

REGULAR ARTICLE

A proteomic analysis of cold stress responses in rice seedlings

Suxia Cui¹, Fang Huang^{2*}, Jie Wang³, Xiao Ma¹, Yongsheng Cheng¹ and Jinyuan Liu¹

¹ Laboratory of Molecular Biology and MOE Laboratory of Protein Science, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, P. R. China

² Department of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, Sweden

³ National Center of Biomedical Analysis, Beijing, P. R. China

Using proteomic analysis, an investigation aimed at a better understanding of the molecular adaptation mechanisms of cold stress was carried out in rice (*Oryza sativa*). The seedlings were exposed to a progressively low temperature stress treatment from normal temperature to 15, 10, and 5°C. Proteins were extracted from the leaves collected from both control and stressed seedlings. By fractionation, approximately 1700 protein spots were separated and visualized on CBB-stained 2-D gels. Sixty protein spots were found to be up-regulated in responding to the progressively low temperature stress and displayed different dynamic patterns. As an initial work, 41 of these proteins were identified using MALDI-TOF MS or ESI/MS/MS. These cold responsive proteins, besides two proteins of unknown function, include four factors of protein biosynthesis, four molecular chaperones, two proteases, and eight enzymes involved in biosynthesis of cell wall components, seven antioxidative/detoxifying enzymes, and proteins linked to energy pathway, as well as a protein involved in signal transduction. The functional proteomes illuminate the facts, at least in plant cell, that protein quality control mediated by chaperones and proteases and enhancement of cell wall components play important roles in tolerance to cold stress. Using TargetP program, the subcellular localization of the identified proteins was analyzed. Proteins (43.9%) were predicted to be located in the chloroplasts, implying that chloroplast proteome is virtually subjective to cold stress. The physiological implications, revealed from the experimental data, are discussed in context of a complex metabolic network in plant cells responsive to cold stress.

Received: June 6, 2004
Revised: September 14, 2004
Accepted: November 18, 2004

Keywords:

Cold stress / Dynamic patterns / Functional proteome / *Oryza sativa*

1 Introduction

Low temperature stress is one of the serious environmental stresses affecting plant growth. A clear understanding of the molecular mechanisms through which plants respond to low temperature is of fundamental importance to plant biology. Knowledge about these mechanisms is also crucial for continued development of rational breeding and transgenic

strategies to improve stress tolerance in crops [1]. Rice is one of the most important cereals determining worldwide sustainable food productivity. As a crop that is highly subjective to cold stress [2], its molecular mechanisms of cold adaptation/resistance acquiring has been of great concern. Numerous studies investigating rice response to cold stress has been reported over the past years. Dramatic changes in gene expression, biomembrane lipid composition and small molecular accumulation have been found to be closely related to

Correspondence: Dr. Jinyuan Liu, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, P. R. China

E-mail: liujy@mail.tsinghua.edu.cn

Fax: +86-10-62772243

* Current address: Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Beijing, P. R. China.

this process (reviews [3–6]). The progress of the complete genome sequence of this plant [7, 8] species has not only updated our knowledge but also provided a plentiful platform to study plant stress physiology. Microarray analysis, for example, has been carried out recently for a global examining of gene expression profile corresponding to cold stress [9]. It was shown that 36 rice genes appear to be induced under cold stress, and gene expression level for several genes reached maximum after cold treatment for 24 h [9]. Although this gene expression profiling under cold stress has deepened our understanding a great deal, it is still intriguing how the transcriptional changes are reflected at the translational level. Changes in transcriptome are not always closely correlated with protein species [10]. Molecular mechanisms underlying in cells coping with environmental perturbation can only be unveiled through integrated analyses of both proteins and mRNAs [11]. Comparing global gene expression reports relevant to cold stress, large amounts of experimental data for identification of corresponding proteins are available.

Proteomic approach is a powerful tool to study plant stress responses. A global protein expression profile can be investigated and compared using a 2-D gel-based protein separation method coupled with protein identification by MS. A number of investigations revealing plant proteomes responding to drought and wound stresses have been carried out in crops using this approach [12–14]. Sixteen and 19 proteins were identified as drought-stress proteins in rice and maize, respectively. In rice, 11 wound-stress proteins were identified. Those stress proteins were in general attributed to a wide metabolic pathway affected by stress in plant. However, it is apparent that the list of stress proteins is far from complete. The main obstacle is due to a loss of proteins in the course of sample preparation, and to numerous relevant proteins detected on 2-D did not show any matches in the current database [12–14].

For plant material, sample preparation is often more difficult due to the rigidity of plant cell walls and interference of large quantities of secondary compounds [6]. The TCA/acetone precipitation method is commonly used for plant protein extraction, alleviating the interference from plant secondary metabolites [16]. This step, however, also bears a risk of selective loss of proteins. It has been shown that a large number of water soluble proteins are missing in the extracts [17]. Indeed some soluble proteins, which were found being stress-responsive characterized by traditional methods were not reported in the proteomic studies mentioned previously.

In the present work, we initiated a functional proteomic investigation of proteins that are responsive to progressively low temperature stress in rice. We adopted a procedure of a prefractional extraction followed by an organic solvent precipitation prior to 2-D gel protein separation. As a result, 41 cold stress proteins were identified. The physiological and biochemical implications of these proteins are discussed in context of a flow of complex events occurring under cold stress condition.

2 Materials and methods

2.1 Chemicals

CHAPS, IPG DryStrip, and IPG buffer were from Amersham Biosciences (Uppsala, Sweden); thiourea from Sigma (St. Louis, MO, USA); sequencing-grade modified trypsin, urea, and acrylamide were obtained from Promega (Madison, USA), and CHCA was purchased from Bruker (Bruker-Franzen, Bremen, Germany).

2.2 Plant growth conditions and cold stress treatments

Rice (*O. sativa* L. ssp. *japonica*) seedlings were grown in the greenhouse with a 16-h light (28°C)/8-h dark (23°C) regime for 2 wk. To provide whole nutrition to the seedlings, Hogland solution was supplied every 2 days. Humidity was maintained at 30%. Cold stress treatments were performed by incubating the seedlings in a growth chamber with decreasing temperatures from 15, 10, and 5°C 24 h for each treatment. The middle portion of the first and the second leaves was collected and frozen in liquid nitrogen, then stored at –80°C for protein extraction. Seedlings grown in normal conditions were used as control.

