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Research article

Development- and cold-regulated accumulation of cold shock domain proteins in wheat

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ABSTRACT

Cold shock domain (CSD) proteins, or Y-box proteins, are nucleic acid-binding proteins that are widely distributed from bacteria to higher plants and animals. Bacterial CSD proteins play an essential role in cold adaptation by destabilizing RNA secondary structures. WHEAT COLD SHOCK DOMAIN PROTEIN 1 (WCSP1) shares biochemical functions with bacterial CSD proteins and is possibly involved in cold adaptation. In this study, the temporal and spatial distribution of the wheat cold shock domain protein family (WCSPs) was serologically characterized with regard to plant development and cold adaptation. Four WCSP genes were identified through database analysis and were classified into three classes based on their molecular masses and protein domain structures. Class I (20 kD) and class II (23 kD) WCSPs demonstrated a clear pattern of accumulation in root and shoot meristematic tissues during vegetative growth. In response to cold, marked increases in WCSP levels were observed but the pattern of accumulation differed by tissue. Accumulation of WCSPs in crown tissue during cold acclimation was observed in the winter cultivar 'Chihokukomugi' but not in the spring cultivar 'Haruyutaka', suggesting a possible function for WCSPs in cold acclimation. During flower and seed development, protein levels of class I and class II WCSPs remained high. The class III WCSP (27 kD) was detected only during seed development. The highest level of class III WCSP accumulation was observed at the milky seed stage. Together, the results of this study provide a view of CSD protein accumulation throughout the life cycle of wheat and suggest that WCSPs function differentially in plant development and cold adaptation.

1. Introduction

Cold shock domain (CSD) or Y-box proteins are widely distributed in bacteria, plants and animals (Matsumoto and Wolffe, 1998; Wolffe, 1994). The CSD, which is the most conserved nucleic acid-binding domain and is capable of binding RNA, ssDNA and dsDNA (Graumann and Marahiel, 1998), contains two consensus RNA-binding motifs (RNP1 and RNP2) that facilitate nucleic acid recognition/binding. In *Escherichia coli* four of the nine CSD proteins, or cold shock proteins (CspA, CspB, CspG and CspI), are highly induced after cold shock treatment. CspA destabilizes RNA secondary structures, a function that is critical for efficient translation of mRNA at low temperatures (Jiang et al., 1997) and transcription anti-termination (Bae et al., 2002). Therefore, *E. coli* CSD proteins are referred to as RNA chaperones. In plants, a gene family encoding CSD proteins has been found in EST and genomic databases (Karlson and Imai, 2003; Sasaki and Imai, 2012), including four CSD proteins (AtCSP1, AtCSP2, AtCSP3, AtCSP4) that were identified in *Arabidopsis thaliana* (Karlson and Imai, 2003).

The *Arabidopsis* CSD proteins commonly destabilize RNA secondary structures and are involved in responses to cold (Kim et al., 2007, 2009; Sasaki et al., 2007). However, their functions in cold response are divergent. Whereas AtCSP3 positively regulates freezing tolerance (Kim et al., 2009), AtCSP2 negatively regulates the cold acclimation process (Sasaki et al., 2013), although both genes are induced by cold. In addition to their function in cold adaptation, it was recently proposed that plant CSD proteins play roles in development. The *AtCSP2* gene is highly expressed in meristematic and developing tissues (Sasaki et al., 2007; Fusaro et al., 2007; Nakaminami et al., 2009). Consistent with its expression patterns, functional analyses

Abbreviations: CSD, cold shock domain; DPA, days post anthesis; DPV, days post vernalization; CT, cold treatment.

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using RNAi knock-down transgenic plants indicated that AtCSP2 regulates flowering time, stamen number and seed development (Fusaro et al., 2007). In addition, overexpression of AtCSP2 affects flowering time and silique length (Sasaki et al., 2013).

Wheat (*Trtticum aestivum* L.) cold shock domain protein 1 (WCSP1), the first plant CSD protein to be functionally characterized (Karlson et al., 2002), is composed of an N-terminal CSD and a C-terminal glycine–rich domain containing three Cys–Cys–His–Cys (CCHC) zinc fingers. Both *WCSP1* mRNA and protein levels are up-regulated by cold in crown tissue. WCSP1 binds to ssDNA, dsDNA and RNA and unwinds double-stranded nucleic acids *in vitro* (Karlson et al., 2002; Nakaminami et al., 2005), as well as unwinding RNA secondary structures *in vivo* (Nakaminami et al., 2006). Expression of *WCSP1* in an *E. coli cspA*, *cspB cspE cspG* quadruple deletion mutant complements its cold-sensitive phenotype (Nakaminami et al., 2006). These findings demonstrate functional conservation of CSD proteins in bacteria and higher plants (Nakaminami et al., 2006).

In order to extend our understanding of wheat CSD proteins, the dynamics of WCSP accumulation during the life cycle of winter wheat was investigated. Our data show that there are three classes of WCSPs that are differentially accumulated in specific tissues and that their patterns of accumulation are associated with cold adaptation and seed development.

