Identification of Winter-Responsive Proteins in Bread Wheat Using Proteomics Analysis and Virus-Induced Gene Silencing (VIGS)*

Ning Zhang‡, Wang Huo‡, Lingran Zhang‡, Feng Chen‡§, and Dangqun Cui‡

Proteomic approaches were applied to identify protein spots involved in cold responses in wheat. By comparing the differentially accumulated proteins from two cultivars (UC1110 and PI 610750) and their derivatives, as well as the F10 recombinant inbred line population differing in cold-tolerance, a total of 20 common protein spots representing 16 unique proteins were successfully identified using 2-DE method. Of these, 14 spots had significantly enhanced abundance in the cold-sensitive parental cultivar UC1110 and its 20 descendant lines when compared with the cold-tolerant parental cultivar PI 610750 and its 20 descendant lines. Six protein spots with reduced abundance were also detected. The identified protein spots are involved in stress-defense, carbohydrate metabolism, protein metabolism, nitrogen metabolism, energy metabolism, and photosynthesis. The 20 differentially expressed protein spots were chosen for quantitative real-time polymerase chain reaction (qRT-PCR) to investigate expression changes at the RNA level. The results indicated that the transcriptional expression patterns of 11 genes were consistent with their protein expression models. Among the three unknown proteins, Spot 20 (PAP6-like) showed high sequence similarities with PAP6. qRT-PCR results implied that cold and salt stresses increased the expression of PAP6-like in wheat leaves. Furthermore, VIGS (virus-induced gene silencing)-treated plants generated for PAP6-like were subjected to freezing stress, these plants had more serious droop and wilt, an increased rate of relative electrolyte leakage, reduced relative water content (RWC) and decreased tocopherol levels when compared with viral control plants. However, the plants that were silenced for the other two unknown proteins had no significant differences in comparison to the BSMV-O-inoculated plants under freezing conditions. These results indicate that PAP6-like possibly plays an important role in conferring cold tolerance in wheat.

Cold/low-temperature stress, a major form of abiotic stress, includes chilling (0–20 °C) and/or freezing (<0 °C) temperatures that can induce ice formation in plant tissues, leading to cellular dehydration, which can limit plant growth and development, resulting in extensive losses to agricultural production worldwide (1). Over time, plants have evolved sophisticated mechanisms to counteract low temperature or freezing stress to survive (e.g. synthesis and accumulation of cryoprotectant substances such as amino acids, sugars, and antifreeze proteins which decrease the freezing point and/or prevent ice nucleation) (2, 3). Plants may also avoid freezing by delaying transition from the vegetative to the reproductive phase (4). In addition, plants can tolerate cold damage by altering expression of a gene involved in photosynthesis, carbohydrate metabolism and energetic pathways, protein metabolism and amino acid metabolism, stress response, and signal transduction (3). Therefore, these cold-tolerant plants are good sources of genes and proteins involved in providing tolerance (3). The study of gene expression at the transcriptome level of many plants, such as Arabidopsis, tomato, barley, wheat, and rice (5–9), have significantly contributed to our understanding of cold tolerance mechanisms and the accumulation of cold-responsive genes (10). However, protein levels are often poorly correlated with their corresponding mRNA profiles (11, 12) because of the regulation of gene expression at transcriptional, post-transcriptional, translational, and post-translational levels (13, 14). Therefore, as a global study of the proteins that comprise the proteome, proteomics can play an important role in addressing plant responses to environmental changes (15). Examining proteome alterations for proteins is crucial; these proteins, unlike transcripts, are direct sources of plant stress responses (16). The effect of cold on protein abundance has been studied in many plants, such as soybean, barley, wheat, Arabidopsis thaliana, rice, and tobacco (4, 17–21).

Until recently, the most common comparisons in plant abiotic stress research have been between proteomes from non-stressed (control) and stressed (treatment) plants. Other comparisons of proteomes have included those examining two different-background genotypes or plant species with contrasting levels of tolerance to a given stress factor (e.g. proteomic analysis of two winter wheat cultivars that differ in low-temperature tolerance response to abrupt low-tempera-
ture stress conditions) (22). The comparison of differentially abundant proteins during cold treatment is mostly performed by conventional two-dimensional electrophoresis (2-DE) or two-dimensional differential gel electrophoresis (2D-DIGE) via MS analysis, as seen in specific tissues or organelles: for example, Arabidopsis cold-and salt-tolerant relative of Thellungiella halophila rosette leaves (15); pea roots, stems and leaves (23); rice seedlings (24); rice leaves (25); rice roots (18); and wheat leaves (4, 22). Generally, these studies have observed that changes in protein abundance during cold treatment were related to abiotic stress responses (26), (e.g. pathogenesis-related protein, cold-regulated protein, cold-responsive LEA/RAB-related COR protein, oxygen-evolving enhancer protein, and oxalate oxidase) (4).

Overwintering stress is one of the most severe abiotic stresses of wheat in China, especially in the Yellow and Huang wheat regions, where the lowest temperatures have been recorded at −10 °C. Frost injury during winter (winterkilling) and early spring can be particularly damaging, causing reductions in wheat yield and severely limiting the growth and productivity of crops (27). Therefore, the main objective of overwintering stress proteomics in crops is to identify winter-responsive proteins, which can potentially be used for crop improvement and breeding (28). In this study, a recombinant inbred line (RIL) \(^1\) winter wheat population was utilized to uncover winter-responsive proteins; a total of 20 candidate winter-responsive proteins were successfully identified. Furthermore, preliminary functional analysis of abiotic stresses by quantitative real-time PCR (qRT-PCR) and virus-induced gene silencing (VIGS) was conducted on three novel candidate cold-responsive proteins (unnamed function). Better understanding of the cold-tolerance mechanisms in bread wheat using these candidate winter-responsive proteins would be beneficial and further studies could be conducted to maximize the potential applications in crop breeding.

