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Expression patterns of the low temperature responsive genes in a dominant ABA-less-sensitive mutant of common wheat

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Abstract

Abscisic acid (ABA) plays important roles in mediating stress responses and in acquiring desiccation tolerance and dormancy of seeds in plant. To study roles of ABA in cold acclimation and development of freezing tolerance in wheat, expression profiles of *Cor/Lea* and their putative transcription factor (TF) genes were analyzed using a dominant mutation line of common wheat lacking seed dormancy. The mutant line showed less sensitivity to exogenous ABA than the original line as judged by the magnitude of ABA inhibition of seedling growth. But expression analysis of *Cor*/*Lea* and TF genes showed that more transcripts except for *Wrab17* were present in the ABA-treated seedlings of mutant line. In developing caryopses, the same tendency as in the seedlings were observed. The mutant line showed no changes in the cold acclimation ability, but it showed a higher level of freezing tolerance than the original line without cold acclimation. No significant differences were observed in the expression profiles of *Cor*/*Lea* and TF genes during cold acclimation between the two lines. Our results imply the presence of an unknown cold responsive pathway, which may be ABA-dependent and enhances the basal level of freezing tolerance by a dominant mutation in EH47-1.

Abbreviations

ABA, abscisic acid; ABI, ABA-insensitive; AREB, ABA-responsive element binding protein; ABRE, ABA-responsive element; bZIP, basic leucine zipper; CBF, C-repeat binding factor; *Cor*/*Lea*, cold-responsive/late-embryogenesis-abundant; DPA, days post anthesis; DREB, dehydration responsive element binding protein; LT, low temperature; PP2C, protein phosphatase 2C; TF, transcription factor

Introduction

Abscisic acid (ABA) regulates important aspects of plant growth and development, including environmental stress tolerance and seed maturation and dormancy (Leung and Giraudat 1998, Finkelstein et al. 2002). Regulatory mechanisms of

ABA-dependent gene expression have been studied using a *vp1* (*viviparous1*) mutant of maize and *abi* (*ABA-insensitive*) mutants of *Arabidopsis thaliana*, both of which showed reduced seed dormancy and low sensitivity to exogenous ABA for inhibition of germination (Leung and Giraudat 1998). ABA biosynthesis is required for seed maturation and dormancy during seed development, while ABA is synthesized *de novo* mainly in response to drought and high salinity stresses in vegetative tissues (Xiong and Zhu 2003, Shinozaki et al. 2003). Many genes as components of stress signaling pathways are induced by exogenous ABA in *Arabidopsis* and rice (Xiong et al. 2002a, Rabbani et al. 2003). These ABA-responsive genes commonly contain *cis*-elements, i.e. ABRE (ABA responsive element), MYBR (MYB recognition site), MYCR (MYC recognition site) and CRT (C-repeat)/DRE (dehydration responsive element), in their promoter regions (Shinozaki and Yamaguchi-Shinozaki 2000, Shinozaki et al. 2003). In *Arabidopsis*, a bZIP-type transcription factor (TF) ABF (ABRE binding factor)/AREB (ABRE binding protein) binds to ABRE and activates ABA-dependent stress responsive genes (Choi et al. 2000, Uno et al. 2000). ABFs/AREBs are classified into the ABI5-homologous subfamily (Jakoby et al. 2002, Kim et al. 2002), but unlike ABI5 they play regulatory function predominantly in vegetative tissues.

Low temperature (LT) induces and/or enhances expression of a number of *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) genes to promote development of freezing tolerance (Thomashow 1999). Induction of *Cor*/*Lea* expression is partly regulated by CBF (CRT binding factor)/DREB1 (DRE binding protein1) (Shinozaki and Yamaguchi-Shinozaki 2000). In *Arabidopsis*, the CBF/DREB1 mediated pathway belongs to ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000, Shinozaki et al. 2003). *Arabidopsis* mutants, *los5* (*low expression osmotically responsive gene5*)/*aba3* (*ABA deficient3*) and *los6*/*aba1*, however, showed severely reduced *Cor*/*Lea* gene expression under LT and reduced levels of freezing tolerance (Xiong et al. 2001, 2002b), suggesting that LT and ABA regulatory pathways are not completely independent. Several *Cor*/*Lea* genes are in fact responsive to exogenous ABA, and their promoter sequences commonly contain ABRE (Lång and Palva 1992, Shinozaki and Yamaguchi-Shinozaki 2000). Expression of the *ABF*/*AREB* family is responsive to various environmental stresses including LT (Choi et al. 2000), and over-expression

