converts it into ADP-glucose, therefore this enzyme participates in starch and sucrose metabolism (Rosa et al. 2009). In this proteomic study, the results show that carbonic anhydrase (spot LT9 and LT10), SHMT (spot U1) and ADP-GPPase (spot LT2) all were up-regulated. This demonstrates that it is necessary to establish a new substance metabolism network for adapting to the cold stress in tobacco.

More also, energy metabolism was altered under chilling stress as revealed by the altered expression of nucleoside diphosphate kinase 2 (NDPK2) (spot CT3) and ATP synthase α and β chains (spot U3). NDPK is believed to be a housekeeping enzyme that maintains the intracellular levels of all deoxyribonucleotide triphosphates (dNTPs) used in biosynthesis, except ATP. Recent studies suggested that NDPK also played a significant role in signal transduction pathways involved in various stress response (Tang et al., 2008). The ATP synthase α and β chains were up-regulated under cold stress, which unavoidably resulted in increasing the ATP production, so as for the cold defense processes.

Cold stress promotes the protein degradation

Our proteomic analysis shows that several type of proteins was partially degraded by cold stress, especially the components of the photosynthesis (Table 1). Similar to our results, the analysis of the rice leaves proteome under cold stress identified some proteins as break-down

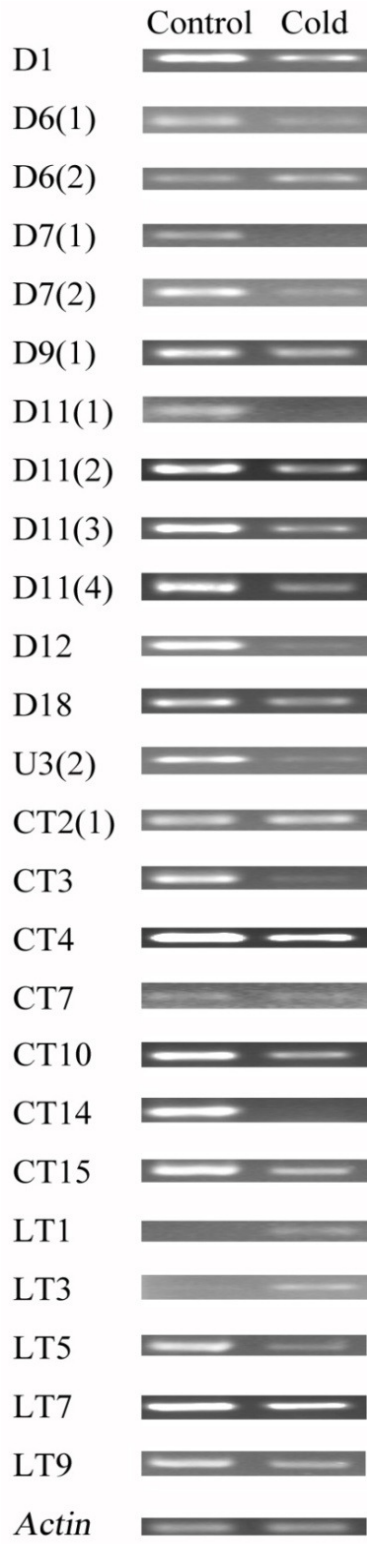

Figure 7. Gene expression analysis by RT-PCR. The genes encoding 25 different proteins from *N. tabacum* in Table 2 were analyzed. The transcript levels of these genes were determined by RT-PCR using specific primers. Tobacco *actin* was used as internal control. Control indicated tobacco seedlings treated at 4°C for 0 h and cold indicated tobacco seedlings treated at 4°C for 4 h. Each experiment was replicated at least three times. RT-PCR, Reverse transcriptase- polymerase chain reaction.

products (Yan et al., 2006). These results indicate that the cold stress promoted protein degradation in plant. Moreover, our results provide evidence for the degradation of photosynthetic proteins such as Rubisco, FBPase, PGK, chlorophyll a/b binding proteins and chloroplast pigment-binding protein, which are mainly located in the chloroplast. These results therefore suggest that the photosynthesis apparatus is susceptible to cold stress and the organelles of chloroplast are more sensitive to the chilling stress, which may be one of the major damage of cold to the tobacco. Furthermore, it was discovered that the proteins related to the RNA processing, translation and protein processing were mainly down-regulated under the cold stress; for example RNA-binding proteins, ribonucleoprotein, ribosomal protein, proteasome subunit, ATP-dependent Clp protease proteolytic subunit and FtsH-like protein Pftf, which may affect the other functional and regulatory proteins biosynthesis, thus resulting in reduced expression of the proteins

Conclusion

In this research, the molecular responses to cold stress were investigated at the protein level in tobacco for the first time. 101 differentially expressed proteins were revealed, and 73 of them were further identified by MS analysis. These proteins were involved in several processes that might work cooperatively to establish a new homeostasis under cold stress.

In summary, the present initial proteomic investigation of tobacco leaf revealed a complex cellular network affected by the low temperature stress. 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.

ACKNOWLEDEGEMENTS

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