Increased freezing tolerance in an ABA-hypersensitive mutant of common wheat

Kobayashi, Fuminori
Takumi, Shigeo
Nakamura, Chiharu

(Citation)
Journal of Plant Physiology,165(2):224-232

(Issue Date)
2008-02

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000537

Freezing tolerance in an ABA-hypersensitive wheat mutant

Chiharu Nakamura
Laboratory of Plant Genetics,
Department of Biological and Environmental Science,
Faculty of Agriculture, Kobe University,
1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
Tel: +81 78 803 5800.
Fax: +81 78 803 5931~5933.
E-mail: **nakamura@kobe-u.ac.jp**

Increased freezing tolerance in an ABA-hypersensitive mutant of common wheat

Fuminori Kobayashi, Shigeo Takumi, Chiharu Nakamura

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

Summary

To study roles of abscisic acid (ABA) in cold-acclimation and cold/freezing tolerance in wheat, we analyzed an ABA-hypersensitive mutant of common wheat, 'Mutant ABA 27' (ABA27). ABA-hypersensitivity in ABA27 was confirmed by bioassay of germination and seedling growth and expression analysis of ABA-responsive genes in comparison with the parental cultivar 'Chihoku-komugi' (Chihoku). ABA27 showed significantly higher freezing tolerance than Chihoku in the seedlings without cold-acclimation. ABA-treated seedlings of ABA27 accumulated more transcripts of ABA-responsive *Cor*/*Lea* genes and their putative transcription factor (TF) genes than Chihoku under both normal and low temperature conditions. Non-ABA-regulated *Cor*/*Lea* transcripts also showed higher accumulation in ABA27 under normal temperature condition. These results suggest that the elevated ABA sensitivity in ABA27 contributes to the improvement of freezing tolerance through the increased expression of the ABA-regulated low temperature signal pathway. Together with the previous results obtained using an ABA-less-sensitive mutant, it is suggested that both positive and negative regulations of ABA response is involved in regulation of the basal level of freezing tolerance in wheat.

Key words ABA, cold-acclimation, *Cor*/*Lea*, freezing tolerance, wheat (*Triticum aestivum*)

Abbreviations

ABA, abscisic acid; ABA27, Mutant ABA 27; ABF, ABA-responsive element binding factor; ABI, ABA-insensitive; AREB, ABA-responsive element binding protein; ABRE, ABA-responsive element; bZIP, basic region leucine zipper; CBF, C-repeat binding factor; *Cor*/*Lea*, cold-responsive/late-embryogenesis-abundant; CRT, C-repeat; DHN, dehydrin; DRE, dehydration responsive element; DREB, dehydration responsive element binding protein; LT, low temperature; LIP, low temperature inducible protein; RAB, responsive to ABA; TF, transcription factor

Introduction

The plant hormone ABA is an important regulator of plant growth and development, affecting diverse processes such as seed maturation and germination, cell division and elongation, and responses promoting tolerance to abiotic stresses (Leuring and Giraudat, 1998; Finkelstein et al., 2002). Genetic screens for ABA-insensitive mutants such as *vp1* (*viviparous1*) of maize and *abi* of *Arabidopsis thaliana* revealed the ABA-regulated gene expression systems (Leuring and Giraudat, 1998; Finkelstein et al., 2002). All of the ABA-hypersensitive mutants including *era1* (*enhanced response to ABA1*), *ein2* (*ethylene insensitive2*), *fry1* (*fiery1*), *abh1* (*ABA-hypersensitive1*), *sad1* (*supersensitive to ABA and drought1*) and *hyl1* (*hyponastic leaves1*) show enhanced response to ABA (Finkelstein et al., 2002). The wild type alleles of these loci are considered to be coding for negative regulators of ABA response (for review, see Himmelbach et al., 2003).

During vegetative growth, an endogenous ABA level is increased upon exposure to water deficit, and the increased ABA level is considered to be an essential mediator in triggering the plant response to dehydration (Leung and Giraudat, 1998). The ABA-mediated signal transduction pathways acting under drought and high-salinity stresses are organized by many components including enzymes of ABA biosynthesis, protein kinases and phosphatases, and TFs regulating a number of *Cor*/*Lea* genes. *Arabidopsis* ABF/AREB are bZIP-type DNA-binding proteins and major TFs regulating the expression of *Cor*/*Lea* genes that contain *cis*-acting elements ABREs in their promoters (Yamaguchi-Shinozaki and Shinozaki, 2005). In addition to ABF/AREB, other TFs like MYC/MYB and CBF4 function in the ABA-dependent stress-signaling pathway (Yamaguchi-Shinozaki and Shinozaki, 2005).

A major pathway for LT stress is non-ABA-regulated in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 2005). LT-responsive *Cor*/*Lea* genes contain *cis*-elements CRTs/DREs in their promoters, and their expression is activated by CBF/DREB1 (Yamaguchi-Shinozaki and Shinozaki, 2005). An endogenous ABA level, however, is transiently increased by LT (Thomashow, 1999). *Arabidopsis* mutants such as *abi1*, *los5* (*low expression osmotically responsive gene5*)/*aba3* (*ABA deficient3*) and *los6*/*aba1* appear to be impaired in their ability of cold-acclimation (Heino et al., 1990; Gilmour and Thomashow, 1991; Mäntylä et al., 1995; Xiong et al., 2001a; 2002). In the *los5*/*aba3* and *los6*/*aba1* mutants, *Cor*/*Lea* expression levels are reduced under LT stress (Xiong et al., 2001a; 2002). These findings suggest that LT and ABA regulatory pathways are not independent.

