Improvement of freezing tolerance in tobacco plants expressing a cold-responsive and chloroplast-targeting protein WCOR15 of wheat

Shimamura, Chisa
Ohno, Ryoko
Nakamura, Chiharu
Takumi, Shigeo

(Citation)
Journal of Plant Physiology,163(2):213-219

(Issue Date)
2006-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000346

Running title:

Wheat *Cor15*-promoted freezing tolerance in transgenic tobacco

Corresponding author:

Shigeo Takumi

Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan

Tel: +81 78 803 5860

Fax: +81 78 803 5859

E-mail: takumi@kobe-u.ac.jp

Improvement of freezing tolerance in transgenic tobacco plants expressing a cold-responsive and chloroplast-targeting protein WCOR15 of common wheat

Chisa Shimamura, Ryoko Ohno, Chiharu Nakamura and Shigeo Takumi*

Laboratory of Plant Genetics, Department of Biological and Environmental Science, Faculty of Agriculture, and Graduate School of Science and Technology, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan

*Corresponding author

Summary

Cold acclimation, an adaptive process for developing freezing tolerance in over-wintering plants, is associated with increased expression levels of a series of *Cor* (cold responsive)/*Lea* (late embryogenesis abundant) genes. To investigate the function of *Wcor15*, a member of the wheat *Cor/Lea* gene family, for improvement of freezing tolerance, two types of transgenic tobacco lines expressing *Wcor15*-containing chimeric genes were produced and characterized. Immunoblot and gene expression analyses of a transgenic tobacco line expressing the *Wcor15-GFP* fusion gene under control of the CaMV35S promoter showed transport and abundant accumulation of the WCOR15 protein in the stromal compartment of the chloroplasts. The 5' upstream region of *Wcor15* induced expression of the *GFP* reporter gene under low-temperature conditions in the transgenic tobacco. Both transgenic lines expressing the *Wcor15-GFP* fusion gene showed a similar and significantly improved level of freezing tolerance compared with the wild-type tobacco plants. Our results demonstrate that the induced expression of the wheat *Wcor15* gene positively contributes to the development of freezing tolerance in the heterologous tobacco plants.

Key words: chloroplast, cold responsive gene, freezing tolerance, green fluorescence protein, wheat (*Triticum aestivum* L.)

Abbreviations: CaMV, cauliflower mosaic virus; CBF/DREB1, CRT-binding factor/DRE-binding protein 1 ; *Cor*, cold responsive gene; CRT/DRE, C-repeat/dehydration responsive element; GFP, green fluorescent protein; GUS, ß-glucuronidase; LT, low temperature; *Lea*, late embryogenesis abundant; M808, Mironovskaya 808; MS, Murashige-Skoog; NA, non-acclimated

Introduction

In over-wintering plant species grown in the temperate regions, exposing to certain periods of low temperature (LT) increases their tolerance to subsequent freezing temperatures. This adaptive process is known as cold or frost acclimation and involves a number of biochemical and physiological changes (Levitt, 1980; Guy et al., 1985). These intracellular changes are regulated by LT through changes in gene expression. In the last decade, a number of LT-responsive genes have been isolated and characterized from a range of both dicotyledonous and monocotyledonous plant species. Among them, the most well characterized gene family designated as the *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) family comprises major LT-signaling components and can contribute to the significant development of freezing tolerance (Thomashow, 1999).

A barley *Cor*/*Lea* gene, *Bcor14b*, encodes an acidic protein BCOR14b (Cattivelli and Bartels, 1990), which is a leaf-specific protein and transported into the stromal compartment of the chloroplasts during cold acclimation (Crosatti et al., 1995, 1999). The *Arabidopsis* genome contains an analogous gene *Cor15a* that encodes a chloroplast-targeted COR15a protein (Lin and Thomashow, 1992). The cold-accumulated COR15a protein alters intrinsic curvature of the inner membrane of the chloroplast envelop, and this alteration results in a decreased incidence of freeze-induced lamellar-to-hexagonal II phase transitions (Steponkus et al., 1998). In wheat, *Wcs19*, a member of the *Cor*/*Lea* gene family and encoding a basic protein transported into the stromal compartment, shows light-stimulated expression during cold acclimation (Chauvin et al., 1993; Gray et al., 1997), in a similar manner to the non-homologous barley *Bcor14b* (Crosatti et al., 1999). The *Wcs19* transcript level is positively correlated with the relative reduction state of photosystem II in wheat (Gray et al., 1997). The WCS19 protein shows a high level of homology with another wheat member WCOR15 (Takumi et al., 2003) but a limited homology with WCOR14, an ortholog of barley BCOR14b (Tsvetanov et al., 2000). BCOR14b and WCOR14 and their non-homologous WCS19 and WCOR15 are all encoded by the *Cor/Lea* genes assigned to the group 2 chromosomes

of barley and common wheat (Takumi et al., 2003). The expression of *Wcor15* is specifically induced by LT in wheat leaves, and light illumination markedly increases the steady-state level of its transcripts. A freezing-tolerant winter wheat cultivar accumulates more *Wcor15* transcripts and corresponding proteins under LT conditions than a sensitive spring cultivar (Takumi et al., 2003; Kobayashi et al., 2004).

