



## Research article

## A comparative proteomic study of cold responses in potato leaves

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## ABSTRACT

The potato is an important food crop worldwide. While potatoes are rich in nutrition, the production suffers from yield loss caused by frost and freezing. This study used a common potato cultivar, 'Zhengshu 6', as the study system to measure the changes in the contents of soluble protein, malondialdehyde (MDA), proline, and chlorophyll after 1, 3, 5, and 7 days of low temperature treatment. We performed two-dimensional electrophoresis (2-DE) in combination with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) technology and identified 52 differentially expressed protein spots among these timepoints. Results showed that levels of soluble protein, MDA, and proline increased as the duration of the low temperature treatment increased, and the chlorophyll content decreased. The 52 identified protein spots were classified by function as involved in defense response, energy metabolism, photosynthesis, protein degradation, ribosome formation, signal transduction, cell movement, nitrogen metabolism, and other physiological processes, thus allowing potato plants to achieve metabolic balance at low temperatures.

## 1. Introduction

The potato (*Solanum tuberosum* L) is one of the world's most important food crops. Potato tubers are highly nutritious; they are rich in starch, vitamins, minerals, and essential amino acids, and are therefore suitable for consumption as a staple food source. Potatoes are also served as animal feed and used as feedstock for many industrial purposes [1]. Despite the agricultural importance of the potato most potato cultivars are not tolerant to frost and freezing conditions; cold injury occurs when the temperature falls below  $-0.8$  °C, and severe freezing injury occurs below  $-2$  °C [2]. Low temperature-induced frost and freezing are two of the main natural hazards that affect potato production. Frost and freezing can cause significant damage to potato plants, leading to reduced yield or even complete yield loss when severe. Annually, all potato-growing regions across the world experience different degrees of cold or freezing damage [3,4,5,6,7,8]. Almost all of the major potato cultivation areas in China suffer from low temperatures, especially in late spring, when potatoes are in the seedling stage. Further damage caused by cold waves and early frosts can occur at the mature stage.

Plant response to low temperature stress is mediated by a series of proteins. Changes in gene expression have been demonstrated to occur in response to low temperature in a wide range of plant species, including

both freezing-tolerant and freezing-sensitive plants. Cold response pathways have been demonstrated to exist in plants that acclimate to low temperatures. Therefore, the characteristic variation analysis of proteome differentiation among different genotypes in response to low temperatures can facilitate analysis of the mechanism underlying cold adaptation [9]. As a result, the study of potato proteomics under low temperature stress has great significance for revealing the plant's cold tolerance mechanism, thereby providing information that may be used to improve its stress-resistance traits in order to reduce the losses caused by low temperature damage.

Upon experiencing low temperature stress, plants display a series of physiological and biochemical changes, which are caused by molecular changes in the plant cells, i.e., changes in the transcriptome, proteome and metabolome. Proline is an important osmoregulation substance. Plant cells lose water after stress. By increasing the content of proline *in vivo*, the concentration of cell fluid is increased, so as to maintain the water holding capacity of cells. Studies have shown that the content of proline is positively related to the cold resistance of the potato [10]. Malondialdehyde (MDA) is one of the main products of plants. For example, a large number of up- and downregulated low temperature-responsive membrane lipid peroxidation in plants under low temperature stress. When freezing injury occurs in plants, the balance of active oxygen

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metabolism in cells is disrupted. The superoxide radicals produced will attack the cell membrane and produce a large amount of malondialdehyde through metabolism. Therefore, malondialdehyde content can be an important indicator of the degree of membrane damage. The mechanism of low temperature tolerance has been studied in responsive genes identified in *Arabidopsis*, wheat, and barley using transcriptomics [11,12,13]. However, due to the regulation of protein translation and degradation, changes in RNA expression under low temperature stress cannot fully indicate the changes at the protein level [14,15,16]. Therefore, proteomics technology is needed to reveal the expression of proteins and to identify those involved in stress tolerance. In recent years, proteomics technology has been developing rapidly along with the completion of genome sequencing for many crops. In rice [17,18], wheat [19], barley [20], and *Arabidopsis* [21], proteomics technology has been successfully applied to study the mechanism underlying low temperature tolerance. Some proteomic studies on crops from the *Solanaceae* family (e.g., tomato, tobacco, and pepper) are also available [22,23]. In potato plants, proteomics has been used to reveal the physiological mechanism underlying tuber development and formation, the pathogenesis of potato late blight, and the mechanism underlying salt stress [24,25]. However, there have been no reports concerning the use of proteomics to determine the mechanism underlying cold tolerance in the potato. Thus, to understand the protein expression profile in potatoes under low temperature stress, we utilized two-dimensional electrophoresis (2-DE) in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to perform proteomics analysis on potatoes under low temperature stress conditions. The results may provide insights into the mechanism of cold tolerance in the potato.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The potato cultivar ‘Zhengshu 6’ [26], bred at the Zhengzhou Vegetable Institute, was used in this study. Potato tubers were planted in plastic pots (height 22 cm × diameter 15 cm), one plant per pot, with six plants total. After seedling emergence, pots were transferred to a growth chamber (22 °C 16 h light, 18 °C 8 h dark, 70% relative humidity, and

light intensity 600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). After growing for four weeks, plants were subjected to low temperature treatment.

### 2.2. Experimental treatment

Four-week old potato plants were placed in the growth chamber for low temperature treatment (16 h light, 8 h dark, 4 °C daytime, 2 °C night time; 65% relative humidity; light intensity 600–1000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The expanded 3rd and 4th leaves were collected before the low temperature treatment (control) and after 1 d, 3 d, 5 d, and 7 d of the low temperature treatment. Samples were frozen in liquid nitrogen and then stored at  $-80\text{ }^{\circ}\text{C}$ .

