A Cold-regulated Nucleic Acid-binding Protein of Winter Wheat Shares a Domain with Bacterial Cold Shock Proteins*

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The molecular mechanisms of cold acclimation are still largely unknown; however, it has been established that overwintering plants such as winter wheat increases freeze tolerance during cold treatments. In prokaryotes, cold shock proteins are induced by temperature downshifts and have been proposed to function as RNA chaperones. A wheat cDNA encoding a putative nucleic acid-binding protein, WCSP1, was isolated and found to be homologous to the predominant CspA of Escherichia coli. The putative WCSP1 protein contains a three-domain structure consisting of an N-terminal cold shock domain with two internal conserved consensus RNA binding domains and an internal glycine-rich region, which is interspersed with three C-terminal CX₄CX₃HXC'C (CCHC) zinc fingers. Each domain has been described independently within several nucleotide-binding proteins. Northern and Western blot analyses showed that WCSP1 mRNA and protein levels steadily increased during cold acclimation, respectively. WCSP1 induction was cold-specific because neither abscisic acid treatment, drought, salinity, nor heat stress induced WCSP1 expression. Nucleotide binding assays determined that WCSP1 binds ssDNA, dsDNA, and RNA homopolymers. The capacity to bind dsDNA was nearly eliminated in a mutant protein lacking C-terminal zinc fingers. Structural and expression similarities to E. coli CspA suggest that WCSP1 may be involved in gene regulation during cold acclimation.

Low temperature is a major environmental limitation on plant geographical distribution and productivity. Many tropical and subtropical plants are less tolerant against low temperature and are easily damaged by chilling temperatures (1). In contrast, overwintering plants are capable of exhibiting high levels of cold tolerance, which is acquired through the process of cold acclimation (CA). The freezing tolerance of such plants increases substantially after a period of exposure to low, but nonfreezing temperature and/or a short photoperiod (2, 3). CA-regulated genes have been identified from numerous plant species including winter wheat (4–6), barley (7, 8), alfalfa (9–11), and Arabidopsis (12–14). Positive correlations of cold-induced genes and freezing tolerance have been observed (10, 15), and functions related to cold acclimation have been suggested for several genes (16, 17).

In prokaryotes, a similar acclimation process termed the “cold shock response” under low temperatures (18) and has been extensively characterized in Escherichia coli (19). CspA, the major cold shock protein of E. coli, accounts for more than 10% of total protein synthesis during the cold acclimation phase (20). The cspA gene has been cloned and sequenced, and primer extension studies have confirmed that cspA transcript levels also increase in response to cold shock (21). E. coli CspA binds to RNA to destabilize secondary structures; therefore, it was proposed that high levels of CspA could facilitate translation at low temperatures by eliminating secondary structures in mRNA (22). Such a function is critical for efficient translation of mRNAs at low temperatures and may also have an effect on transcription. In addition, recent reports revealed that CspA functions as a transcription anti-terminator and is responsible for the expression of a set of cold-responsive genes (23).

In this paper, we describe the isolation and characterization of a wheat CA-related gene, WCSP1, which encodes a putative protein with high sequence similarity with the bacterial Csp protein family and retroviral CCHC-type zinc finger proteins. We demonstrate that WCSP1 is a novel eukaryotic cold-regulated nucleic acid-binding protein capable of binding ssDNA, dsDNA, and RNA homopolymers. Collectively, the in vitro nucleotide binding functions, structural similarity to CspA, and responsiveness to low temperature suggest that WCSP1 may be involved in the regulation of CA-related genes.