of *ABF3* and *ABF4*/*AREB2* increases the levels of chilling and freezing tolerance (Kim et al. 2004). These findings suggest that the ABF/AREB mediated pathway is involved in cold acclimation and development of freezing tolerance in *Arabidopsis*. Other ABA-signaling components such as ABI1 and ABI5 are also associated with the development of freezing tolerance (Mäntylä et al. 1995, Tähtiharju and Palva 2001, Brocard et al. 2002). The *ABI1* encoding protein phosphatase 2C (PP2C) respond to cold treatment and act as a negative regulator of ABA signaling (Schweighofer et al. 2004).

In wheat and its related species, a number of *Cor*/*Lea* and their TF genes including *CBF*/*DREB* homologs and bZIP-type genes have been isolated and characterized (Thomashow 1999, Kobayashi et al. 2004a). However, information on roles of ABA in the regulation of these genes under LT conditions is still limited. To study roles of ABA in the cold acclimation and development of freezing tolerance, we used an ABA-insensitive, non-dormant mutant line of common wheat, EH47-1. EH47-1 was derived from EMS (ethylmethane sulfonate)-treated seeds of an ABA sensitive and dormant line, 'Kitakei-1354' (Kitakei), and the ABA-insensitive phenotype was due to a single dominant mutation (Kawakami et al. 1997). Embryos of the mutant line lose sensitivity to ABA during the later half of seed maturation process, while embryos of the parental line maintain the sensitivity even after maturity. In this study, we analyzed expression profiles of *Cor*/*Lea* and TF genes in ABA- and LT-treated seedling leaves and developing caryopses. In spite of no alteration of *Cor/Lea* and TF gene expression under LT, EH47-1 showed significantly higher freezing tolerance than Kitakei. In ABA-treated seedling and developing caryopses of EH47-1, however, drastic alterations of ABA-responsive gene expression were observed. These findings suggest that ABA insensitivity contributes to the basal level of freezing tolerance in wheat without affecting cold acclimation process.

Materials and methods

Plant materials and bioassay conditions for ABA sensitivity and freezing tolerance

An ABA-insensitive, non-dormant mutant line EH47-1 of common wheat (*Triticum*

aestivum L.) was derived from EMS-induced mutagenesis of a highly ABA sensitive, dormant and red-grained line Kitakei, which is a winter-type wheat (Kawakami et al. 1997). In bioassay for ABA sensitivity based on post-germination growth, 10 seeds from each line were placed in plastic petri dish (90 mm in diameter and 15 mm in depth) containing two sheets of filter paper (82 mm in diameter) wetted with 6 mL of distilled water or 20 μM ABA solution and incubated at 20˚C in the darkness. On the sixth day, lengths of shoots and primary roots were recorded. Two common wheat cultivars, spring type 'Chinese Spring' (CS) and winter-type 'Mironovskaya 808' (M808), were additionally used in bioassay for ABA sensitivity. For evaluation of freezing tolerance, 7-day-old seedlings of the mutant and the parental line were subjected to bioassay according to Kobayashi et al. (2004b). The whole experiments were repeated three to four times and the data were statistically analyzed.

ABA and LT treatments and RNA extraction

ABA treatment (up to 24 h) was performed by spraying 7-day-old seedlings of CS, M808 and the mutant and the parental line grown under standard conditions (Ohno et al. 2001) with a solution of 20 μM ABA containing 0.1% (w/v) Tween 20. LT treatment (up to 10 days) was given by transferring 7-day-old seedlings from the standard conditions to the cold acclimation condition at 4˚C. After indicated periods, bulk RNA was extracted by guanidine thiocyanate from leaves of 10 seedlings at different time points. At 3-18 DPA (days post anthesis), about 0.5 g of the whole caryopses from the primary and secondary florets of the mutant and the parental line, grown in a greenhouse, were collected from two spikes and ground to a fine powder in liquid nitrogen. Total RNA was extracted using an SDS-Phenol method (Kawakami et al. 1992)