### 2.3. Measurements of the physiological and biochemical parameters

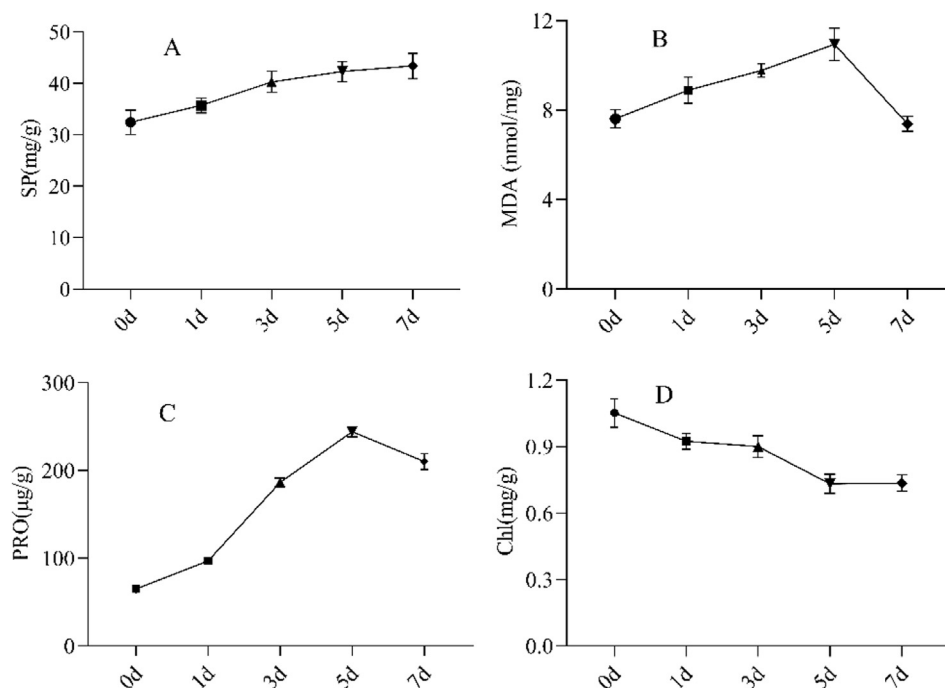
The content of soluble protein (SP) was measured using the bicinchoninic acid (BCA) method [27]. Proline (PRO) content was measured using the ninhydrin method [28]. Malondialdehyde (MDA) content was measured using the thiobarbituric acid (TBA) method [29]. Chlorophyll content was measured using an acetone and ethanol mixture.

### 2.4. Protein sample preparation

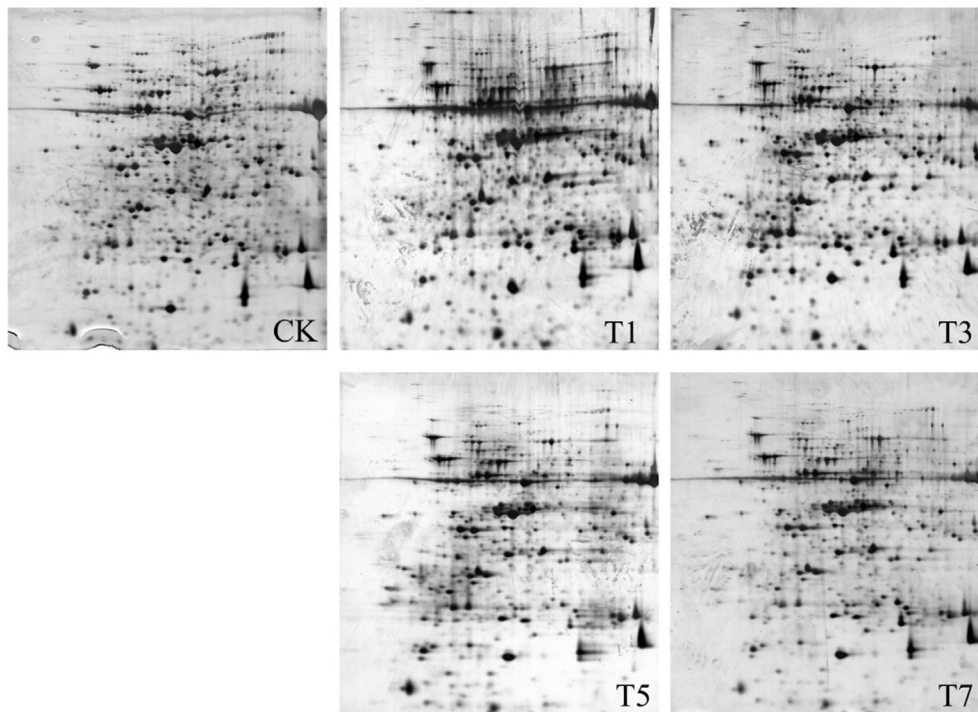
Total protein extraction from potato leaves was performed using trichloroacetic acid/acetone (TCA/A) precipitation [30]. A modified Bradford method was used for protein quantification with bovine serum albumin as a standard. Quantified protein was then used for further analysis [31].

### 2.5. 2-DE, gel staining, and image analysis

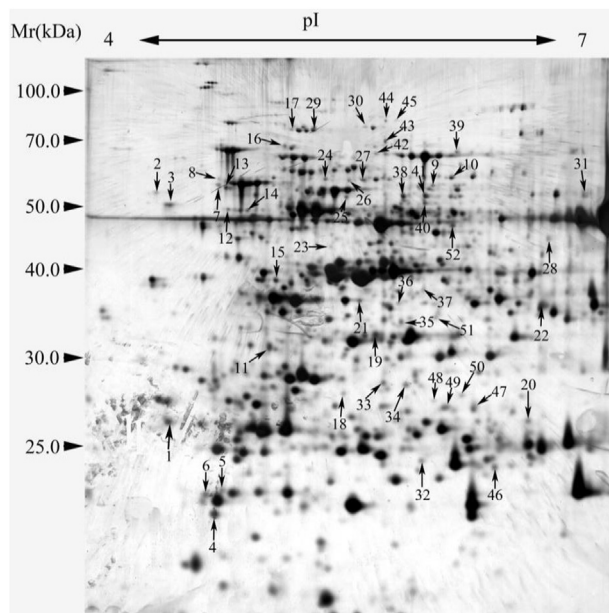
Isoelectric focusing in the first dimension was carried out on the Bio-Rad PROTEAN IEF Cell. Protein samples were diluted with a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS (W/V), 50 mM dithiothreitol (DTT), and 0.5% (V/V) IPG buffer (pH 4–7) to a concentration of 800  $\mu\text{g/mL}$  for sample loading. After centrifugation at 10,000 g for 15 min, 300  $\mu\text{L}$  supernatant was loaded onto the 17 cm gel strip. The isoelectric focusing program was as follows: active rehydration at 50 V



**Figure 1.** Changes in soluble protein, malondialdehyde, proline, and chlorophyll during low temperature stress.



**Figure 2.** Representative spot maps of potato cv. Zhengshu 6 at different stages.



**Figure 3.** Identification of 52 differentially expressed protein spots from potato leaves by 2-DE.

for 13 h (20 °C) followed by a rapid increase of the voltage to 100 V applied for 1 h, 500 V for 1 h, and 1000 V for 2 h, followed by a linear increase of the voltage to 8,000 V for 4 h. Subsequently, isoelectric focusing was operated at 8,000 V for 7.5 h, reaching 60,000 Vh. Finally, a rapid decrease of voltage to 500 V was applied and maintained for 10 h. Following the first-dimension isoelectric focusing (IEF) separation, the strips were equilibrated in a buffer containing 6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (W/V) sodium dodecyl sulfate (SDS), 20% glycerol (V/V), and 2% (W/V) DTT for 15 min to unfold the proteins. In the second step, an equilibration buffer with 2% (W/V) DTT replaced by 2.5% (w/v)

iodoacetamide was used for re-equilibration and was applied for 15 min to remove extra DTT.