2.3 Protein extraction/fractionation

Protein extraction/fractionation was performed according to Giavalisco *et al.* [17] with modifications. Frozen leaf tissue was ground to a fine powder in liquid nitrogen and homogenized in ice-cold extraction buffer containing 50 mM Tris-HCl, pH 7.8, 10% glycerol, 2% β -mercaptoethanol, 1 mM PMSF, and 1 mM EDTA. After 30 min, the homogenate was centrifuged for 30 min at $20\,800 \times g$. The resulting supernatant constituted fraction I. The pellet was washed twice with the same buffer, ground, and centrifuged; the resulting supernatant was combined into fraction I. The remaining pellet was extracted in 100 mM phosphate buffer, pH 7.1, containing 0.2 M KCl, 10% glycerol, 2 mM MgSO_4 , 4% w/v CHAPS, 2% β -mercaptoethanol, 1 mM PMSF, and 1 mM EDTA. After stirring at 4°C for 40 min, 30 mM DTT, 7 M urea, and 2 M thiourea were added and stirred for additional 40 min at room temperature. After centrifugation (17°C) at $20\,800 \times g$ for 30 min the supernatant was collected as fraction II. Protein content was estimated according to Peterson [18] using BSA as a standard.

2.4 2-DE and image analysis

Proteins from each fraction were precipitated with methanol/chloroform according to the method described by Wessel *et al.* [19], respectively. After lyophilization, 250 μL of an IEF solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 0.5% v/v IPG buffer, pH 3–10 (Amersham Biosciences) was added to the precipitants containing 400 μg of

proteins. To enhance protein solubilization of fraction II, 2% of IPG buffer, pH 3–10 was used. After centrifugation at $9000 \times g$ for 3 min, the supernatant was applied onto a linear IPG strip (pH 4–7, 13 cm). Rehydration loading and IEF were performed as described by Huang *et al.* [20]. For second dimension, the strips were incubated in an equilibration buffer (6 M urea, 30% v/v glycerol, and 2% SDS in 0.05 M Tris-HCl buffer, pH 8.8) containing 15 mM DTT for 15 min as the first step, then replaced by 2.5% iodoacetamide as the second step. The strips were placed on top of an SDS-polyacrylamide gel (13% polyacrylamide) prepared according to Laemmli [21] and sealed with 0.8% agarose. The electrophoresis was carried out at 6°C and 5 mA/gel for 1 h and then 10 mA/gel using a Hoefer SE 600 apparatus (Amersham Biosciences). Proteins were detected by CBB R-250. The 2-D gels were scanned using a UMAX PowerLook 2100XL scanner (Willich, Germany). Triplicates were applied to each treatment. A total of 12 2-D gels for each fraction were analyzed using ImageMaster 2-D Elite software version 4.01 (Amersham Biosciences). Spots detecting parameters were set according to the manufacturer's instructions, as follows: sensitivity 8000, operator size 45, noise factor 5, background factor 8000. To verify the autodetected result, all spots were manually edited. A total of 1330 spots in fraction I and 660 spots in fraction II were reproducibly detected and included for analysis.

2.5 MALDI-TOF and ESI-MS/MS analysis

Protein spots showing at least 1.5-fold increase in abundance during the treatments were selected and excised manually for protein identification. In-gel digestion of protein spots followed by peptide extraction was performed according to Fulda [22]. All mass spectra of MALDI-TOF MS were obtained on a Bruker Reflex III mass spectrometer (Bruker-Franzen Bremen, Germany) in positive ion mode as described by Jin *et al.* [23]. The spectra were internally calibrated using trypsin autolysis products. All obtained peptide-mass fingerprints were analyzed using MASCOT (<http://www.matrixscience.com>) searching NCBI nr database. To denote a protein as an unambiguous identification the following criteria were used: coverage of the mature protein by the matching peptides must reach a minimum of 15%, and at least five independent peptides should match, with a mass tolerance of ± 0.2 Da and one missed cleavage site.

For proteins that could not be identified by MALDI-TOF, ESI-MS/MS was performed on a Q-TOF2 hybrid quadrupole/TOF mass spectrometer (Micromass, UK) with a nano-flow Z-spray source. Peptide sequencing was performed using palladium-coated borosilicate electrospray needle (Protana, Denmark) according to the method of Yan *et al.* [24]. The mass spectrometer was operated in the positive ion mode with a source temperature of 80°C, and a potential of 800–10 000 V applied to the nanospray probe. The peptide sequences were deduced with MasSeq program. The peptide tags were submitted to Sequence Query in MASCOT (<http://www.matrixscience.com>) for database search. The search

parameters used were: monoisotopic peptide masses, ± 0.2 Da peptide mass tolerance; one missed cleavage, modifications allowed for oxidation of methionine and carboxyamidomethylation of cysteine.

3 Results

3.1 Rice leaf proteome scale estimated from the fractionated extraction

Using fractionated extraction, approximately 1330 strained spots were resolved in the range pH 4–7 from fraction I (Fig. 1) and 660 strained spots were resolved from fraction II (Fig. 2), respectively. Fraction I contained soluble proteins whereas fraction II consisted of mainly structure-associated proteins. Overlay analysis revealed that approximately 300 spots are common to both fractions (data not shown). As a result, the proteome of rice leaf deduced from the present work is at least 1700 proteins.

3.2 Rice leaf proteomes in response to a progressive exposure to low temperature stress

Figure 1 shows four reproducible gel maps of fraction I in accordance with control and low temperature stress conditions. Among the 1330 spots, 37 strained spots were found to be up-regulated under cold stress as annotated in Fig. 1. Figure 2 shows a representative 2-D gel for fraction II. Twenty-three strained spots were up-regulated under cold stress as labeled in Fig. 2. As a result, the expression of 60 proteins was increased in response to the progressively low temperature stress, which altered in abundance more than 1.5-fold in at least one point of the cold stress treatment (Table 1). Among these proteins, 38 spots exhibit a greater than two-fold enhancement (Table 1).