Constitutive expression of the *Arabidopsis Cor15a* gene using the cauliflower mosaic virus (CaMV) 35S promoter results in a significant increase of freezing tolerance in *Arabidopsis* (Artus et al., 1996). Similarly, *Arabidopsis* plants constitutively expressing the wheat *Wcs19* gene showed significant increases in their tolerance against freezing stress and photoinhibition (NDong et al., 2002). We herein report the improvement of freezing tolerance by induced expression of the wheat *Wcor15-GFP* fusion gene in transgenic tobacco plants using both the CaMV35S promoter and the native promoter. Our results demonstrated a limited but significant function of the WCOR15 protein in the development of freezing tolerance in plant cells.

Materials and methods

Plant materials, vector construction and transformation of tobacco plants

A winter cultivar 'Mironovskaya 808' (abbreviated as M808) of common wheat (*Triticum aestivum* L.) was grown in a controlled-climate cabinet at 25 ˚C with a 16 h photoperiod at a light intensity of 110-120 µm photons m^{-2} s^{-1} provided by cool white fluorescence lamps (the standard conditions).

The CaMV35S promoter of pBI121 (Clontech) was exchanged for the 1.7-kb 5' upstream sequence of *Wcor15* amplified from the selected genomic clone to construct the *Wcor15::GUS* chimeric gene (Takumi et al., 2003). The GUS reporter gene of pBI121 was replaced by a *Wcor15*-GFP fusion gene to produce the *35S::Wcor15-GFP* construct. *sGFP*(*S65T*) encoding a modified

version of GFP (Chiu et al., 1996), was used for the plasmid construction. The GUS gene of *Wcor15::GUS* was also exchanged for the *Wcor15-GFP* fusion gene for the construction of the *Wcor15::Wcor15-GFP* plasmid.

These two different plasmid constructs, *35S::Wcor15-GFP* and *Wcor15::Wcor15-GFP*, were introduced into the tobacco genome by *Agrobacterium*-infection method. Leaf discs of *Nicotiana tabacum* cv. 'Petit Havana' were infected with *Agrobacterium tumefaciens* LBA4404. Transformed cells were selected in the Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing 250 mg L^{-1} kanamycin, and T_0 generation plants were regenerated on hormone-free MS medium containing 50 mg L^{-1} kanamycin. These transformants were self-pollinated to produce T_1 and T_2 generations.

Immunoblot analysis

Polyclonal antibodies against WCOR15 and WCOR14 were previously constructed (Kobayashi et al., 2004). For the immunoblot analysis, a soluble protein fraction was extracted from the seedling leaves of M808 and transgenic tobacco plants. The seedlings were grown for 7 d under the standard conditions. Conditions for cold acclimation were according to Ohno et al. (2001). Briefly, 7-d-old wheat seedlings and 2-week-old tobacco seedlings were placed at 4 ± 0.5 ˚C under the standard light condition or under different light/dark regimes including complete dark. Protein extraction, immunoblotting and signal detection were performed according to Kobayashi et al. (2004).

For chloroplast preparation and fractionation, wheat leaves were homogenized with buffer A containing 0.44 M mannitol, 50 mM Tris-HCl (ph 8.0), 3 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 % (w v^{-1}) BSA. After centrifugation at 1900 x g for 10 min, the pellet (crude chloroplasts) was re-suspended in buffer A. The chloroplast suspension was layered on a sucrose discontinuous gradient (15, 40 and 60 %) with buffer B (buffer A without 2-mercaptoethanol). After centrifugation for 30 min, intact chloroplasts were collected from the 40-60 % sucrose interface,

diluted gently with two volumes of buffer B, and finally centrifuged for 10 min. The pellet (whole chloroplast) was suspended in buffer C (25 mM Hepes-KOH, ph 7.5, 10 mM EDTA), and re-centrifuged for 15 min. The pellet (whole thylakoids) was suspended in the Laemmli buffer. The supernatant containing the soluble fraction (stroma) was concentrated before western blot analysis.