The second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the Ettan™ DALT SIX System (GE Healthcare, Washington, USA) with 12% gels (26 cm × 20 cm). SDS-PAGE was performed with 1.0 W/gel for 40 min (20 °C) followed by a 10 W/gel until the loading dye reached 1 cm above the bottom of the gel (20 °C).

A 2-DE gel staining was performed using the silver nitrate method [32]. A gel imaging system, the UMAX PowerLook 2100XL scanner (UMAX Systems GmbH, Willich, Germany), was used to scan the gel image. PDQuest software version 7.1 (Bio-Rad, Hercules, CA, USA) was used to analyze the gel image to determine the protein isoelectric point, molecular weight, and relative expression, and to match the set of protein spots on the gels with three replicates for each treatment. Protein quantity was normalized. Compared to the control, protein spots with significantly different expression greater than 2-fold were considered differentially expressed protein (DEP) spots (Student's *t*-test,  $P < 0.05$ ).

#### 2.6. In-gel digestion, mass spectrometry analysis, and database searching

In-gel enzyme digestion of protein spots was carried out as described in [33]. Triple TOF 5600 LC-MS/MS high resolution liquid chromatography coupled with a mass spectrometry system (AB Sciex) was used for the analysis. The Triple TOF 5600 mass spectrometer was used for first and second MS scans. IDA was used for data analysis. LC separation conditions were as follows: Thermo C18 column (4.6 × 150), mobile phase 90% acetonitrile/0.1% formic acid, speed 0.3 mL min<sup>-1</sup>. The range of the MS scan was 300–2000 *m/z*, followed by the secondary scan (MS/MS). Maximum charge of the precursor ions was 80 eV. The obtained MS or MS/MS data were subjected to database searching using ProteinPilot™ 5.0 software (AB SCIEX, Framingham, MA, USA) for protein identification. The fixed modification was cysteine acetylation, the variable modification was methionine oxidation, the maximum number of enzyme cleavage sites allowed was two, and mass tolerance of parent ions was 0.05 Da. Potato protein sequences downloaded from the potato genome database (<http://solanaceae.plantbiology.msu.edu/pg>

**Table 1.** Identification of 52 differently expressed protein spots from potato leaves.

Spot No. <sup>a)</sup>	Protein Name <sup>b)</sup>	AccessionNo. <sup>c)</sup>	Unused <sup>d)</sup>	MP <sup>e)</sup>	SC(% <sup>f)</sup>	Theor. Mr/pI <sup>g)</sup>	Exp. Mr/pI <sup>h)</sup>	Subcellular localization <sup>i)</sup>	Fold changes <sup>j)</sup>				
									FC <sub>T1/CK</sub>	FC <sub>T3/CK</sub>	FC <sub>T5/CK</sub>	FC <sub>T7/CK</sub>	
<b>01 Metabolism</b>													
P11	11	thiamine thiazole synthase 1, chloroplastic [Solanum tuberosum]	gi 565388645	11.38	7	28.6	37.8/5.40	30.6/5.21	Chloroplast	1.22	1.44	4.61↑	3.25↑
P34	34	magnesium protoporphyrin IX methyltransferase, chloroplastic [Solanum tuberosum]	gi 565348979	10.05	5	11	35.5/6.61	28.3/5.85	Extracellular	0.68	0.46↓	0.43↓	0.61
P38	38	ketol-acid reductoisomerase, chloroplastic [Solanum tuberosum]	gi 565366716	48.19	47	35.6	110.7/6.07	54.0/5.83	Nucleus	1.00	0.68	0.53	0.44↓
P40	40	glutamine synthetase [Solanum tuberosum]	gi 565371595	10.05	5	11	53.4/5.26	51.3/5.92	Plasma membrane	1.40	3.11↑	2.04↑	2.10↑
P51	51	ferredoxin-dependent glutamate synthase 1, chloroplastic/ mitochondrial [Solanum tuberosum]	gi 565396313	40.15	23	19.6	77.2/6.18	33.6/5.97	Plasma membrane	0.89	0.89	0.31↓	0.49↓
P54	9	putative dihydroxy-acid dehydratase, mitochondrial [Solanum tuberosum]	gi 565359581	13.23	9	12.9	66.3/6.17	58.0/5.75	Chloroplast	0.59	0.44↓	0.28↓	0.39↓
P58	22	fructose-bisphosphate aldolase, cytoplasmic isozyme 1 [Solanum tuberosum]	gi 565358575	64.47	71	45.7	78.2/5.94	35.9/6.45	Chloroplast	1.20	0.83	0.41↓	0.72
P60	28	fructose-bisphosphate aldolase, cytoplasmic isozyme 1 [Solanum tuberosum]	gi 565358575	16	8	30	38.2/5.94	44.5/6.49	Chloroplast	0.75	0.59	0.46↓	0.37↓
<b>02 Energy</b>													
P4	4	ATP synthase delta chain, chloroplastic [Solanum tuberosum]	gi 565389084	25.29	28	38.4	27.2/8.61	21.4/4.68	Chloroplast	0.32↓	1.57	1.31	1.09
P8	8	ruBisCO large subunit-binding protein subunit alpha, chloroplastic [Solanum tuberosum]	gi 565346319	6	3	6.5	61.9/5.37	59.3/4.68	Nucleus	0.43↓	0.49↓	0.41↓	0.42↓
P13	13	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [Solanum tuberosum]	gi 88656812	6.27	5	12.8	52.7/6.59	56.7/4.98	Cytoplasm	0.48↓	0.59	0.86	0.47↓
P19	19	ATP synthase CF1 beta subunit (chloroplast) [Solanum tuberosum]	gi 329124676	32.51	26	52.4	53.4/5.26	32.3/5.71	Chloroplast	1.55	1.55	0.79	1.03
P23	23	glyceraldehyde-3-phosphate	gi 565386971	16.07	10	22.3	42.7/8.46	42.7/5.50	Cytoplasm	0.43↓	0.45↓	0.24↓	0.48↓

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Table 1 (continued)