Information on roles of ABA in the regulation of ABRE-containing genes under LT remains limited in wheat and its related species. We previously studied cold-acclimation and freezing tolerance of a dominant ABA-less-sensitive wheat mutant ‘EH47-1’ (Kobayashi et al., 2006). Although EH47-1 showed reduced ABA-sensitivity, the observed transcript levels of ABA-responsive *Cor*/*Lea* and TF genes were higher in EH47-1 than in the parental line after ABA

treatment. EH47-1 showed higher freezing tolerance than the parental line under non-acclimated condition, but no significant differences were observed in the expression profiles of *Cor*/*Lea* and TF genes during cold-acclimation. These results suggest that the basal level of freezing tolerance is under control of ABA sensitivity and independent of the *CBF*-mediated *Cor*/*Lea* expression in wheat.

To obtain further information on roles of ABA in cold-acclimation and freezing tolerance in wheat, we analyzed an ABA-hypersensitive mutant line ABA27. ABA27 showed higher ABA sensitivity during germination and post-germination growth and higher freezing tolerance in seedlings than the parental cultivar, and it also accumulated more transcripts of some *Cor*/*Lea* and TF genes under both normal condition and after LT treatment. These results suggest that an elevated ABA sensitivity positively contributes to the level of freezing tolerance in wheat.

Materials and methods

Plant materials

The common wheat (*Triticum aestivum* L.) mutant line, ABA27 (strain ID, KT020-133), is registered in the database of KIBR (Kihara Institute for Biological Research) genetic strains of NBRP (National BioResourse Project) KOMUGI program (http://shigen.lab.nig.ac.jp/wheat/komugi/strains/aboutNbrp.jsp). This line was derived from the Japanese cultivar Chihoku after ethylmethane-sulfonate (EMS) mutagenesis (Dr. Noda, personal communication).

Bioassay for ABA sensitivity during germination and post-germination growth

Seed germination was studied in four replications of 40 seeds of each line and the reciprocal F_1s between ABA27 and Chihoku. The seeds were placed in plastic petri dishes (90 mm in diameter and 15 mm in depth) containing two sheets of filter paper (82 mm diameter) wetted with 6 mL of distilled water or 20 μM ABA solution, and incubated at 20˚C in the darkness. Germination was scored up to 5 d after imbibition. In bioassay of ABA sensitivity based on post-germination growth, seeds from ABA27, Chihoku and their F_1 plants were imbibed under tap water for 5 h and kept overnight at 4˚C. Imbibed seeds were placed in a glass petri dishe containing filter papers wetted with distilled water, and incubated for 24 h at 20˚C in the darkness. Ten synchronously germinated seeds were further treated with distilled water or 20 μM ABA solution in the same condition as the germination assay. After 3 d, the length of primary roots was recorded. The whole experiments were repeated four times and the data were statistically analyzed.

Bioassay for freezing tolerance

For the evaluation of freezing tolerance, 20 imbibed seeds from each line were sown in the same pot with soil. Seven-d-old seedlings of the mutant and the parental line were cold-acclimated (4˚C for 7 d), and then treated with a freezing temperature at –15˚C for 6 h in the dark (Kobayashi et al., 2004a). The frozen seedlings were thawed overnight at 4˚C and transferred back to the normal temperature condition at 25˚C. On the 7th d after transfer, numbers of the seedlings showing growth recovery were recorded. The whole experiments were repeated three to four times and the data were statistically analyzed.

ABA and low temperature treatments and RNA extraction

ABA treatment (up to 24 h) was performed by spraying 7-d-old seedlings grown under the standard conditions at 25˚C (Ohno et al., 2001) with a solution of 20 μM ABA containing 0.1% (w/v) Tween 20. LT treatment up to 14 d was given by transferring 7-d-old seedlings from the standard condition to the cold-acclimation condition at 4˚C. After indicated periods, total RNA was extracted by guanidine thiocyanate from leaves of 10 seedlings as previously described (Kobayashi et al., 2006).

RNA gel blot and RT-PCR analyses

Steady state levels of transcripts of seven wheat *Cor*/*Lea* genes (*Wcor14*, *Wcor15*, *Wlt10*, *Wdhn13*, *Wrab15*, *Wrab17* and *Wrab18*) were studied by RNA gel blot analysis using the corresponding cDNA clones as ^{32}P-labelled probes. These cDNA clones were previously characterized (Tsvetanov et al., 2000; Tsuda et al., 2000; Ohno et al., 2001; 2003; Takumi et al., 2003; Kobayashi et al., 2004a). RNA gel blot analysis was according to Kobayashi et al. (2004a). For RT-PCR, first strand cDNA was synthesized from 1 μg of DNaseI-treated RNA with oligo-dT primers using Rever Tra Ace (TOYOBO, Osaka, Japan). Gene-specific primer sets listed in Kobayashi et al. (2006) were designed based on the nucleotide sequences of *Cor*/*Lea* genes and their putative TFs including *Wcbf2* (accession number AB178166), *Wdreb2* (AB193608), *Wlip19* (AB193552) and *Wabi5* (AB193553) (Kobayashi et al., 2004a; 2004b; Kume et al., 2005; Egawa et al., 2006). Ubiquitin gene was used as an internal control, and RT-PCR conditions were carefully manipulated to measure the steady state levels of transcripts at the exponential phase of amplification. RT-PCR products were separated by electrophoresis through 1.5 % agarose gel and stained with ethidium bromide.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using a Line Gene Fluorescent Quantitative Detection System (Bio Flux, Tokyo, Japan) and gene-specific primer sets (Kobayashi et al., 2006). As an internal control, the ubiquitin gene was used. The rate of amplification was monitored by SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) according to the manufacture's protocol. Results were presented according to Goodman et al., (2004).