Northern blot and semi-quantitative RT-PCR analyses

Steady state levels of transcripts of eight wheat *Cor*/*Lea* genes (*Wcor14*, *Wcor15*, *Wdhn13*, *Wlt10*, *Wrab17*, *Wrab18* and *Wrab19*) were studied by northern 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. 2003a, Kobayashi et al. 2004b). Northern blot analysis was according to Kobayashi et al. (2004b). For RT-PCR analysis, 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 Table 1 were designed based on the nucleotide sequences of *Cor*/*Lea*, *WP5CS* (accession number AB193551), *Wcbf2* (AB178166), *Wdreb2* (AB193608), *Wlip19* (AB193552) and *Wabi5* (AB193553) (Takumi et al. 2003b, Kobayashi et al. 2004a, 2004b, Kume et al. 2005). Expression patterns of three wheat TF genes, *TaABF* (Johnson et al. 2002), *TaVP1* (Nakamura and Toyama 2001) and *EmBP-1* (Guiltinan et al. 1990), and two EST clones encoding putative PP2C, whyd2h10 (BJ306288) and whf1b10 (BJ245536 and BJ251421), were additionally studied. 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. The PCR products were separated by electrophoresis through 1.2% agarose gel and stained with ethidium bromide.

Differential display with RAPD primers

Differential display method (Liang and Pardee 1992) was performed using first strand cDNA synthesized from total RNA samples of non-acclimated Kitakei and EH47-1 seedling leaves as templates. A total of 201 random 10-mer primers (Operon Technologies, Inc., California, USA) were used for identification of transcripts abundant in EH47-1. PCR amplification was initiated at 95˚C for 1 min, followed by 40 cycles of 94˚C for 1 min, 40˚C for 1min, 72˚C for 1 min, and terminated at 72˚C for 1 min. After amplification, the resulting fragments were separated on a 1.5% agarose gel. These fragments were cloned into pGEM-T Easy vector (Promega, WI, USA) and sequenced. Sequence analysis was according to Kobayashi et al. (2004b). Expression analysis of these clones was studied by RT-PCR using gene-specific primers listed in Table 1.

Results

ABA sensitivity evaluated by bioassay and *Cor*/*Lea* transcript accumulation in CS and M808

ABA sensitivity of two wheat cultivars, CS and M808, which show significantly different levels of freezing tolerance after cold acclimation (Ohno et al. 2001,

Kobayashi et al. 2004b), was evaluated based on inhibition of seedling growth by exogenous ABA. Exogenous ABA at a concentration of 20 μM greatly reduced shoot and root lengths in both cultivars (Fig. 1a, b). The magnitude of inhibition estimated by the relative growth rate (% growth in the presence of ABA relative to growth in the absence of ABA) was greater in M808 than in CS (Fig. 1c). The result indicated that the freezing tolerant winter cultivar M808 was more sensitive to exogenous ABA than the freezing susceptible spring cultivar CS at the seedling stage.

To further examine ABA sensitivity of the cultivars, we studied transcript accumulation of four ABA-responsive genes (*Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*) after application of 20 μM ABA. All these genes were temporarily induced by exogenous ABA and the amount of their transcripts reached maximum levels within 2 h under the standard temperature condition in both cultivars (Fig. 1d, e). M808 accumulated more abundant *Cor*/*Lea* transcripts than CS after the ABA treatment. The result confirmed that ABA sensitivity evaluated by *Cor*/*Lea* gene expression was higher in M808 than in CS, agreeing with the bioassay data.

ABA sensitivity evaluated by bioassay and expression patterns of ABA-responsive genes in Kitakei and EH47-1

ABA sensitivities of Kitakei and EH47-1 were also compared in post-germination growth (Fig. 2a-c). Shoots of Kitakei and EH47-1 were similar in length under the ABA-free condition, and exogenous ABA inhibited the shoot growth in both lines (Fig. 2a). Root growth was also inhibited by ABA (Fig. 2b). Root length was significantly longer in EH47-1 than that in Kitakei under the ABA-treated condition (Fig. 2b). The magnitude of ABA inhibition of shoot and root growth were lower in EH47-1 than those in Kitakei (Fig. 2c). These results indicated that the ABA sensitivity of EH47-1 was less than that of Kitakei during post-germination growth.