Spot No. <sup>a)</sup>	Protein Name <sup>b)</sup>	AccessionNo. <sup>c)</sup>	Unused <sup>d)</sup>	MP <sup>e)</sup>	SC(% <sup>f)</sup>	Theor. Mr/pI <sup>g)</sup>	Exp. Mr/pI <sup>h)</sup>	Subcellular localization <sup>i)</sup>	Fold changes <sup>j)</sup>				
									FC <sub>T1/CK</sub>	FC <sub>T3/CK</sub>	FC <sub>T5/CK</sub>	FC <sub>T7/CK</sub>	
	dehydrogenase A, chloroplastic [Solanum tuberosum]												
P24	24	plastidic phosphoglucomutase [Solanum tuberosum]	gi 8250622	27.68	29	18.5	61.2/5.26	60.6/5.46	Chloroplast	0.29↓	0.42↓	0.52	0.40↓
P25	25	glyceraldehyde-3-phosphate dehydrogenase (chloroplast) [Solanum tuberosum]	gi 327198779	18.61	16	32.2	48.0/7.06	54.4/5.54	Nucleus	1.58	0.83	3.09↑	2.09↑
P26	26	plastidic phosphoglucomutase [Solanum tuberosum]	gi 8250622	39.33	16	26.9	61.1/5.26	60.7/5.65	Nucleus	1.08	0.66	1.29	1.19
P27	27	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [Solanum tuberosum]	gi 565402329	29.45	16	19.9	61.2/5.37	60.7/5.65	Cytoplasm	0.34↓	0.54	0.64	0.51
P30	30	ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic [Solanum tuberosum]	gi 565359707	46.65	25	25.4	102.2/5.99	80.7/5.65	Nucleus	0.18↓	0.33↓	0.56	0.47↓
P32	32	ribulose biphosphate carboxylase/oxygenase activase 1, chloroplastic isoform X2 [Solanum tuberosum]	gi 565343801	18.93	18	26.6	48.3/8.49	23.1/5.94	Endoplasmic reticulum	0.69	0.61	0.33↓	0.63
P35	35	ribulose biphosphate carboxylase/oxygenase activase 1, chloroplastic isoform X2 [Solanum tuberosum]	gi 565343801	18.02	15	29.8	48.3/8.49	33.6/5.83	Nucleus	0.58	0.57	0.49↓	0.67
P36	36	glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic [Solanum tuberosum]	gi 565351180	12.34	8	23.1	47.93/7.53	35.5/5.81	Nucleus	0.85	0.52	3.7↑	2.31↑
P41	41	glyceraldehyde-3-phosphate dehydrogenase, cytosolic [Solanum tuberosum]	gi 565361313	8.05	5	18.7	36.6/6.98	54.0/5.92	Plasma membrane	0.71	0.60	0.53	0.55
P46	46	ATP synthase CF1 beta subunit (chloroplast) [Solanum tuberosum]	gi 329124676	72.63	73	83.1	53.4/5.26	23.7/6.23	Nucleus	1.26	0.90	2.92	1.67
P47	47	ATP synthase CF1 alpha subunit (chloroplast) [Solanum tuberosum]	gi 88656789	35.55	23	44.8	55.4/5.14	27.0/6.14	Nucleus	0.66	0.41↓	0.42↓	0.68
P48	48	oxygen-evolving enhancer protein 1,	gi 565355906	41.19	29	61.4	35.0/5.89	27.3/5.98	Nucleus	0.49↓	0.49↓	0.43↓	0.80

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Table 1 (continued)

Spot No. <sup>a)</sup>	Protein Name <sup>b)</sup>	AccessionNo. <sup>c)</sup>	Unused <sup>d)</sup>	MP <sup>e)</sup>	SC(% <sup>f)</sup>	Theor. Mr/pI <sup>g)</sup>	Exp. Mr/pI <sup>h)</sup>	Subcellular localization <sup>i)</sup>	Fold changes <sup>j)</sup>				
									FC <sub>T1/CK</sub>	FC <sub>T3/CK</sub>	FC <sub>T5/CK</sub>	FC <sub>T7/CK</sub>	
	chloroplatic [Solanum tuberosum]												
P49	49	chlorophyll a-b binding protein 3C, chloroplatic isoform X2 [Solanum tuberosum]	gi 565391644	33.99	32	43.1	28.3/5.47	27.0/6.02		0.93	0.56	0.44↓	0.80
P50	50	ATP synthase CF0 subunit I (chloroplast) [Solanum tuberosum]	gi 88656790	23.51	17	60.3	20.9/8.76	27.8/6.10	Nucleus	0.59	0.38↓	0.23↓	0.52
P53	52	enolase [Solanum tuberosum]	gi 565343656	44.86	29	54.1	47.9/5.79	47.3/6.06		2.36↑	0.65	4.83↑	1.71
P55	10	NADP-dependent malic enzyme [Solanum tuberosum]	gi 565357492	12	6	10	64.1/5.71	61.3/6.06	Chloroplast	0.65	0.91	0.72	0.96
P61	31	dihydrolipoyl dehydrogenase 1, mitochondrial [Solanum tuberosum]	gi 565378559	49	44	41.8	52.9/6.90	54.0/6.64	Cytoplasm	5.41↑	4.76↑	3.34↑	1.69
P62	39	transketolase, chloroplatic [Solanum tuberosum]	gi 565357366	49.81	48	29.9	80.2/6.22	68.9/6.08	Chloroplast	0.61	0.33↓	0.39↓	0.48↓
<b>05 protein synthesis</b>													
P1	1	28 kDa ribonucleoprotein, chloroplatic [Solanum tuberosum]	gi 565369617	40.72	49	48.3	33.3/4.67	27.1/4.61	Chloroplast	0.75	0.94	0.65	0.57
P15	15	P0 ribosomal protein [Solanum tuberosum]	gi 82623393	42.13	41	44.1	33.9/5.11	37.7/5.23	Chloroplast	4.88↑	1.70	2.92↑	3.56↑
P17	17	elongation factor G, chloroplatic [Solanum tuberosum]	gi 565378095	13.36	8	10.8	86.4/5.40	78.6/5.31	Chloroplast	0.96	0.50	0.88	0.48↓
P18	18	29 kDa ribonucleoprotein A, chloroplatic [Solanum tuberosum]	gi 565395354	8.29	5	13.4	31.8/6.34	27.8/5.33	Vacuole	0.44↓	0.30↓	0.30↓	0.44↓
P29	29	elongation factor G, chloroplatic [Solanum tuberosum]	gi 565378095	58.29	43	40.2	86.4/5.40	78.6/5.42	Mitochondrion	0.49↓	0.52	0.53	0.46↓
<b>06 protein destination and storage</b>													
P2	2	26S proteasome non-ATPase regulatory subunit 4 homolog isoform X3 [Solanum tuberosum]	gi 565347475	28.08	22	48.3	42.7/4.46	55.3/4.59	Nucleus	0.46↓	0.26↓	0.28↓	0.74
P7	7	2-Cys peroxiredoxin BAS1, chloroplatic [Solanum tuberosum]	gi 565344108	6	3	14.2	52.7/4.78	57.5/4.68	Chloroplast	0.27↓	0.48↓	0.52	0.37↓
P16	16	luminal-binding protein [Solanum tuberosum]	gi 565353800	44.4	26	10.8	70.7/4.99	70.0/5.38	Cytoplasm	0.38↓	0.31↓	0.48↓	0.63
P45	45	ATP-dependent zinc metalloprotease FTSH,	gi 565350308	62.07	42	56.6	76.0/6.19	82.9/5.79	Plasma membrane	0.44↓	0.27↓	0.77	0.58

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Table 1 (continued)