Results

Morphological characteristics of ABA27

The ABA27 was isolated as a dormant mutant derived from EMS treated seeds of Chihoku in 1986 and then was registered in the genebank of 'NBRP KOMUGI' (see Materials and methods). Morphological differences between Chihoku and ABA27 were observed in their spikes. ABA27 had longer spikes (10.08 ± 0.45 cm, mean ± standard deviation, n=20) than Chihoku (8.85 ±0.97 cm, n=18). This line difference was due to the different length of rachis because of no significant change in the number of spikelet between Chihoku and ABA27.

Germination of ABA27 seeds

A previous germination test using developing caryopses showed that ABA sensitivity in ABA27 was higher than in its parent Chikoku (Dr. Noda, personal communication). In the present study, we compared the time-course of germination of the mature seeds among ABA27, Chihoku and their reciprocal F_1s under both ABA and non-ABA conditions. In the absence of exogenous ABA, ABA27 showed delayed germination in comparison with Chihoku (Fig. 1A), indicating an enhanced sensitivity of ABA27 to endogenous ABA. Under the presence of 20 μM ABA, germination of ABA27 was more markedly delayed, whereas germination of Chihoku was not significantly affected by the ABA treatment (Fig. 1B). These results indicated that ABA27 was more sensitive to ABA during seed germination than Chihoku. Moreover, germination of the F_1 seeds under both conditions was similar to that of Chihoku throughout the tested period (Fig. 1A, B), indicating that the ABA-hypersensitivity in ABA27 is caused by a recessive mutation.

ABA sensitivity during post-germination growth

To study ABA sensitivity during early seedling development, levels of inhibition of seedling growth by 20 μM of exogenous ABA was compared between ABA27 and Chihoku. Root growth was greatly inhibited by ABA treatment in both lines (Fig. 2A, B). The magnitude of inhibition in root growth estimated by the relative growth rate (% growth in the presence of ABA relative to growth in the absence of ABA) was significantly greater in ABA27 than in Chihoku (Fig. 2B, C). These results showed that ABA27 was more sensitive to the exogenous ABA than Chihoku during post-germination growth. The root growth of the F_1 seedlings was also inhibited by the exogenous ABA (Fig. 2A, B). The relative root growth rate of the F_1 seedlings did not show a significant difference from that of Chihoku (Fig. 2C). These results indicated that the recessive mutation causing hypersensitivity to ABA in ABA27 affected not only seed germination but also seedling growth.

Expression profiles of ABA-responsive genes in ABA-treated seedlings

ABA sensitivity in the seedlings was studied based on the levels of ABA-responsive gene expression after ABA application. RNA gel blot analysis of two *Cor*/*Lea* genes, *Wdhn13* and *Wrab17*, indicated their higher induction by ABA in ABA27 than in Chihoku (Fig. 3A). Time-courses of the transcript accumulation of ABA-responsive *Cor*/*Lea* genes (*Wdhn13*, *Wrab17*, *Wrab15* and *Wrab18*) and their putative TF genes (*Wdreb2*, *Wabi5* and *Wlip19*) were studied by RT-PCR. The levels of *Wdhn13* and *Wrab17* transcripts increased after ABA application and reached maxima within 2-5 h under the standard temperature condition in both lines (Fig. 3B). The amount of transcripts of other tested genes (*Wrab18* and *Wlip19*) also increased by ABA treatment in both lines (data not shown). ABA27 accumulated more transcripts of most *Cor*/*Lea* and TF genes examined than Chikoku (Fig. 3B). Although the accumulation pattern of the *Wabi5* transcript was fluctuated during the tested period, the transcript was more abundantly accumulated in ABA27 than in Chihoku. The expression pattern of *Wrab15* showed a remarkable line difference: the accumulated level of the transcript was much greater in ABA27 than in Chihoku, irrespective of the ABA treatment. These results were consistent with the enhanced ABA sensitivity of ABA27 shown by the seedling bioassay (Fig. 2).

Freezing tolerance in non- and cold-acclimated seedlings

The level of freezing tolerance was compared between ABA27 and Chihoku by scoring the numbers of the recovered seedlings from freezing damage (Fig.4). LT treatment at 4˚C for 7 d significantly increased the level of freezing tolerance in both lines (Fig. 4A). ABA27 showed significantly higher freezing tolerance than Chihoku in both non- and cold-acclimated seedlings. The result indicated that the mutation in ABA27 did not affect the cold-acclimation ability but caused improvement of the basal level of freezing tolerance. An increased ABA sensitivity thus appeared to be positively related to the development of higher freezing tolerance in ABA27.

Expression profiles of low temperature-responsive genes

LT responsiveness of seven *Cor*/*Lea* genes (*Wcor14*, *Wcor15*, *Wlt10*, *Wdhn13*, *Wrab15*, *Wrab17* and *Wrab18*) and four TF genes (*Wcbf2*, *Wdreb2*, *Wabi5* and *Wlip19*) was compared between ABA27 and Chihoku after LT treatment up to 14 d by RNA gel blot and RT-PCR analyses. All of the tested *Cor*/*Lea* genes were induced within 1 d in both lines, and the amounts of their transcripts reached maximum levels during 3-7 d of LT treatment (Fig. 5A, B). The induction of TF genes occurred within 8 h, and the amounts of transcripts reached maximum levels within 1-3 d of LT treatment in both lines (e.g. *Wabi5* in Fig. 5B). The expression patterns of all examined genes in the two lines resembled those in the previously studied common wheat lines (Kobayashi et al., 2004a; 2006; Kume et al., 2005; Egawa et al., 2006), and agreed with the development of freezing tolerance after

cold-acclimation. The levels of these transcripts after 3 d of LT treatment were similar in both lines. At the earlier period, however, the amounts of *Wabi5*, *Wrab15* and *Wrab18* transcripts were higher in ABA27 than in Chihoku (Fig. 5C). Quantitative RT-PCR analysis confirmed the much higher amounts of *Wabi5* and *Wrab15* transcripts in ABA27 than in Chihoku under LT (Fig. 6).