MATERIALS AND METHODS

**Plant Materials—** An F\(_2\) RIL population (derived from a cross of UC 1110 × PI 610750), composed of 187 lines, was provided by Prof. Jorge Dubcovsky from the University of California, Davis. The RIL population was planted on October 15, 2013 and October 16, 2014 in Anyang (N36.1°, E114.5°); and on October 6, 2013 and October 8, 2014 in Zhengzhou (N34.9°, E113.6°). The average monthly growing degree days (GDD) from October of 2013 and 2014 to March of 2014 and 2015 in Anyang and Zhengzhou are shown in Table IV. Frost injury during winter was investigated in Anyang and Zhengzhou on March 1, 2014 and February 25, 2015, respectively. The damage extents were divided into four ranks (3, 2, 1, 0) from high to low based on the phenotypic changes according to the standards of the wheat cultivar approval committee of the Yellow and Huang wheat region (i.e. sensitive, moderate sensitive, moderate tolerant, cold tolerant, respectively). The wheat parental cultivars UC 1110 (Rank 3) and PI 610750 (Rank 0) differed in cold-tolerance, therefore, the descendants displayed segregation of cold tolerance. Leaves were collected after the investigation of phenotype in Zhengzhou, including the cold-sensitive cultivar UC 1110, cold-tolerant cultivar PI 610750, two cold-sensitive pools (CSP-1 and CSP-2), and two cold-tolerant pools (CTP-1 and CTP-2). Each cold-sensitive or cold-tolerant pool was composed of an equivalent mixture of leaves from 10 lines of the RIL population with level 3 or 0 under the four environments. Sampled leaves were rapidly frozen in liquid nitrogen, and stored at −80 °C for protein and RNA extractions.

**Protein Preparation—** For 2-DE, protein samples with three biological replicates were prepared. Proteins from leaves were extracted using the trichloroacetic acid (TCA)/acetone method (14). Leaf samples of 500–700 mg were pulverized in liquid nitrogen with a mortar and pestle. Ten volumes of ice-cold 10% (w/v) TCA in acetone plus 0.07% (v/v) 2-mercaptoethanol, followed by incubation at −20 °C for 2 h. After centrifugation at 14,000 \(g\) for 30 min at 4 °C, the supernatants were discarded and the pellets were washed three times with ice-cold acetone plus 0.07% (v/v) 2-mercaptoethanol. The pellets were vacuum-dried and resuspended in lysis buffer (29) for 2 h. The suspension was centrifuged at 14,000 \(g\) for 40 min at 25 °C to remove insoluble materials. Concentrations of total protein were determined by the Bradford assay (Bio-Rad, California) based on a bovine serum albumin (BSA) standard (29).

**2-DE and Image Analysis—** For 2-DE, 800 μg of protein samples were loaded onto an ReadyStrip\(^{TM}\) IPG Strip (24 cm, pH 4–7, Bio-Rad) and hydrated passively with 450 μl of protein solution containing 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH 4–7) for 12–18 h at 20 °C using a PROTEAN IEF Cell (Bio-Rad). The first-dimension isoelectric focusing (IEF) was performed in six steps: 250 V for 2 h, 250 V for 2 min, 500 V for 90 min, 500 V for 90 min, 1000 V for 2 h, 9000 V for 5 h, and 9000 V for 10 h with a total of 99 kVh and a constant 500 V for the last 12 h. After IEF, the strips were incubated for 15 min in “equilibration buffer II” consisting of 6 M urea, 2% (w/v) SDS, 1.5 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 2% (w/v) dithiothreitol (DTT); then, in “buffer II” consisting of 6 M urea, 2% (w/v) SDS, 1.5 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 2.5% (w/v) iodoacetamide for 15 min.

For second-dimension electrophoresis, the strips were transferred to 12% vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. All leaf samples were performed in triplicate to obtain statistically reliable results. After electrophoresis, fixation and Coomassie brilliant blue (CBB) staining of gels were carried out (30), wherein gels were fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 40 min. To visualize the gels, they were stained with a staining solution consisting of 0.12% (v/v) CBB G-250, 20% (v/v) alcohol, 10% (v/v) phosphoric acid, and 10% (w/v) ammonium sulfate; then, destained in double-distilled H\(_2\)O (dd H\(_2\)O). The 2-DE images were scanned at 300 dpi with a UMAX Power Look 2, 100XL scanner (Maximum Tech, Taiwan, China); a quantitative intensity analysis was performed using the PDQuest software (version 8.0.1, Bio-
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**Table I**

| Spot no. | Forward primer | Reverse primer | Produce length (bp) |
|----------|----------------|----------------|--------------------|
| 4        | TGTGCAGAATTGTGGGAG | GCCAGATCGAGGTATCCGC | 194                |
| 6        | TTGGTGGCTGTCCACCTGC | GGACAGGTTCGCGCAAGAC | 171                |
| 7/10     | TCCAGGTAGCTCCAGGACTG | TTGGTGGCTTCCTCCAGCAA | 170                |
| 8/9      | GCCAGGACGCAGAAGAAGAG | GGCTTCCCCTATATGTGCTG | 216                |
| 11       | GAGTAGAGCTCCAGCCTGGG | TGTAGAGACGTAAGGCTTT | 280                |
| 13/14    | CGGAGTACATCCGACTCTGCA | TGGAAAGCTTCTGCAGTGGT | 334                |
| 15       | ACATGACATGCAACTCTACGT | AGAAGACTCCCGCATCATAA | 100                |
| 16       | CACCTGGGAACACTACCTGTC | ACCATGTCCTCTCCTGCTA | 217                |
| 17       | TTACCCCTCAGCCAACCTCACA | GGCGGAGAACACTACATTCC | 139                |
| 18       | GCTGCCAGATCTCTCAGGGAG | CGGCGGTTCCTGATCTCCTT | 253                |
| 19       | CACGGGCTTGGCTTGGGAC | CGGAGCTCAAGGTTAAGGAGG | 147                |
| 20       | GAGGAAGAGAGACAGGAGAG | TGATTTTCAGGCGAGGAGAG | 111                |
| 21       | CCTCAACACGCTCAAGCAGGTG | GGTTTCCGCGCTCCAGTAA | 226                |
| 23       | CCACCTGGCCTCTCTCGAGAT | GGTGGAAGGTGTTAGAGACT | 254                |
| 24       | ACGAAGGAGGGTCTCGCTGCT | GGCGTAACTCCGGTCTGGAAG | 241                |
| 3/27     | GGACCAAGAGGGATGATCTG | AGCTATGCGAGGATTCAGGAG | 274                |
| β-actin  | GTTCCACTTATGAGGGATACACG | GAAACTCCACCTGAGAACACATTACC | 422                |
| V-20     | CGTCCCTCCGCTGGAAATCCA | GAGAAGGCGCTGCTCGTAGAC | 198                |
| V-6      | TGTAGAAGAAAGCCACCCA | TGTGCTGCTAGATCTGCCC | 171                |
| V-21     | GCATGTGATCCCTCCTGGGACT | CACAACTGCGAACATGGCGAG | 191                |
| V-PDS    | TCACAGATTAGCAGGACGCTCA | GTTTACTGGCCGCTTACCT | 211                |

a Spot no.: the numbering corresponds to the 2-DE gels in Fig. 2. Spot no. and protein name correspondingly are the same as shown in Table II (except for β-actin and PDS).