** Experimental Procedures

** Plant Material—Winter wheat plants (Triticum aestivum L. cv. Chi-baka) were used as the source of plant tissue for all experiments. Prolonged temperature experiments for RNA and protein analyses utilized germinated seeds that were planted in commercial potting mix, irrigated with tap water, and grown in a growth chamber that was maintained under 22 °C/18 °C (16 h day/8 h night) cycles for 14 days. Cold acclimation was stimulated by transferring plants to 6 °C/2 °C (8 h day/16 h night) cycles in an environmentally controlled growth room. Plants were harvested prior to and after 1, 3, 6, 10, 14, and 18 days of cold treatment. Crown tissue was harvested, frozen immediately in liquid nitrogen, and stored at −80 °C until processed for RNA extraction as described below. For protein analysis, seedlings were shifted (21 days after germination) from non-acclimating conditions to 4 °C (constant) with 8 h day/16 h night photoperiods. Crown tissue was harvested prior to and 2, 5, 10, 20, and 47 days after transfer to cold.

** glutathione S-transferase; ssDNA, single-stranded DNA; GRP, glycine-rich proteins; RRM, RNA-recognition motif.
acclimating conditions and immediately plunged in liquid nitrogen. Samples were stored at −80 °C until processed for total protein extraction as described below.

All plants used to monitor the response to short term stress treatments were grown hydroponically. Surface-sterilized seeds were imbibed in the dark for 12 h and evenly distributed atop a plastic mesh for 21 h and every 3 days until full-grown seedlings were harvested. After growing for 7 days, wheat seedlings were subjected to environmental stress treatments. Low temperature, heat, ABA, salinity, and dehydration treatments were conducted by transferring mesh grids to separate containers with tap water (4 or 42 °C), 50 μM ABA or 300 mM NaCl, and no water, respectively. Root and shoot tissue was harvested at 4 °C until processed for total protein extraction.

Protein extraction and slot-blot analysis—Total protein was isolated from wheat root, shoot, and crown tissues using TRIzol reagent (Invitrogen). Samples were maintained at 200 mM NaCl, and no water, respectively. Root and shoot tissue was subjected to agarose gel electrophoresis (0.8% agarose) and were resolved with 50 volts for 60 min. DNA gel shifts were subsequently visualized by ethidium bromide staining. Total RNA was isolated from wheat root, shoot, and crown tissues using TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was separated in 1.0% formamide-agarose gels and subsequently transferred onto Hybond N+ nylon membranes (Amersham Biosciences) with a vacuum immunoblotter (ATTO; Tokyo, Japan). The membranes were subsequently UV-fixed and used for differential hybridization with total RNA, which was isolated from cold- and non-acclimated crown tissue (as described below). Double-stranded cDNA was synthesized from mRNA isolated from both cold- and non-acclimated wheat tissue using SMARTer RACE cDNA amplification kit (Clontech). Membranes were subsequently UV-fixed and used for blot hybridization. Hybridization and washes were performed according to standard protocols (24). Signals from arrayed DNA were quantified with a BAS1000 image analyzer (Fuji Film; Tokyo, Japan), and clones that interacted strongly with cold-acclimated RNA were subsequently sequenced as described below.

DNA Sequencing—The cloned DNA insert identified from library screening was completely sequenced using a Thermo Sequenase v2.0 kit (Amersham Biosciences) and a 373A DNA sequencer model (Applied Biosciences; San Jose, CA). DNA sequence analysis was performed with DNASIS software (Hitachi; Yokohama, Japan), and the sequence alignment was generated with CLUSTAL X (25). The phylogenetic tree was calculated by the Neighbor-Joining method and displayed using TreeView software (26).

RNA Blotting and Hybridization—Total RNA was isolated from wheat root, shoot, and crown tissues using TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was separated in 1.0% formamide-agarose gels and subsequently transferred onto Hybond N+ membrane (24). Rapid-Hyb buffer (Amersham Biosciences) containing salmon testes DNA (10 μg/ml) was used for both pre-hybridization and hybridization at 65 °C overnight. RNA blots were subsequently washed once with 2× SSC, 0.1% SDS for 15 min and twice with 0.1× SSC, 0.1% SDS for 20 min at 65 °C. Blots were exposed to Kodak BioMax MR x-ray film (Kodak; New Haven, CT) with an intensifying screen at −80 °C.