Monitoring of GFP expression and bioassay for freezing tolerance

GFP activity was assessed using the kanamycin-resistant homozygous T_2 progeny of the transgenic tobacco lines. The progeny plants were grown for 7 d at 27 ˚C under a 16 h photoperiod at a light intensity of 110-120 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps. The plants were then cold acclimated for 7 d at 4 ± 0.5 ˚C under the same photo-intensity and photoperiod conditions. The autofluorescence (red) and the GFP images of chloroplasts were observed under fluorescence light microscopy BX60 (Olympus, Japan). The GFP fluorescence image was detected with excitation at 488 nm and emission at 530 nm. A confocal laser microscopy IX71 with FV1000 (Olympus) was used for the observation at a high magnification. To monitor the kinetics of GFP fluorescence levels during bioassay for freezing tolerance, individual transgenic plants were grown for 2 weeks in a 24-well microplate (IWAKI, Japan), cold acclimated for 1 to 3 d and then frozen at -10 ˚C for 2 h or 5 h. The GFP fluorescence levels were monitored with excitation at 485 nm and emission at 535 nm and quantified by a fluorescence plate reader LB970 (Berthhold technologies, Tokyo, Japan). Changes of the GFP levels were recorded using the same individual plants, and the means of 10 individual transformants were compared at each time points.

Two-week-old seedlings of the transgenic tobacco plants were cold acclimated at 4˚C for 3 d and then frozen at -10 ˚C for 2-6 h in dark. The frozen seedlings were thawed overnight at 4 ˚C and transferred back to the standard conditions. At the 7th d after the transfer, survival of seedlings was recorded. The whole bioassay experiment was repeated 3 times.

Results and Discussion

Chloroplast-targeting of the WCOR15 protein

In our previous study, we showed the leaf-specific expression of *Wcor15* and highly conserved structure of the putative chloroplast transit peptides at the N-terminal of WCOR15 (Takumi et al., 2003). More than 80% identity was observed between WCOR15 and WCOR14 in this region, although their homology was limited (34%) in the downstream main region. We also showed that the fusion protein WCOR15-GFP was targeted into chloroplast of a spiderwort epidermal tissue as revealed by a transient expression assay using particle bombardment (Takumi et al., 2003). To investigate the intracellular localization of the WCOR15 protein in wheat cells, chloroplasts were collected from 5-d cold-acclimated seedling leaves and the stromal compartments were separated from the thylakoid fraction. The immunoblot analysis clearly showed that both WCOR15 and WCOR14 were accumulated in the stromal compartments of chloroplasts isolated from cold-acclimated wheat leaves (Fig. 1A). These two COR proteins were thus induced by LT treatment, transported into chloroplasts and accumulated in the chloroplast stromal compartments. In the thylakoid fraction, weak signals of both proteins were observed, indicative of their weak association with the thylakoid membranes.

Totally 10 tobacco transgenic plants with the *35S::Wcor15-GFP* construct were recovered in the kanamycin-containing medium. Out of them, three stably GFP-expressing T_2 lines (#2, 6 and 7) were established. GFP fluorescence images, which were completely overlapped with autofluorescence images of chloroplasts in these lines clearly showed the chloroplast localization of WCOR15 (Fig. 1B). Further observations were made at a high magnification in mesophyll cells of mature leaves (Fig. 1C). Strong GFP signals were detected in the central part of the chloroplasts in the mesophyll cells. The localization of GFP signals in the mesophyll cells of the transgenic tobacco plants agreed with the stromal localization revealed by immunoblot analysis in the wheat

cells (Fig. 1A). These results clearly show that the transit peptide of the wheat COR protein can precisely transport the fused protein into the chloroplast stroma of not only wheat plants but also heterologous tobacco plants.

Cold-inducibility of the *Wcor15* promoter and improvement of freezing tolerance in transgenic tobacco plants

Promoter regions of *Cor*/*Lea* genes commonly contain a CCGAC core motif called CRT (C-repeat)/DRE (dehydration responsive element) in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). The CRT/DRE motif is recognized by CBF (CRT-binding factor)/DREB1 (DRE-binding protein 1) transcription factors (Stockinger et al., 1997; Liu et al., 1998). In the 5' upstream region of *Wcor15*, three CRT/DRE core motifs were identified, similar to another wheat *Cor*/*Lea* gene *Wcs120* (Takumi et al., 2003; Vazquez-Tello et al., 1998). The *Wcor15* promoter region functions for cold-responsive and light-stimulated expression as revealed by histochemical GUS-staining assay of transgenic tobacco plants (Takumi et al., 2003). A recently identified barley transcription factor HvCBF2, which is one of the barley CBF homologs, tends to bind preferably to a GTCGAC motif (Xue, 2003). Two GTCGAC sequences were found in the 5' upstream region of the wheat *Wcor15* (Fig. 2A).