Rad). First, 2-DE gels of leaf samples from PI 610750 and CTP-1/CTP-2 were selected as the reference gels; gels of UC 1110 and CSP-1/CSP-2 matched to the reference gels, respectively. Automatic groups were formed and single spots that differed between replicates were manually checked and corrected when necessary. Spots that existed in three independent sample sets were selected (Additional file 3). Image quantitative analysis revealed significant differences in protein spot abundance by Student’s t test (abundance variation at least two-fold, p < 0.05). In addition, amino acid sequences were compared using the DNAMAN Version 6.0 software (http://NSw.softlandsl.com/free/dnaman_v6+free.html).

**Two-dimensional Gel Excision, Tryptic Digestion, and Desalting**—Protein extracts were separated on preparative gels and 20 proteins of interest were recovered from the gels for identification. Proteins (800 μg) from samples were resolved on separate preparative polyacrylamide gels and were visualized by staining with a modified silver staining method that was compatible with subsequent mass spectrometric analysis (31). Protein spots of interest were cut from the prepared gels, destained for 20 min in 30 mM potassium ferricyanide/100 mM sodium thiouisulfate (1:1 v/v) and washed with Milli-Q water until the gels were destained. The spots were incubated in 0.2 M NH4HCO3 for 20 min and then lyophilized. Each spot was digested with trypsin in 25 mM NH4HCO3 and 5% acetonitrile, and oxidation of methionine allowed as a variable modification. Automatic tryptic digestion allowed, carbamido methylation set as a fixed modification, acetylation of lysine residues, formic acid and TFA were added (Additional file 3). Peptide B ((M+H) + 842.5100 and 2, 211.1046) were used as the internal calibrates, and ten of the most intense ion signals were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. In MS/MS positive ion mode, for one main MS spectrum, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern. Collision energy was 2 kV, the collision gas was air, and the default calibration was set using the GIu1-Fibrinopeptide B (M+H + 1,570.6696) spotlighted onto Cal 7 positions of the MALDI target. Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed using the Mascot search engine 2.2 (Matrix Science Ltd., London, UK) that was embedded into the GPS-Explorer Software 3.6 (Applied Biosystems) on the NCBI database, and 51829 sequences generated from protein sequences of Tricium (downloaded March 2015) with the following parameter settings: 100 ppm mass accuracy, with trypsin cleavage and one missed cleavage allowed, carbamido methylation set as a fixed modification, and oxidation of methionine allowed as a variable modification. Additionally, MS/MS fragment tolerance was set to 0.4 Da. A GPS explorer protein confidence index ≥ 95% was used for further manual validation.

**Transcriptional Expression Analysis by qRT-PCR**—Twenty of the differentially abundant proteins were chosen by qRT-PCR. The specific primers were designed using Primer 3.0 (Table II). Total RNA from UC 1110, PI 610750, CTP-1, CTP-2, CTP-1 and CTP-2 was extracted using the total RNA kit (TaKaRa, Dalian, China). Two-Step PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time; Takara) was used for the RT reactions. The temperature program was adjusted as follows: 2 min at 42 °C, 15 min at 37 °C, 5 s at 85 °C, and then 4 °C. For each candidate, three biological replicates were performed. qRT-PCR was conducted using a Bio-Rad IQ5 Real-Time PCR Detection System. Each reaction included 20 μl of the products from the diluted RT reactions, 0.4 μl of each primer (forward and reverse), 10 μl of GoTaq® qPCR Master Mix (Perfect Real Time; Promega, Madison), and 7.2 μl of nuclease-free water. The reactions were incubated in a 96-well plate at 95 °C for 3 min, followed by 40
cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicates for each sample. The β-actin gene (GenBank accession no. AB181991) served as the endogenous control.

**Abiotic Stress Treatments**—Yunong 201 seeds were sterilized by immersion in 10% (w/v) H2O2 for 0.5 h and then thoroughly washed with distilled water. Sterilized seeds were grown in glass dishes (9 cm diameter) with double distilled H2O (dd H2O). Seedlings were maintained in an illuminated incubator at 25/15 °C day/night temperatures under 16/8 h light/dark photoperiod and 250 μmol m−2 s−1 light intensity. Two-week-old seedlings with similar heights were used to analyze the response of the unnamed protein product (i.e., protein spot 20) under different abiotic stresses. Some seedlings were transferred to an illuminated incubator at 4 °C or 42 °C for low or high-temperature stress treatments, respectively. Some seedlings were exposed to 200 mM NaCl (salt treatment) and 18% PEG 6000 (osmotic treatment) by root immersion. The leaves of the wheat cultivar were collected at 0, 1, 2, 6, 12, and 24 h after the different treatments and then stored at −80 °C.

**System of Virus-induced Gene Silencing (VIGS)**—The weak spring-summer wheat cultivar Zhengmai 9023, kindly provided by Henan Academy of Agricultural Sciences, was used for the VIGS experiment because of its high sensitivity to barley stripe mosaic virus (BSMV) according to our pretest. Primers were designed using Primer 3.0 (Table I) and 198-bp, 171-bp, and 191-bp fragments were generated for spot 20 (PAP6-like), spot 6 (spot N°6), spot 21 (spot N°21), respectively. Vector constructs were performed as previously described (32). The α, β, and γ RNAs of the BSMV genome were synthesized from linearized plasmids (33), using RiboMAXTM Large Scale RNA Production System-T7 (Promega, Madison). The *in vitro* transcripts of each RNA segment were mixed in an equimolar ratio and added to an abrasive FES buffer (34). Each silencing construct was transfected onto an illuminated incubator at 4 °C or 42 °C for low or high-temperature stress treatments, respectively. Some seedlings were exposed to 200 mM NaCl (salt treatment) and 18% PEG 6000 (osmotic treatment) by root immersion. The leaves of the wheat cultivar were collected at 0, 1, 2, 6, 12, and 24 h after the different treatments and then stored at −80 °C.

