Identification and Analysis of Differentially Expressed Proteins in Leaves of Winter Turnip Rape (Brassica rapa) 'Longyou 7' Under Low Temperature Stress: Postprint

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Abstract

To investigate the cold resistance mechanism of the super cold-resistant winter turnip rape (Brassica rapa) variety 'Longyou 7' from a proteomics perspective, this study employed the TCA (trichloroacetic acid)-acetone precipitation method to extract total leaf proteins before and after low temperature stress (4 °C, 7 d). The protein extraction method, IPG (immobilized pH gradient) strip types, and other steps were optimized, and two-dimensional electrophoresis and mass spectrometry analysis techniques were used to identify the differential expression patterns of total leaf protein components in 'Longyou 7' at the five-leaf stage under low temperature stress. The results showed that the improved protein extraction buffer (containing DTT, dithiothreitol) and PVPP (polyvinylpolypyrrolidone) yielded an average protein concentration 3.42 g · L 1 higher than before improvement, with a desalting time 1.14 h shorter than before improvement. Simultaneously, the protein extraction buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) obtained rich protein species, with 661 protein spots detectable in the gel map, representing an 11.2% increase compared with the detectable protein spots before improvement (587). Electrophoresis using 17 cm IPG strips with pH 4–7 better separated proteins and produced reproducible, high-resolution proteome maps. Using PDQuest 8.0 software, the protein expression profiles of the super cold-resistant variety 'Longyou 7' before and after low temperature stress were analyzed and compared, revealing 15 differentially expressed protein spots before and after low temperature stress, which are speculated to be associated with the response to low temperature stress. Further mass spectrometry analysis of the differentially expressed protein spots identified 11 protein spots related to low temperature stress, including photosynthesis-related proteins, carbohydrate

metabolism-related proteins, substance transport-related proteins, and stress response-related proteins. Moreover, significant differences existed in the expression levels of leaf proteins in 'Longyou 7' before and after low temperature stress treatment, and these differential proteins may play important roles in the cold stress response of winter turnip rape.

Full Text

Identification and Analysis of Differentially Expressed Proteins in Leaves of Brassica campestris 'Long-you No. 7' in Response to Low Temperature Stress

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Abstract

To investigate the cold resistance mechanism of the super cold-resistant winter rapeseed variety *Brassica campestris* 'Long-you No. 7' from a proteomic perspective, this study employed the TCA (trichloroacetic acid)-acetone precipitation method to extract total leaf proteins before and after low temperature stress (4 °C for 7 days). The protein extraction method, IPG (immobilized pH gradient) strip types, and other procedures were optimized. Two-dimensional electrophoresis and mass spectrometry were used to identify differential expression patterns of total leaf proteins in 'Long-you No. 7' at the five-leaf stage under low temperature stress. The results showed that the improved protein extraction solution containing DTT (dithiothreitol) and PVPP (polyvinylpolypyrrolidone) yielded an average protein concentration 3.42 $g \cdot L^{1}$ higher than before improvement, with desalting time shortened by 1.14 h. Additionally, protein extraction solution containing the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) yielded rich protein varieties, detecting 661 protein spots in the gel map—an 11.2% increase compared to the 587 spots detectable before improvement. Electrophoresis using 17 cm IPG strips with pH 4–7 better separated proteins and produced high-quality proteomic maps with good repeatability. PDQuest 8.0 software was used to compare proteomic expression profiles of the super cold-resistant 'Long-you No. 7' before and after low temperature stress, revealing 15 differentially expressed protein spots that were presumably related to low temperature stress response. Further mass spectrometry analysis of these protein spots identified 11 proteins associated with low temperature stress, including proteins related to photosynthesis, sugar metabolism, material transport, and stress response. Moreover, obvious differences in expression levels of

leaf proteins were observed before and after low temperature stress treatment in 'Long-you No. 7' , suggesting these differentially expressed proteins may play important roles in the cold resistance response of winter rapeseed.