Polycyclonal Anti-peptide Antibody Production—Seventeen consecutive amino acids (CGFISPEDGSEDLFVHQS) were selected from within the RNP-1 and RNP-2 regions of WCSP1 (see Fig. 1A) and produced as a synthetic peptide (an N-terminal cysteine residue was added for affinity purification of immune serum). The peptide (CGFISPEDGSEDLFVHQS) was conjugated to keyhole limpet hemocyanin and injected into rabbits for the production of polyclonal antibodies. The peptide was coupled to Sepharose 4B via the N-terminal cysteine residue and used for affinity purification of immune serum. Peptide synthesis, polyclonal antibody production, and affinity purification were performed by Alpha Diagnostics International (San Antonio, TX).

Total Protein Extraction and Protein Blot Analysis—Harvested crown tissue (grown as described above) was ground with liquid nitrogen, and −200 mg was boiled for 5 min with SDS-extraction buffer and supplemented with 1× Complete Mini protease inhibitor mixture (Roche Molecular Biochemicals). Extracted total proteins were centrifuged at maximum speed for 10 min in a microcentrifuge. Five volumes of ice-cold acetone were added to the collected supernatant, and samples were maintained at −20 °C for 1 h. Precipitated proteins were resuspended in SDS-sample buffer and used for subsequent SDS-PAGE and Western blot analysis (15 μg/ml per lane). Anti-peptide primary antibodies and preimmune sera conjugated anti-rabbit secondary antibodies (Amersham Biosciences) were used (1:2,000) for detection of WCSP1-like translation product with the ECL system (Amersham Biosciences). Recombinant Protein Production and Purification—A full-length WCSP1 fusion protein construct (GST:WCSP1) was created by incorporating an in-frame N-terminal BamHI site and utilizing a pre-existing C-terminal Xho site for cloning into the pGEX6P-3 expression vector. A GeneEngineer in vitro site-directed mutagenesis kit was used (Promega; Madison, WI) for the creation of the N-terminal restriction site with a mutagenic primer (5′-GCCTCCGTTTTCGGGATCCAAGTGTTGAGAGG-3′). Plasmid DNA was digested with BamHI and XhoI and ligated into pGEX6P-3 vector (GST: TGA-132) lacking C-terminal zinc fingers was created by introducing a stop codon at amino acid position 132 with a mutagenic primer (5′-CCGGATGATGACAGTGCT-3′) and utilizing the cloned GST:WCSP1 construct as the template for mutation. Mutant constructs were confirmed by sequencing to confirm the deoxydeoxynuclease I site-directed chain termination reaction. E. coli BL21-DE3 (Novagen; Madison, WI) competent cells that were transformed with either GST:WCSP1, GST:TGA-132, or control pGEX6P-3 vector were grown in 2× YT media until the OD reached 0.6, and recombinant protein expression was induced by adding isopropyl-β-d-thiogalactopyranoside to a final concentration of 0.5 mM. Cultures were subsequently grown for 1 additional hour at 37 °C, after which cells were pelleted, resuspended, in 1× phosphate-buffered saline, and disrupted with sonication. Lysed samples were centrifuged at 12,000 × g for 10 min at 4 °C. Recombinant proteins were purified from the soluble fraction by batch purification with glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. Eluted GST, GST:WCSP1, and GST:TGA-132 fusion proteins were applied to Centricron YM-30 spin columns (Millipore; Bedford, MA), concentrated, and washed five times with an equal volume of gel filtration buffer (7.0 M ammonium sulfate). Protein concentration was estimated using the D_, protein assay (Bio-Rad). Protein samples were separated by SDS-PAGE (27) and stained with Coomassie Brilliant Blue dye for visualization of recombinant protein purity.