It is noteworthy that different patterns were displayed for a number of proteins (Table 1). Some proteins, *i.e.*, spots 107, 111, 128, 209, 210, and 215, increased steadily as the temperature decreased. Other proteins, *i.e.*, spots 114, 120, 131, 137, 214, and 220, showed a peak at one stage (15 or 10°C) of the low temperature process, followed by a decrease in the level of the proteins. Interestingly, some proteins, *i.e.*, spots 103, 104, 105, 109, 110, 111, 119, 124, 132, 203, 205, 208, 216, and 221, were found to be strongly induced under mild stress conditions (15°C), then maintained at a constant level even if exposed to a higher stress intensity (10 and 5°C). Figure 3 shows examples representing the dynamics of different proteins in response to cold stress conditions. This implies that plant cells were able to monitor different levels of stress intensity by modulating corresponding protein expression.

3.3 Identification of cold responsive proteins

Sixty protein spots up-regulated by low temperature stress were analyzed by MALDI-TOF MS and/or ESI-MS/MS. Proteins identified from the 41 spots are listed in Table 2. Four

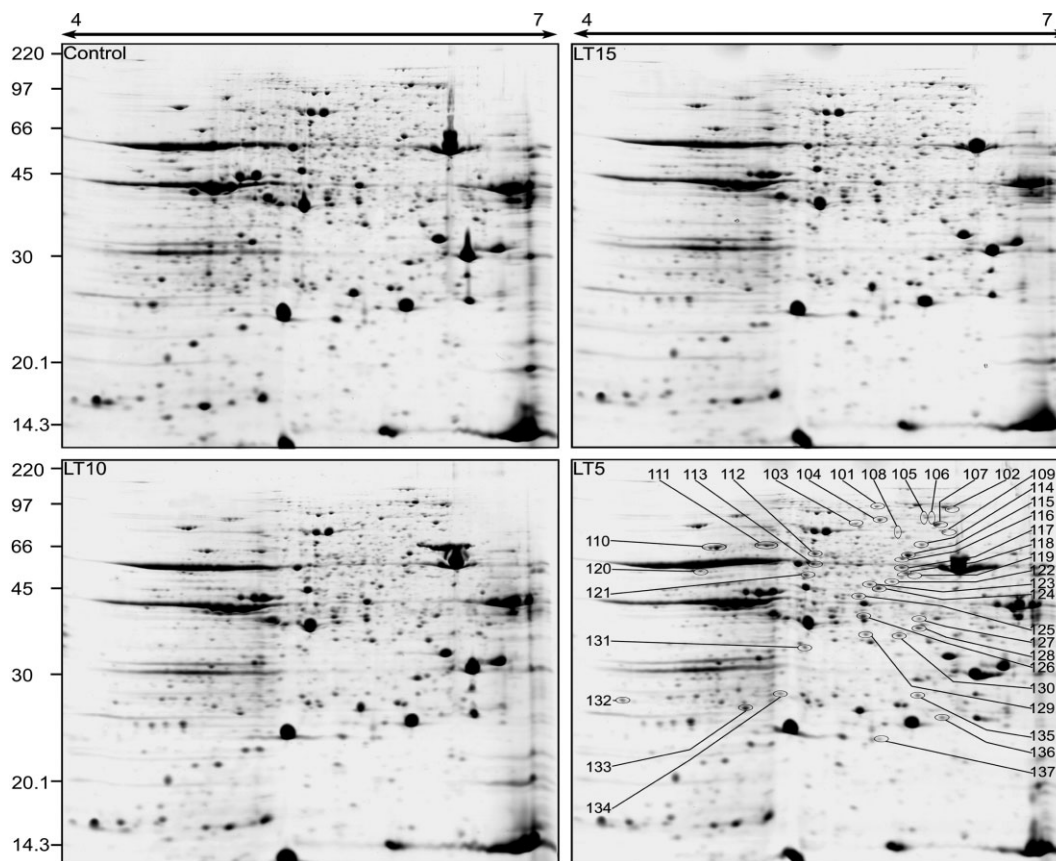


Figure 1. Protein expression profiles of fraction I. Proteins were extracted from rice leaves collected from seedlings grown at normal temperature (control) or exposed to the progressively low temperature within a range of 15°C (LT15), 10°C (LT10), and 5°C (LT5), respectively. Proteins were separated by 2-DE. In the first dimension (IEF), 400 µg of protein was loaded on a 13 cm IPG strip with a linear gradient of pH 4–7. In the second dimension, 13% SDS-PAGE gels were used. Proteins were visualized using CBB R-250. Proteins that increased under cold stress in fraction I are numbered on the 2-D map marked as LT5.

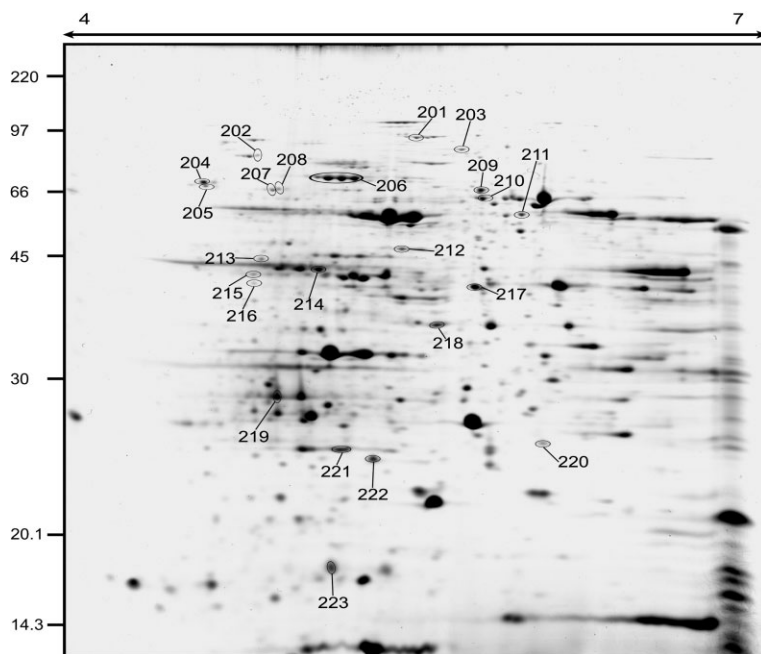


Figure 2. Protein expression profiles of fraction II. Proteins were extracted from rice leaves collected from seedlings exposed to low temperature at 5°C (LT5). Proteins were separated by 2-DE. In the first dimension (IEF), 400 µg of protein was loaded on a 13 cm IPG strip with a linear gradient of pH 4–7. In the second dimension, 13% SDS-PAGE gels were used. Proteins were visualized using CBB R-250. Proteins that increased under cold stress in fraction II are numbered on the 2-D map.