Wdhn13 and *Wrab17* were temporarily induced by exogenous ABA in EH47-1 and Kitakei (Fig. 2d, e). The amount of *Wrab17* transcripts reached maximum levels within 2 h, while a maximum level of *Wdhn13* transcript reached 5 h after ABA treatment in both lines (Fig. 2e). The higher accumulation of *Wrab17* transcript in Kitakei agreed with the observation that ABA sensitivity of the mutant was lower than that of Kitakei. At the 2nd h after ABA treatment, the accumulation

of *Wdhn13* transcript was higher in Kitakei than in EH47-1, but the accumulation level increased drastically at the 5th h in EH47-1 and was higher than in Kitakei (Fig. 2e). Expression analysis of other ABA-responsive genes including *Wrab15*, *Wrab18*, TF genes (*Wdreb2*, *Wlip19* and *Wabi5*) and putative PP2C encoding genes (whyd2h10 and whf1b10) was studied by RT-PCR. *Wdreb2* encode the EREBP (ethylene responsive element binding protein)/AP2 (APETALA2)-type TF (Takumi et al. 2003b) and is responsive to exogenous ABA (unpublished data). Both *Wlip19* and *Wabi5* encode the bZIP-type TFs and show ABA-responsive expression (Kobayashi et al. 2004a). Two wheat EST clones, whyd2h10 and whf1b10, are selected from EST library of spikelet at late flowering and spike at flowering date, respectively (http://shigen.lab.nig.ac.jp/wheat/komugi/ests/tissueBrowse.jsp) and these deduced proteins shows high homology with rice ABI2 (AC130728-11) and maize PP2C (AY621066), respectively. The results of RT-PCR indicated that these genes showed different expression patterns from that of *Wdhn13* and *Wrab17*, and were disagree with the line difference in ABA sensitivity. The transcript accumulation of *Wdreb2*, *Wlip19*, *Wabi5* and *Wrab15* was maintained high levels during 0–24 h in EH47-1 (Fig. 2e). The expression of *Wrab18*, whyd2h10 and whf1b10 reached to a maximum level within 2 h of ABA treatment and maintained high levels up to 24 h in EH47-1 (Fig. 2e). In Kitakei, however, no increases or down-regulation by ABA treatment in the transcript accumulation of these genes were observed during this period (Fig. 2e). These expression profiles in Kitakei were different from those in CS and M808.

Freezing tolerance after cold acclimation

Developmental time-course of freezing tolerance was monitored under the LT condition in EH47-1 and Kitakei. Cold acclimation at 4˚C for 5 to 35 days significantly increased the level of freezing tolerance in both lines (Fig. 3), indicating that the mutation did not impair the cold acclimation ability of EH47-1. EH47-1 showed much higher levels of freezing tolerance than Kitakei throughout the tested period of cold acclimation. It was however noted that EH47-1 showed a significantly higher level of freezing tolerance than Kitakei in the non-acclimated seedlings, indicating that the higher freezing tolerance was due to the elevated basal level of freezing tolerance independent of cold acclimation.

LT-responsive expression of *Cor*/*Lea*, PP2C-like genes and *WP5CS*

Expression patterns and transcript levels of seven *Cor*/*Lea* genes (*Wcor14*, *Wcor15*, *Wdhn13*, *Wlt10*, *Wrab15*, *Wrab17* and *Wrab18*) genes were compared between EH47-1 and Kitakei during the cold acclimation period up to 10 days. The cold acclimation induced expression of all *Cor*/*Lea* genes in both lines, and the amount of transcripts reached maximum levels within 1-5 days of LT treatment depending on the genes (Fig. 4a, b). These expression patterns in Kitakei and EH47-1 resembled those in CS and M808 (Ohno et al. 2001, Kobayashi et al. 2004b) and were correlated with the development of freezing tolerance. However, no differences were observed between Kitakei and EH47-1 in the *Cor*/*Lea* expression profiles.

LT responsiveness of two PP2C-like genes (whyd2h10 and whf1b10) and wheat *P5CS* (Δ^1-pyrroline-5-carboxylate synthetase, which involved in the proline biosynthesis) homolog, *WP5CS* (Kobayashi et al. 2004a), was also studied by RT-PCR (Fig. 4b). These genes were rapidly induced by LT treatment and reached maximum levels within 2-24 h of LT treatment in both lines. We found that the level of LT-induced expression of these genes was nearly equal in both lines.

