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Gene Note

Nuclear localization of sweet cherry DREB1/CBF ortholog (CIG) and low temperature-inducible activity of *CIG* promoter

Hiroyasu Kitashiba[1,a,*], Yusuke Ban[2], Chikako Honda[1], Takaya Moriguchi[1,2]

[1] National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan; [2] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
*E-mail: kite@bios.tohoku.ac.jp Tel: +81-22-717-8651 Fax: +81-22-717-8654

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Abstract The fluorescent signal of GFP fused to CIG-B, which is one of the three *DREB1*/*CBF* (dehydration responsive element binding protein 1/C-repeat binding factor) homologs isolated from sweet cherry, was observed only in nucleus. The expression of *GFP* driven by *CIG-B* promoter was up-regulated by low-temperature. These results suggested that nuclear localization signal (NLS) motif in the N-terminal region of CIG-B protein and inducer of CBF expression (ICE)-like motifs in *CIG-B* promoter could be responsible for nuclear targeting and low temperature perception, respectively.

Key words: DREB/CBF, green fluorescence protein, nuclear localization signal, sweet cherry.

Plant growth and seed production are greatly affected by environmental factors. Recent climate-related events suggest that the area of land that is unsuitable for agriculture may increase due to the prevalence of undesirable environmental factors such as drought, salinity and extreme temperatures unfavorable for plant growth. In recent decades, considerable advances in improving tolerance of plants to environmental stresses have been achieved through genetic transformation. Thus, the isolation and characterization of genes that are involved in environmental stress tolerance are becoming increasingly important for future crop production.

The C-repeat dehydration responsive element (CRT/DRE) is a plant *cis*-acting DNA element that stimulates gene transcription in response to low temperature and drought stress. Yamaguchi-Shinozaki and Shinozaki (1994) first demonstrated that CRT/DRE plays an important role in the responses of plants to dehydration and low temperature. Subsequently, transcriptional activators that bind to the CRT/DRE were identified and designated as DREB1A/CBF3 (dehydration responsive element binding protein 1A/C-repeat/DRE binding factor 3), DREB1B/CBF1, and DREB1C/CBF2, which are actually induced by cold stress (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). To date, *DREB1*/*CBF* ortholog genes have been characterized in many plants such as rice, maize, *Brassica napus* and grape (Jaglo et al. 2001; Dubouzet et al. 2003; Qin et al. 2004; Xiao et al. 2006).

Constitutive overexpression of the *DREB1*/*CBF* genes in transgenic *Arabidopsis* plants resulted in a higher tolerance to multiple stresses including cold, salt and drought (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). In addition, similar results were observed in transgenic *Brassica napus* (Jaglo et al. 2001) and maize (Qin et al. 2004) by the overexpression of their respective *DREB1*/*CBF* ortholog genes. We have previously reported on the identification of three *DREB1*/*CBF* homologs (designated as *CIG-A*, *-B* and *-C*) from sweet cherry (Kitashiba et al. 2002, 2003), and shown increased salt and freezing tolerance in transgenic *Arabidopsis* by overexpression of *CIG-B* (Kitashiba et al. 2004). In addition, nuclear localization signal (NLS) motif and some characteristic sequences such as Myb-/Myc-recoginition sites and G-box (E-box/ABRE-like motif) were found in the N-terminal region of CIG-B protein and in the 5′-upstream region of *CIG-B* (Kitashiba et al. 2002). In this paper, we investigated the localization of CIG-B in onion cells to elucidate the possible function of the NLS motif. Furthermore, we also analyzed the response of the *CIG-B* promoter to cold using the *GFP* gene as a reporter.

Firstly, to examine the possible function of NLS motif in the *CIG-B* protein, a *CIG-B* DNA fragment (CIGB390; from +1 to +390), which included the NLS motif and ERF/AP2 domain (Figure 1A), was amplified

Abbreviations: CBF, C-repeat binding factor; DREB1, dehydration responsive element binding protein 1; ICE, inducer of CBF expression; GFP, green fluorescence protein; NLS, nuclear localization signal; DIG, digoxigenin-dUTP

[a] Present address: Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981–8555, Japan

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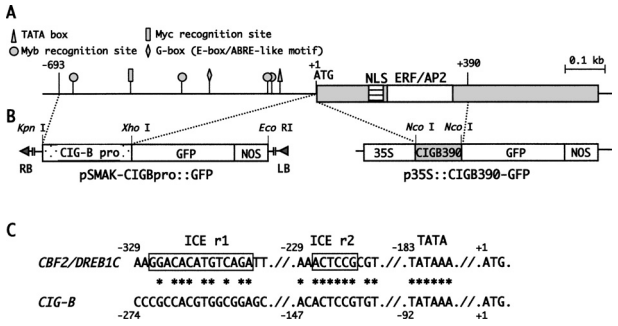