**RESULTS**

**Phenotypic Comparison of UC1110/CSPs and PI610750/CTPs**—Wheat frost injury during the winter of 2013–2014 and 2014–2015 cropping seasons was investigated in Zhengzhou and Anyang, respectively. The amount of plants corresponding to the four levels (0, 1, 2, and 3) of frost damage in Zhengzhou were 27, 4, 15, and 34 lines during the 2013–2015 two cropping seasons, respectively; the numbers in Anyang were 26, 7, 19, and 36 lines during the 2013–2015 cropping seasons, respectively. In this study, the sampled 27 and 25 wheat lines, respectively, showed level 3 and 0 in frost injury during winter investigation for all four environments tested (in 2013 and 2014 at Anyang and Zhengzhou, respectively). The phenotypes of the cold-tolerant cultivar PI610750 (Fig. 1A), cold-sensitive cultivar UC1110 (Fig. 1B), and partial lines composed of cold-tolerant pools (CTPs; Fig. 1C) and cold-sensitive pools (CSPs; Fig. 1D) were shown.

**Identification and Classification of Differentially Accumulated Proteins**—More than 1000 protein spots were detected on each gel. Of these, 28 spots displayed altered abundance between UC 1110 and PI 610750, and there were 23 differentially accumulated protein spots between the CSPs (CSP-1 and CSP-2) and the CTPs (CTP-1 and CTP-2) (Fig. 2). Further analysis indicated that 20 out of 28/23 spots above-mentioned were simultaneously present in the two parents and four constructed pools (CTPs and CSPs), and these 20 spots were then analyzed by mass spectrometry. Finally, the 20 spots, representing 16 unique proteins, were successfully identified using MALDI-TOF/TOF analysis (Additional files 1 and 2). Among the identified 20 protein spots, 14 spots were up-regulated and the remaining six protein spots were downregulated in the cold-sensitive cultivar UC 1110 and CSPs when compared with the cold-tolerant cultivar PI 610750 and CTPs. According to the differential functions, the 20 identified protein spots were classified into six main groups: stress/defense (5%, 1), carbohydrate metabolism (5%, 1), protein metabolism (10%, 2), nitrogen metabolism (10%, 2), energy production and transportation (15%, 3), photosynthesis (40%, 8), and unknown function (15%, 3) (Table II). Some identified spots from different positions of the same gel and with the same isoelectric point (pl) and molecular mass (Mw) were expected to have the same name. These spots referred to four different groups: ribulose 1,5-bisphosphate...
carboxylase/oxygenase large subunit, Rubisco (i.e. protein spots 7 and 10), ribulose-1 (chloroplast) (i.e. protein spots 8 and 9), ATP synthase CF1 alpha subunit, chloroplast (i.e. protein spots 13 and 14), and plastid glutamine synthetase 2 (i.e. protein spots 3 and 27) (Table II). Those protein spots may be recognized as different products because of nucleotide gene polymorphisms, alternative splicing, proteolytic cleavage, or post-translational modifications of a single gene or protein; thus indicating associations with different cellular functions (40). Three identified proteins (15%, 3) were listed as either unknown or hypothetical proteins in the database (Table II). In order to gain functional information about these proteins, their homologs were searched by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) using the amino acid sequences as search queries (41).

The corresponding homologs with the highest homology are shown in Table III. These proteins shared more than 86% positive relationship with homologs at the amino acid level, such as an unnamed protein product (i.e. protein spot 6), which shared 82% identity with protein plastid transcriptionally active 16, chloroplastic (chloroplastic) at the amino acid level, indicating a similarity in their function.

Comparison of Abundance Patterns of Identified Proteins at the mRNA and Protein Levels—Six of the downregulated proteins (i.e. protein spots 7, 8, 9, 10, 11, and 24) and 14 of the up-regulated proteins (i.e. protein spots 3, 4, 6, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23, and 27) were chosen by qRT-PCR to investigate corresponding expression changes at the RNA level that could be correlated with cold tolerance. A comparison of the expression patterns at the mRNA and protein levels indicated that the transcriptional expression patterns of 11 genes were consistent with the protein expression patterns, whereas the remaining nine genes showed poor consistency between the transcriptional and translational levels in the cold-sensitive cultivar UC 1110 and CSP-1 when compared with the cold-tolerant cultivar PI 610750 and CTP-1 (Fig. 4 Table V).

Stress Resistances of Protein Spot 20 in Response to Abiotic Stresses—Based on the BLAST search performed and the putative functional information related to the three unnamed or hypothetical proteins (protein spots 6, 20 and 21) obtained based on existing research results (42–47), it was found that protein 20 (designated PAP6-like) is possibly related to cold response. Therefore, in order to investigate the relationship between PAP6-like (which shared the same conserved domains “PAP-fibrillin” with plastid-lipid-associated protein 6, chloroplastic, PAP6) with stress response (cold, heat, drought, and salt), the expression patterns in the Yunong 201 wheat cultivar were examined. In response to cold treatment, gene expression of PAP6-like was displayed up-regulated; and the expression abundance reached 4.2-fold at 6 h. During 0–24 h under salt stress, the gene of PAP6-like was up-regulated and reached the highest peak at 2 h (Fig. 5). However, when wheat was exposed to drought and heat stresses, the gene was downregulated during the two stress periods, and was further downregulated under heat stress in comparison to drought stress.

Virus-induced Gene Silencing of PAP6-like and the Other Two Unknown Protein Genes in Wheat—VIGS was performed in order to further evaluate the role of PAP6-like, as well spot
6 (spot N°6) and spot 21 (spot N°21) in the response of wheat to cold stress. qRT-PCR was performed to determine the transcript levels of PAP6-like, spot N°6, and spot N°21 in silenced plants, the viral controls, nonstressed nonsilenced (NS) and freeze-stressed nonsilenced (FS) plants at day 14 after viral inoculation, respectively. The transcript levels of the genes were significantly reduced in silenced plants in comparison to plants inoculated with only BSMV0 (Fig. 6). The average PAP6-like, spot N°6 and spot N°21 transcript levels were reduced 7.1-fold, 4.3-fold, and 14.9-fold in BSMV\textsubscript{PAP6-like}, BSMV\textsubscript{spot N°6}, and BSMV\textsubscript{spot N°21}-inoculated plants and were increased 3.3-fold, 2.8-fold, 3.1-fold in FS when they compared with the BSMV\textsubscript{0}-treated plants, respectively. The transcript levels of PAP6-like, spot N°6, and spot N°21 in NS (0.89-fold, 1.07-fold, and 1.06-fold, respectively) were not significantly different from the viral control plants.