Table 1. Abundance ratio^{a)} of up-regulated proteins in response to progressively low temperature stress in rice leaf

Spot	Abundance ratio			Spot	Abundance ratio		
	LT15	LT10	LT5		LT15	LT10	LT5
1.5–2 folds up-regulated spots							
Fraction I				135*	1.70±0.23	1.79±0.27	1.87±0.35
112	1.70±0.03	1.72±0.01	1.69±0.02	136*	1.81±0.23	1.89±0.31	1.53±0.15
113*	1.56±0.04	1.22±0.19	1.49±0.17	Fraction II			
116*	1.32±0.18	1.45±0.21	1.85±0.28	201*	1.63±0.11	1.34±0.07	1.19±0.16
117*	1.42±0.05	1.69±0.15	1.54±0.18	204	1.40±0.07	1.61±0.11	1.79±0.02
118*	1.35±0.02	1.71±0.10	1.67±0.18	206*	1.36±0.10	1.51±0.06	1.27±0.11
121*	1.69±0.06	1.46±0.11	1.87±0.07	207	1.82±0.12	1.43±0.07	1.76±0.20
125*	1.41±0.07	1.33±0.21	1.58±0.10	211*	1.75±0.14	1.76±0.16	1.59±0.10
126*	1.61±0.02	1.70±0.27	1.63±0.21	212*	1.63±0.09	1.26±0.05	1.08±0.23
128*	1.35±0.12	1.56±0.06	1.92±0.12	213	1.41±0.05	1.85±0.25	1.88±0.07
129	1.38±0.04	1.66±0.26	1.55±0.10	218*	1.77±0.20	1.77±0.29	1.54±0.16
133*	1.76±0.24	1.63±0.22	1.89±0.29	223*	1.73±0.13	1.63±0.19	1.85±0.10
Greater than 2-fold up-regulated spots							
Fraction I				130	1.84±0.09	1.93±0.20	2.16±0.18
101*	1.72±0.37	2.67±0.30	2.17±0.12	131*	3.13±0.09	1.78±0.16	1.63±0.11
102*	1.63±0.21	2.04±0.29	1.93±0.17	132	5.10±0.68	5.66±0.89	8.98±0.81
103	2.54±0.54	2.44±0.42	3.72±0.77	134*	1.49±0.10	1.57±0.23	2.30±0.17
104*	3.06±0.63	3.44±0.51	3.63±0.60	137*	1.97±0.29	2.61±0.69	1.46±0.24
105*	4.85±0.86	6.59±0.86	7.00±1.18	Fraction II			
106*	1.18±0.16	2.82±0.41	2.75±0.69	202*	1.76±0.21	1.80±0.20	2.48±0.49
107*	1.92±0.17	2.36±0.12	3.76±0.68	203*	2.63±0.18	3.15±0.19	2.79±0.08
108	1.98±0.50	3.40±0.46	3.62±0.67	205*	2.99±0.39	5.15±0.15	4.64±0.44
109*	5.48±0.61	4.44±0.82	5.22±0.23	208	2.14±0.39	2.01±0.40	2.00±0.15
110*	2.35±0.16	2.30±0.38	3.13±0.30	209*	1.39±0.30	1.84±0.41	2.39±0.57
111*	2.40±0.20	2.72±0.09	3.36±0.32	210*	1.42±0.09	1.72±0.08	2.22±0.23
114*	1.48±0.14	2.16±0.24	1.83±0.37	214*	2.52±0.12	1.91±0.14	1.38±0.34
115*	0.99±0.11	1.35±0.18	2.13±0.42	215	1.62±0.11	1.86±0.16	2.70±0.13
119	2.39±0.36	2.20±0.41	2.68±0.49	216	2.11±0.41	3.08±0.65	2.99±0.19
120*	2.56±0.55	1.84±0.29	1.34±0.32	217	1.59±0.36	2.08±0.31	1.72±0.20
122*	1.61±0.06	2.33±0.33	2.38±0.17	219	1.08±0.06	3.71±0.49	3.35±0.53
123*	1.67±0.09	2.22±0.27	2.19±0.28	220	2.08±0.13	1.11±0.15	1.07±0.06
124*	2.28±0.10	3.04±0.54	3.07±0.50	221	2.33±0.30	2.15±0.11	2.21±0.38
127	1.27±0.13	1.76±0.16	2.04±0.04	222	2.26±0.25	1.63±0.18	2.10±0.17

a) Spots abundance is expressed as the ratio of intensities between stress and control. Each value represents the mean ± SE of triplicates. LT means low temperature, and the following number represents temperature for stress treatment.

* Represents proteins identified by MALDI-TOF and/or ESI-MS/MS.

proteins (s102, s104, s123, s201) were annotated in the database as OSJNBa0020P07.3 (s102, s104), OSJNBa0011P19.5, and OSJNBa0039C07.4 (s201). From a BLAST search, these proteins were assigned as elongation factor (s102, s104), S-adenosylmethionine synthetase 2 (s123), and ATP-binding subunit of the ATP-dependent Clp protease (s201), respectively (Tables 2, 3).