To evaluate the chloroplast-targeted GFP activity under control of the native promoter, the *Wcor15::Wcor15-GFP* construct were introduced into tobacco plants. From 10 transgenic regenerants recovered in the kanamycin-containing medium two stably GFP-expressing T_2 lines (#17 and 18) were established. Using the fluorescence microplate reader, changes of GFP activity during cold acclimation and freezing treatment were *in vivo* monitored in the two *Wcor15::Wcor15-GFP* transgenic lines (Fig. 2B). The GFP fluorescence levels of these transgenic lines were higher than that of the wild-type tobacco plants and increased during the cold acclimation and freezing treatment after the cold acclimation. This increase of the GFP activity controlled by the *Wcor15* promoter showed a good agreement with the corresponding increase revealed by the immunoblot

analysis of the WCOR15 protein (Fig. 3). The GFP-detection system using the 5' upstream region of the *Wcor15* gene, therefore, should serve as a useful means in directly monitoring the kinetics of *in vivo* expression patterns of the *Cor* gene in both monocotyledonous and dicotyledonous plants. However, the induction level of the *Wcor15-GFP* fusion gene under control of the *Wcor15* native promoter was much lower than that by the CaMV35S promoter-driven fusion gene (Fig. 2C). The much higher levels of WCOR15-GFP accumulation was confirmed by the immunoblot analysis using the WCOR15 antibody in all three *35S::Wcor15-GFP* transgenic plants (Fig. 3). The kinetics of GFP fluorescence levels monitored by the fluorescence microplate reader also agreed with the levels observed by the immunoblot analysis. The line #2 of the *35S::Wcor15-GFP* transgenic tobacco showed cold-responsive accumulation of the WCOR15-GFP fusion protein, which seemed to be due to the positional effect of the integrated transgene.

Constitutive expression of the chloroplast-targeted *Arabidopsis* COR15a and wheat WCS19 proteins improved the freezing tolerance of *Aabidopsis* protoplasts and plants, respectively (Artus et al., 1996; NDong et al., 2002). In our bioassay, the wild-type tobacco plants could survive the 2-h freezing treatment at -10˚C after cold acclimation for 3 d, but even cold-acclimated transgenic plants were killed by the 6-h freezing treatment (Fig. 4). We, however, observed significantly improved freezing tolerance in both types of transgenic lines with the *35S::Wcor15-GFP* or *Wcor15::Wcor15-GFP* chimeric gene when they were treated by 4-h freezing after 3-d cold acclimation, under which condition all the wild-type tobacco plants were killed (Figs. 1D and 4). No significant differences were observed in the level of freezing tolerance between the two types of transgenic lines, which showed contrasted levels of WCOR15 accumulation (Fig. 3). Moreover, the transgenic lines with the *35S::Wcor15-GFP* gene that show markedly increased levels of the WCOR15 protein also required the cold acclimation for developing the freezing tolerance similar to those expressing much lower levels of the native promoter-driven *Wcor15::Wcor15-GFP* gene. Our observation is consistent with that reported in the transgenic *Arabidopsis* plants over-expressing the wheat *Wcs19* gene (NDong et al., 2002). The level of freezing tolerance in the

transgenic plants therefore did not directly correlate with the accumulated levels of the WCOR15-GFP fusion protein. Restricted but significantly improved levels of freezing tolerance were also reported in the transgenic *Arabidopsis* plants constitutively expressing the *Cor15a* and *Wcs19* genes (Artus et al., 1996; NDong et al., 2002). Taken together, these results likely suggest that effective threshold levels of freezing tolerance can only be attained by the collective action and cumulative effect of individual COR/LEA proteins, each with a limited effect. Nevertheless, our results demonstrate that the wheat gene *Wcor15* can play a recognizable role in conferring freezing tolerance in both monocotyledonous and dicotyledonous plants.

Acknowledgements

We thank Dr. Y. Niwa, Shizuoka University, Japan, for his gift of the *sGFP*(*S65T*) plasmid. We also thank F. Kobayashi and Dr. N. Mori for their kind help. The work was supported in part by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology of Japan to CN (no. 13306002) and ST (no. 17780005). Contribution number 170 from the Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University.

References

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF. Constitutive expression of the cold-regulated *Arabidopsis thaliana COR15a* gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA 1996;93:13404-09.

Baker SS, Wilhelm KS, Thomashow MF. The 5'-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol 1994;24:701-13.

Cattivelli L, Bartels D. Molecular cloning and characterization of cold-regulated genes in barley. Plant Physiol 1990;93:1504-10.

Chauvin LP, Houde M, Sarhan F. A leaf-specific gene stimulated by light during wheat acclimation to low temperature. Plant Mol Biol 1993;23:255-65.