Keywords: *Brassica campestris*; Long-you No. 7; Low temperature stress; Two-dimensional electrophoresis; Proteomics

Introduction

Low temperature is a common and serious natural disaster in agricultural production that can cause plant cell dehydration, crystallization, and irreversible gelation within the protoplast, resulting in mechanical damage or cell death. Compared with *Brassica napus*, *Brassica campestris* possesses excellent traits such as strong cold resistance and tolerance to barren conditions, playing an irreplaceable role in cold and arid regions of northern China [1]. Since Sun et al. [2] proposed the feasibility of northward expansion of winter rapeseed to cold and arid regions of northwestern China, the cultivation area of winter rapeseed has been increasing, generating substantial ecological and economic benefits. The successful northward expansion of winter rapeseed primarily depends on the successful breeding of super cold-resistant varieties. The 'Longyou' series of *B. campestris* varieties exhibit excellent cold resistance, but the underlying mechanism remains incompletely understood. Current research on *B. campestris* has mainly focused on morphological and physiological characteristics of cold resistance [3] and cloning of cold-responsive genes [4-6], while proteomic studies on its cold resistance mechanism are scarce. Pu [7] conducted preliminary research only on comparison of protein loading amounts and optimization of isoelectric focusing (IEF), without in-depth investigation of differential proteins before and after low temperature stress.

Proteins are the executors of physiological functions and direct manifestations of life phenomena. Therefore, comprehensive and in-depth understanding of complex life activities requires proteomic research [8]. Two-dimensional gel electrophoresis (2-DE) technology has become the preferred technique in proteomics research, particularly for analyzing and identifying stress-responsive differential proteins. Using 2-DE technology enables comparative analysis of dynamic changes in proteomes of biological samples under different conditions, thereby screening and identifying function-specific proteins and their corresponding genes. In recent years, 2-DE technology has been widely applied in agriculture, medicine, and microbiology [9-11]. For example, Cui et al. [12] analyzed the leaf proteome of cotton (*Gossypium hirsutum*) under salt stress and found significant changes in abundance of 24 protein spots, identifying some salt stress-responsive proteins. Wang et al. [13] treated hydroponically grown *B. napus* seedlings with phosphorus-sufficient and phosphorus-deficient nutrient solutions for 6 days, discovering 62 significantly differentially expressed protein spots between treatments and control, with 25 proteins upregulated and 37 downregulated by the stress. Wang et al. [14] studied the leaf proteome of maize (*Zea mays*) under low temperature stress and obtained five new

protein spots presumed to be related to cold resistance. Under low temperature stress, plants undergo genetic, physiological, and biochemical responses that alter expression levels of metabolism-related and transport-related proteins, thereby inducing stress resistance reactions to cope with external stress [15]. Therefore, this study applied proteomic methods and techniques to analyze differential protein expression changes in leaves of *B. campestris* 'Long-you No. 7' under low temperature stress versus normal growth conditions, screening and identifying proteins related to low temperature stress response to deepen understanding of the molecular mechanism of low temperature stress response in *B. campestris* and provide a foundation for further utilization of genetic engineering to breed new cold-resistant varieties and screen for cold-resistant germplasm resources.

Materials and Methods

1.1 Experimental Materials and Design

The super cold-resistant (tolerating -32 °C with over 85% overwintering survival rate) *B. campestris* variety 'Long-you No. 7' [16] was used as experimental material. On September 24, 2015, full and uniformly sized rapeseeds were selected, treated with 10% hydrogen peroxide for 30 min, rinsed 2-3 times with sterile water, and placed in petri dishes lined with two layers of filter paper for germination (14 h light at 30 °C; 10 h dark at 28 °C). After seed germination, seeds were sown in pots (14 cm × 13 cm) containing equal amounts of nursery substrate, with the substrate uniformly placed 2 cm below the pot rim. A total of 20 pots were prepared with 4 seedlings per pot and cultivated in an artificial incubator (14 h light at 25 °C; 10 h dark at 20 °C). At the five-leaf stage, seedlings were divided into two groups of 10 pots each. One group continued growing under the above conditions as control (CK), while the other treatment group (T) was subjected to low temperature stress at 4 °C in an artificial incubator (14 h light, 10 h dark). After 7 days of continuous treatment, mixed leaf samples were collected from both control and treatment groups in triplicate, snap-frozen in liquid nitrogen, and stored at -80 °C for total protein extraction. Nutrients for experimental materials were provided by the nursery substrate composed of vermiculite, perlite, peat, and organic fertilizer, with nitrogen-phosphorus-potassium content 6% and organic matter 45%, purchased from Gansu Academy of Agricultural Sciences. Materials were watered every 1-2 days with the maximum water-holding capacity of pots and managed routinely.