Because of the observed higher freezing tolerance in ABA27 than in Chihoku under the non-acclimation condition (Fig. 4), transcript levels LT-responsive genes were compared using the non-acclimated seedlings. RT-PCR showed that the levels of transcripts of four ABA-responsive genes (*Wdhn13*, *Wrab15*, *Wrab18* and *Wabi5*) were higher in ABA27 than in Chihoku (Figs. 5D). Quantitative RT-PCR analysis showed that ABA27 accumulated over four-fold of *Wabi5* and *Wrab15* transcripts than Chihoku under the non-acclimated condition (Fig. 6). The amounts of transcripts of non-ABA-responsive genes (*Wcor14* and *Wcor15*) were also higher (Fig. 5D).

Discussion

A role of ABA sensitivity in abiotic stress signaling remains controversial. In *Arabidopsis*, ABA-insensitive *abi1* and ABA-deficient *los5*/*aba3* and *los6*/*aba1* mutants showed reduced levels of drought/cold-induced and ABA-regulated accumulation of *Cor*/*Lea* transcripts, which resulted in their reduced freezing tolerance (Mäntylä et al., 1995; Xiong et al., 2001a; 2002). On the other hand, while a number of ABA-hypersensitive mutants of *Arabidopsis* showed enhanced expression of ABA-responsive genes (Finkelstein et al., 2002), they exhibited contrasting responses to drought and NaCl. Mutants of *era1* (Pei et al., 1998) and *abh1* (Hugouvieux et al., 2001) exhibited increased tolerance due to reduction in transpirational water loss, but *sad1* (Xiong et a., 2001b) and *fry1* (Xiong et al., 2001c) showed increased sensitivity due to defective stomatal regulation.

Much less information is available on the roles of ABA sensitivity in cold-acclimation and freezing tolerance. To study roles of ABA in cold-acclimation and freezing tolerance in wheat, we examined an ABA-hypersensitive mutant ABA27 that was selected based on the elevated ABA sensitivity in developing caryopses. ABA27 showed ABA-hypersensitivity during seed germination and post-germination seedling growth (Figs. 1, 2). The observed ABA-hypersensitivity in ABA27 is caused by a recessive mutation according to the bioassays of germination and root growth of the reciprocal F_1s made between ABA27 and the parental Chihoku (Figs. 1, 2). ABA27 also showed significantly higher freezing tolerance than Chihoku even without LT treatment (Fig. 4). In the non-acclimated seedlings, ABA27 accumulated more transcripts of *Cor*/*Lea* and TF genes including *Wdhn13*, *Wrab15*, *Wrab18* and *Wabi5* than in Chihoku (Figs. 5D, 6). These results at least agree with the previous reports in the ABA sensitivity mutants of *Arabidopsis*, i.e., the increased ABA sensitivity results in the increased expression of ABA-responsive *Cor/Lea* genes (Xiong et al., 2001c). The

relationship between ABA sensitivity and freezing tolerance observed in ABA 27 suggests that ABA sensitivity plays a positive role in the regulation of freezing tolerance in wheat.

In a previous study (Kobayashi et al., 2006), we examined an ABA-less-sensitive mutant of common wheat EH47-1, which was selected by the reduced level of ABA sensitivity in developing caryopses (Kawakami et al., 1997). EH47-1 showed a reduced sensitivity to ABA as judged by the magnitude of ABA inhibition of seedling growth, but showed higher accumulation of *Cor/Lea* and TF transcripts in ABA-treated seedlings than the parental line under the standard temperature condition (Kobayashi et al., 2006). EH47-1 retained an ability of cold-acclimation and showed an elevated level of freezing tolerance even without LT treatment. No differences were observed in the expression profiles of LT-responsive genes between EH47-1 and the parental line under the LT condition. It was thus suggested that a dominant mutation in EH47-1 was not associated with the cold-acclimation process but caused a significant improvement of the basal level of freezing tolerance due to the loss of function of unknown negative regulator of an ABA-responsive pathway.

ABA27 showed higher accumulation of transcripts of some ABA-responsive genes such as *Wabi5*, *Wrab15* and *Wrab18* than Chihoku after cold-acclimation (Figs. 5, 6), which was in contrast to EH47-1. This observation indicates that the ABA signaling is not independent of the LT-responsive *Cor*/*Lea* gene expression in wheat, and that at least some ABA-responsive genes are involved in the ABA-dependent LT signal pathway like the *Arabidopsis RAB18* gene, whose expression is reduced in *abi1* under LT stress (Lång and Palva 1992; Mäntylä et al., 1995). A limited level of enhancement of the ABA-responsive genes under LT stress, however, suggests that the ABA-dependent pathway has a minor role in cold-acclimation.

ABA27 accumulated, under the non-LT condition, more transcripts of *Wcor14* and *Wcor15* (Fig. 5D), which are members of the non-ABA-responsive *Cor/Lea* genes in wheat (Tsvetanov et al., 2000; Takumi et al., 2003). A reason for this constitutive enhancement remains unclear, but this might suggest that an ABA-dependent pathway can indirectly activate expression of non-ABA-regulated genes, contributing to the improvement of freezing tolerance without cold-acclimation. Our results showing the increased levels of freeing tolerance in both of the ABA-hypersensitive and less-sensitive mutants under the non-acclimation condition together with the increased expression of *Cor/Lea* and TF genes suggest a dual presence of positive and negative regulations of ABA response in wheat.