Using ESI-MS/MS three proteins were identified from spots s107, s133, and s218 (Tables 2, 3). Figure 4 shows one of the peptide sequence tags from s107 used in MASCOT sequence query search, with 100% match to methionine synthase in potato, and 90% match to maize, sorghum, and *Arabidopsis*. In rice, the gene of VB₁₂-independent methionine synthase has been cloned and expressed [25]. The peptide sequence tag shows 100% match to the cloned gene product. Therefore, we annotate the rice protein s107 as VB₁₂-independent methionine synthase (Tables 2, 3). Using two peptide

sequence tags (YTSIKPLGDR, TAGGLLTETTK) generated from spot 133, an *Arabidopsis* homolog of 20 kDa chaperonin (Tables 2, 3) was identified. Another peptide sequence tag (LVYTNDQGEIVK) from s218 leads to an identification of putative ferredoxin-NADP(H) oxidoreductase (Tables 2, 3).

Spot 124, a putative epimerase/dehydratase, shares 90.2% sequence identity with an epimerase/dehydratase in *Arabidopsis* (Fig. 5). This protein has been characterized as 43-kDa GDP-mannose 3', 5'-epimerase in *Arabidopsis* after being subjected to an *in silico* tryptic digestion using the MS-Digest algorithm [26]. Spot s203, a putative reductase, has high homology to mitochondrial NADH-ubiquinone oxidoreductase [27]. Therefore, we annotate these two proteins (s107 and s203) as 43-kDa GDP-mannose 3', 5'-epimerase and NADH-ubiquinone oxidoreductase, respectively. Table 3 is a summary of reannotations suggested from the present work.

Table 2. Leaf proteins responsive to progressively low temperature stress identified by PMF and peptide sequence tags

Spot	Protein name	Accession	Peptides Matched	Sequence Coverage (%)	Theoretical/Experimental		Species	Location ^{a)}	Possible function
					Mass (kDa)	pI			
Fraction I									
101	putative aconitate hydratase	BAD05751	38	32	98.02/103.07	5.67/5.72	rice	–	metabolism
102	OSJNBa0020P07.3	CAE01286	32	34	95.52/98.13	5.85/6.09	rice	–	protein biosynthesis
104	OSJNBa0020P07.3	CAE01286	35	42	93.92/77.82	5.85/5.74	rice	–	protein biosynthesis
105	sucrose synthase 2	N9909830	31	35	92.85/81.99	5.94/5.94	rice	–	metabolism
106	sucrose synthase 1	S23543	22	24	92.07/82.74	5.96/6.00	rice	mit	metabolism
107*	VB ₁₂ -independent methionine synthase	AAF74983			84.61/73.40	5.93/6.03	potato	–	metabolism
109	phenylalanine ammonia-lyase	S06475	22	26	75.74/67.26	8.53/6.07	rice	–	defense response
110	60 kDa chaperonin alpha subunit	AAP44754	11	26	61.61/59.06	5.36/4.86	rice	chl	protein folding and degradation
111	60 kDa chaperonin beta subunit	NP_910308	26	48	64.05/59.56	5.60/5.15	rice	chl	protein folding and degradation
113	UDP-glucose pyrophosphorylase	BAB69069	14	34	51.90/54.98	5.43/5.41	rice	–	metabolism
114	putative oxalyl-CoA decarboxylase	BAB33274	23	41	60.79/61.88	5.95/5.96	rice	–	metabolism
115	ATP synthase CFI alpha chain	NP_039380	32	57	55.63/58.91	5.95/5.88	rice	chl	energy pathway
116	unnamed protein product	CAA34060	23	37	5527/57.72	5.70/5.82	wheat	–	unknown
117	aldehydde dehydrogenase ALDH2b	BAB19052	23	47	59.27/54.89	6.33/5.86	rice	mit	metabolism
118	putative cytosolic 6-phosphogluconate dehydrogenase	NP_910282	20	52	52.69/52.67	5.85/5.87	rice	s	metabolism
120	unknown protein	NP_921715	17	46	51.53/52.16	5.42/4.77	rice	chl	unknown
121	eukaryotic initiation factor 4A	P35683	29	42	46.90/50.32	5.29/5.38	rice	–	protein biosynthesis
122	S-adenosylmethionine synthetase 1	P46611	13	40	43.19/47.71	5.74/5.70	rice	–	metabolism
123	OSJNBa0011P19.5	NP_908684	13	41	42.87/49.00	5.68/5.81	rice	–	metabolism
124	putative epimerase/dehydratase	NP_921492	28	57	42.75/46.50	5.75/5.74	rice	–	metabolism
125	putative glutamate-1-semialdehyde 2,1-aminomutase	BAD11647	22	40	50.21/43.56	6.48/5.64	rice	chl	metabolism
126	putative mRNA binding protein precursor	BAC83225	19	39	40.98/37.22	7.68/5.66	rice	chl	transcription
128	guanine nucleotide-binding protein beta subunit-like protein	NP_916988	9	50	36.21/36.15	5.97/5.95	rice	–	signal transduction
131	putative thiamine biosynthetic enzyme	BAC45141	21	44	36.93/32.82	5.44/5.38	rice	chl	metabolism
133*	20 kDa chaperonin	NP_197572			26.79/26.49	8.86/5.03	arabidopsis	chl	protein folding and degradation
134	ferritin	AAM74943	11	48	28.14/28.40	5.47/5.30	rice	s	defense response
135	glutathione S-transferase II	NP_916246	7	33	24.42/28.46	5.77/5.94	rice	s	defense response
136	probable photosystem II oxygen-evolving complex protein 2 precursor	NP_911136	9	44	26.92/26.34	8.66/6.06	rice	chl	photosynthesis
137	putative 33 kDa oxygen evolving protein of photosystem II	NP_918587	9	33	34.84/24.01	6.10/5.71	rice	chl	photosynthesis
Fraction II									
201	OSJNBa0039C07.4	CAE05148	23	25	98.44/98.23	5.79/5.43	rice	mit	protein folding and degradation
202	70 kDa heat shock protein	T10248	10	17	75.64/76.76	5.15/7.78	cucumber	chl	protein folding and degradation
203	putative reductase	AAL58200	23	35	81.07/84.49	5.86/5.51	rice	mit	respiration metabolism
205	glutelin	1603218A	16	27	56.90/62.09	8.96/4.52	rice	s	metabolism
206	FtsH-like protein Pftf precursor	AAD17230	13	26	74.34/64.94	6.00/5.05	tobacco	chl	protein folding and degradation
209	ATPase alpha subunit	NP_922436	21	41	55.15/62.45	5.95/5.65	rice	–	energy pathway
210	ATP synthase CFI alpha chain	NP_039380	31	60	55.63/60.56	5.95/5.63	rice	chl	energy pathway
211	putative phytoene dehydrogenase precursor	AAK92625	21	38	64.73/56.40	7.95/5.76	rice	chl	metabolism
212	translational elongation factor Tu	AAL37431	13	36	50.38/48.71	6.19/5.37	rice	chl	protein biosynthesis
214	phosphoribulokinase precursor	AAM94337	12	40	44.84/43.25	5.68/4.98	rice	chl	metabolism
218*	putative ferredoxin-NADP(H) oxidoreductase	BAD07826			38.72/35.74	7.98/5.49	rice	chl	electron transport
223	ATP synthase CFI epsilon chain	NP_039389	5	51	15.29/17.96	5.03/5.12	rice	chl	energy pathway