1.2 Protein Extraction and Separation Optimization

1.2.1 Screening of Optimal pH Range for IPG Strips in Total Protein Gel Maps To screen for the optimal pH range of IPG strips that would produce clearly observable high-abundance protein spots with high resolution for low-abundance proteins and uniform protein spot distribution in total protein gel maps of 'Long-you No. 7' leaves, 17 cm pH 3-10 and 17 cm pH 4-7 IPG

strips were used to examine protein separation in gel maps and determine the optimal pH range.

1.2.2 Extraction of Total Leaf Proteins and Protein Concentration Determination Total leaf proteins from winter rapeseed were extracted using the TCA-acetone precipitation method [17-20].

Improved method: (1) Rapeseed leaves were rinsed with distilled water, dried with filter paper, placed in a sterilized, pre-chilled mortar with a small amount of PVPP, ground thoroughly with liquid nitrogen into fine powder, and transferred to several liquid nitrogen-pre-chilled 2 mL centrifuge tubes. (2) Each tube was added with TCA-acetone (containing 10% TCA, 0.07% DTT, and 0.015% PMSF) pre-chilled at -20 °C, vortexed and mixed, stored overnight at -20 °C, then centrifuged at 4 °C, 20,000×g for 30 min, with supernatant discarded and pellet retained. (3) Each tube was added with approximately 2 mL of 100% cold acetone (containing 0.07% DTT and 0.015% PMSF), vortexed and mixed, left standing at -20 °C for 1 h, then centrifuged at 4 °C, 20,000×g for 20 min, with supernatant discarded and pellet retained. This step was repeated 1-2 times until the pellet appeared milky white and the supernatant became transparent. (4) Approximately 2 mL of 80% cold acetone (containing 0.07% DTT and 0.015% PMSF) was added, vortexed and mixed, left standing at -20 °C for 30 min, then centrifuged at 4 °C, 20,000×g for 15 min, with supernatant discarded and pellet retained. This operation was repeated 1-2 times. (5) The centrifuge tubes containing pellets were placed in a freeze dryer until white powder formed, then a certain amount of protein lysis solution (with DTT added fresh) was added for room temperature lysis for 2 h, with vortex mixing every 30 min, followed by centrifugation at 4 °C, 20,000×g for 30 min. The supernatant was collected as total leaf protein, quantified, aliquoted, and stored at -70 °C.

Before improvement: The protein extraction solution did not contain PVPP, DTT, or PMSF, with all other steps identical to the improved method.

1.2.3 Protein Quantification Protein concentration was determined using the Bradford method [20].

1.2.4 Two-Dimensional Electrophoresis Two-dimensional electrophoresis was performed on total leaf proteins from rapeseed under low temperature stress and control (CK) conditions. The first-dimension isoelectric focusing (IEF) used IPG strips of pH 3-10 and pH 4-7, following the GE Healthcare 2-DE operation manual. The sample loading amount was 1,000 g, with samples mixed with rehydration solution at a 1:4 volume ratio to a total volume of 500 L. The procedure followed the program shown in . The second-dimension used 12% acrylamide gel for SDS-PAGE. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, and images were acquired using a UMAX Powerlook 2100XL scanner. PDQuest 8.0 analysis software was used for standardized processing of gel maps, protein spot matching, and biological

statistics to identify differentially expressed protein spots. Each protein sample was analyzed in triplicate.

