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Arabidopsis COLD SHOCK DOMAIN PROTEIN2 is a RNA chaperone that is regulated by cold and developmental signals

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Abstract

Bacterial cold shock proteins (CSPs) are RNA chaperones that unwind RNA secondary structures. *Arabidopsis* COLD SHOCK DOMAIN PROTEIN2 (AtCSP2) contains a domain that is shared with bacterial CSPs. Here we showed that AtCSP2 binds to RNA and unwinds nucleic acid duplex. Heterologous expression of *AtCSP2* complemented cold sensitivity of an *Escherichia coli csp* quadruple mutant, indicating that AtCSP2 function as a RNA chaperone in *E. coli*. AtCSP2 mRNA and protein levels increased during cold acclimation, but the protein accumulation was most prominent after 10 days of cold treatment. AtCSP2 promoter::GUS transgenic plants revealed that AtCSP2 is expressed only in root and shoot apical regions during vegetative growth but is expressed in reproductive organs such as pollens, ovules and embryos. These data indicated that AtCSP2 is involved in developmental processes as well as cold adaptation. Localization of AtCSP2::GFP in nucleolus and cytoplasm suggested different nuclear and cytosolic RNA targets.

Keywords: *Arabidopsis*; Cold acclimation; Cold shock domain; RNA chaperone

Cold shock domain (CSD) is a nucleic acid-binding domain that is widely distributed in bacteria, plants, and animals [1,2]. In *Escherichia coli*, four out of the nine CSD proteins (CspA, CspB, CspG and CspI) are highly induced after cold shock and are involved in cold adaptation. A quadruple mutation in *cspA*, *cspB*, *cspE,* and *cspG* resulted in growth defect at low temperature [3,4]. *E. coli* CspA destabilizes RNA secondary structures, a function which is critical for efficient translation of mRNA at low temperatures [5] and transcription anti-termination [6].

In plants, a gene family of CSD proteins has been found in EST and genome databases [7]. We have isolated and characterized a CSD protein (WCSP1) from wheat. *WCSP1* mRNA is up-regulated by cold and the corresponding protein accumulated to high levels during cold acclimation. WCSP1 binds to ssDNA, dsDNA, and RNA [8,9]. Constitutive expression of *WCSP1* in the *E. coli cspA*, *cspB*, *cspE*, *cspG* quadruple deletion mutant complemented its cold sensitive phenotype. Transcription anti-termination activity was demonstrated for WCSP1 using an *E. coli* strain that has a hairpin loop upstream of a chloramphenicol resistance gene [10]. In addition, *in vitro* melting assays clearly demonstrated that WCSP1 unwinds dsDNA [10]. These data suggested that WCSP1 shares a function with *E. coli* CSPs during the process of cold adaptation.

In *Arabidopsis,* four CSD proteins were identified in the genome sequence [7]. One of the genes, AtCSP2/AtGRP2 (At4g38680) encodes a 19-kDa protein that is comprised of a N-terminal CSD and a C-terminal glycine-rich/CCHC zinc finger (GR/ZnF) domain containing two ZnF motifs (Fig 1A). *AtCSP2* mRNA is induced during cold acclimation [7], however, detail biochemical and biological functions of AtCSP2 are yet to be determined.

In this paper, we characterized biochemical activity of AtCSP2 using *in vivo* and *in vitro* systems and demonstrated that AtCSP2 functions in destabilizing

Abbreviations: CSD, cold shock domain; GR, glycine rich region; ZnF, zinc finger.