Chiu W-I, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J. Engineered GFP as a vital reporter in plants. Curr Biol 1996;6:325-30.

Crosatti C, de Laureto PP, Bassi R, Cattivelli L. The interaction between cold and light controls the expression of the cold-regulated barley gene *cor14b* and the acclimation of the corresponding protein. Plant Physiol 1999;119:671-80.

Crosatti C, Soncini C, Stanca AM, Cattivelli L. The accumulation of a cold-regulated chloroplastic protein is light-dependent. Planta 1995;196:458-63.

Gray CR, Chauvin LP, Sarhan F, Huner NPA. Cold acclimation and freezing tolerance: a complex interaction of light and temperature. Plant Physiol 1997;114:467-74.

Guy CL, Niemi KJ, Brambl R. Altered gene expression during cold acclimation of spinach. Proc Natl Acad Sci USA 1985;82:3673-77.

Kobayashi F, Takumi S, Nakata M, Ohno R, Nakamura T, Nakamura C. Comparative study of the expression profiles of the *Cor*/*Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. Physiol Plant 2004;120:585-94.

Levitt J. Responses of Plants to Environmental Stresses, 2nd edn. New York: Academic Press, 1980. Vol. 1, p 166-222.

Lin C, Thomashow MF. DNA sequence analysis of a complemantary DNA for cold-regulated *Arabidopsis* COR15 and characterization of the COR15 polypeptide. Plant Physiol 1992;115:171-80.

Liu Q, Sakuma Y, Abe H, Kasuga M, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 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 1998;12:165-78.

Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.

NDong C, Danyluk J, Wilson KE, Pocock T, Huner NPA, Sarhan F. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol 2002;129:1368-81.

Ohno R, Takumi S, Nakamura C. Expression of a cold-responsive *Lt-Cor* gene and development of freezing tolerance during cold acclimation in wheat (*Triticum aestivum* L.). J Exp Bot 2001;52:2367-74.

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF. Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 1998;95:14570-75.

Stockinger EJ, Gilmour SJ, Thomashow MF. *Arabidopsis thaliana CBF1* encodes an AP2 doomain-containing transcription 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 1997;94:1035-40.

Takumi S, Koike A, Nakata M, Kume S, Ohno R, Nakamura C. Cold-specific and light-stimulated expression of a wheat (*Triticum aestivum* L.) *Cor* gene *Wcor15* encoding a chloroplast-targeted protein. J Exp Bot 2003;54:2265-74.

Thomashow MF. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Ann Rev Plant Physiol Plant Mol Biol 1999;50:571-99.

Tsvetanov S, Ohno R, Tsuda K, Takumi S, Mori N, Atanassov A, Nakamura C. A cold-responsive wheat (*Triticum aestivum* L.) gene *wcor14* identified in a winter-hardy cultivar 'Mironovska 808'. Genes Genet Syst 2000;75:49-57.

Vazquez-Tello A, Quellet F, Sarhan F. Low temperature-stimulated phosphorylation regulates the binding of nuclear factors to the promoter of *Wcs120*, a cold-specific gene in wheat. Mol Gen Genet 1998;257:157-66.

Xue GP. The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. Plant J 2003;33:373-83.

Yamaguchi-Shinozaki K, Shinozaki K. A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature or high salt stress. Plant Cell 1994;6:251-64.

Legends of figures

Figure 1. Chloroplast targeting of the WCOR15-GFP fusion protein and improvement of freezing tolerance in transgenic tobacco plants. (A) Immunoblot analysis using the specific antibodies against WCOR14 and WCOR15 in M808 seedling leaves. Total protein and chloroplast protein were obtained from 5-d cold-acclimated leaves, and the tylakoid and stromal compartments were fractionated. NA, non-acclimated. (B) Transport of the fusion protein into chloroplasts of mature leaves of the transgenic tobacco plants with *35S::Wcor15-GFP*. Green and red images show GFP fluorescence and chlorophyll autofluorescence, respectively. A right-most photo represents the merged image (yellow). (C) GFP fluorescence at the high magnification in a single mesophyll cell of transgenic tobacco plants with *35S::Wcor15-GFP*. A right-most photo represents the merged image. (D) Effect of the WCOR15-GFP fusion protein on freezing tolerance in transgenic tobacco plants. Transgenic tobacco lines expressing the WCOR15-GFP fusion protein were used for bioassay of the freezing tolerance after cold acclimation. The *Wcor15* gene was regulated either by CaMV35S promoter or by the 5' upstream region of the *Wcor15* gene. A picture was taken at the 10th day of recovery after freezing treatment at -10˚C for 4 h.