1.2.5 Mass Spectrometry Identification of Differential Protein Spots
PDQuest 8.0 software was used to analyze three replicate gel maps. Protein spots appearing at the same position in all three maps were identified as differential protein spots. Differential protein spots were recovered and digested [21] before being sent to Shanghai Applied Protein Technology Co., Ltd. for MALDI-TOF-TOF MS analysis. The obtained peptide fragment mass data were analyzed using PMF analysis software MASCOT 2.2 to identify relevant protein spots. Protein information was retrieved from databases (NCBI: http://www.ncbi.nlm.nih.gov/protein/ and EMBL-EBL: http://www.ebi.ac.uk/services/proteins).

1.3 Data Analysis

Microsoft Excel 2010 was used for data processing and table preparation, and SPSS 17.0 software was used for significance analysis.

Results

2.1 Effects of Extraction Method on Protein Concentration and Desalting Time

As shown in , after multiple repeated experiments, the average total leaf protein concentration obtained by the pre-improvement extraction method (without PVPP and DTT) was 8.11 $g \cdot L^{1}$, with an average IEF desalting time of 6.51 h. The improved method (with PVPP and DTT) yielded an average total protein concentration of 11.53 $g \cdot L^{1}$ and an average desalting time of 5.37 h. indicates that differences in protein concentration and desalting time between the improved and pre-improvement methods reached extremely significant levels ($P<0.01$). Therefore, through improvement of the protein extraction solution, proteins in leaves were more fully dissolved, resulting in higher protein concentrations that better met the loading requirements for 2-DE. Additionally, adding PVPP to the extraction solution effectively removed impurities such as phenolic compounds, improved protein purity, reduced salt content in samples, and facilitated the IEF process in 2-DE.

2.2 Comparison of Protein Spots Separated by Different pH IPG Strips

As shown in [Figure 1: see original paper] (A, B), when total leaf proteins of winter rapeseed 'Long-you No. 7' were separated using wide-range (17 cm, pH 3-10) nonlinear IPG strips, protein spots under low temperature stress were mainly concentrated in the acidic range (pH 4-7). Gel map analysis using PDQuest 8.0 software detected 800-900 protein spots. To achieve clearer protein spot

separation, narrow-range (17 cm, pH 4-7) IPG strips were used for protein separation ([Figure 2: see original paper], [Figure 3: see original paper]). Compared with wide-range (pH 3-10) nonlinear strips, the narrow-range (pH 4-7) strips showed better separation effects, higher resolution, and more uniform protein spot distribution for 'Long-you No. 7' leaf proteins, detecting 600-700 protein spots on gel maps. Thus, 17 cm, pH 4-7 linear IPG strips were more suitable for separating total leaf proteins of rapeseed.

2.3 Effect of Protease Inhibitor (PMSF) on 2-DE Map Separation of Rapeseed Leaf Proteins

After plant cell disruption, released proteases degrade proteins, causing loss of some protein spots in 2-DE gel maps ([Figure 2: see original paper]A). Therefore, this study added the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) to the sample extraction solution to effectively prevent protein degradation ([Figure 2: see original paper]B). Analysis by PDQuest 8.0 software showed that gel maps without PMSF detected 587 protein spots, while those with PMSF detected 661 spots—an increase of 74 spots. Therefore, during sample preparation, protein degradation should be avoided as much as possible to prevent reduction in protein varieties and analytical errors.

2.4 Analysis of Differential Protein Spot Maps in Rapeseed Leaves Under Low Temperature Stress

Clear 2-DE maps with distinct protein spots were obtained from rapeseed leaves before and after low temperature stress, with three replicate electrophoresis maps for each sample being basically consistent. PDQuest 8.0 software was used to automatically match Coomassie-stained images ([Figure 3: see original paper]A, B), combined with visual observation and manual adjustment. An average of 672 protein spots were detected before low temperature stress with a matching rate of 93%, while 689 spots were detected after stress with a matching rate of 90%. Detection of newly appearing protein spots with expression changes greater than 2-fold revealed 15 qualitatively differentially expressed new protein spots between before and after low temperature stress in 'Long-you No. 7' leaves.