The result of bioassay for freezing tolerance showed that the basal level of freezing tolerance of EH47-1 was superior to that of Kitakei (Fig. 3), therefore transcript accumulation levels of *Cor*/*Lea*, PP2C-like and *WP5CS* genes were compared between the two lines in the non-acclimated seedlings by RT-PCR (right panels in Fig. 4a, b). The accumulation levels of *Wlt10* and *Wrab15* transcripts were higher in EH47-1 than in Kitakei, and these observations agreed with the result of bioassay. But the accumulation pattern of *Wrab18* transcript was contrary to that of *Wlt10* and *Wrab15*, which was against the bioassay data.

LT responsive expression of putative TF genes

Expression profiles of four putative TF genes (*Wcbf2*, *Wdreb2*, *Wlip19* and *Wabi5*) in response to LT were compared between cold-acclimated seedlings of EH47-1 and Kitakei. Wheat *CBF*/*DREB1* homolog, *Wcbf2*, encode the EREBP/AP2-type TF and is ABA-independent (Kume et al. 2005). In semi-quantitative RT-PCR analysis, some low amounts of TF transcripts were detected in the untreated seedlings of both lines (Fig. 5). The amount of *Wcbf2* transcript showed a temporal

drop and increased again to a high plateau level. The expression pattern of *Wlip19* was similar to that of *Wcbf2*. The expression pattern of *Wdreb2* and *Wabi5* was different from that of *Wcbf2* and *Wlip19* but similar to each other; the transcripts accumulated to maximum levels within 2 to 8 h of LT treatment and then decreased. No differences were observed in the expression patterns of TFs between the two lines during cold acclimation periods. These TF gene expression patterns thus did not explain the difference of freezing tolerance between Kitakei and EH47-1.

TaABF encoding a bZIP-type TF is seed specific (Johnson et al. 2002), and *TaVP1* functioning in mature embryos is a wheat ortholog of maize *VP1* and *Arabidopsis ABI3* (Nakamura and Toyama 2001). We additionally studied expression profiles of these two TF genes during the cold acclimation period. Although very little amounts of transcripts were detected, their LT responsiveness was indicated in both lines, and their expression patterns resembled to those of *Wdreb2* and *Wabi5*.

Detection of genes increasing expression levels in EH47-1 under the normal condition by differential display

To further study the enhancement of the basal level of freezing tolerance in EH47-1 under the normal condition, identification of genes specifically expressing or increasing the expression level in EH47-1 was carried out by the differential display method. In this analysis, cDNA synthesized from non-acclimated seedlings and 201 random 10-mer primers were used. The PCR was performed with only one primer or mixed two primers and repeated two times. Total 185 transcripts were identified and included 8 fragments increasing transcript accumulation level in EH47-1, but EH47-1 specific fragment was not detected. These 8 fragments were isolated by RT-PCR and sequenced. The primer sets for amplification, length of these fragment and results of BLAST search are shown in Table 2. Based on the sequence data, gene-specific primer sets of each fragments were designed and used for expression analysis by RT-PCR. The results indicated that transcript accumulation of three fragments (designated as TW1, TW4#18 and TW4#21) was higher in EH47-1 than in Kitakei under the normal condition (Fig. 6a), but no differences were observed between the lines in the other genes expressions. These three fragments responded to exogenous ABA (Fig. 6b). The transcript accumulation of TW1 slightly increased in Kitakei but was down-regulated in

EH47-1. TW4#18 was maintained the high level in EH47-1, while in Kitakei the accumulation decreased to a low level, which patterns were similar to that of *Wdreb2* and *Wlip19* (Fig. 2d). As to TW4#21, the levels of transcript accumulation reduced in both lines after ABA treatment (Fig. 6b). TW1 and TW4#18 expression also responded to the LT treatment (Fig. 6c). The expression of TW1 gradually increased during cold acclimation in both lines. TW4#18 showed unique expression pattern comparing with those of other genes during cold acclimation; the transcripts accumulated to maximum levels within 30 min of LT treatment and then increased again to a high level.