Spot No. <sup>a)</sup>	Protein Name <sup>b)</sup>	AccessionNo. <sup>c)</sup>	Unused <sup>d)</sup>	MP <sup>e)</sup>	SC(%) <sup>f)</sup>	Theor. Mr/pI <sup>g)</sup>	Exp. Mr/pI <sup>h)</sup>	Subcellular localization <sup>i)</sup>	Fold changes <sup>j)</sup>				
									FC <sub>T1/CK</sub>	FC <sub>T3/CK</sub>	FC <sub>T5/CK</sub>	FC <sub>T7/CK</sub>	
	chloroplastic [Solanum tuberosum]												
P57	20	proteasome subunit alpha type-6 [Solanum tuberosum]	gi 565356827	12.63	8	45.7	27.3/6.11	26.5/6.39	Nucleus	0.58	0.48↓	1.08	0.66
<b>10 signal transduction</b>													
P3	3	calreticulin [Solanum tuberosum]	gi 565355763	43.18	35	33.6	47.6/4.50	51.3/4.69	Mitochondrion	0.27↓	0.36↓	0.21↓	1.10
<b>11 Disease/defense</b>													
P5	5	2-Cys peroxiredoxin BAS1, chloroplastic [Solanum tuberosum]	gi 565344108	10	5	18	29.4/6.34	22.7/4.95	Chloroplast	0.05↓	0.90	0.75	0.31↓
P6	6	2-Cys peroxiredoxin BAS1, chloroplastic [Solanum tuberosum]	gi 565344108	6	3	14.2	29.4/6.34	22.4/4.86	Cytoplasm	0.12↓	1.45	1.52	0.96
P12	12	trigger factor protein TIG [Solanum tuberosum]	gi 565358747	20.41	11	20.3	61.2/5.08	50.0/5.00	Chloroplast	0.32↓	0.41↓	0.48↓	0.34↓
P14	14	trigger factor protein TIG [Solanum tuberosum]	gi 565358747	91.59	74	68	61.2/5.08	50.0/5.06	Cytoplasm	0.42↓	0.25↓	0.49↓	0.64
P42	42	protein TIC 62, chloroplastic [Solanum tuberosum]	gi 565365922	20.92	15	15.7	78.6/5.40	68.7/5.71	Vacuole	0.52	1.44	0.33↓	0.77
P43	43	thimet oligopeptidase [Solanum tuberosum]	gi 565382894	44.87	27	24.4	9.54/6.37	74.3/5.75	Vacuole	0.38↓	0.20↓	0.30↓	0.44↓
P44	44	M1 family aminopeptidase isoform X2 [Solanum tuberosum]	gi 565354114	97.35	68	50.5	110.7/6.07	82.9/5.75	Vacuole	0.87	0.26↓	0.59	0.39↓
<b>20 Secondary metabolism</b>													
P21	P21	hyoscyamine 6-dioxygenase [Solanum tuberosum]	gi 565383140	22	11	22	37.9/5.54	35.9/6.45	Nucleus	1.05	1.14	3.24↑	2.70↑
<b>12 unknown</b>													
P33	33	uncharacterized protein At2g37660, chloroplastic [Solanum tuberosum]	gi 565348015	28.99	18	50.3	32.5/8.91	28.3/5.75	Plasma membrane	0.62	0.46↓	0.35	0.44↓
P37	37	uncharacterized oxidoreductase At1g06690, chloroplastic [Solanum tuberosum]	gi 565383154	41.76	33	35.6	40.4/6.78	37.3/5.92	Nucleus	0.83	0.82	0.24↓	0.51

<sup>a)</sup> Spot No, Spot number.

<sup>b)</sup> Names and species of proteins obtained via the ProteinPilot™ 5.0 software from potato protein sequences downloaded from the NCBI database.

<sup>c)</sup> Accession No, Accession number.

<sup>d)</sup> Score probability (protein score) for the entire protein and for ions complemented by the percentage of the confidence index (C.I.).

<sup>e)</sup> MP indicate the number of matched peaks for the PMF data, respectively.

<sup>f)</sup> SC, Sequence coverage.

<sup>g)</sup> The subcellular localization prediction of 52 differently abundant proteins based on Plant-PLOC.

<sup>h)</sup> Theor. Mr/pI shows theoretical molecular weight and pI isoelectric point.

<sup>i)</sup> Exp. Mr/pI shows experimental molecular weight and isoelectric point.

<sup>j)</sup> Fold change was calculated from T1, T3, T5 and T7 over the CK gels, which '↑', '↓' and 'ns' stand for up-regulated, down-regulated and no significant change, respectively.

sc\_download.shtml) were used for data searching. Protein spectra with confidence intervals of 0.95 were considered reliable. Peptide sequences were manually matched to the database for protein identification.

### 2.7. Functional classification and subcellular localization of identified protein spots

Functional annotation of the identified protein spots was done as described in Bevan et al. [34]. Plant-PLoc software (<http://www.csbio.sjt.u.edu.cn/bioinf/plant/>) was used for the prediction of subcellular localization [35].

## 3. Results

### 3.1. Analysis of physiological and biochemical parameters

During the process of low temperature treatment, the total contents of SP, MDA, PRO, and chlorophyll in the leaves at 0 d, 1 d, 3 d, 5 d, and 7 d were measured; as the duration of treatment increased, SP content rapidly increased. However, from 5–7 d, the rate of increase in SP content slowed, and the SP content reached its maximum at 7 d (Figure 1A). Similar patterns were observed for MDA and PRO content; both increased at first and then decreased, reaching the maximum values at 5 d and then beginning to decrease at 7 d (Fig. 1B, C). Under low temperature stress conditions the potato cell membrane was damaged, causing the MDA content to increase. In contrast, the total chlorophyll content decreased as the treatment duration increased (Figure 1D), indicating that chlorophyll synthesis was inhibited during low temperatures.

### 3.2. Bioinformatic analysis of the 52 identified protein spots

Figure 2 shows a representative 2-DE gel image before low temperature stress and after 1, 3, 5, and 7 d of low temperature stress. 52 DEP spots were identified (Figure 3 and Table 1) and classified into eight groups (Figure 4).

### 3.3. Analysis of the differences among the 52 DEPs

Figure 4 shows the diagram of the protein expression profiles at the four treatment time points. Compared to the control, samples treated for 1 d, 3 d, 5 d, and 7 d exhibited protein spots with different abundances (Table 1). At 1 d of low temperature treatment, we found 3 upregulated and 19 downregulated DEPs. At 3 d of treatment, 2 DEPs were upregulated and 22 were downregulated. At 5 d of treatment, 9 DEPs were upregulated and 23 were downregulated. At 7 d of treatment, 7 DEPs were upregulated and 19 were downregulated. This result indicated that as the duration of low temperature treatment increased, more proteins began to respond to low temperature stress. The number of downregulated proteins rapidly increased, peaking at 5 d, followed by a decrease at 7 d. This pattern was the same as for the changes in MDA and PRO content, which leveled off after the plants adapted to the low temperature and then began to decrease (see Figure 5).