2. Methods

2.1. Plant material and growth conditions

The winter wheat (*Triticum aestivum* L.) cultivar 'Chihokukomugi' was utilized for most of the experiments. The spring cultivar 'Haruyutaka' was utilized to test differential accumulation in response to cold. Seeds were surface-sterilized with 70% ethanol for 5 min and 1% sodium hypochlorite for 10 min. Seeds were then washed thoroughly with water to remove excess sodium hypochlorite. The sterilized seeds were placed on a paper towel soaked with distilled water and were subsequently maintained at 20 °C in a plastic container. Radicles and coleoptiles were collected three days post germination. For sampling of young seedlings, germinated seeds were placed on a plastic mesh grid with a mesh size of 3 × 3 mm. The mesh grid was placed in a container with tap water and plants were grown under 16 h light/8 h dark at 22/18 °C for up to 7 d. Seven-day-old plants were used for the sampling of roots, shoot apices and leaf tissues. Roots were washed with tap water and divided into three parts: tip (1 cm from the tip), base (1 cm from the base), and middle (rest of the tissue, 2–3 cm).

Long-term cold treatment for cold acclimation and vernalization was applied to soil-grown three-week-old plants. Before cold acclimation, the plants were grown in a chamber operated under a 22/18 °C, 16 h light/8 h dark regime. Cold treatment at 4/2 °C, 10 h light/14 h dark was applied to the plants. Tissue samples from root parts (tip, middle and upper), crowns and leaves were harvested after 0, 7, 14, 21, 28 and 35 d of cold treatment. Following 35 d of cold treatment (vernalization), the plants were transferred to normal temperature conditions (22/18 °C, 16 h light/8 h dark) in order to induce the reproductive stage. Sampling of crown tissue including shoot apical meristems was conducted at 0, 5, 12, 17, 22, 33 and 40 days post-vernalization. The developmental phase of the meristem was determined according to Prasil et al. (2004). Developing seeds were harvested at 3, 7, 15, 20 and 34 DPA.

2.2. Immunoblot analyses

Total proteins were extracted from collected tissues using sodium phosphate buffer (50 mM, pH 7.5) supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF) (Christova et al., 2006). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). Samples (10 μg protein per slot) were separated by 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked for 1 h at room temperature in 1 × PBS buffer supplemented with 5% dry milk and 0.1% Tween 20. After washing in 1 × PBST buffer, the membrane was incubated with polyclonal antibody (1: 10000) for 1 h at room temperature. The peptide antibody utilized (Karlson et al., 2002) was produced against highly conserved RNA-binding motifs of WCSP1 and has been used to detect CSD proteins from different plant species (Sasaki et al., 2007; Chaikam and Karson, 2008). After washing with 1 × PBST buffer, the membranes were incubated with anti-rabbit IgG peroxidase-linked secondary antibody (1: 10000 v/v, GE Healthcare). Chemiluminescent detection of the signal was carried out using an ECL kit (GE Healthcare) according to the manufacturer's instructions.

To detect dehydrin proteins, membranes were incubated with a rabbit anti-plant dehydrin polyclonal antibody (1: 5000, Stressgen Bioreagents) prepared against a synthetic peptide containing the conserved sequence EKKGIMDKIKELPG (Close, 1996). The subsequent steps were the same as the protocol for WCSP detection.

Gels were stained with coomassie brilliant blue after transfer in order to confirm equal loading (Supplemental Figs. 1–4).

3. Results

3.1. The WCSP proteins in wheat

To elucidate the functions of CSD proteins in wheat, we set out to identify and characterize the entire WCSP family in wheat. Two WCSP1-like protein sequences (AB161682 and AB161683) were registered in Genbank as WCSP2 and WCSP3, respectively. A search of the expressed full-length cDNA database at the KOMUGI website (http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp) identified another WCSP1-like protein (tplb0005l05) that was distinct from the other WCSP1-family proteins previously identified. Accordingly, the sequence was named WCSP4. An alignment of the four WCSP sequences is shown in Fig. 1A. The deduced molecular masses of the proteins encoded by *WCSP1*, *WCSP2*, *WCSP3* and *WCSP4* are 21.4, 19.2, 21.5 and 24.5 kD, respectively. The four WCSPs all consist of an N-terminal CSD and a C-terminal Gly-rich/CCHC zinc finger region. The amino acid sequences are highly conserved within the CSD (Fig 1A). Based on their molecular masses and the number of CCHC zinc finger repeats, it appears that there are three subgroups of WCSPs in wheat (Fig. 1B).

3.2. WCSP accumulation during germination and vegetative growth in winter wheat

We utilized peptide antibodies against a highly conserved motif within the CSD (Karlson et al., 2002) to monitor accumulation of the WCSP family proteins during growth and development in winter wheat. In dry seeds, three bands corresponding to 20-, 23- and 27-kD polypeptides were detected (Fig. 2). In our analyses of recombinant proteins, WCSP1 produced in *E. coli* and transgenic rice migrated as a 23 kD protein in SDS-PAGE (data not shown). Based on molecular masses deduced from the DNA sequences, it was therefore assumed that the 20-kD and 27-kD bands corresponded to WCSP2 and WCSP4, respectively, and that WCSP3 migrated together with WCSP1 at 23 kD. The reason for the discrepancy between the calculated and apparent molecular masses on SDS-PAGE can be attributed to the highly biased amino acid composition of the proteins due to the Gly-rich region, as reported for LEA-like proteins (Sarhan et al., 1997). Hereafter, we