Under low temperature stress, the content of original proteins in plants changed significantly to adapt to adverse stress, with 2-DE gel maps differing from the control, indicating that low temperature stress affected changes in soluble protein structure and composition in rapeseed leaves. When plants are stimulated by low temperature stress, related genes are induced to express and synthesize new proteins ([Figure 3: see original paper]B, spots 6-15; [Figure 4: see original paper])—i.e., induced expression protein spots. These results indicate that under normal conditions, some proteins are expressed at low abundance or not at all, but under low temperature stress, these proteins are expressed and function, causing a series of physiological and biochemical changes that enable plants to tolerate or resist low temperature stress. Simultaneously, low temperature obstructs some normal gene expression pathways, suppressing expression

of some original proteins (spots 1-5 in [Figure 3: see original paper]A; [Figure 4: see original paper])—i.e., suppressed expression protein spots. Upregulation and downregulation of related genes under low temperature stress represent adaptive responses of plants to adverse environmental conditions.

2.5 Mass Spectrometry Identification Results of Differentially Expressed Proteins

Among the 15 differential protein spots detected after low temperature stress, 11 were successfully identified and 4 were not. The mass spectrometry data of the 11 successfully identified proteins were compared with the NCBI protein database to obtain relevant information on differentially expressed protein spots (). The 11 identified differential proteins included photosynthesis-related proteins (spots 1, 5, 8, 15), sugar metabolism-related proteins (spot 7), material transport-related proteins (spots 2, 6), and stress response proteins (spots 9, 10, 13, 14).

Discussion

3.1 Sample Preparation and Protein Hydration Loading Amount as Primary Factors for Successful 2-DE

To completely obtain protein types and quantities expressed by a tissue or organ at a specific stage, maximizing avoidance of protein loss and degradation is the primary factor for successful 2-DE [22]. Since winter rapeseed leaves contain large amounts of phenolic compounds, quinones, polysaccharides, lipids, pigments, and other plant secondary metabolites, these substances severely interfere with the first-dimension IEF [23-25]. Therefore, this study added appropriate amounts of PVPP during sample extraction to effectively reduce the impact of these substances on the focusing process. Increasing centrifugal force and extending centrifugation time could remove salts from samples to avoid problems of failing to reach preset voltage due to excessively high salt content. Wang et al. [26] found that 80% acetone produced optimal protein precipitation effects; therefore, this study used 80% cold acetone for final extraction to more thoroughly clean inorganic ions from the extraction solution, reduce conductivity caused by inorganic ions, and achieve impurity removal. Since plant cells contain proteolytic enzymes that are released into solution during sample grinding, causing protein hydrolysis [27], this study added 0.015% PMSF protease inhibitor to the protein extraction solution to effectively prevent hydrolysis of total rapeseed proteins. 2-DE requires that proteins in samples be completely dissolved, depolymerized, denatured, and reduced to enable effective separation in the first-dimension IEF. Since -mercaptoethanol has relatively poor reducing capacity and strong volatility with a special odor, this study used DTT with stronger reducing capacity and lower volatility to fully disrupt disulfide bonds between proteins for better separation. In proteomics, when studying differential proteins, total proteins under two conditions being compared should be loaded at the same level. Generally, protein loading amounts for 2-DE range between 0.5-1.2 mg. Excessive loading leads to inadequate sample absorption

by strips, producing vertical and horizontal streaks and poor protein spot separation, while also masking low-abundance protein spots with high-abundance ones, making it difficult to accurately excise target protein spots and resulting in numerous protein types for mass spectrometry analysis and inaccurate identification results. Insufficient loading fails to meet Coomassie brilliant blue staining requirements [28] and causes blurry protein spots in gel maps, leading to false positives and difficulties in subsequent analysis. Therefore, this study used a protein loading amount of 1 mg, obtaining clear protein spots without streaks and producing high-quality 2-DE maps conducive to subsequent analysis.