Nucleic Acid Binding Analysis—Gel retardation analysis was performed as described previously (28, 29), with minor modifications. 150 ng of either single-stranded (M13mp8) or double-stranded (M13mp8 RFII) DNA (Nippon Gene, Toyama, Japan) was incubated with GST fusion proteins (GST:WCSP1 or GST:TGA-132), which were added to binding reactions in amounts ranging from 0, 7, 70, 350, to 700 pmol. Purified GST was used as a negative control and incorporated as 700 pmol in binding reactions. Nucleotides and proteins were incubated in 15 μl of binding buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 4 mM MgCl2, and 50 mM sodium acetate) at either 0 °C or 37 °C on ice for 30 min. To test relative affinities of ssDNA and dsDNA binding, 350 pmol of GST:WCSP1 was added to assays and incubated with nucleotides (150 ng) in binding buffer with variable final concentrations of KCl (0, 50, 100, 200, and 400 mM). All binding reactions were subjected to agarose gel electrophoresis (0.8% agarose) and were resolved with 50 volts for 60 min. DNA gel shifts were subsequently visualized by ethidium bromide staining.

Ribohomopolymer RNA Binding Assay—Ribohomopolymer binding assays were performed as described previously (30, 31) with a modification in the scale of the binding reactions. 5 pmol of either GST:WCSP1, GST:TGA-132, or GST alone was added to 5 μl of ribohomopolymer-agarose beads (prepared in the same concentration as described previously, (32), in 20 μl of RHPA binding buffer (10 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100) with a final NaCl concentration of either 125 or 250 mM and 1 mg/ml heparin. Samples were incubated on ice for 10 min and subsequently washed three times in 1× RHPA buffer with final NaCl concentrations of either 125 or 250 mM. After the last wash, samples were dried in an Iwaki HVC-500 halogen vacuum concentrator (Tokyo, Japan) and resuspended in 100 μl of 1× SDS sample buffer. For each construct, 10% of input protein (0.5 pmol) was resuspended in 100 μl of 1× SDS sample buffer to serve as a control for the affinity of recombinant proteins to ribohomopolymer-agarose beads. 20 μl of boiled samples (input controls and recovered proteins) were utilized for SDS-PAGE electrophoresis and subsequent protein blot analyses with goat-anti-GST primary antibodies (1:2,000) and horseradish peroxidase-labeled donkey-anti-goat secondary antibodies (1:2,000). Protein blot analysis was performed with the ECL detection system.

RESULTS

Isolation of WCSP1-encoding cDNA—A cDNA clone that accumulates in cold-acclimated crown tissue was isolated by differential screening of a cDNA macro-array with cold- and non-acclimated radio-labeled total RNA. The clone contained a 690-bp open reading frame that encodes a putative 21.4-kDa translation product (WCSP1). The deduced amino acid sequence was high in glycine content (43%) and lacked any possible sorting sequences such as nuclear localization and secre-
tion signals (Fig. 1A). Interestingly, WCSP1 is characterized by multiple distinct functional domains (Fig. 1). The N-terminal CSD of WCSP1 is underlined, and the C_2X_C_2X_H_4X_C (CCHC) zinc finger motifs are boxed. The peptide region that was selected for the production of polyclonal antibodies is shaded by a gray box. The location for the single base pair site directed mutation (C → A) of a premature stop codon (TGA) is indicated at amino acid 132 as an overhead asterisk. The accession number for the full-length WCSP1 DNA sequence is AB066265. B, schematic representation of the multi-domain structure of the deduced amino acid sequence of WCSP1 and the truncated TGA-132 mutant protein, which lacks all three C-terminal zinc fingers. C, schematic representation of individual C-terminal zinc fingers in association with zinc atoms.

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ments of the GRP-2 proteins of Arabidopsis and N. sylvestris; however, they contain two CCHC-type zinc fingers interspersed within the glycine-rich regions (Fig. 2A). Striking homology was also found with the E. coli CspA cold shock protein family (Fig. 2A). The CSD has been identified in many RNA/DNA-binding proteins from both prokaryotes and eukaryotes (38), and CSD sequences of several CSD proteins including bacterial cold shock proteins have been compared. A phylogenetic tree revealed that WCSP1 belongs to the same group as the previously characterized GRPs of plants (Fig. 2B). In addition, there appears to be an additional group of CSD proteins in plants because the Arabidopsis genome sequence revealed two