* Refers to the proteins identified by ESI-MS/MS.

a) Location of identified proteins were predicted by TargetP ([http:// www.cbs.dtu.dk/services/TargetP](http://www.cbs.dtu.dk/services/TargetP)) [29]. chl: chloroplast; mit: mitochondrion; _: any other location; s: secretory pathway.

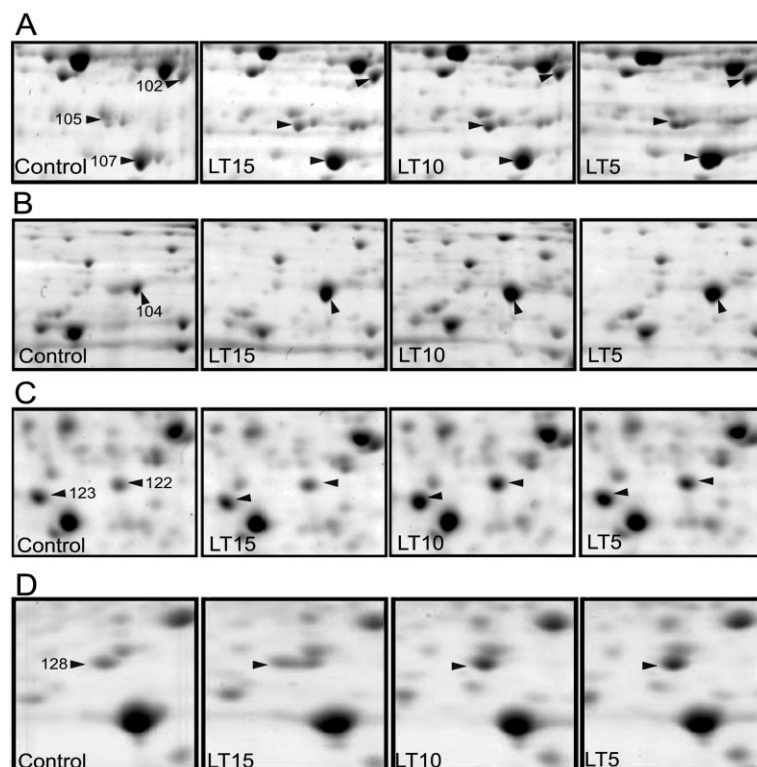


Figure 3. Examples of induction of some protein spots during the progressively low temperature process from normal temperature to 15, 10, and 5°C (marked as control, LT15, LT10, LT5). (A) s102, elongation factor; s105, sucrose synthase 2; s107, VB₁₂-independent methionine synthase. (B) s104, elongation factor. (C) s122, S-adenosylmethionine synthetase 1; s123, S-adenosylmethionine synthetase 2. (D) s128, guanine nucleotide-binding protein beta subunit-like protein, showing a migration under cold stress.

Table 3. Protein annotation and proteins identified by ESI-MS/MS

Spot	protein name (from NCBI)	Suggested protein name		
Proteins annotated				
102	OSJNBa0020P07.3	elongation facor		
104	OSJNBa0020P07.3	elongation facor		
123	OSJNBa0011P19.5	S-adenosylmethionine synthetase 2		
124	putative epimerase/dehydratase	GDP-mannose 3', 5'-epimerase		
201	OSJNBa0039C07.4	ATP-dependent Clp protease ATP-binding subunit		
203	putative reductase	NADH-ubiquinone oxidoreductase		
Proteins identified by ESI-MS/MS		ESI-MS/MS		
		Sequence tag	%Identify ^{a)}	Score ^{b)}
107	VB ₁₂ -independent methionine synthase	IPSTEEIADR	100	135
133	20 kDA chaperonin	YTSIKPLGDR	100	141
		TAGGLLLTETTK	100	157
218	putative ferredoxin-NADP(H) oxidoreductase	LVYTNDQGEIVK	100	164

a) Percentage of identity between the amino acids present in MS/MS tag and the sequences in databases

b) Scores greater than 75 are significant ($p < 0.05$).