Phenotypic and Physiological Changes of Wheat Silenced for PAP6-like and the Other Two Unknown Protein Genes Under Freezing Stress—Phenotypes of the plants were observed during the entire course of the experiment (from 1 to 19 days). Slight chlorosis was observed in the viral controls and in all of the silenced plants after rub inoculation with BSMV constructs because of the plant’s response to virus infection (Fig. 7A). This has been previously reported in VIGS studies in wheat (37, 48). The droop symptoms were observed in the viral controls and in all of the silenced plants after rub inoculation with BSMV constructs because of the plant’s response to virus infection (Fig. 7A). This has been previously reported in VIGS studies in wheat (37, 48). The droop symptoms were observed in plants after freezing stress for 5 days (Fig. 7B). In comparison with the freeze-stressed viral control plants, there were no remarkable phenotypic differences in leaves from the
## Table II

List of winter-responsive leaf protein spots from 2-DE gels identified by MALDI-TOF/TOF-MS

| Spot no. | Protein species | Accession no. | Score | Protein M/pI | Coverage (%) | Protein score C.1.% | NP* | UC 1110/PI 610750 ratio/ p value | CSP-1/CTP-1 ratio/ p value | Plant species |
|----------|----------------|---------------|-------|--------------|---------------|---------------------|-----|----------------------------------|--------------------------|----------------|
| 6        | Unnamed protein product | gi 660272771 | 1060  | 52.59/5.58   | 56            | 100                 | 24  | 1.72 0.0023 1.41 0.0021 | Triticum aestivum |
| 20       | Unnamed protein product | gi 257389844 | 566   | 29.52/9.51   | 39            | 100                 | 11  | 1.12 0.0027 1.81 0.0034 | Triticum aestivum |
| 21       | Hypothetical protein TRIU3_22780 | gi 473759990 | 253   | 33.20/8.75   | 26            | 100                 | 8   | 2.02 0.0015 1.20 0.0054 | Triticum aestivum |
| 17       | Aminoacyl tRNA synthase complex-interacting | gi 473910726 | 168   | 21.16/5.42   | 29            | 100                 | 5   | 1.63 0.0021 1.35 0.0012 | Triticum urartu |
| 24       | Elongation factor Tu | gi 474198705 | 1050  | 45.79/4.61   | 64            | 100                 | 22  | 0.63 0.0032 0.20 0.0014 | Triticum urartu |
| 23       | DRP6 protein, partial | gi 85857190  | 257   | 16.71/8.54   | 65            | 100                 | 8   | 1.97 0.0111 1.24 0.0027 | Triticumturgidum subsp. durum |
| 7        | Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, Rubisco, partial (chloroplast) | gi 713664233 | 852   | 51.84/6.32   | 60            | 100                 | 25  | 0.67 0.0018 0.42 0.0027 | Triticum monococcum |
| 8        | Ribulose-1 (chloroplast) | gi 73955481  | 863   | 53.45/6.22100 | 58            | 100                 | 26  | 0.43 0.0024 0.45 0.0031 | Triticum macha |
| 9        | Ribulose-1 (chloroplast) | gi 73955481  | 863   | 53.45/6.22100 | 59            | 100                 | 26  | 0.36 0.0014 0.28 0.0061 | Triticum macha |
| 10       | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, Rubisco, partial (chloroplast) | gi 713664233 | 852   | 51.84/6.32   | 63            | 100                 | 25  | 0.60 0.0028 0.27 0.0023 | Triticum monococcum |
| 11       | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, Rubisco, partial (chloroplast) | gi 713664251 | 446   | 51.79/6.36   | 58            | 100                 | 23  | 0.41 0.0029 0.39 0.0041 | Triticum dicoccon |
| 16       | Chlorophyll a/b binding protein, chloroplastic | gi 473952980 | 150   | 28.46/5.15   | 19            | 100                 | 6   | 1.16 0.0034 2.78 0.0017 | Triticum urartu |
| 18       | Chloroplast light-harvesting chlorophyll a/b binding protein | gi 302566696 | 318   | 28.37/5.14   | 37            | 100                 | 6   | 1.57 0.0009 3.12 0.0031 | Triticum aestivum |
| 19       | Chlorophyll a/b binding protein 1, chloroplastic | gi 473965828 | 318   | 30.44/5.52   | 16            | 100                 | 4   | 1.61 0.0012 3.81 0.0018 | Triticum urartu |
| 4        | ATP synthase CF1 beta subunit | gi 73955480  | 1300  | 53.88/5.06   | 75            | 100                 | 31  | 2.11 0.0018 3.20 0.0021 | Triticum macha |
| 13       | ATP synthase CF1 alpha subunit (chloroplast) | gi 73955470  | 911   | 55.32/6.11   | 57            | 100                 | 27  | 2.31 0.0051 1.84 0.0041 | Triticum macha |
| 14       | ATP synthase CF1 alpha subunit (chloroplast) | gi 73955470  | 940   | 55.32/6.11   | 54            | 100                 | 27  | 1.50 0.0041 2.02 0.0031 | Triticum macha |
| 15       | Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta | gi 474195362 | 427   | 61.33/7.01   | 45            | 100                 | 22  | 3.40 0.0033 1.77 0.0061 | Triticum urartu |
| 3        | Plastid glutamine synthetase 2 | gi 251832986  | 552   | 47.00/5.75   | 37            | 100                 | 11  | 2.02 0.0015 1.45 0.0071 | Triticum aestivum |
| 27       | Plastid glutamine synthetase 2 | gi 251832986  | 544   | 47.00/5.75   | 38            | 100                 | 12  | 3.50 0.00120 1.17 0.0037 | Triticum aestivum |

*Spot no.: corresponds to protein spot on gels shown in Fig. 2.
Accession no.: predicted protein in NCBI database.
Scores were searched against the database NCBInr.
M/pI: M, of molecular mass of predicted protein/pl of predicted protein.
NP*: Number of matched peptides.
Ratio: the ratio between intensity of identified protein spots in UC 1110/CTP-1 vs. control PI 610750/CTP-1. Ratio changes were from three biologically independent experiments of 2-DE at p < 0.05.
freeze-stressed of BSMV$_{\text{spotN}^0}$, and slight phenotypic difference of leaves from the freeze-stressed of BSMV$_{\text{spotN}^0}$21 was shown. However, leaves of the freeze-stressed BSMV$_{\text{PAP6-like}^-}$-treated plants displayed distinctly more serious droop and wilt in comparison to plants from the other freeze-stressed treatments. Therefore, PAP6-like protein was the only gene product investigated for further assessment of physiological parameters.