**Fig. 2.** Comparison of the WCSP1 primary structure with E. coli CspA protein and glycine-rich proteins from plants. A, alignment of amino acid sequences for wheat WCSP1 (WCSP1), E. coli CspA (cspA), N. sylvestris GRP-2 (nsGRP2), A. thaliana GRP-2 (atGRP2), and A. thaliana GRP-2b (atGRP2b). Cold shock domains are boxed, and consensus RNA binding domains (RNP1 and RNP2) and CCHC zinc finger motifs are shaded in gray. Perfectly matched residues, conserved residues, and less conserved residues are indicated by an asterisk (*), a colon (:), and a period (.), respectively. B, phylogenetic tree of the CSD sequences from diverse organisms. The bar represents evolutionary distance, expressed in the number of substitutions per amino acid. Accession numbers of the CSD proteins are: E. coli CspA (P15277), CspB (P36995), CspG (Q71530), Lactococcus lactis CspB (CAA76695), Bacillus subtilis CsbB (P32081), Xenopus laevis YB3 (CA-A2778), Human YB-1 (I39382), C. elegans LIN-28 (AAC47476), WCSP1 (BA-B7535), AtGRP2 (AA24707), nsGRP2 (CA-A282), and A. thaliana (T05494, T00837).
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Additional clones, T05494 and T00837 (Fig. 2B).

**WCSP1 Transcript and Protein Levels during Cold Acclimation**—Northern blot analyses were performed to assess WCSP1 expression during a prolonged period of CA (Fig. 3A). WCSP1 transcript accumulation in crown tissue was observed at low levels before initiation of cold acclimation and WCSP1 expression steadily increased throughout the 18-day testing period (Fig. 3A). Short term cold treatment was used to refine the critical time period for WCSP1 induction in response to low temperature (Fig. 3B). Shoot and root samples from hydroponically cold-treated seedlings determined that WCSP1 mRNA induction occurs within hours subsequent to low temperature exposure. WCSP1 mRNA levels were slightly higher in roots than shoots, but their induction patterns were similar during the 24-h testing period (Fig. 3B).

Hydroponically grown seedlings were utilized to reveal the response of WCSP1 to multiple environmental stresses (Fig. 4). As compared with low temperature-induced expression patterns, no detectable cumulative induction pattern was found for WCSP1 transcript within the 24-h testing period for environmental stress applications (Fig. 4). Collectively, these data support the notion that WCSP1 may function in association to low temperature response and may act independently of other major environmental stresses.

For the investigation of WCSP1 protein levels in response to cold acclimation, we utilized an affinity-purified polyclonal antibody that was raised against an amino acid sequence within the RNA binding domain of WCPS1 (Fig. 1A). Western blot analysis of equally loaded total protein extracts revealed two protein bands of 27 and 25 kDa, which were detected with antiserum (Fig. 5). Both protein bands demonstrated a gradual increase in response to prolonged periods of cold acclimation.

**Functional Analysis of WCSP1**—Striking homology was found within the CSD and CCHC zinc finger motifs of WCSP1 (Fig. 2A) to the well characterized *E. coli* Cap family proteins (22) and retroviral transcription factors, both of which have been found to bind ssDNA and RNA. Because of the unique nature of multiple nucleotide binding domains in WCSP1, interspersed glycine-rich regions, and multiple CCHC-type zinc fingers, the capacity to bind dsDNA was tested also. Recombinant GST:WCSP1 and a mutant fusion protein (GST:TGA-132), which lacked C-terminal zinc fingers (Fig. 1B), were purified to near homogeneity. An empty pGEX6P-3 vector was used for the purification of GST and as a negative control in the DNA and RNA binding assays (Fig. 6).