3.4 Functional classification and subcellular localization prediction of cold responsive proteins

All protein sequences detected and identified were searched against gene ontology tool (www.geneontology.org) and TargetP program (www.cbs.dtu.dk/services/TargetP) for func-

tional classification and subcellular localization prediction [28, 29]. These identified cold responsive proteins were found to be involved in diverse biological processes, covering signal transduction (s128), transcription (s126), protein biosynthesis (s102, s104, s121, s212), protein folding, assembly and degradation (s110, s111, s133, s202, s201, s206), defense response (s109, s134, s135) [30–34], energy pathway (s115,

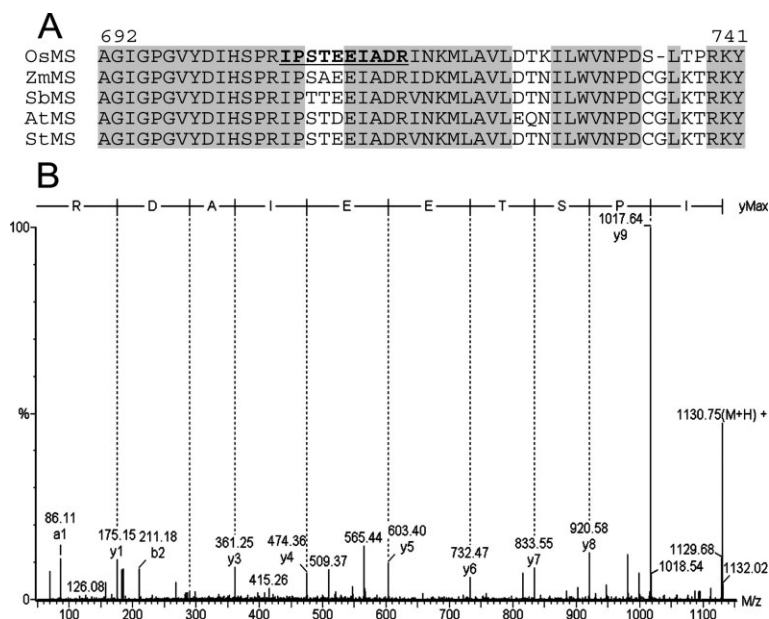


Figure 4. Identification of methionine synthase (MS) based on peptide sequence tag derived from ESI-MS/MS. (A) Multiple sequence alignment of MS (showing only a fragment of the entire sequence) in five species. Identity: 92.89%. The peptide sequence tag is underlined. OsMS, MS from rice; ZmMS, MS from maize; SbMS, MS from sorghum; AtMS, MS in *Arabidopsis*; StMS, MS of potato. (B) NanoESI-MS/MS spectrum of the m/z 1130.75 ($M + H$)⁺ peptide ion derived from in-gel tryptic digestion of s107.

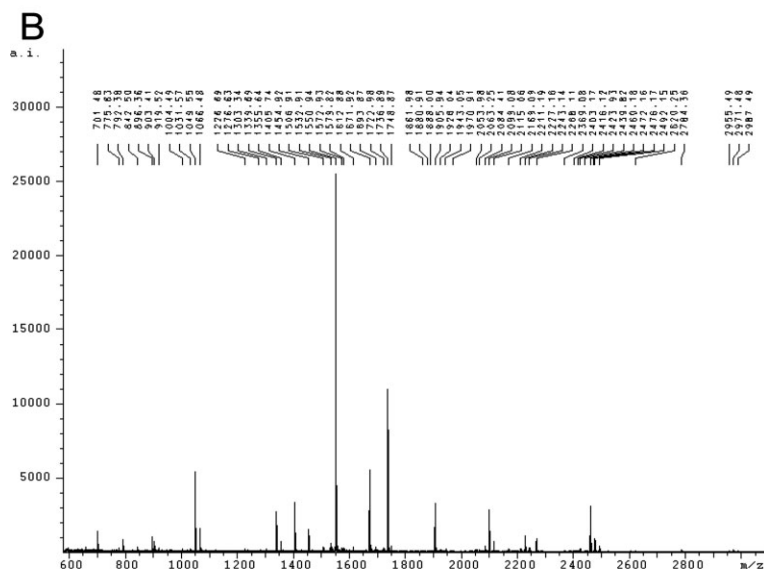


Figure 5. Identification of putative epimerase/dehydratase (s124). (A) Sequence alignment of putative epimerase/dehydratase in rice (OsEPIM) with GDP-mannose 3', 5'-epimerase from *A. thaliana* (AtEPIM). Identity: 90.24%. The sequence of the fragments is deduced from MALDI analysis. The sequence coverage is 52%. (B) MALDI-TOF spectrum of tryptic digestion from s124.

s209, s210, s223), and metabolism. Two proteins with unknown function (s116, s120) were also identified as cold response proteins in this study. Among proteins responsible for metabolism, three enzymes are involved in synthesis of methionine and *S*-adenosylmethionine (s107, s122, s123), three enzymes are related to formation of UDP-glucose (s105, s106, s113), and two proteins are linked to oxygen-evolving reaction of photosynthesis (s136, s137) (Tables 2, 3). These results indicate that the biochemical pathways mentioned above are subjective to cold stress. Using TargetP program, prediction of subcellular localization of identified proteins based on their *N*-terminal amino acid sequence was performed [29]. The result of prediction shows that as much as 43.9% of the up-regulated proteins are located in the chloroplasts (Table 2). This implies that chloroplasts are one of the organelles inside cells mostly influenced by cold stress.

4 Discussion

In this work, we present a carefully performed proteomic analysis of rice leaves subjected to progressively low temperature treatments. Using a fractional extraction approach, 1700 protein spots were visualized on 2-D gels for two protein fractions extracted from the leaves. To distinguish cold responsive proteins from the proteins synthesized due to cellular injury caused by shock conditions [35] and to obtain the dynamic protein expression patterns responsive to different cold stress intensity, we adopted a treatment process of progressively low temperature stress. As a result, we found 60 cold responsive proteins, their expression was significantly increased after exposure to the progressively low temperature stress (Table 1).

Forty-one out of the 60 proteins were identified using MALDI-TOF MS or ESI/MS/MS coupled with database searching (Tables 2, 3). A number of proteins that were found to be cold stress-responsive proteins previously are verified in the present investigation using proteomic approach. These proteins include: phenylalanine ammonia-lyase [30, 36], ferritin [37], and sucrose synthase [38]. Other proteins that are recognized as general stress-inducible proteins, such as GSTs [33, 39, 40], *S*-adenosylmethionine synthetase [41], and molecular chaperons were also evident in this specific study examining cold stress response. Furthermore, our results also reveal a wider scope of cold responsive proteins and/or enzymes, such as GDP-mannose 3', 5'-epimerase, oxalyl-CoA decarboxylase, thiamine biosynthetic enzyme, glutelin, methionine synthase, and two proteins of oxygen-evolving complex. Based on their putative physiological functions, proteins identified in the present work can be divided into four subgroups in correlation to their specific biological functions.