3.2 pH 4-7 Nonlinear IPG Strips as Optimal for Separation and Identification of Total Leaf Protein Maps in *B. campestris*

In preliminary experiments, wide-range (17 cm, pH 3-10) nonlinear IPG strips were used for preliminary protein separation, revealing that protein spots were mainly concentrated in the acidic region (pH 4-7), with many spots appearing in clusters that failed to separate effectively. Gel map analysis using PDQuest 8.0 software could detect 800-900 protein spots. However, most functional proteins involved in plant life activities (such as photosynthesis, respiratory metabolism, and stress response) are low-abundance proteins [29]. Therefore, this study selected 17 cm, pH 4-7 IPG strips for protein separation, detecting 600-700 protein spots. Although some protein spots were lost, the separation effect was significantly superior to the former, with separated protein spots distributed more uniformly and clearly on gel maps, laying a foundation for finding and identifying proteins related to winter rapeseed cold resistance functions.

3.3 Functional Analysis of Differentially Expressed Proteins

3.3.1 Photosynthesis-Related Proteins Oxygen-evolving enhancer protein (spot 1) primarily functions to catalyze water splitting to release oxygen, reduce plastoquinone, and generate transmembrane proton electrochemical potential gradients. Sugihara et al. [30] and Yamada et al. [31] found that abiotic stresses such as low temperature, drought, and salt cause degradation of photosynthesis-related proteins, reducing plant light use efficiency and increasing susceptibility to photoinhibition. Ribulose-1,5-bisphosphate carboxylase/oxygenase (spot 5) is a key enzyme in plant photosynthesis, with its activity closely related to photosynthetic rate [32]. This study found that this protein spot was suppressed after low temperature stress, indicating that the photosynthetic apparatus in 'Long-you No. 7' leaves was damaged under low temperature, with proton transport and photophosphorylation processes inhibited. The 23 kD peripheral protein of photosystem II (spot 15), bound to the lumen side of thylakoid membranes, functions to maintain PSII water-splitting activity [33]. After low temperature stress, 'Long-you No. 7' leaves induced expression of peripheral proteins to resist low temperature stress and maintain stable photosynthetic rates. Carbonic anhydrase (spot 8) is a zinc-containing metalloenzyme that catalyzes the reversible hydration of CO

in cells and is closely related to photosynthesis. Deng et al. [33] demonstrated that carbonic anhydrase participates in biochemical pathways of stress response, while Chen et al. [34] identified CA protein in longan (*Dimocarpus longan*) using differential proteomics, showing at the protein level that CA protein was upregulated under low temperature stress, indicating a relationship between its gene and low temperature stress. The induced expression of carbonic anhydrase in 'Long-you No. 7' after low temperature stress may reflect the plant' s need for energy to maintain growth under stress.

3.3.2 Sugar Metabolism-Related Proteins Fructose-1,6-bisphosphate aldolase (spot 7) is a key enzyme in glycolysis and gluconeogenesis, primarily involved in soluble sugar synthesis and induced expression in response to low temperature stress. As osmotic regulators, soluble sugars prevent intracellular condensation and ice crystal formation that causes mechanical damage, while maintaining intracellular osmotic pressure, increasing tissue water content, and lowering cytoplasmic freezing point to enhance plant low temperature tolerance. This study showed that this enzyme is an inducible protein that was rapidly expressed and accumulated after low temperature stress, thereby ensuring normal sugar metabolism pathways in 'Long-you No. 7' leaves during low temperature stress response. Tang [35] found through phenotypic analysis and gene chip detection in *Arabidopsis thaliana* that expression of the fructose-1,6-bisphosphate aldolase gene (*CpFBA*) was significantly upregulated in low temperature lethal mutants. Sun [36] found that low temperature stress within certain thresholds induced expression of *CpFBA* in different wheat (*Triticum aestivum*) varieties, with significantly upregulated expression leading to continuous accumulation of corresponding proteins, similar to results of this study.