DNA binding assays that utilized M13 mp8 phage DNA determined that WCSP1 is capable of binding both ssDNA and dsDNA (Fig. 7) with high affinity. Gel shifting was apparent when 7 pmol of purified GST:WCSP1 proteins was added to binding reactions. When the quantity of GST:WCSP1 was increased in individual binding reactions, successive retardation of nucleotide migration was observed with both ssDNA and dsDNA (Fig. 7A). Conversely, when 700 pmol of GST was added to the binding reaction as a negative control, neither ssDNA nor dsDNA shifted (Fig. 7, A and C). Relative affinity of nucleotide-protein complexes was assessed by incorporating a range of salt concentration into binding reactions. GST:WCSP1 fusion proteins maintained association to both ssDNA and dsDNA in KCl concentrations up to 400 mM. GST:TGA-132, which contained a premature stop codon immediately before C-terminal zinc fingers, was used to separately evaluate the contribution of the cold shock domain and CCHC zinc fingers for the affinity to ssDNA and dsDNA. When increasing amounts of GST:TGA-132 fusion proteins were added to ssDNA, nucleotide-protein complex retardation was shifted in a fashion similar to that of GST:WCSP1. However, the affinity of GST:TGA-132 fusion proteins to dsDNA was severely compromised (Fig. 7C).

Usage of ribohomopolymer-agarose beads determined that GST:WCSP1 and the truncated GST:TGA-132 are both capable of binding RNA. Both fusion proteins displayed a preference for binding poly(G) and poly(U) and were detected above 10% control inputs (Fig. 8). Alteration of salt concentration in binding and washing buffers did not compromise the fusion protein affinity for ribohomopolymers. GST, which was used as a negative control in binding reactions, failed to associate with RNA.

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**Fig. 3. Expression of WCSP1 during cold acclimation.** Northern blot analysis of total RNA from wheat crown tissue (A) and shoot and root tissue (B) demonstrated cold responsiveness of transcript under prolonged and short term low temperature exposure, respectively. rRNAs were visualized by staining with ethidium bromide to serve as equal loading controls. The environmental treatment of samples is described under “Experimental Procedures.”

**Fig. 4. WCSP1 expression during various stresses.** Time course expression of WCSP1 transcript in shoot and root tissues during short term salinity, drought, heat shock, and 50 μM ABA treatments, respectively, are shown. rRNAs were visualized by staining with ethidium bromide to serve as equal loading controls. Stress treatments are described in detail under “Experimental Procedures.”
Collectively, these functional analyses indicate that the cold shock domain of WCSP1 is likely critical for ssDNA/RNA binding affinity. Secondly, multiple C-terminal CCHC zinc fingers and flexible interspersed glycine-rich regions facilitate binding to dsDNA.

**DISCUSSION**

Winter wheat has the capacity to become highly freeze-tolerant during the cold acclimation phase; however, the molecular mechanisms of this process are still largely unknown. Because of the intricacies of CA, it is important to increase the number of identified CA-related genes/proteins in an attempt to eventually obtain a comprehensive understanding of CA. Here within, we describe a member of a novel class of plant cold acclimation-related genes, WCSP1, and an initial functional characterization of the gene product.

In *E. coli*, downshifts in growth temperature induce cellular adaptation to low temperature (18). Cold-induced proteins, including a ribosome-associated protein (CsdA), a ribonuclease (PNP), a recombination factor (RecA), and a nucleoid-associated protein (H-NS) as well as CspA family proteins have been identified and extensively characterized (18, 19). When comparing characterized cold-induced proteins between plants and *E. coli*, there are few similarities. This observation, however, is in contrast to the highly conserved heat shock protein family (39). As a result, it is of great interest to determine whether there is a common mechanism of cold acclimation exhibited by both bacteria and plants. The CspA protein family members are ~7 kDa and contain only a CSD. Eukaryotic homologues, however, contain an N-terminal CSD and additional carboxy-terminal domains such as basic/aromatic islands, Arg-Gly repeat motifs, and CCHC-type zinc fingers (40). *E. coli* CspA binds to ssDNA/RNA without apparent sequence specificity and has been proposed to function as an RNA chaperone that facilitates efficient translation at low temperature (22). Two internal highly conserved consensus RNA-binding motifs (RNP-1 and RNP-2) are critical for CSD nucleotide binding activity (41). A recent paper reported that the CspA protein family acts as transcription anti-terminators and regulates mRNA levels of cold-induced genes in the *metY-rpsO* operon (23).