The major subgroup in response to cold stress was found to be attributed to protein metabolism. Four proteins identified are factors of protein biosynthesis (s102, s104, s121, s212), and six proteins are involved in protein

folding, assembly, and degradation. They are 60 kDa chaperonin alpha and beta subunits (s110, s111), 20 kDa chaperonin (s133), 70 kDa heat shock protein (s202), ATP-binding subunit of ATP-dependent Clp protease (s201), and FtsH-like protein Ptf precursor (s206). These molecular chaperones and proteases have been intensively studied and are known to be induced under various stress conditions [42–45]. Our data demonstrate that the expression of these proteins responsible for protein synthesis and quality control is enhanced remarkably under cold stress, suggesting that novel protein biosynthesis was required under cold stress, and an active protein quality control system inside the cells is playing an important role in plant tolerance to cold stress.

The minor subgroup in response to cold stress is assigned to eight proteins that are involved in a flow of event in biosynthesis of cell wall components. They include sucrose synthase 1 and 2 (s105, s106), UDP-glucose pyrophosphorylase (s113), phenylalanine ammonia-lyase (s109), methionine synthase (s107), two isoenzymes of *S*-adenosylmethionine synthetase (s122 and s123), and glutamate semialdehyde aminotransferase (s125). Sucrose synthase and UDP-glucose pyrophosphorylase catalyze sucrose and glucose-1-phosphate into UDP-glucose, respectively. UDP-glucose is directly used for cellulose synthesis [46]. Phenylalanine ammonia-lyase is a key regulatory enzyme in phenylpropanoid metabolism. The pathway produces a large amount of phenolic compounds, which are the precursor molecules of suberins and lignins [47, 48]. Methionine synthase catalyzes the formation of methionine, which further catalyzed into *S*-adenosylmethionine by *S*-adenosylmethionine synthetase. *S*-adenosylmethionine is a major methyl group donor for many cellular molecules, particularly for the methylation of several derivatives of the phenylpropanoid pathway, including the methylation of cinnamic acids, an enzyme product of phenylalanine ammonia-lyase [49]. Cinnamic acid is one of the intermediates in biosynthesis of lignin and suberin. Therefore, we assume that the response of methionine synthase and *S*-adenosylmethionine synthetase to low temperature may stimulate the biosynthesis of lignin and suberin. However, it should be pointed out that methionine synthase and *S*-adenosylmethionine synthetase are also involved metabolically in the biosynthesis of ethylene and polyamines, and the phytohormones that are tightly correlated with plant response to stress environments including low temperature [50–52]. Therefore, the specific roles of these enzymes in response to the progressively low temperature stress remain to be established. As for glutamate semialdehyde aminotransferase, its role in plant stress response was thought to be relevant to lignification of cell wall [5].

The third subgroup in response to cold stress comprises seven proteins, which are linked to antioxidative/detoxifying reaction. For example, ferritin (s134), an iron-binding protein, is proposed to protect plants from oxidative damage induced by a wide range of stresses [31]. GDP-mannose 3', 5'-epimerase (s124) and 6-phosphogluconate dehydrogenase

(s118) may apply their antioxidative effect by affecting level of ascorbic acid and glutathione, two main antioxidants in cells [26, 53]. GST (s135) with activity of GSH peroxidase is an enzyme catalyzing the conjugation of the glutathione to a variety of toxic substrates arising from oxidative stress, thereby reducing their toxicity [32–34]. Another two enzymes, aldehyde dehydrogenase (s117) and oxalyl-CoA decarboxylase (s114), are the enzymes responsible for degradation of aldehydes arising from reactions of reactive oxygen species with lipids, proteins [54], and oxalic acid, a byproduct accumulated under environmental stress, respectively. In addition, we identified thiamine biosynthetic enzyme (s131) in this subgroup because thiamin pyrophosphate is coenzyme of oxalyl-CoA decarboxylase.

The fourth subgroup in response to cold stress is obviously related to energy pathway. Three proteins, *i.e.*, alpha and epsilon chains of ATP synthase CF1 (s115, s223) and ATPase alpha subunit (s209) were up-regulated under cold stress. This implies that more energy is required for reinforcing plant resistance to cold stress. The enhancement of the elongation factors (s102, s104, s212), and initiation factor 4A (s121) is evident of the energy acquired in biosynthesis of stress responsive proteins. In addition, we found some enzymes involved in photosynthesis and respiration, such as four proteins served as probable components of electron transport chain, *i.e.*, two oxygen-evolving complex proteins (s136 and s137), ferredoxin-NADP(H) oxidoreductase (s218), and NADH-ubiquinone oxidoreductase 75 kDa subunit (s203). Two enzymes involved in carbohydrate metabolism, *i.e.*, aconitate hydratase (s101) and phosphoribulokinase (s214) were also found. The enhancement of expression of these proteins implies that cold stress results in a foundational metabolic alteration.

In summary, the present initial proteomic investigation of rice leaf crude extractions reveals a complex cellular network affected by the low temperature stress. The network covers a broad metabolic process, including protein biosynthesis and quality control system, biosynthesis of cell wall components, antioxidative/detoxifying reaction and energy production, and metabolites supply persisting to plant cold stress tolerance. It is noteworthy that although the application of the fractionation of protein extraction procedures allowed us to obtain the significantly extended proteome in rice exposed to cold stress, most of the identified proteins are soluble proteins. For substantial proteins resolved from the water-insoluble fraction (fraction II), we failed to obtain any match in the current database and therefore virtually only a handful of membrane proteins were identified in the work presented in this paper. To uncover more insight into how this intricate network is operating within the cells encountering unfavorable growth conditions, an entire list of stress-responsive protein identification is essential. Using a complementary proteomic strategy [55], one can not doubt attempt in combination, a more comprehensive database of rice, towards such a desirable goal.

This work was supported by the National Natural Science Foundation of China (nos. 30370847, 30170080, and 30270753), and the State Key Basic Research and Development Plan of China (200 CB 117303).

5 References

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