Figure 2. *In vivo* monitoring of cold induction of the *GFP* reporter gene under control of the 5' upstream region of *Wcor15* in transgenic tobacco plants. (A) Structure of the *Wcor15* genomic region. Boxes indicate two exons. A putative chloroplast signal peptide is represented by a shaded box. Putative *cis*-elements were indicated. (B) Kinetics of the GFP fluorescence levels during cold-acclimation and the following freezing treatment in the transgenic tobacco plants with *Wcor15::Wcor15-GFP*. Means with standard error bar were shown. WT, wild-type tobacco; NA, non-acclimated control; 4˚C-1d, 1-d acclimated at 4˚C; 4˚C-2d, 2-d acclimated; 4˚C-3d, 3-d acclimated; -10˚C-2h, treated by freezing at -10˚C for 2 h

after 3-d acclimation; -10˚C-5h, treated by freezing for 5 h after 3-d acclimation; after, incubation under the standard temperature condition for 1-d after 6-h freezing. (C) Kinetics of the GFP fluorescence levels during cold-acclimation and the following freezing treatment in the transgenic tobacco plants with *35S::Wcor15-GFP*.

Figure 3. Levels of the WCOR15 protein in transgenic tobacco lines. Immunoblots were performed using WCOR15 antibodies and the total protein fraction extracted from seedling leaves of the transgenic lines. NA, non-acclimated; 4˚C-1d, 1-d acclimated at 4˚C; 4˚C-3d, 3-d acclimated; -10˚C-2h, treated by freezing at -10˚C for 2 h after 3-d acclimation at 4˚C; -10˚C-5h, treated by freezing for 5 h after 3-d acclimation.

Figure 4. Summary of the bioassay for freezing tolerance of wild-type and transgenic tobacco lines. Transgenic tobacco lines expressing the WCOR15-GFP fusion protein were used for bioassay of the freezing tolerance after 3 d cold acclimation. The *Wcor15* gene was regulated either by CaMV35S promoter or by the 5' upstream region of the *Wcor15* gene.