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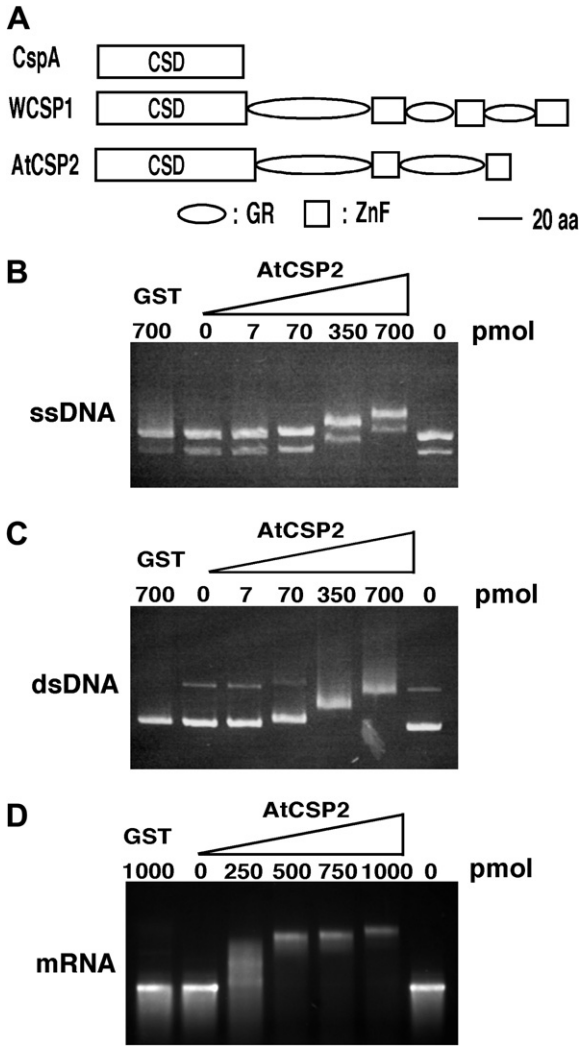

Fig. 1. Nucleic acid binding activity of AtCSP2 revealed by gel shift assay. (A) Structural features of CSD proteins (*E. coli* CspA, wheat WCSP1 and Arabidopsis AtCSP2). Recombinant AtCSP2 protein was incubated with either ssDNA (B), dsDNA (C), or *in vitro*-transcribed luciferase mRNA (D) and separated by agarosegel electrophoresis. Gel shifts were subsequently visualized by EtBr staining.

double-stranded nucleic acids and is regulated developmentally and in response to cold.

Materials and methods

Plant materials, growth conditions, and treatments. *Arabidopsis thaliana* Col-0 was used in this study. A T-DNA insertion knockout mutant of *AtCSP4* (*atcsp4-1*) was obtained from GABI-KAT (BX657199). Prior to germination, seeds sown on the Murashige and Skoog (MS) medium were left for 2 days at 4 °C for stratification. Plants were grown at 22 °C under continuous illumination. Low temperature treatments were performed by transferring seedlings to a growth chamber set to 4 °C under continuous illumination.

Recombinant protein production and purification. A full-length *AtCSP2* cDNA was amplified with 5′-CCAGGATCCATGAGCGGAGAC-3′ and 5′-GCGCTCGAGTTAACGTCCACC-3′ (underlines denote restriction sites) and cloned into the BamHI–XhoI site of pGEX6P-3 (GE Healthcare) to produce a gluthathione *S*-transferase (GST)-fused protein (pGEX-AtCSP2). Construction of pGEX-WCSP1 and pGEX-CspA was described previously [7,10]. *E. coli* cells containing pGEX-AtCSP2, pGEX-WCSP1, pGEX-CspA, and pGEX6P-3 were cultured in 2 × YT until OD_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was subsequently added to a final concentration of 0.5 mM and recombinant proteins were induced for 2 h. The bacterial cultures were centrifuged and the pellets were disrupted with sonication. The cell lysate was centrifuged and the total soluble fraction (supernatant) was affinity purified with a glutathione–Sepharose 4B column (GE Healthcare). Subsequent to washing, bound GST-fused protein was digestion with PreScission protease (GE Healthcare) according to the manufacture's instructions. The resulting recombinant proteins without GST tag were washed and concentrated with buffer (10 mM Tris–HCl, pH 7.5).