Acknowledgements We thank Dr. K. Noda for providing us with information on Chihoku and ABA27. This work was supported by Grants-in-Aid from JSPS Research Fellowships for Young Scientists to FK and from the Ministry of Education, Culture, Sports, Science and Technology of Japan to CN (no. 13306002) and ST (no. 17780005).

References

Egawa C, Kobayashi F, Ishibashi M, Nakamura T, Nakamura C, Takumi S. Transcript accumulation and alternative splicing of a *DREB2* homolog are differentially regulated by cold and drought stresses in common wheat. Genes Genet Syst 2006;81:77-91.

Finkelstein RR, Gampala SS, Rock CD. Abscisic acid signaling in seeds and seedlings. Plant Cell 2002;14 (Suppl):S15-S45.

Gilmour S, Thomashow MF. Cold acclimation and cold regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol 1991;17:1233-40.

Goodman CD, Casati P, Walbot V. A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 2004;16:1812-26.

Heino P, Sandman G, Lång V, Nordin K, Palva ET. Abscisic acid deficiency prevents development of freezing tolerance in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 1990;79:801-9.

Himmelbach A, Yang Y, Grill E. Relay and control of abscisic acid signaling. Curr Opin Plant Biol 2003;6:470-9.

Hugouvieux V, Kwak JM, Schroeder JI. An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. Cell 2001;106:477-87.

Kawakami N, Miyake Y, Noda K. ABA insensitivity and low ABA levels during seed development of non-dormant wheat mutant. J Exp Bot 1997;48:1415-21.

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 2004a;120:585-94.

Kobayashi F, Takumi S, Nakamura C. Regulation of cold-responsive *Cor*/*Lea* genes and their transcription factors by the major freezing tolerance locus *Fr-1* in wheat. Recent Res Devel Plant Sci 2004b;2:249-66.

Kobayashi F, Takumi S, Egawa C, Ishibashi M, Nakamura C. Expression patterns of low temperature responsive genes in a dominant ABA-less-sensitive mutant line of common wheat. Physiol Plant 2006;127:612-23.

Kume S, Kobayashi F, Ishibashi M, Ohno R, Nakamura C, Takumi S. Differential and coordinated expression of *Cbf* and *Cor*/*Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes Genet Syst 2005;80:185-97.

Lång V, Palva ET. The expression of a *rab*-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 1992;20:951-62.

Leung J, Giraudat J. Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol 1998;49:199-222.

Mäntylä E, Lång V, Palva ET. Role of abscisic acid in drought-induced freezing tolerance, cold

acclimation, and accumulation of LTI78 and RAB18 proteins in *Arabidopsis thaliana*. Plant Physiol 1995;107:141-48.

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.

Ohno R, Takumi S, Nakamura C. Kinetics of transcript and protein accumulation of a low-molecular-weight wheat LEA D-11 dehydrin in response to low temperature. J Plant Physiol 2003;160:193-200.

Pei ZM, Ghassemian M, Kwak CM, McCourt P, Schroeder JI. Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science 1998;282:287-90

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. Annu Rev Plant Physiol Plant Mol Biol 1999;50:571-99.

Thomashow MF. So what's new in the field of plant cold acclimation? Lots! Plant Physiol 2001;125:89-93.

Tsuda K, Tsvetanov S, Takumi S, Mori, N, Atanassov, A, Nakamura C. New members of a cold-responsive group-3 *Lea/Rab*-related *Cor* gene family from common wheat (*Triticum aestivum* L.). Genes Genet Syst 2000;75:179-88.

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.

Xiong L, Ishitani M, Lee H, Zhu JK. The *Arabidopsis LOS5*/*ABA3* locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell 2001a;13:2063-83.

Xiong L, Gong Z, Rock CD, Subramanian S, GuoY, Xu W, Galbraith D, Zhu JK. Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis*. Dev Cell 2001b;1:771-81.

Xiong L, Lee BH, Ishitani M, Lee H, Zhang C, Zhu JK. *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. Genes Dev 2001c;15:1971-84.

Xiong L, Lee H, Ishitani M, Zhu JK. Regulation of osmotic stress-responsive gene expression by the *LOS6*/*ABA1* locus in *Arabidopsis*. J Biol Chem 2002;277:8588-96.

Yamaguchi-Shinozaki K, Shinozaki K. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci 2005;10:88-94

Figure legends

Figure 1. ABA inhibition of seed germination in Chihoku, ABA27 and their reciprocal F_1s. Seeds (n=40) were germinated with and without 20 μM ABA at 20˚C under the darkness. Germinated seeds were counted daily up to 5 d after the start of imbibition at 20˚C. (A) Germination rate in the absence of ABA (distilled water only). (B) Germination rate in the presence of 20 μM ABA.

Figure 2. Inhibition of seedling growth by ABA (20 μM) in Chihoku, ABA27 and their reciprocal F_1s. (A) Effect of exogenous ABA on root length. Imbibed seeds were germinated at 20˚C under the darkness and then incubated with and without 20 μM ABA at 20˚C under the darkness. Lengths of primary roots were measured at the 3rd d of the treatment. The experiments were independently performed four times. The small bars represent standard errors. (C) Comparison of the relative root growth (%) (root lengths with/without ABA). The relative root growth indicates the magnitude of inhibition by ABA treatment. An asterisk indicates statistical significance at the 5% level (Student's *t*-test). (B) A picture showing the inhibition of seedling growth by ABA was taken at the 3rd d of ABA treatment.