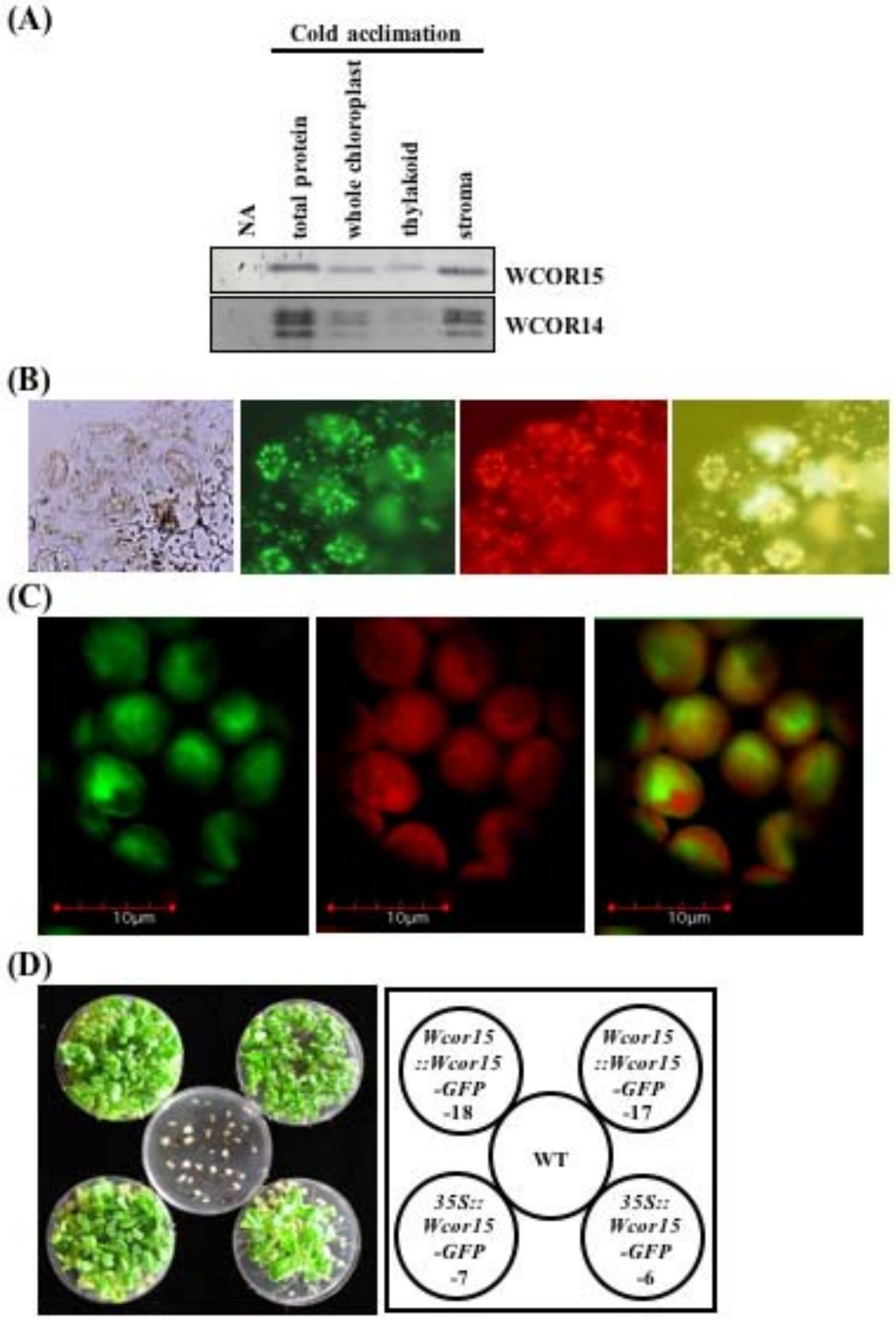

Fig. 1 (Shimamura et al.)

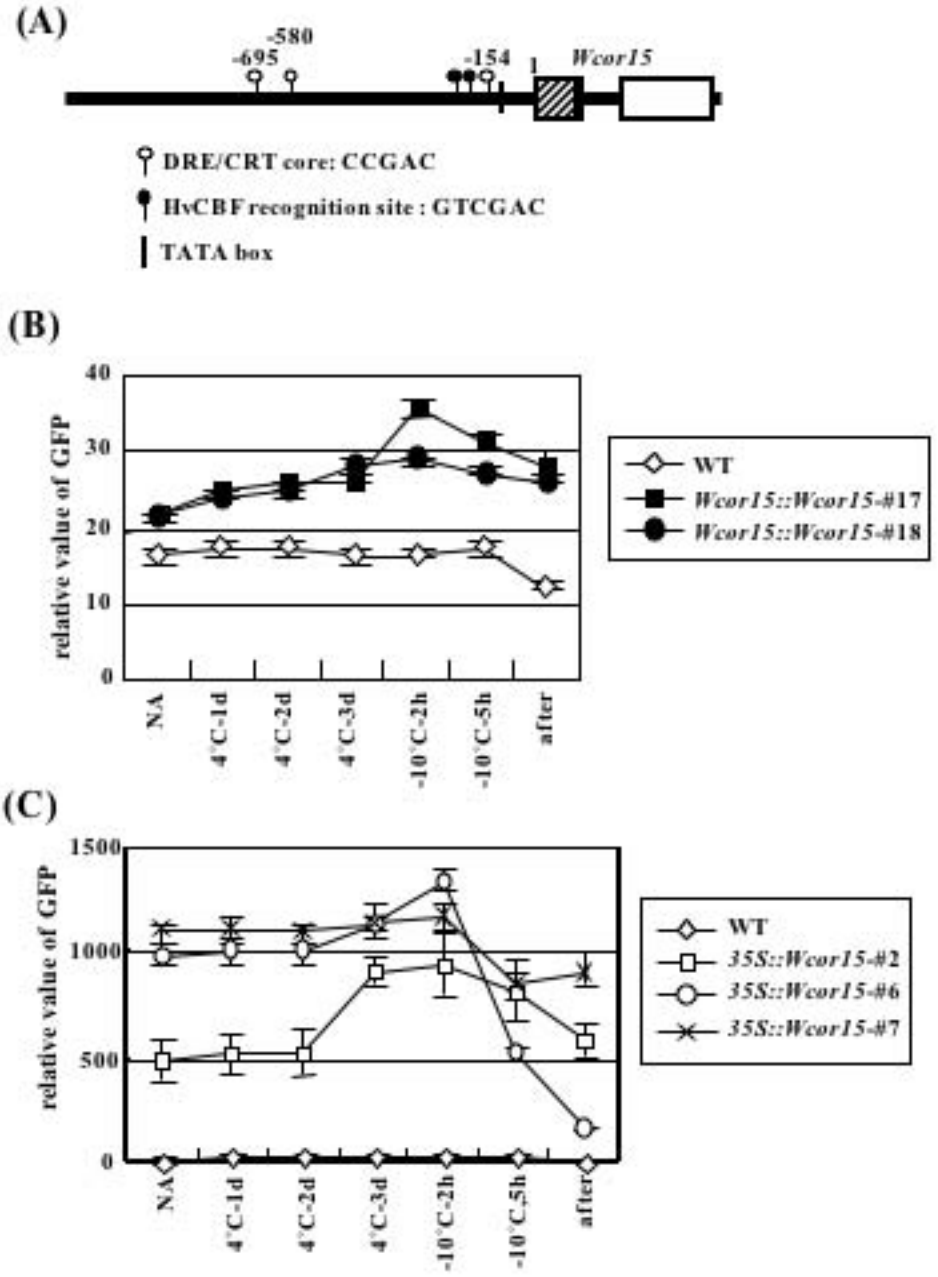

Fig. 2 (Shimamura et al.)