Expression of *Cor*/*Lea* and their putative TF genes in developing caryopses

To further evaluate ABA sensitivity of the mutant line, expression profiles of the ABA responsive TF genes were studied in developing caryopses. More transcripts of *Wdreb2* were detected in Kitakei than in EH47-1 at 3 DPA (Fig. 7a). The expression profile of *Wabi5* was similar to that of *Wdreb2*, but contrary to *Wdreb2*, more transcripts of *Wabi5* were accumulated in EH47-1 than in Kitakei throughout the experimental period until 18 DPA (Fig. 7a). It was reported that transcripts of *TaABF* showed accumulation during seed maturation and dormancy acquisition (Johnson et al. 2002). We thus studied expression of *TaABF* in the developing caryopses and found that EH47-1 accumulated more transcript than Kitakei during 3-7 DPA (Fig. 7a). In EH47-1, the amount of *TaABF* transcript decreased during 10-14DPA but increased again at 18 DPA. The expression pattern of *TaVP1* resembled to that of *TaABF* in both lines (Fig. 7a). *EmBP-1* encoding a bZIP-type TF, which binds to the *cis*-elements Em1a and Em1b with ACGT ABRE-core sequences (Guiltinan et al. 1990, Niu and Guiltinan 1994, Razik and Quatrano 1997), increased the amount of its transcript in EH47-1 towards the later stage (18 DPA), while a much lower transcript level was observed in Kitakei throughout the tested period (Fig. 7a). All examined genes except for *Wdreb2* therefore showed higher expression levels in EH47-1 than in Kitakei during this early phase of seed maturation. The transcript accumulation of two PP2C-like genes, TW1, TW4#18 and TW4#21 was also detected in the developing caryopses and these levels except for TW4#21 were higher in EH47-1 than in Kitakei (Fig. 7a). The high accumulation levels of whyd2h10, TW1 and TW4#18 were observed at 3-7 DPA. The expression profiles of whf1b10 and TW4#21 resembled that of *EmBP-1* but the

increase was observed in both lines contrary to *EmBP-1*.

Expression patterns of five *Cor*/*Lea* genes in the developing caryopses were studied by northern blot analysis (Fig. 7b). No transcripts of *Wdhn13* and *Wrab17* were detected by northern blot and RT-PCR in both lines (data not shown). Transcripts of ABA-responsive *Cor/Lea* genes, *Wrab15*, *Wrab18* and *Wrab19*, became detectable within 10 DPA in EH47-1, while the transcript accumulation delayed in Kitakei until 14 DPA. In both lines, the levels of transcripts increased until 18 DPA.

Discussion

The expression profiles of wheat *Cor*/*Lea* and their putative TF genes are correlated with the time-dependent development of freezing tolerance after cold acclimation in two wheat cultivars, CS and M808 (Takumi et al. 2003b, Kobayashi et al. 2004b). M808 develops much higher levels of freezing tolerance than CS throughout cold acclimation periods (Ohno et al. 2001, Kobayashi et al. 2004b). During the cold acclimation periods, M808 accumulates more *Cor*/*Lea* transcripts and corresponding proteins (Ohno et al. 2001, 2003, Kobayashi et al. 2004b). The cultivar difference in the level of freezing tolerance therefore likely results from different levels of transcript and protein accumulation of *Cor*/*Lea* genes. ABA-responsive expression of *Cor*/*Lea* and TF genes also reflected the difference of ABA sensitivity between CS and M808 (Kobayashi et al. 2004b, Fig. 1). In *Arabidopsis*, *abi1*, *los6*/*aba1* and *los5*/*aba3* mutants showed reduced levels of cold-induced and ABA-dependent accumulation of *Cor*/*Lea* transcripts compared to the wild-type, which appeared to be associated with the reduced freezing tolerance (Mäntylä et al. 1995; Xiong et al. 2001, 2002b). These results suggest that ABA sensitivity is related to development of freezing tolerance, i.e., the higher the sensitivity the higher the freezing tolerance. Contrary to the expectation, however, an ABA-less-sensitive mutant line of wheat, EH47-1, showed a higher level of freezing tolerance than the wild-type Kitakei (Fig. 3). On the other hand, no differences were observed in the expression profiles of *Cor*/*Lea* and TF genes between Kitakei and EH47-1 during cold acclimation period (Fig. 4, 5), though some of *Cor*/*Lea* and TF genes such as *Wrab15*, *Wrab18*, *Wdreb2*, *Wlip19* and