Figure 3. Expression profiles of some ABA-responsive genes in ABA-treated seedlings of Chihoku and ABA27. (A) Comparison of the levels of *Wdhn13* and *Wrab17* transcripts between the two lines. Seedlings were treated with 20 μM ABA, and total RNA was isolated 2 h after the treatment. RNA gel blots were probed with ^{32}P-labelled cDNAs of the *Wdhn13* and *Wrab17* genes. rRNA was used as an internal control. (B) Time-course of the transcripts accumulation of *Cor*/*Lea* genes (*Wdhn13*, *Wrab17* and *Wrab15*) in the lines. Cycle numbers of PCR are indicated at the right sides of the electrophoregrams. Ubiquitin gene (*Ubi*) was used as controls for RT-PCR.

Figure 4. Comparison of the levels of freezing tolerance after non-acclimation (NA) and cold-acclimation (7A) in Chihoku and ABA27. (A) Effect of cold acclimation on the development of freezing tolerance. Bioassay was performed under the standard assay conditions; 4˚C for 7 d for cold-acclimation and –15˚C for 6 h for freezing. Data are represented as means ± standard deviation (n=3-4). An asterisk indicates statistical significance at the 5% level (Student's *t*-test). (B) A picture showing the line difference in the level of freezing tolerance after 7 d of cold-acclimation was taken at the 10th d of recovery after freezing.

Figure 5. Expression profiles of *Cor*/*Lea* and *Wabi5* in Chihoku and ABA27 during cold-acclimation. The transcript accumulation in the seedling leaves was monitored for 14 d by RNA gel blot analysis (A) or by RT-PCR analysis (B). (C) Comparison of the transcript levels of *Wabi5*, *Wrab15* and

Wrab18 in the cold-acclimated seedlings by RT-PCR. (D) Comparison of the transcript levels of *Cor*/*Lea* and *Wabi5* in the non-acclimated (NA) seedlings by RT-PCR. Cycle numbers of PCR are indicated at the right sides. rRNAs and ubiquitin gene (*Ubi*) were used as controls for RNA gel blot and RT-PCR, respectively.

Figure 6. Quantitative RT-PCR analysis of *Wabi5* (A) and *Wrab15* (B). Real-time RT-PCR was performed using gene-specific primer sets and RNA samples extracted from seedlings of Chihoku and ABA27 with and without cold-acclimation. Ubiquitin gene was used as an internal control. The transcript levels were shown as relative values compared to the mRNA levels at the non-acclimated (NA) condition in Chihoku.

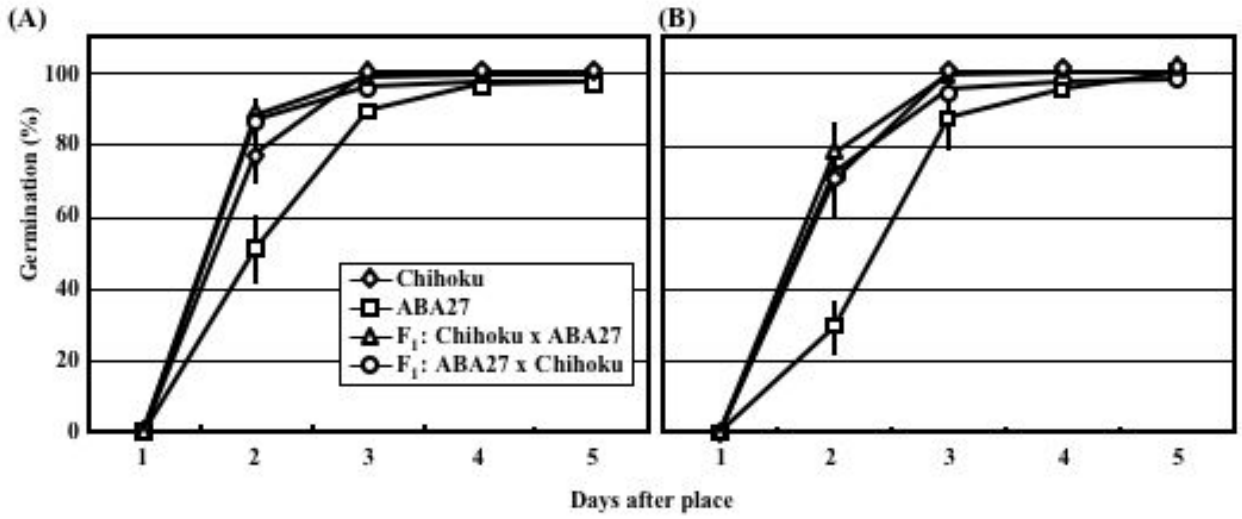

Fig. 1 (Kobayashi et al.)