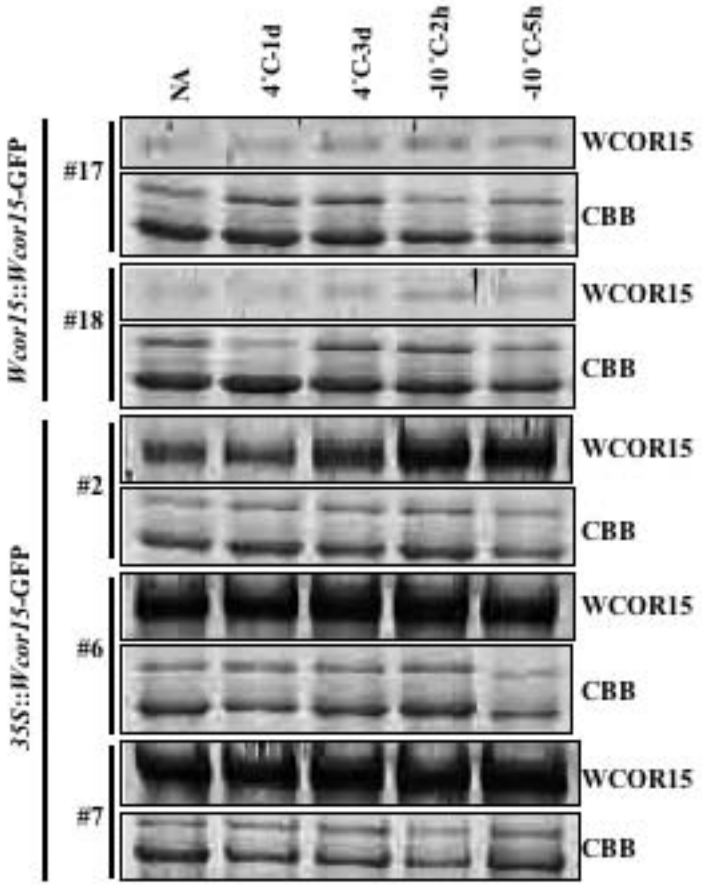

Fig. 3 (Shimamura et al.)

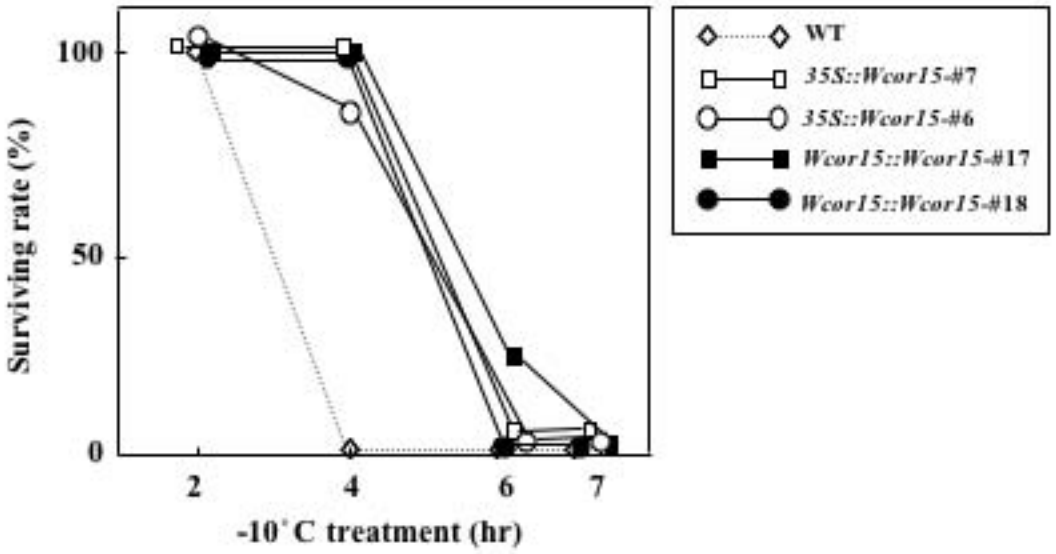

Fig. 4 (Shimamura et al.)