Expression and protein analyses determined that WCSP1 mRNA and protein levels, respectively, steadily increased in crown tissue during cold acclimation. Accumulation of the WCSP1 transcript initiated within 10 h of cold treatment in shoots and roots of young seedlings (Fig. 3A). This contrasts those of *E. coli* CspA, whose induction by low temperature is more rapid and transient (21, 42). The expression of WCSP1 transcript was not induced by environmental stress treatments such as salinity, drought, or ABA, all of which have been independently determined to induce the expression of various cold acclimation-related genes (Fig. 4). This observation supports the notion that WCSP1 gene regulation is confined within a low temperature signaling pathway and that WCSP1 functions in association to CA in cold-tolerant winter wheat. Two protein bands (25 and 27 kDa) were detected with the polyclonal antibody, which indicates that wheat contains an additional immunologically related protein (Fig. 5B). Incomplete DNA sequence data available from wheat expressed sequence tags databases support this supposition, whereas wheat contains at least two additional WCSP1 homologues (data not available).

**FIG. 5.** Western blot analysis of cold-acclimated wheat total protein extracts. Total proteins from a time-coursed cold acclimation treatment were separated by SDS-PAGE (A), and a duplicate gel was electro-transferred to a nylon membrane for Western blot analysis (B). Arrows indicate two cold-regulated polypeptides (27 and 25 kDa), which were detected by the CSD peptide antibody.

**FIG. 6.** Purification of recombinant proteins expressed within *E. coli* cells. *E. coli* BL21(DE3) cells were transformed with either pGEX6P-3, pGEX6P-3:WCSP1, or pGEX6P-3:TGA-A-132 and induced with isopropyl-β-D-thiogalactopyranoside for recombinant protein purification. Soluble protein extracts (S), flow-through (F), and eluted purified recombinant proteins (E) are designated by corresponding abbreviations. Estimated molecular mass (kDa) for the migration of GST:WCSP1, GST: TGA-132, and GST recombinant proteins was 50.8, 43.0, and 28.0 respectively.
FIG. 7. Analysis of DNA binding activity of WCSP1 by gel shift assay. Purified GST:WCSP1 fusion proteins were incubated with either ssDNA or M13mp8 dsDNA RFI DNA (dsDNA) to analyze the effect of WCSP1 (A) or salt concentration (B) on the formation of nucleotide-protein complexes. A range of WCSP1 fusion proteins from 0 to 700 pmol was used for analyses, and purified GST (700 pmol) was loaded for a negative control (A). Final concentrations of 0–400 mM KCl were utilized within binding reactions to assess electrostatic effects of nucleotide-protein associations (B). A negative control with 400 mM KCl and nucleotides only (no WCSP1 fusion proteins) was loaded to serve as a negative control for the effect of KCl on DNA migration during agarose gel electrophoresis (B). Functional analysis for the contribution of multiple C-terminal zinc finger motifs (C) utilized a truncated WCSP1 fusion product (GST:TGA-132) (Fig. 1B). Purified recombinant GST:TGA-132 was incubated in increasing quantities (0–700 pmol) with either ssDNA or dsDNA and subsequently separated with agarose gel electrophoresis (C). Agarose gel-shift analyses determined that the mutant protein has the capacity to effectively bind ssDNA, whereas the previously observed wild-type affinity for dsDNA was nearly abolished.