### 3.4. GO classification and enrichment analysis of DEPs

We also carried out a gene ontology (GO) functional enrichment analysis on all DEPs (Figure 6). The DEPs in potato leaves under low temperature stress at the four different points are widely involved in 29 subcategories among the three GO categories of biological process (BP), cellular component (CC), and molecular function (MF).

As shown in Figure 6, DEPs in potato leaves at 1 d, 3 d, 7 d of low temperature stress involved 23 GO subcategories, and those at 5 d of low temperature stress involved 24 GO subcategories. These GO subcategories included biological regulation, response to stimuli, and metabolic processes in the BP category; organelles and cell membrane in the CC category; and catalytic activity, antioxidant activity, and binding

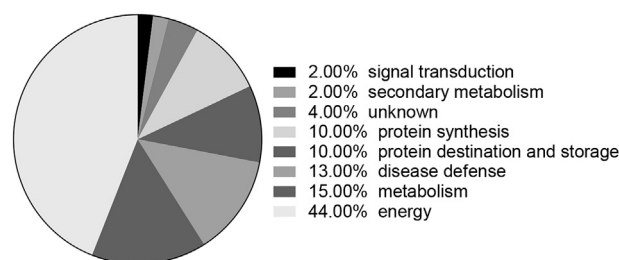


Figure 4. Functional groups of 52 differently expressed protein spots from potato leaves under treatments.

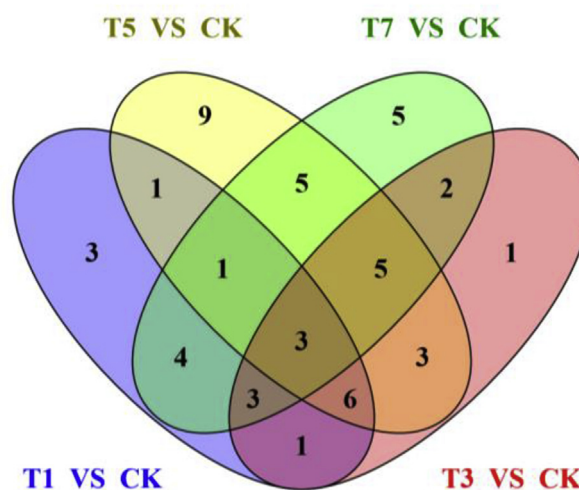


Figure 5. Differences in protein abundance at 1 d, 3 d, 5 d, and 7 d of low temperature treatment in comparison with the control.

in the MF category. A pathway enrichment analysis using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database was performed to determine the major biochemical metabolic pathways, photosynthetic pathways, and the biosynthesis secondary metabolites that DEPs were involved in. The results showed that at all time points during low temperature stress (Fig. 7A, B, C, D), the pathways with the highest number of DEPs were photosynthesis, amino acid biosynthesis, alanine metabolism, and starch and sugar metabolism. DEPs significantly enriched in the pathway of photosynthetic ATP synthase. Many DEPs were also enriched in pathways related to phosphate metabolism, oxidative phosphorylation, amino acid biosynthesis, and starch and sugar metabolism. Secondary metabolites produced by these metabolic pathways can alleviate or eliminate injuries caused by reactive oxygen species (ROS) induced by stresses such as low temperature. These results also indicated that the response to low temperature stress in the potato was a complex physiological and biochemical process controlled by multiple genes, pathways, and metabolites. Consistent with the results of the GO enrichment analysis, significantly enriched KEGG metabolic pathways of the DEPs were mainly biochemical metabolic pathways, photosynthesis, and secondary metabolite biosynthesis.

## 4. Discussion

### 4.1. Changes in physiological characteristics in potatoes upon low temperature stress

When plants are challenged by low temperature stress a series of changes in physiological characteristics occur, including inhibited plant growth and photosynthesis, peroxidation of membrane lipids, cell metabolism disorders, massive accumulation of free radicals and reactive oxygen species, inhibited chlorophyll synthesis, increased permeability



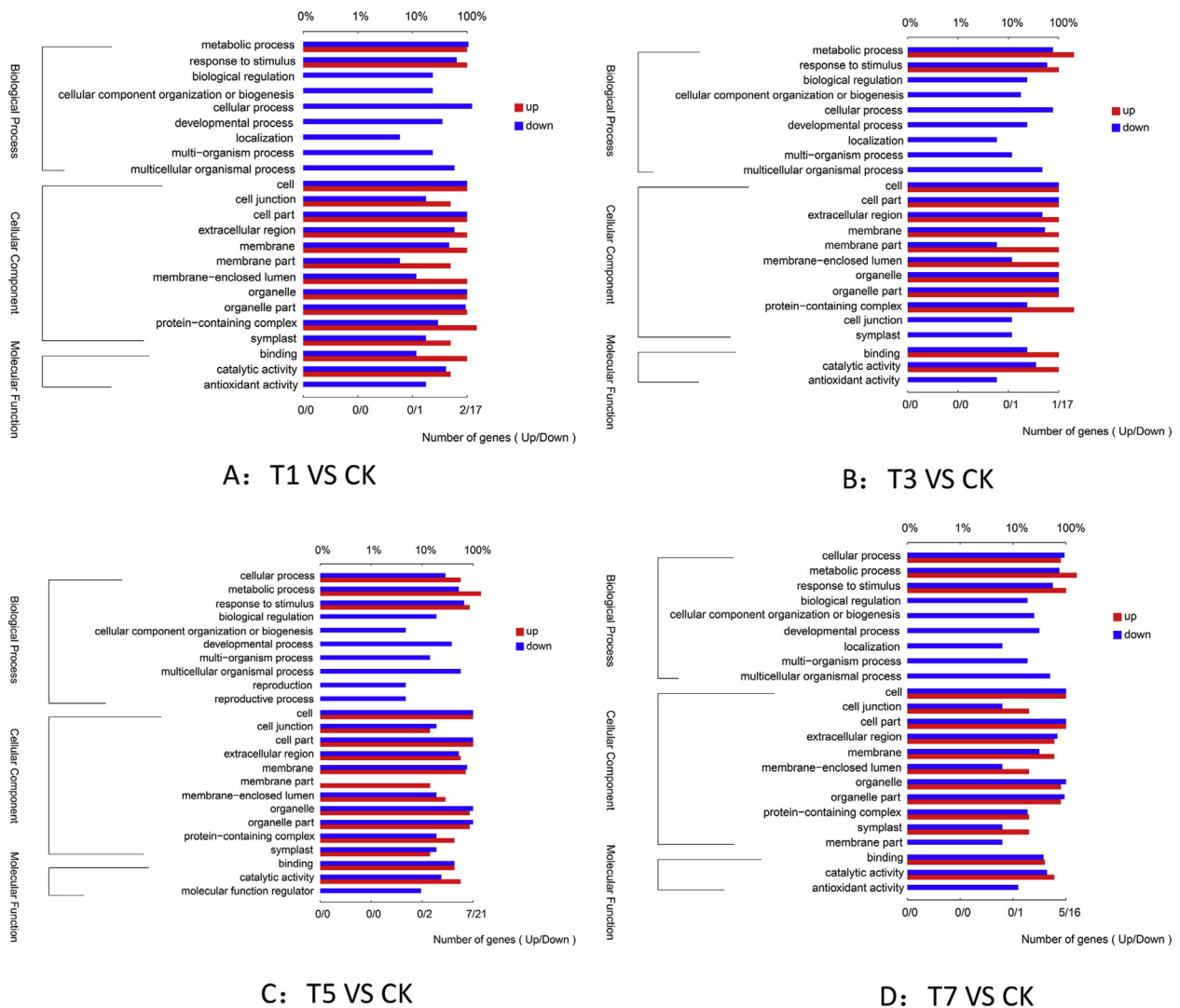


Figure 6. Gene ontology classification of the differently expressed proteins under low temperature stress.