Wabi5 showed the higher expression levels in EH47-1 than in Kitakei with ABA treatment (Fig. 2e), indicating that ABA signaling seems to be not involved in cold acclimation. In *Arabidopsis*, loss-of-function analysis indicated that AtPP2CA act as a negative regulator in the cold acclimation through an ABA-dependent pathway (Tähtiharju and Palva 2001). Freezing tolerance is improved due to increased *Cor*/*Lea* expression levels in *AtPP2CA*-antisense transgenic plant. The expression analysis of wheat PP2C-like genes showed no differences in the expression patterns between Kitakei and EH47-1 during testes periods, suggesting that the enhancement of freezing tolerance in EH47-1 is not contributed by these putative PP2Cs in cold acclimation pathway. Taken together, these findings indicate that a single dominant mutation in EH47-1 seems not to be associated with cold acclimation including the *Cor*/*Lea* gene expression patterns and that the mutation causes a significant improvement of the basal level of freezing tolerance without affecting the cold acclimation ability and the expression profiles of *Cor*/*Lea* and TFs. The ABA sensitivity might be a determinant of the basal level of freezing tolerance in wheat.

Some of *Cor*/*Lea* genes, such as *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*, are activated by ABA in the seedlings of CS and M808 (Tsuda et al. 2000, Kobayashi et al. 2004b, Fig. 1d, e). Changes in expression of these genes in response to exogenous ABA (Fig. 1d, e) agreed with the differences in ABA sensitivity of these cultivars as measured by root and shoot elongation (Fig. 1a-c). EH47-1 was isolated as wheat ABA-insensitive mutant line during later seed maturation (Kawakami et al. 1997) and showed less ABA sensitivity than the wild-type Kitakei during post-germination growth (Fig. 2a-c). ABA responsiveness of *Wrab17* in Kitakei and EH47-1 was similar to that in CS and M808 and the expression pattern agreed with ABA sensitivity in the both lines (Fig. 2d, e). But expression profiles of other ABA responsive *Cor*/*Lea* and TF genes were different from that of *Wrab17*. In Kitakei, the transcript accumulation of these genes was down-regulated by exogenous ABA treatment, while the accumulation of transcripts was maintained during the tested periods in EH47-1 (Fig. 2e). We also observed the expression profiles of ABA-responsive genes in the developing caryopses during 3-18 DPA. The expression profiles during this stage show the effect of endogenous ABA on the ABA-responsive gene expressions. All examined *Cor*/*Lea* and TF genes except for *Wdreb2* showed higher expression levels in EH47-1 than in Kitakei (Fig. 7).

The ABA-induced transcript accumulation of these genes tends to be high levels in both developing caryopses and seedlings of EH47-1, which are in contrast to the ABA sensitivity of seedlings and disagrees with the loss of ABA sensitivity in EH47-1 during the seed maturation (Kawakami et al. 1997). These results indicate that the ABA-responsive gene expression patterns in Kitakei are different from those in other wheat accessions such as CS and M808, and the down-regulation of many ABA responsive genes in Kitakei is recovered by a mutation in EH47-1. Kitakei is a highly ABA sensitive and dormant cultivar (Kawakami et al. 1997), and thus the characteristics of Kitakei might cause the unusual gene expression patterns.

Higher freezing tolerance was observed even in non-acclimated seedlings of EH47-1 compared with those of Kitakei (Fig. 3). The gene expression levels under non-acclimated condition showed that the transcripts of *Wlt10* and *Wrab15* accumulated more in EH47-1 than in Kitakei, but the accumulation level of *Wrab18* transcript was higher in Kitakei than in EH47-1 (Fig. 4). These observations are not enough to explain the enhancement of freezing tolerance in EH47-1. We further demonstrated the differential display using cDNA synthesized from the RNA extracted from non-acclimated seedlings of the both lines and identified new three cDNA fragments abundantly expressed in EH47-1 (Fig. 6, Table 2). The TW4#18 expression was down-regulated by exogenous ABA treatment in Kitakei, while a high accumulation level was observed in EH47-1 (Fig. 6b). In the developing caryopses, the accumulation levels of TW1 and TW4#18 transcripts were also higher in EH47-1 than in Kitakei (Fig. 7). The LT-responsive expression of TW1 and TW4#18, however, showed nearly equal levels between Kitakei and EH47-1 (Fig. 6c). The TW1 and TW4#18 expression profiles were similar to those of other examined genes. That is, even though Kitakei and EH47-1 showed the differential expression levels of many of examined genes under non-acclimated- and ABA-treated conditions, no significant difference was observed under the LT conditions. Although it is not clear about relationships between the reduced ABA sensitivity and the ABA responsive gene expression profiles in EH47-1, it is suggested that the drastic changes of expression profiles of some ABA-responsive *Cor*/*Lea* and TF genes by the dominant mutation might contribute the increase of freezing tolerance ability independently of the cold acclimation. The altered gene expression patterns might cause increase of the basal level of freezing tolerance in EH47-1. Further studies are required to clarify the mutation locus in EH47-1 and