3.3.3 Material Transport-Related Proteins Transcriptional regulator SUPERMAN zinc finger protein (spot 2) is a transcription factor that responds to external environmental stress by specifically binding to cis-regulatory elements in promoter regions of cold target genes through specific domains [37]. Expression of such proteins enables plants to more effectively cope with low temperature stress. Currently, such genes obtained from cotton, petunia (*Petunia hybrid*), and other plants generally participate in plant growth, development, and stress responses [38-39]. However, this protein was suppressed after low temperature stress in this study, suggesting that after prolonged low temperature stress, protein structural domains in 'Long-you No. 7' leaf tissues were damaged, preventing specific binding of cold target genes to protein domains. Annexin (spot 6) is a class of Ca^2 and phospholipid-binding proteins. Research on plant annexins started relatively late, but annexin proteins have been found to be widely distributed in plants and play important roles in plant growth, development, and environmental stress responses, primarily participating in cold resistance reactions, cell secretion, Ca^2 metabolism, salt tolerance, drought stress, and ABA signal transduction [39]. He et al. [40] found that annexins possess peroxidase activity in cells, suggesting induced expression of this protein spot.

Thus, transcriptional regulator SUPERMAN zinc finger protein plays an important role in clearing reactive oxygen species accumulated in 'Long-you No. 7' leaves under low temperature stress, thereby ensuring normal material transport under adverse stress.

3.3.4 Stress Response-Related Proteins Cysteine proteinase inhibitors (spots 9, 10) protect cells, tissues, and organs from exogenous proteolytic enzyme hydrolysis [41]. Additionally, studies have shown that overexpression of protease inhibitor genes can enhance transgenic plant resistance to abiotic stresses such as low temperature and drought. *Ammopiptanthus mongolicus* is an important stress-resistant plant resource in northwestern China, and Liu et al. [42] cloned the low temperature-induced *AmpI* gene identified as a cysteine proteinase inhibitor gene. Thaumatin-like protein (spot 13) is a plant defense protein with multiple biological activities and important functions. When plants suffer biotic or abiotic stress, thaumatin-like proteins are rapidly expressed and accumulated in plant tissues to quickly enhance plant resistance [43]. Hiilovaara-Teijo et al. [44] found that thaumatin-like proteins secreted by winter rye (*Secale cereale*) leaf apoplast under low temperature induction had antifreeze activity identical to antifreeze proteins. Virus-resistance protein (spot 14) can induce expression of pathogenesis-related proteins, which accumulate under low temperature stress to induce plant cold resistance [45], and can also induce production of other proteins or substances to generate systemic resistance in plants against external stress damage [46]. In this study, expression and accumulation of cold-responsive proteins in 'Long-you No. 7' under low temperature effectively prevented low temperature damage to cell physiology and structure, thereby greatly enhancing its cold resistance.

Conclusion

The 17 cm, pH 4-7 IPG strips were most suitable for separating protein spots in rapeseed leaves. After low temperature stress, expression of some photosynthesis-related proteins in leaves of the super cold-resistant *B. campestris* variety 'Long-you No. 7' was suppressed, while other photosynthesis-related proteins were induced. Suppressed proteins may result from irreversible damage to plant tissues under prolonged low temperature, while plants induced expression of key photosynthetic enzymes such as carbonic anhydrase to maintain normal photosynthesis and growth. Expression of material transport-related proteins such as annexin helped maintain cell structure, promote transmembrane ion transport, and maintain homeostasis. Additionally, low temperature-induced thaumatin-like proteins and other stress-responsive proteins synergistically functioned to resist low temperature stress. It can be inferred that stable photosynthesis, efficient material metabolism systems, and powerful stress defense mechanisms play important roles in maintaining growth of the super cold-resistant variety 'Long-you No. 7' under low temperature conditions.

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