of the plasma membrane, and a rapid increase in MDA. Studying the changes in physiological characteristics could help assess plant cold tolerance and elucidate the mechanism of cold tolerance. Potato cultivars are not tolerant to frost and freezing. found that under low temperature stress, the PRO level increased in potato plants; the more tolerant to low temperature stress the plants were, the higher the PRO level was found to be [10]. Chen et al. found that after cold acclimation the level of SP in potato leaves was high, and the increase in SP content was positively correlated with cold resistance [36]. Changes in the levels of SP, MDA, PRO, and chlorophyll after low temperature treatment measured in [37] this study exhibited the same pattern as in previous studies. Studies have shown that spraying ABA could improve crop resistance to cold [37,38]. Therefore, spraying a certain amount of exogenous hormones can reduce the content of MDA, increase the content of chlorophyll, SP and PRO, enhance the activity of enzymes in the potato, thereby improving the cold resistance of potatoes.

#### 4.2. Proteins related to energy metabolism

Proteins for energy metabolism are crucial for maintaining plant cell growth and development; they provide ATP and other metabolic intermediates necessary for plants. Abiotic stress affects physiological reactions in plants, among which the most important are physiological

reactions related to energy. ATP synthase is a key enzyme for oxidative phosphorylation [39,40] as it catalyzes ATP synthesis and hydrolysis. In the present study 31 DEPs were identified as related to energy metabolism, accounting for 59% of total proteins identified. Among those proteins, ATP synthase  $\delta$  chain (P4), ATP synthase  $\beta$  subunit (P17, P46), ATP synthase  $\alpha$  subunit (P47), and ATP synthase CF0 subunit (P50) are involved in energy metabolism. Protein spots P4, P47, and P50 exhibited downregulated expression by different degrees at 5 d of low temperature treatment, indicating that the photophosphorylation in potato leaves was affected by low temperature stress. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) is a key enzyme in glycolysis; it is also an enzyme necessary for the survival of organisms. A lack of this enzyme can trigger a metabolic disorder in the organism. In this study, we found that GADPH A (P23) was downregulated under low temperature stress, while GADPH B (P36) was upregulated at 5 d and 7 d of low temperature stress, indicating that GADPH A was the main GADPH involved in the potato's stress response to low temperature. Downregulated expression of GADPH A decreases normal metabolism, thereby enhancing the adaptivity of the potato to low temperature. It has been shown that dilute alcoholase is involved in the response of plants to abiotic stresses such as extreme temperature, salt stress, hypoxia stress, and drought [41,42]. The identified enolase (p53) was downregulated under low temperature stress, indicating that enolase participated in the corresponding process of low

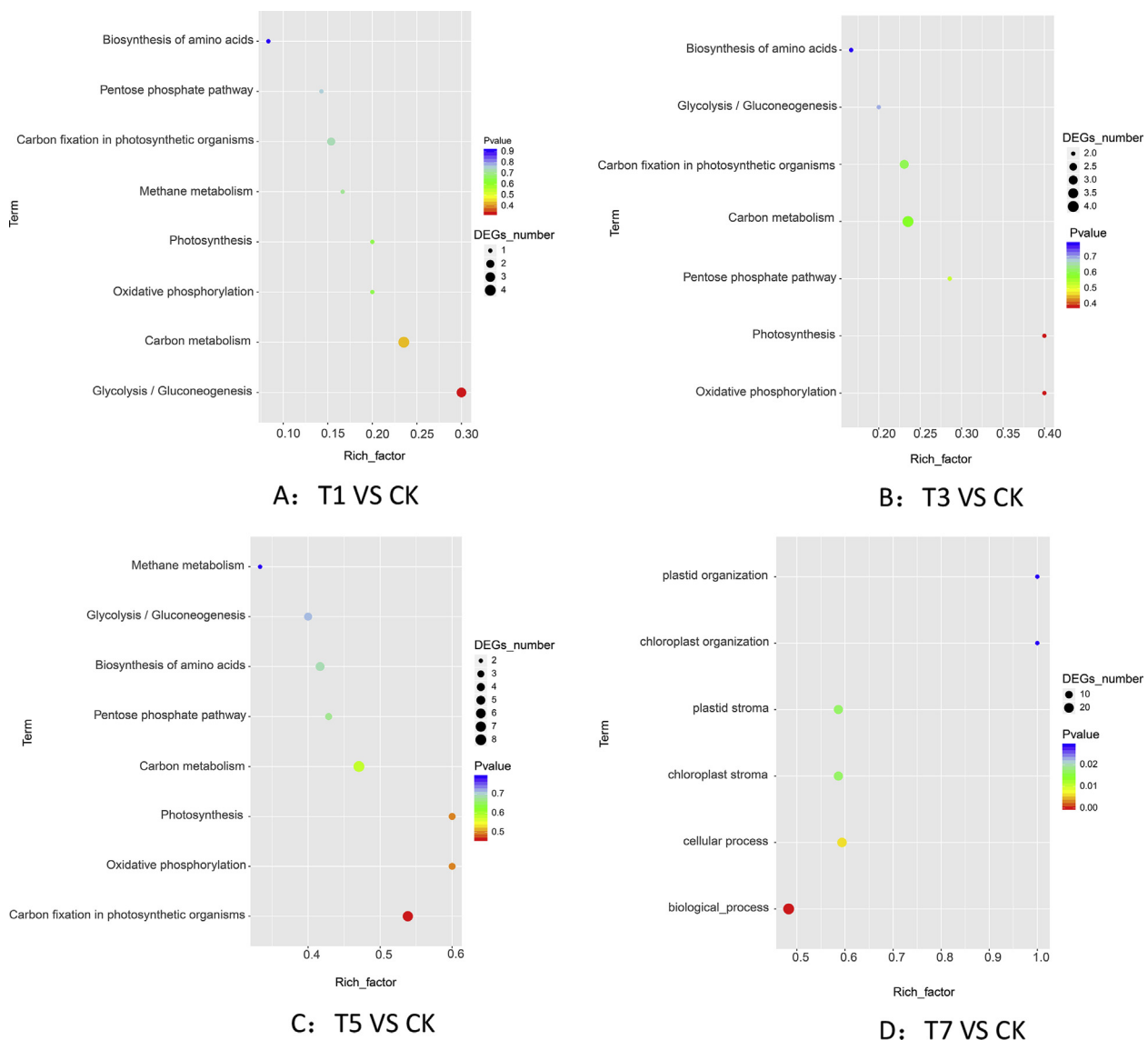


Figure 7. KEGG analysis of differentially expressed proteins under low temperature stress.