Nucleic acid binding analysis. Gel shift assay with ds/ssDNA substrates was performed as previously described [8]. Single-stranded (M13mp18) DNA (of 0.1 μg) was incubated with AtCSP2 or GST in 15 μl of binding buffer (10 mM Tris–HCl, pH 7.5) and was maintained on ice for 15 min. The samples were then separated on a 1% agarose gel and stained with ethidium bromide for visualization of gel shifts. Gel shift with mRNA substrates was performed as previously described [9]. Luciferase mRNA was *in vitro* transcribed with the RiboMAX kit (Promega).

In vitro DNA-melting assay. *In vitro* DNA-melting assay was similarly performed as described [10]. Two partially complementing oligonucleotides labeled with FITC and BHQ1 (black hole quencher), respectively, were mixed and denatured at 95 °C for 1 min, which was followed by incubation on ice for annealing. Annealed substrates were incubated for 2 h on ice with GST, AtCSP2, WCSP1 or CspA proteins, respectively. Fluorescence measurements were visualized on an LAS-3000 image analyzer (Fujifilm, Japan). Fluorescence intensity was calculated with Image Gauge software (Fujifilm, Japan).

Bacterial complementation. Complete ORF of *AtCSP2* was cloned into the pINIII expression vector by adding an in-frame N-terminal NdeI site with the primer 5′-GAGATTCATATGATGAGCGGA-3′ and a C-terminal BamHI site by using 5′-CCGGCGGGATCCTTAACGTCC-3′. Construction of pINIII-WCSP1 and pINIII-CspA was described previously [10]. These pINIII constructs were transformed into *E. coli* BX04 (Δ*cspA*, Δ*cspB*, Δ*cspE*, Δ*cspG*) cells. BX04 cultures were grown in liquid medium and spotted onto LB-ampiciline plates and incubated at either 37 °C or 17 °C.

RT-PCR analysis. Total RNA was extracted from plant tissues using RNeasy Plant Mini Kit (Qiagen) and subsequently used for first-strand cDNA synthesis with Gene Amp RNA PCR core kit (Applied Biosystems). PCR amplifications were performed with Expand High Fidelity PCR System (Roche), using gene-specific primers for *AtCSP2*: 5′-GTGGAGAAGAGTGAGTTG-3′ and 5′-CGATGATCTCTCAAGATTTAAG-3′. β-tubulin was performed with 5′-CGTGGATCACAGCAATACAGAGCC-3′ and 5′-CCTCCTGCACTTCCACTTCGTCTTC-3′.

Western blot analysis. Plant tissues were homogenized and extracted with a protein extraction buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 2% β-mercaptoethanol). The extracts were cleared by centrifugation at 13,000*g* for 15 min. After centrifugation, the supernatant was collected and protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad). Extracted total protein (15 μg) was separated on SDS–PAGE and transferred onto Hybond-C extra membrane (GE Healthcare). The membranes were hybridized with custom rabbit polyclonal antibodies against cold shock domain of WCSP1 (1:5000 v/v, Hokudo, Hokkaido, Japan) and anti-rabbit IgG peroxidase-linked secondary antibodies (1:10,000 v/v; GE Healthcare). Chemiluminescent detection of the signal was carried out using the ECL kit (GE Healthcare) according to the manufacturer's instructions.

Analysis of AtCSP2 promoter-GUS expression. The promoter region (1 kb upstream from the initiation codon) of *AtCSP2* was PCR-amplified with 5′-ACATGCCTGCAGTTCGAATTATAA-3′ and 5′-GTCTCC GGATCCTTTGATTGGAAT-3' primers. The amplified products were cloned into the PstI–BamHI-digested pBI121 vector (Clontech) to create the *AtCSP2* promoter-GUS construct (ProCSP2::GUS). *Agrobacterium tumefaciens* strain GV3101 harboring ProCSP2::GUS was used to transform *Arabidopsis* Col-0 plants according to the floral dip method [11]. To measure β-glucuronidase (GUS) activity, tissues from transgenic plants