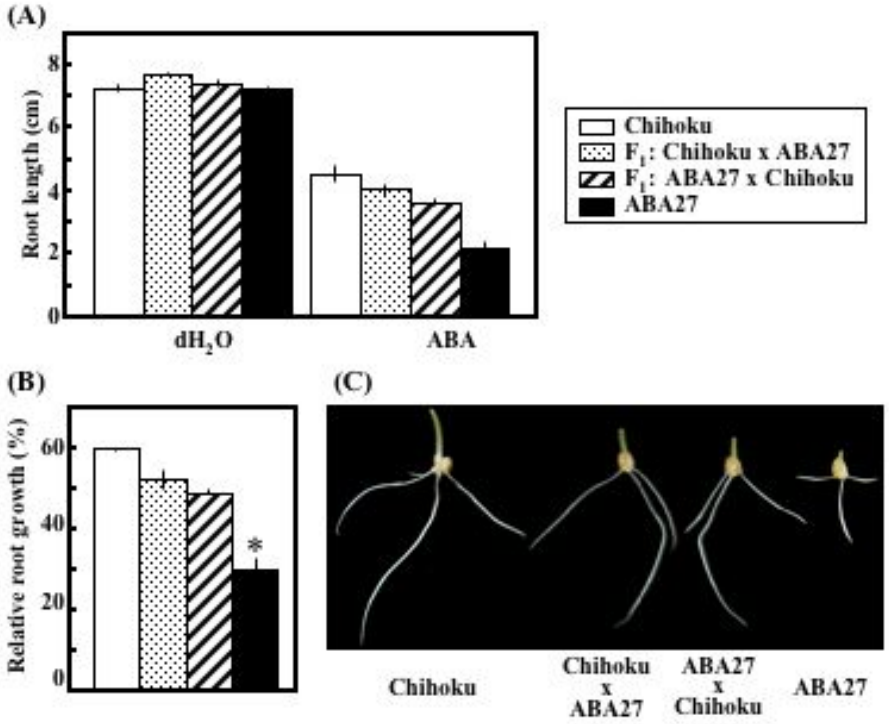

Fig. 2 (Kobayashi et al.)

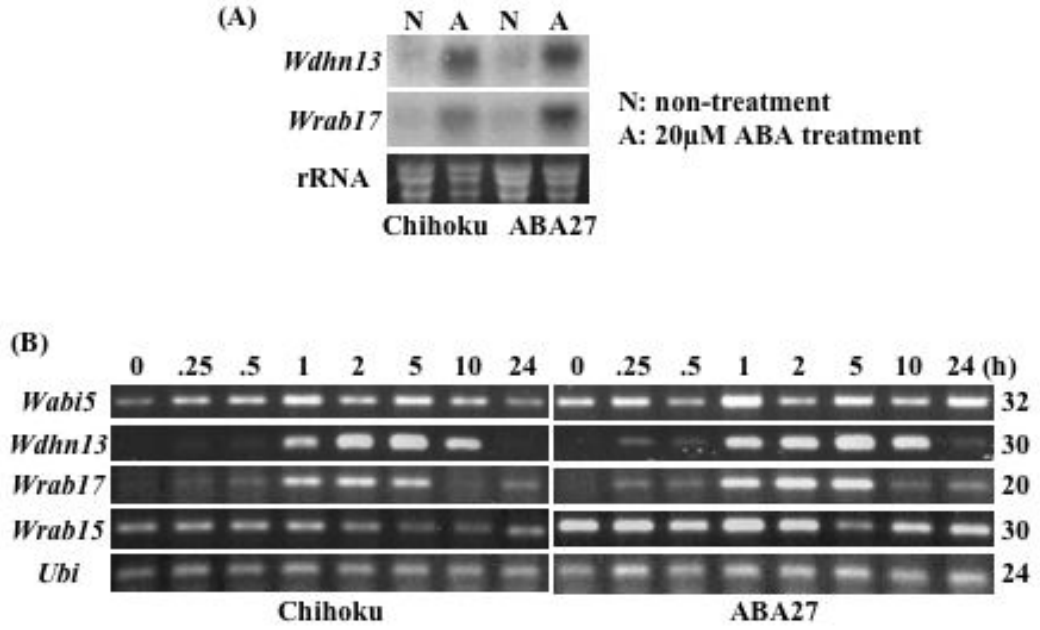

Fig. 3 (Kobayashi et al.)

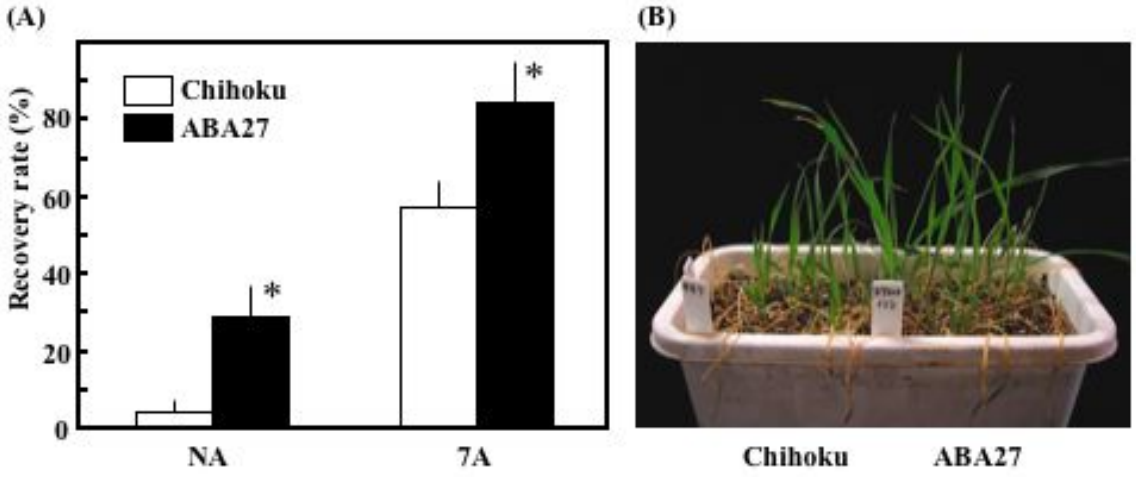

Fig. 4 (Kobayashi et al.)