FIG. 8. Analysis of RNA binding activity of WCSP1 with ribohomopolymer-Sepharose binding assay. GST:WCSP1, GST:TGA-132 fusion proteins, or GST alone were added to ribohomopolymer-agarose beads to assess the capacity for ribonucleotide binding. Western blot analysis of proteins association to agarose beads revealed that GST:WCSP1 and GST:TGA-132 displayed a preference for binding poly(G) and poly(U) ribohomopolymers. Under the least stringent conditions, GST did not bind to any ribohomopolymer.
shown). Although the accumulation patterns were not synchronized, both proteins displayed up-regulation during cold treatments. Observed molecular weights, calculated by comparison to molecular weight standards migration, were higher than predicted for both wheat total protein (Fig. 5) and recombinant protein extracts (Fig. 6). These data indicate that WCSP1 may undergo post-translation modification.

Although it was first identified within prokaryotic cold shock proteins, interpretations from phylogenetic analyses have suggested that the CSD is an ancient progenitor molecule that was present at the origin of single cell evolution (38). Unlike the RNA-recognition motif (RRM), the CSD is the most conserved nucleic acid binding domain (43) and is contained within many eu-eukaryotic RNA/DNA-binding proteins of diverse function (38). For example, human YB-1 binds to Y-box sequence CTGATTGGCCAA and was considered to function in transcriptional regulation (44, 45). A Xenopus X-box protein, FRGY2, is involved in transcriptional activation and mRNA masking in oocytes (45, 46). A CSD protein from Caenorhabditis elegans, LIN-28, is involved in the regulation of L2/L3 cell fate in larval development (47). Within plants, however, it is surprising that only a few reports have described the presence of the cold shock domain (36, 37). Furthermore, no studies have analyzed the involvement of these plant proteins to cold temperature stress. For the first time, the identification of WCSP1 as a cold acclimation-specific protein in winter wheat has correlated a common structure-function relationship between bacterial and plant CSD proteins to the process of cold adaptation.

In addition to the CSD, WCSP1 contains three C-terminal CCHC-type zinc fingers, which are interspersed by glycine-rich domains. This RNA-binding motif was identified previously in retroviral nucleocapsid proteins (33), TFIIIA (48), and more recently in plant proteins such as AtGRP2 of Arabidopsis (49), GRP2 (49), and RZ-1 (50) of N. sylvestris and AG5 of wheat (51). The CCHC motif has been described previously to bind solely RNA (48); however, deletion of three CCHC motifs and flexible glycine-rich linker regions in WCSP1 nearly eliminated binding to dsDNA (Fig. 7C). The glycine-rich domain is also found in many plant RNA-binding proteins (52). In contrast to other RNA-binding motifs, this domain does not contain fixed consensus sequences. Although it is not known whether the glycine-rich domain is directly involved in RNA binding, deletion of this domain resulted in a loss of poly(G)RNA binding activity (48); however, deletion of three CCHC motifs and flexible glycine-rich linker regions in WCSP1 severely inhibited dsDNA binding (Fig. 7C). These data indicate that the glycine-rich and CCHC domains are likely recruited for enhanced dsDNA binding activity.

Available information on WCSP1 may suggest a possible function of WCSP1. Because the CSD of WCSP1 is more similar to E. coli CspA family proteins than eu-eukaryotic Y-box proteins, and because WCSP1 contains no possible functional domains besides those for nucleic acid binding, the in vivo function of WCSP1 may be similar to that of CspA. It has been proposed that CspA acts as an RNA chaperone to facilitate efficient translation under low temperature by destabilizing secondary structures in mRNA (22). It was recently demonstrated that CspA functions as a transcription anti-terminator to regulate expression of metY-rpsO operon genes at the transcription level (23). It seems less possible that WCSP1 is involved in recovery from general translational arrest at low temperature because expression of WCSP1 was not induced immediately after temperature downshifts but was steadily induced during the cold acclimation process (Fig. 3A). However, it is still possible that WCSP1 is involved in the regulation of cold acclimation-associated genes at the levels of transcription or translation. Increasing numbers of reports are demonstrating the importance of RNA-binding proteins in the regulation of developmental and physiological processes such as flowering time (63), leaf development (64), meiosis (65), and circadian rhythms (66). Thus, it is of great interest to determine the function of WCSP1 in the regulation of the cold acclimation process.

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