Figure 1. Gene structure. (A) Schematic structure of the *CIG-B* genome. Box region represents ORF including NLS motif (stripe) and ERF/AP2 DNA binding domain (white). (B) Constructs for transformation of tobacco (pSMAK-CIGBpro::GFP) and particle bombardment assay (p35S::CIGB390-GFP). 35S, CaMV 35S promoter; LB, left border of T-DNA; RB, right border of T-DNA; GFP, green fluorescence protein (C) Comparison of the 5′-upstream sequences between *CBF2*/*DREB1C* and *CIG-B*. Regions designated ICEr1 and ICEr2 are boxed. Stars represent identical sequences.

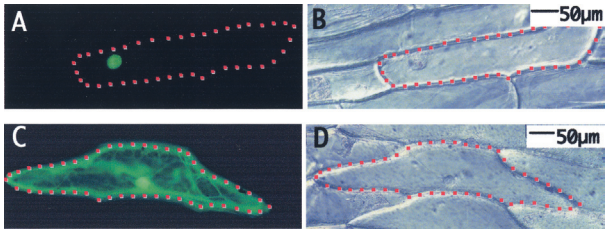

Figure 2. Localization of the GFP protein. Fluorescent microscopy analysis of onion epidermal cells transformed with the p35S::CIGB390-GFP plasmid (A) or a control p35S::GFP plasmid (C). The bright field images are shown in (B) and (D) corresponding to cells in (A) and (C), respectively.

by PCR with a set of primers containing the *Nco* I recognition site at the 5′-ends. After digestion with *Nco* I, the resulting DNA fragments (CIGB390) were ligated into a *Nco I* site located between the *CaMV35S* promoter and the engineered *GFP* gene (Chiu et al. 1996), *sGFP*(S65T) which was kindly provided by Dr. Niwa in Shizuoka University, resulting in a plasmid carrying the construct p35S::CIGB390-GFP (Figure 1B). Negative controls consisted on the *sGFP*(S65T) plasmid without the CIGB390 fragment. For transient assays, onion epidermal cell layers were peeled and placed on MS basal medium. The p35S::CIGB390-GFP plasmid DNA was introduced into the onion cells with 1.1 μm gold microcarriers using a helium-driven PDS-1000 particle delivery system (Bio-Rad) operating with a vacuum of 28 inch Hg, a helium pressure of 1100 psi and 6 cm target distance. After bombardment, tissues were incubated for one day at 22°C. Onion cells were observed with incident light fluorescence microscopy (Zeiss Axioskop) using a UV filter and blue filter combination (Okada and Toriyama 2001).

Fluorescence of the CIGB390-GFP was detected only in the nucleus (Figure 2A), while the fluorescent signal in the negative control cells was detected not only in the cytosol but also in the nucleus (Figure 2C). These results point at the function of the NLS motif of the CIG-B protein in nuclear localization. Recently, it was reported that GFP fused to the grape-DREB1/CBF or to the *Arabidopsis*-DREB2A, both which contain NLS motifs in the N-terminal regions, were also localized only in the nucleus (Sakuma et al. 2006; Xiao et al. 2006). Therefore, CIG-B may play an important role as a transcription factor in the nucleus of the plants, including sweet cherry.

Furthermore, we examined the response of the *CIG-B* promoter to low temperature in transgenic tobacco plants. A DNA fragment from −693 to −1 in relation to the putative translation start, which contained typical promoter elements such as a TATA-box, Myb-/Myc-recognition sites and G-box (E-box/ABRE-like motif) (Figure 1 and Kitashiba et al. 2002), was re-amplified using a set of primers containing either an *Kpn* I or a *Xho* I site at the 5′-ends (Figure 1B). The amplified DNA fragments were digested with *Kpn* I and *Xho* I, and then ligated into the 5′ upstream of *sGFP(S65T)* gene of pBlue-sGFP(S65T)-NOS KS plasmid (Chiu et al. 1996) which was kindly provided by Y. Niwa, Shizuoka University. The *Kpn* I-*Eco* RI fragment of CIG-B promter::sGFP::NOS was excised and ligated into the cassette Ti-vector pSMAK312-Blue (kindly provided by H. Ichikawa, National Institute of Agrobiological Sciences). The resultant construct (pSMAK-CIGBpro::GFP; Figure 1B) and the pSMAK312-Blue (as a negative control) were introduced into *Agrobacterium tumefaciens* strain LBA4404. These recombinant strains were used to transform *Nicotiana tabacum* cv. Petit Havana SR1 according to the leaf disk method (Horsch et al. 1989). Transgenic plants were selected based on kanamycin resistance. Three CIGBpro::GFP T_0 lines (#4, 10 and 11) were selected and propagated on MS-HF medium under illumination of ~2500 lux in a 16 h light/8 h dark cycle at 25°C. To confirm the presence of the transgene in these transgenic lines, DNA gel blot analysis was carried out using a 435 bp fragment between 152nd bp and 587th bp in *GFP* gene as a probe, which was synthesized by PCR using PCR DIG Probe Synthesis Kit (Roche Diagnostics). One (#11) or two copies (#4 and 10) of *GFP* were observed in CIGBpro::GFP transgenic lines (Figure 3A). No signal was detected in negative control plants (#6; Figure 3A). The CIGBpro::GFP transgenic lines were kept at 25°C until they were transferred to 4°C in the Constant Low Temperature Chamber (model THD101PA, ADVANTEC) for cold treatment where they were kept for 3 h. After treatment, the plants were harvested and total RNA was extracted using ISOGEN (NipponGene) according to the manufacture's instructions, and expression analysis was performed by RNA gel blot