At the end of 5 days exposure to −5 °C, the rates of relative electrolyte leakage were examined in all treatment groups (Fig. 8A). The FS plants were markedly increased in the rates of relative electrolyte leakage relative to the NS plants. The FS plants did not differ remarkably from the stressed viral control, indicating that virus inoculation had no effect on the rates of relative electrolyte leakage in the plants. Additionally, plants silenced for PAP6-like had a significant increase in the rates of relative electrolyte leakage in comparison to FS and viral control plants. The impact of silencing on plant water status under cold limitations was also examined (Fig. 8B). Freeze-stressed BSMV$_{\text{o}}$-treated plants and FS plants did not have significant differences in RWC, whereas the FS plants drastically reduced in RWC when compared with the NS plants. Similarly, in comparison to freeze-stressed BSMV$_{\text{o}}$-treated plants, the freeze-stressed BSMV$_{\text{PAP6-like}^-}$-treated plants had a significant reduction in RWC. Furthermore, total tocopherol contents in leaves of FS plants and freeze-stressed viral control did not differ significantly. Plants silenced for PAP6-like were dramatically decreased in total tocopherol contents when compared with FS and viral control plants.

**DISCUSSION**

In this study, a F$_{10}$ RIL wheat population (UC 11110 × PI 610750) composed of 187 lines was selected to identify protein spots that contributed to the differences in cold tolerance between cultivars including descendant lines based on 2-DE and MALDI-TOF/TOF-MS. The candidate winter-responsive proteins were found to be involved in several physiological processes including stress/defense, carbohydrate metabolism, protein metabolism, nitrogen metabolism, energy metabolism, and photosynthesis.

Plants typically respond to cold stress by lowering the energy metabolism-related processes to manage the oxidative burst imposed by low temperatures (4). Photosynthesis is usually the first process to be influenced by cold stress (41), which can essentially disrupt all major components of photosynthesis including thylakoid electron transport, carbon reduction cycle, and stomatal conductance control. Moreover, proteomic analyses have revealed that photosynthesis-related proteins were modulated by the cold stress (49). In this study, which was the largest functional group affected by cold stress, ~40% of the cold-responsive proteins were involved in the Calvin cycle and electron transport (Fig. 3).

Leaf proteins contain one of the purest and highly-nutritive components, Rubisco (ribulose 1,5-bisphosphate carboxylase), which accounts for up to 30–70% of soluble leaf proteins (SLP) (50). This proteins catalyzes the first step in net photosynthetic CO$_2$ assimilation (51), which is the rate-limiting step in synthesizing the majority of the world’s biomass. Rubisco, as one of the key enzymes in the Calvin cycle (the dark phase of photosynthesis), is considered to be a protein reserve that can be mobilized at leaf senescence or when the plant experiences deficits of either carbohydrates or nitrogen (41). As such, Rubisco activity is modulated by environmental stress and can inhibit photosynthesis and carbon assimilation through reduction of stomatal conductance (52). Cold has been suggested to damage the Rubisco protein itself (53). For example, Rubisco was downregulated after 4 weeks of cold acclimation (4–6 °C) and subsequent treatment for 12 h at −2 °C in wheat leaves (4). In this study, ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, Rubisco, partial (chloroplast) (i.e. protein spots 7, 10, and 11), ribulose-1, (chloroplast) (i.e. protein spots 8 and 9) was significantly decreased in cold-sensitive cultivar UC 1110 and CSPs compared with cold-tolerant cultivar PI 610750 and CTPs. This effect agreed with previous research results on wheat (4, 41, 54) and rape (55). Thus, cold-induced changes in Rubisco may indicate a modulation in Rubisco activity under cold-stress conditions.

The light-harvesting chlorophyll a/b-binding (LHCb) proteins are the apoproteins of the light-harvesting complex of photosystem II (56). Chlorophyll a/b-binding-like protein genes were up-regulated in higher plants that were exposed to −12 °C-freeze stress (57); after a 3-day exposure to −5 °C-simulated spring freeze stress, chlorophyll a/b-binding-like protein genes were up-regulated in wheat (41), implying that they play a protective role in cold-stress tolerance. Extreme low temperatures have been suggested to disrupt thylakoid...
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Average monthly growing degree days from October 2013/2014 to March 2014/2015 in Anyang and Zhengzhou

| Month       | Location (°C) | Anyang | Zhengzhou |
|-------------|---------------|--------|-----------|
| 2013.10     | 15.4 °C       | 17.3 °C|
| 2013.11     | 7.8 °C        | 9.7 °C |
| 2013.12     | 2.3 °C        | 4.1 °C |
| 2013.01     | 3.1 °C        | 4.2 °C |
| 2014.02     | 2.8 °C        | 3.1 °C |
| 2014.03     | 12.4 °C       | 12.9 °C|
| 2014.10     | 17.1 °C       | 17.6 °C|
| 2014.11     | 9.4 °C        | 9.9 °C |
| 2014.12     | 2.5 °C        | 3.9 °C |
| 2015.01     | 2.8 °C        | 3.9 °C |
| 2015.02     | 4.5 °C        | 5.8 °C |
| 2015.03     | 10.6 °C       | 11.2 °C|

electron transport, reduce Rubisco activity, and induce stomatal closure, which reduces CO₂ uptake and induces water loss (53). In this study, an increased abundance of chlorophyll a-b binding protein, chloroplastic (i.e. protein spot 16), chloroplast light-harvesting chlorophyll a/b binding protein (i.e. protein spot 18), chlorophyll a-b binding protein 1, chloroplastic (i.e. protein spot 19) implies that these proteins have putative roles in cold-stress response and in maintaining normal or even high levels of chlorophyll biosynthesis.