temperature stress. Glutamine synthetase (GS) is involved in metabolism and amino acid synthesis. In this study, the identified GS (P40) was upregulated in response to low temperature stress, indicating that when the potato was under low temperature stress, the upregulation of GS promoted the synthesis of amino acids related to stress to increase the ability to adapt to the environment [43,44]. In this study, protein spots P22 and P28 corresponded to fructose-bisphosphate aldolase, which exhibited downregulated expression during low temperature stress, indicating that the sugar metabolism pathway was involved in the response to low temperature.

#### 4.3. Proteins related to photosynthesis

Photosynthesis is the process that fixes light energy and synthesizes sugar in higher plants. Abiotic stress can damage the thylakoid membrane in the chloroplast and can affect the photosynthetic metabolism, thereby affecting normal plant growth and development [45,46]. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) (P13, P32, and P35) is the key enzyme that fixes CO<sub>2</sub>. This enzyme greatly influences the photosynthetic rate in plants. Studies have shown that the large subunit of RuBisCo, as a photosynthetic protein, is prone to degradation under abiotic stress. In this study, protein spot P8 was identified as the large subunit of Rubisco. Protein spot P8 exhibited downregulated expression upon low

temperature stress. This indicated that RuBisCo activase had degraded, which led to a decrease in photosynthetic rate in the leaf and inhibited growth and development. Chlorophyll a/b-binding protein (P49) is a key protein in the light-harvesting complex. Plant photosystem I (PSI) and the light-harvesting complex of photosystem II (PSII) act as light receptors that can harvest light energy and transfer light energy to the reaction center. Protein spot P49 exhibited downregulated expression under low temperature stress. Its expression stabilized at 7 d, which was helpful for energy transfer to defend against cold damage. Oxygen-evolving enhancer protein (P48) is one of the peripheral proteins of the oxygen evolution complex; the protein functions in stabilizing PSII. Under low temperature stress P48 expression was downregulated, indicating that the photosynthetic rate in potato leaves was reduced and that chloroplasts were damaged, leading to inhibited chlorophyll synthesis. Studies have shown that UV-C can mitigate the effects of low temperatures on chlorophyll content generated [47]. Through specific measures to avoid potato damage the photosynthetic system to reduce the impact of low temperature on chlorophyll content produced.

#### 4.4. Proteins related to protein synthesis and protein destination

In this study there were 10 protein spots involved in protein synthesis and destination, accounting for 20% of all identified proteins. The

ribosomal protein P0 (P15, P18) exhibited upregulated expression under low temperature stress, indicating that low temperature stress increased the rate of protein synthesis for adaptation to the low temperature environment [48]. This may be due to the fact that different proteins have different regulatory pathways, which has been reported for abiotic stress responses in other plant species. The 26S proteasome involved in protein destination is a multi-subunit complex that has the activity of a proteolytic enzyme and is ubiquitin dependent. It plays important roles in DNA repair, proteolysis, and the regulation of the physiological functions of the cell [49]. 26S proteasome exhibited downregulated expression under low temperature stress, indicating that the metabolic rate in the potato was reduced and that proteolysis was affected.

#### 4.5. Proteins related to signal transduction

Proteins related to signal transduction identified in this study include calreticulin (P3). Calreticulin plays important roles in maintaining the dynamic Ca<sup>2+</sup> balance in plants and is involved in plant growth, development, and stress responses. Recently, many studies have confirmed the functions of calreticulin in plant stress resistance and disease resistance. Studies have shown that calreticulin is highly expressed under stressors, and that this increased expression enhances plant stress resistance [50,51,52]. The calreticulin P3 identified in this study exhibited downregulated expression upon low temperature stress followed by a normal expression level, indicating that upon low temperature stress, the abundance of water-soluble calreticulin in the potato was reduced, while the abundance of fat-soluble calreticulin increased. It is possible that after the potato was exposed to low temperature stress, calreticulin was transported to the endoplasmic reticulum, thereby being involved in low temperature stress signal transduction.

#### 4.6. Proteins related to disease defense

Under low temperature stress high levels of ROS are accumulated in plants, which causes the oxidation of protein, lipids, and DNA. This can damage the structure of the cell membrane and lead to injuries in plants [45,53]. To defend the organism from oxidative stress, plants produce a series of ROS-scavenging enzymes. One study showed that the antioxidant enzymes SOD, CAT, and APX are crucial to plant defense from disease [54]. In this study, the expression of 2-cysteine peroxidase (P5, P6) related to disease defense was downregulated, indicating that during low temperature stress, the disease defense system in the potato was enhanced to defend against the damage from low temperature. Under low temperature stress, enhanced oxygen scavenging activity in vivo potato ability to reduce the accumulation of reactive oxygen species, can reduce the impact of cold damage caused.

### 5. Conclusion

This study investigated the changes in proteins in potato plants during low temperature stress via measuring the changes in physiological parameters and proteomics. Results from this study provide a reference for studying the mechanism of cold tolerance in the potato. Combining 2-DE and LC-ESI-MS/MS technology, we identified a total of 52 protein spots involved in eight categories of biological processes including energy, metabolism, and protein synthesis. As the duration of low temperature stress increased, the number of upregulated and downregulated protein spots increased. After adapting to the low temperature stress for some time, the number of upregulated protein spots increased, while the number of downregulated protein spots decreased, which may be related to stress responses during low temperature stress in potato plants. The downregulation of RuBisCO, ribonucleoprotein, 26S protease ATP, and calreticulin, as well as the upregulation of glyceraldehyde-3-phosphate dehydrogenase and glutamate decarboxylase, may be associated with the defense responses against low temperature stress in the potato. The

changes in protein expression indicated that the adjustment of potato plants to low temperature stress involved multiple metabolic pathways.

### Declarations

#### Author contribution statement

Huawei Li: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Wenbin Luo: Performed the experiments.

Rongchang Ji, Guochun Xu: Analyzed and interpreted the data.

Yongqing Xu: Contributed reagents, materials, analysis tools or data.

Sixin Qiu, Hao Tang: Conceived and designed the experiments.

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#### Data availability statement

Data included in article/supplementary material/referenced in article.

#### Declaration of interests statement

The authors declare no conflict of interest.

#### Additional information

No additional information is available for this paper.

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