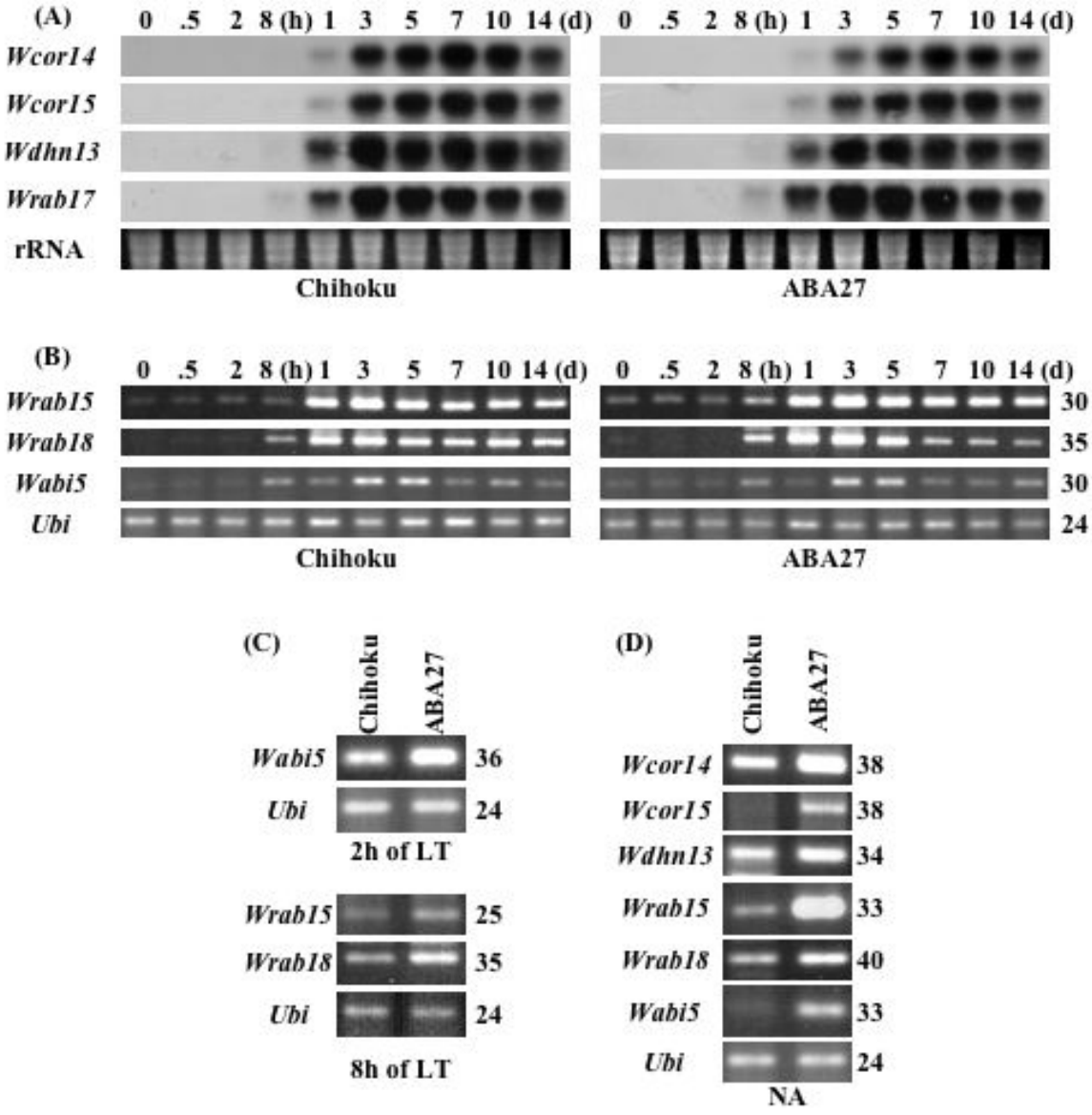

Fig. 5 (Kobayashi et al.)

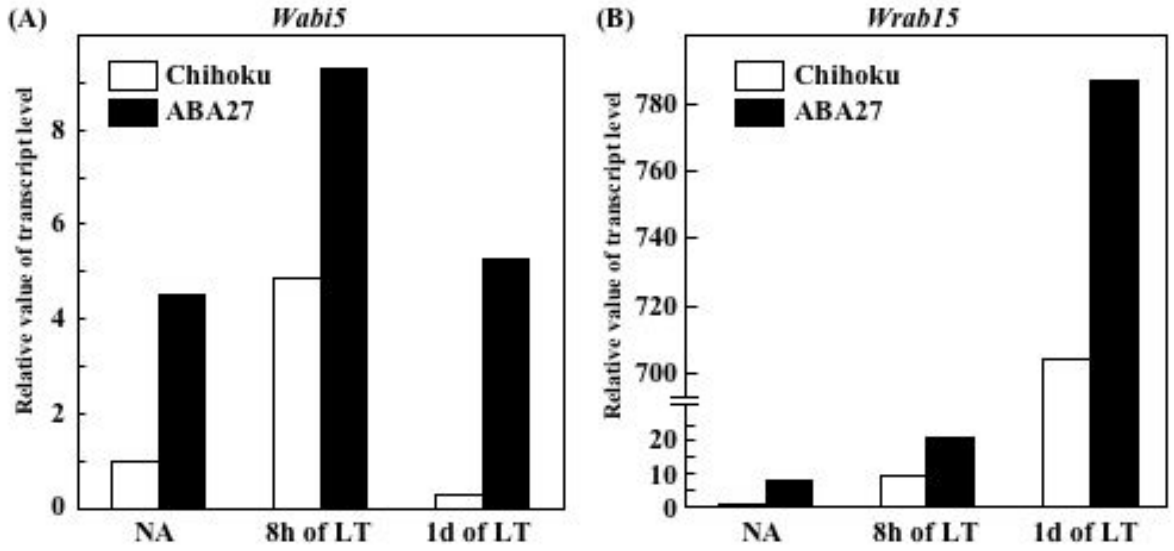

Fig. 6 (Kobayashi et al.)