Glutamine synthetase 2 (GS2) is expressed abundantly in leaf mesophyll cells; its major roles are to assimilate NH₃ produced by the reduction of NO₃⁻ and to reassimilate NH₃ released during photorespiration (58). Overexpression of GS2 in transgenic rice plants showed increased photorespiration capacity as well as increased tolerance to stress (59). Pyrophosphate, fructose-6-phosphate 1-phosphotransferase (PFPP), catalyzes the reversible interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate, a key step in regulating the metabolic flux toward glycolysis or gluconeogenesis, which plays a vital role in carbohydrate metabolism and other cellular processes. A previous study demonstrated that this enzyme was induced by cold stress in potatoes (60). ATP synthases are membrane-bound enzyme complexes/ion transporters that connect ATP synthesis and/or hydrolysis with the proton transport across a membrane. ATP-dependent synthases/proteases play essential roles in controlling the availability of short-lived regulatory proteins, as well as in removing abnormal or damaged proteins; these enzymes play a critical role in the removal of damaged proteins and in the fine control of some key cellular components, combining peptidase and chaperone activities (61). After short-term (3 days) and long-term (21 days) cold treatments in wheat, the plants showed increased ATP biosynthesis (62). Overexpression of the ATP synthase gene in the transgenic Arabidopsis caused an increased resistance to salt, drought, and cold stresses (63). In this study, plastid glutamine synthetase 2 (i.e. protein spots 3 and 27), which is involved in the biosynthesis of organic nitrogenous compounds (i.e. amino acids), was up-regulated in the cold-sensitive cultivar UC 1110 and CSPs. Additionally, the change in abundance of one enzyme involved in carbohydrate metabolism was observed: the up-regulation of pyrophosphate-fructose 6-phosphate1-phosphotransferase subunit beta (i.e. protein spot 15). Moreover, three of the candidate cold-responsive proteins were found to be involved in energy metabolism including ATP synthase CF1 beta subunit (i.e. protein spot 4), ATP synthase CF1 alpha subunit (chloroplast) (i.e. protein spots 13 and 14); all of which displayed up-regulation in the cold-sensitive cultivar UC 1110 and CSPs. The up-regulation of nitrogen metabolism, carbohydrate catabolism, and energy pathway-related proteins in the cold-sensitive cultivar UC 1110 and CSPs may contribute to stabilizing the cellular osmotic pressure, and was a result of the increasing demand for energy in plants exposed to cold stress.

An increase (14 spots) or a decrease (6 spots) in the abundance of protein spots corresponding to protein biosynthesis or degradation after exposure to cold treatment is co-existent. Under abiotic stresses, some proteins could be damaged, misfolded, inefficient or unnecessary, and needed to be de-
graded, e.g. Rubisco (i.e. protein spots 7, 10, and 11). Some proteins could be synthesized or induced to cope with the abiotic damages, e.g. glutamine synthetase (i.e. protein spots 3 and 27), ATP synthase (i.e. protein spots 4, 13, and 14), LHCB protein (i.e. protein spots 16 and 18). It is associated with an increase in the abundance of aminoacyl tRNA synthase complex-interacting (i.e. protein spot 17) and a decrease in abundance of the elongation factor Tu (i.e. protein spot 24).

Traditional 2-DE approaches have difficulty in separating membrane proteins (64), therefore, some membrane proteins related to cold stress may not be uncovered by the technique utilized in this study, and several of these proteins will be discussed. In order to withstand extracellular stimuli, plant cells alter the proteomic properties of the plasma membrane to adapt to stresses (65). The alterations of plasma membrane proteins during cold stress have been recognized as crucial adaptation mechanisms in response to low temperatures (66). Several of these cold-responsive plasma membrane proteins are: dehydration-inducible proteins, which may protect proteins and membranes against freeze-induced dehydration (66, 67); plant synaptotagmin-associated membrane resealing...

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**Fig. 4.** Transcriptional expression profiles of 20 candidate winter-responsive protein genes by qRT-PCR. A, Parental cultivar UC 1110 compared with parental cultivar PI 610750. B, CSP-1 compared with CTP-1.

**TABLE V**

| Plant            | α | β | γ   | δ   | Total |
|-----------------|---|---|-----|-----|-------|
| NS              | 23.35 (b) | 0 | 25.25 (b) | 3.80 (c) | 52.40 (b) |
| FS              | 29.56 (a) | 0 | 28.07 (a) | 5.82 (a) | 63.45 (a) |
| BSMV_0          | 28.90 (a) | 0 | 27.53 (a) | 4.79 (b) | 61.20 (a) |
| BSMV_{PAP6-like}| 18.74 (c) | 0 | 16.66 (c) | 3.01 (d) | 38.41 (c) |

*NS, nonstressed nonsilenced; FS, freeze-stressed nonsilenced; BSMV_0, freeze-stressed viral control plants; BSMV_{PAP6-like}, freeze-stressed silenced plants.*

*α, β, γ, and δ indicate α-, β-, γ-, and δ-tocopherol, respectively.*

*b 0 indicates that the compound was below detection.*

*Letters (a, b, c, and/or d) indicate statistical differences among NS, FS, BSMV_0, and BSMV_{PAP6-like} using one-way analysis of variance least significant difference analysis.*
protein, involved in plant freezing tolerance mechanism (68); clathrins and dynamin-related protein that are associated with functional changes of endocytosis activity and increased during cold acclimation (69). Furthermore, some identified plasma membrane proteins have been verified to act in cold tolerance. For example, overexpression of phospholipase D resulted in increased freezing tolerance, whereas the knockout resulted in decreased freezing tolerance (70). A lipocalin-like protein (temperature-induced lipocalin) is a plasma membrane protein that responds to cold treatment, and the overexpression of this protein results in higher survival rates at freezing temperatures (66, 71). Additionally, one quantitative trait locus COLD1, which encodes a regulator of G-protein signaling that localizes on the plasma membrane and endoplasmic reticulum, conferred chilling tolerance in japonica rice. Rice lines with a deficiency or down-regulation of COLD1 (jap) were sensitive to cold, whereas overexpression of COLD1 (jap) significantly enhanced chilling tolerance (72).

In this study, protein spots were identified with unknown functions possibly because of the absence of genomic sequence of this grass species until recently (29). Nevertheless, the corresponding proteins with the highest homology (the same conserved domains) were obtained by a BLAST search, and these homologs which may have similar functions. Among the 20 identified protein spots, the unnamed protein product (i.e. protein spot 20) shared the same conserved domains “PAP-fibrillin” with plastid-lipid-associated protein 6, chloroplastic (PAP6) in Brachypodium distachyon (Table III) (designated PAP6-like), which shared 94% sequence similarities with PAP6 in conserved domains, indicating similar functions between the two proteins. Plastid lipid-associated protein (PAP) is also known as fibrillin, or chromoplast-specific protein (42). Members of the PAP/fibrillin family have been known to be associated with plastoglobules, which is a dominant structural protein associated with carotenoids and other neutral lipids in plastids, is encoded by a single nuclear gene in several species, is present in leaves and other organs (47), and has been shown to be induced upon environment stress.