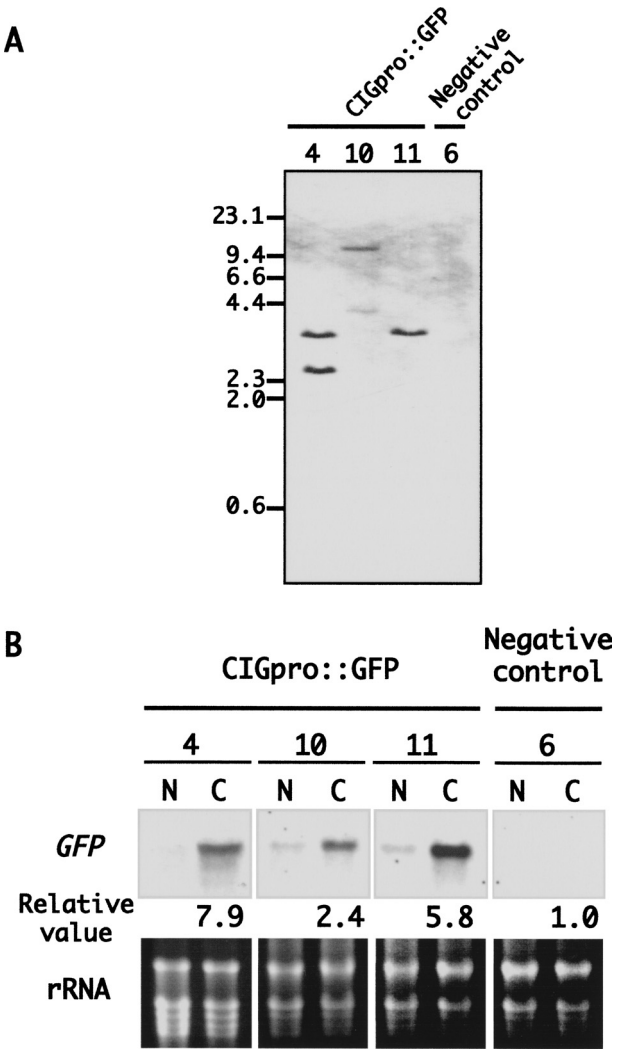

Figure 3. Analyses of transformants. (A) DNA gel blot analysis of transformants (#4, 10 and 11) with pSMAK-CIGBpro::GFP and negative control transgenic plant (#6) with pSMAK312Blue. Five μg of genomic DNAs were digested with *Eco* RI and then were electrophoresed in 1.0% (w/v) agarose gel and transferred to a Hybond N membrane (Amersham Biosciences) by blotting with 10× SSC. The *GFP* probe between 152nd bp and 587th bp was synthesized by PCR using PCR DIG Probe Synthesis Kit (Roche Diagnostics). Hybridization, washing, and detection were performed using the DIG DNA Labeling and Detection Kit (Roche Diagnostics). (B) RNA gel blot analysis in CIGBpro::GFP transformants and negative control plants which were grown under normal conditions (N, i.e. 25°C) or exposed to 4°C for 3 h (C). Relative values of expression level were normalized to the ribosomal RNA (rRNA) level by densitometric analysis; the hybridization signal of each plant under normal condition was set to 1.0 and the signal under low temperature was quantified. For RNA gel blot analysis, 10 μg of total RNA were electrophoresed on a 1% agarose gel and then transferred to a Hybond N membrane (Amersham Biosciences) by blotting with 20× SSC. Hybridization, washing, and detection were performed with the same method as in DNA gel blot analysis.