In 1994, fibrillin (molecular weight of ~32 kDa) was isolated from bell pepper fruit (73), renamed PAP (74), and was found to have high sequence similarity with the chloroplastic drought-induced stress protein CDSP 34 of potato (Solanum tuberosum) thylakoids (45). After several decades of study, PAPs were discovered to be a part of a large protein family present in photosynthetic organisms ranging from cyanobacteria to higher plants, and are involved in central processes of plant physiology, particularly plastid architectural development and plant responses to stress (46). Earlier studies have shown that high light, drought, low temperature, high salinity, oxidative stress and senescence induce the expression of CDSP34 in S. tuberosum (42–45). Mechanical wounding and drought induced the expression of PAP (FIB1) in pepper and tobacco leaves (75), whereas overexpression of pepper FIB1 in tobacco leads to the slightly faster development in response to stress (76). The FIB1 promoter has been demon-

![Fig. 5. qRT-PCR analysis of PAP6-like in response to different abiotic stresses. Four (A, B, C, and D) abiotic stresses: cold, salt, drought, and heat. Bars represent standard errors of triplicate experiments.](image-url)
strated to be activated in leaves of transgenic tobacco exposed to wounding, drought, or methyl viologen treatment (75). Additionally, photooxidative stress imposed by high light combined with cold stress reduced levels of (FIB1a, -1b, -2) in Arabidopsis, whereas the visible phenotype of FIB1–2 RNA interference lines included retarded shoot growth under stress (77). The increase in PAP/fibrillin expression indicates that, when stressed, leaves synthesize more PAP to modulate the activity of carotenoids in response to increased light reaction activity and photoprotection or to stabilize accumulated neutral lipids (47). Only recently were fibrillins documented in wheat (78), and the role of structural protein PAP6 from the PAP/fibrillin family is entirely unknown in wheat. In this study, an increased abundance of protein PAP6-like was observed in the cold-sensitive cultivar UC 1110 and CSPs compared with the cold-tolerant cultivar PI 610750 and CTPs, implying that this protein was induced under cold-stress conditions. The results at transcriptional level in this study showed an increase of the PAP6-like gene in cold and salt stresses, whereas, drought and high-temperature stresses decreased the expression of this gene. The different effects of stresses on PAP6-like expressions are not exact consistent with earlier findings, possibly because of different members of protein family, different species, growth conditions, and levels of stress applied.

**Fig. 6.** Relative expression of PAP6-like in differently treated wheat plants. Expression of PAP6-like in nonstressed nonsilenced (NS), freeze-stressed nonsilenced (FS), and silenced plants BSMV_PAP6-like at 14 dpi were calibrated to the mean level of expression of the gene in the BSMVv_treated plants. Bars represent standard errors of triplicate experiments.

**Fig. 7.** Phenotypes of the virus-infected wheat plants with BSMV RNA transcripts (A) and subjected to freezing stress at day 5 (B). A. From left to right, nonsilenced plant served as control, BSMVv, BSMV_PDS, BSMV_spotN°6, BSMV_spotN°21, and BSMV_PAP6-like plant compared with the control. B. Freeze-stressed BSMVv-inoculated plants served as positive control monitoring time course of VIGS. Nonstressed nonfreeze-stressed (−5 °C) plants, and freeze-stressed BSMV_spotN°6, BSMV_spotN°21, and BSMV_PAP6-like plants were included for comparison of phenotypes. Note the depressed vigour of plants silenced for PAP6-like compared with the viral control plants.
VIGS provides an alternative strategy for gene functional analysis through the simultaneous knockdown of expression of multiple related gene copies, which can overcome the inherent problems of polyploidy and limited transformation potential that hamper functional validation studies in wheat (37). Since this technology was first successfully used in bread wheat (34), VIGS has been widely used for the analysis of gene function in resistance against wheat pathogen and wheat aphid, as well as water stress (34, 35, 37, 48, 79). However, few studies have been performed in analyzing cold stress response genes by VIGS in wheat. In this study, in order to further evaluate the role of the novel gene PAP6-like, VIGS was utilized to functionally validate this candidate cold-responsive gene. The level of silencing (7.1-fold) achieved for the targeted PAP6-like genes was comparable to other VIGS wheat studies (34, 37, 48). The consistent phenotype across the silenced plants also neutralized the concern of the stress response to BSMV masking the effect of cold stress effects, suggesting that cold trait functional studies can be carried out using VIGS in bread wheat. Stress is associated with structural cell damage and reduced moisture content in plants (80) and these changes may lead to plant lodging or leaves wilting (81). The change in cell membrane permeability and the degree of cell destruction caused by the stresses can be reflected directly through the change in electrolyte permeability, which was measured by the rate of relative electrolyte leakage (82). Moreover, enhanced tocopherol accumulation also occurs in response to a variety of abiotic stresses including high light, drought, high salinity, and cold and may provide an additional line of protection from oxidative damage (38, 83–85). In this study, leaves from the freeze-stressed BSMV_{PAP6-like}-treated plants had distinctly more severe droop and wilt than the freeze-stressed viral controls. This phenotypic result was confirmed by quantitative analysis, which revealed markedly increased rates of relative electrolyte leakage, but decreased RWC and tocopherol accumulation in the freeze-stressed BSMV_{PAP6-like}-treated plants. These results indicate an important role of PAP6-like in conferring cold stress in wheat. Furthermore, the other two unnamed protein genes (i.e. spots N°6 and spot N°21) had the highest homology with protein plastid transcriptionally active 16 (chloroplastic), and rhodanese-like domain-containing protein 4 (chloroplastic) from Brachypodium distachyon (Table III), respectively, and these were also silenced by VIGS. However, silenced wheat plants did not show significant phenotypic differences when compared with BSMV_{0}-treated control plants under freezing stress. Previously, functional validation of three Arabidopsis potential drought stress responsive genes in wheat by VIGS was carried out, and the results indicated that one of the genes had no improvement over BSMV_{0}-inoculated plants under limited water conditions (37), suggesting that additional copies of the gene could exist in wheat. The DNA sequences of these other copies could potentially be different enough to cause ineffective silencing with the VIGS construct used, thus resulting in compensatory gene function by the remaining active genes. Alternatively, spots N°6 and spot N°21 might not play the key role in modulating cold response in wheat plants.

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§ To whom correspondence should be addressed: Agronomy College, Henan Agricultural University, 95 Wenhua Road, Zhengzhou 450002, China. Tel.: +86-371-63558537; E-mail: chf0088@163.com.

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