analysis with the same *GFP* probe as in DNA gel blot analysis described above. *GFP* gene expressions in the CIGBpro::GFP lines were obviously up-regulated by 2.4 to 7.9 folds in response to low temperature compared with those maintained at 25°C (Figure 3B). In contrast, no expression was observed in negative control line (Figure 3B). No correlation was observed between the copy number of the transgene and the relative expression ratios of the *GFP* gene. This result show that the DNA fragment (−693 to −1 bp) upstream from the putative translation start possesses the promoter region of *CIG-B* and suggests its role in responding to low temperature. Similar role was proposed for the promoters of DREB1/CBF genes (Shinwari et al. 1998; Zarka et al. 2003). In order to examine the low temperature-inducible activity of *CIG* promoter visually through GFP fluorescent, the transgenic plants were exposed to low 4°C for 3 hr. Unfortunately, however, no differences in the accumulation of GFP fluorescence were observed between CIGBpro::GFP and negative control plants. Although the reason for that still remains unclear, it is possible that the duration of cold treatment was not long enough to allow GFP driven by the *CIG-B* promoter to accumulate. We then tried to explain how the promoter region of *CIG-B* can respond to low-temperature. Zarka et al. (2003) identified key motifs for cold-responsiveness, i.e. ICEr1 (induction of CBF expression region 1) and ICEr2, in the *CBF2* promoter region of *Arabidopsis*. Interestingly, a sequence motif CGCCACGTGGCGGA, in which nine of fourteen nucleotides matched those of ICEr1 (GGACACATGTCGA), was present at −272 to −259 in the sweet cherry *CIG-B* promoter (Figure 1C). Furthermore, the ICEr2 sequence (ACTCCG) was perfectly conserved between −145 and −140 in the *CIG-B* promoter; whereas marked divergency in a stretch of the distance was not found between the putative TATA-box and ICEr2 among *CIG-B* and *CBF2* (Figure 1C). These features supported the cold-inducible activity of *CIG-B* promoter, possibly under the co-operation with the other motifs such as Myb-/Myc-recognition sites and G-box, although further analysis of this region, including deletion and/or mutational analyses, would be needed to confirm that. Thus, the correlation between up-regulation by low temperature treatment and the existence of ICEr1 and ICEr2 motifs in plants including sweet cherry and *Arabidopsis* provide useful information to understand the mechanisms of cold response in other plants.

In this paper, we focused on low temperature response for assessing the role of the *CIG-B* promoter. Although the responses of *CIG-B* promoter to other stresses such as salt and drought remain unclear, Sinwari et al. (1998) reported that the *DREB1B* promoter is induced by salt stress. By analogy, it is possible that the *CIG-B* promoter may also be involved in tolerance to multiple environmental stresses.

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References

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* 6: 325–330

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J* 33: 751–763

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J* 16: 433–442

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1989) Leaf disc transformation. In: Gelvin SB, Schilperoort RA (eds) *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordrecht, Netherlands, pp A5/1–A5/9

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol* 127: 910–917

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* 280: 104–106

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotech* 17: 287–291

Kitashiba H, Matsuda N, Ishizaka T, Nakano H, Suzuki T (2002) Isolation of genes similar to DREB1/CBF from sweet cherry (*Prunus avium* L.). *J Japan Soc Hort Sci* 71: 651–657

Kitashiba H, Matsuda N, Ishizaka, T. Nakano H, Suzuki T (2003) Isolation of two genes similar to *DREB1*/*CBF* from the sweet cherry and their analysis by transformation into *Arabidopsis*. In: Tanino, K.K. et al. (eds) *Acta Horticulturae*: *Environmental Stress and Horticulture Crops*. International Society for Horticultural Science, Leuven pp 39–45

Kitashiba H, Ishizaka T, Isuzugawa K, Nishimura K, Suzuki T (2004) Expression of a sweet cherry DREB1/CBF ortholog in *Arabidopsis* confers salt and freezing tolerance. *J Plant Physiol* 161: 1171–1176

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10: 1391–1406

Okada T, Toriyama K (2001) Pollen vegetative cell-specific expression of *Bra r 1*: useful tool for observation of the vegetative nucleus and identification of transgenic pollen by nuclear-targeted GFP. *Sex Plant Reprod* 13: 301–307

Qin F, Sakuma Y, Li J, Liu Q, Li Y-Q, Shinozaki K, Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol* 45: 1042–1052

Sakuma Y, Murayama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* 18: 1292–1309

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem Biophys Res Commun* 250: 161–170

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc Natl Acad Sci USA* 94: 1035–1040

Xiao H, Siddiqua M, Braybrook S, Nassuth A (2006) Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. *Plant Cell Environ* 29: 1410–1421

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* 6: 251–264

Zarka DG, Jonathan T, Cook D, Thomashow MF (2003) Cold induction of Arabisopsis CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol* 133: 910–918