gene(s) resulting in high ABA-sensitivity and dormancy in Kitakei.

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Table 1 Gene-specific primer sets used in this study.

Gene (tissue)	Forward (F) and reverse (R) primer sequences	Product length (bp)	annealing temp
Wcor14	F: 5'-TTCTTCTTCCGTGCTGCTCG-3'	415	62˚C
	R: 5'-TTTGCTCACATCCTCGACCG-3'		
Wcor15	F: 5'-ACAACCTACCCTACCCTACC-3'	666	55˚C
	R: 5'-TTTCTTTATTGCGTTTGACA-3'		
Wdhn13	F: 5'-TGCGGGATCCCGGGAGCACCAGGGGCA-3'	397	68˚C
	R: 5'-TCCCCCGGGGGATTAGTGCTGCTCAGGC-3'		
Wlt10	F: 5'-CAGAGCCTCCCAGTTAGCAATG-3'	410	67˚C
	R: 5'-CAGACGCTCATCAAGGAAGGAA-3'		
Wrab15	F: 5'-GGGATTCTTTCTTCGCGTCT-3'	507	62˚C
	R: 5'-AGCCTCGGCCTTGAGTATGT-3'		
Wrab17	F: 5'-TCCATCAACTTCAAAAATG-3'	546	56˚C
	R: 5'-TGTGGTCTTCTTGGTGGCA-3'		
Wrab18	F: 5'-TCGATTCATCCAAGCCAGAG-3'	658	62˚C
	R: 5'-ACCAAACGAGTAAAGGAAGCAA-3'		
WP5CS	F: 5'-CCAAGTGAACCTTCATCGAA-3'	1038	52˚C
	R: 5'-AACAAGCAACGTCTCCATTG-3'		
Wcbf2	F: 5'-CTCAAACCAACCTGCAAC-3'	790	57˚C
	R: 5'-AAGCGTTTTTGACATTACATTA-3'		
Wdreb2	F: 5'-AAGAAAACAGGCGACAAGAT-3'	1249	58˚C
	R: 5'-ACGAAGCACAAAAAACTAGC-3'		
Wlip19	F: 5'-TCCCACTCCTTCTCCGTCGC-3'	676	62˚C
	R: 5'-CGCCGTGGCATGACTTGTCT-3'		
Wabi5	F: 5'-GAGGGGGTCATGGACTTCAG-3'	1228	61˚C
	R: 5'-GCCTACAGGTCAGCGGTCTC-3'		
EmBP-1	F: 5'-CAGGCGCACGCGGAGTGG-3'	404	63˚C
	R: 5'-GCGGATGATGATGAGCCCTTCTGA-3'		
TaABF (leaf)	F: 5'-GAACCACACACACCACAACCAC-3'	1363	60˚C
	R: 5'-TGACATTCGGGTCCTTATGGTT-3'		
TaABF (seed)	F: 5'-CTGACGCTGGACGAGCTGCA-3'	526	63˚C
	R: 5'-ACATCATTGGGCCAGGCTGC-3'		
TaVP1 (leaf)	F: 5'-CGCGGCACTCACAGGGGGAC-3'	1951	65˚C

	R: 5’-CCGTCTTCTCCGCCCGCCTT-3’		
TaVP1 (seed)	F: 5’-CGCGGCACTCACAGGGGGAC-3’	611	66˚C
	R: 5’-TCGACGGAGGCGGATGCTGC-3’		
whyd2h10	F: 5’-CAAAGGATGACGAGTGTCTTA-3’	473	56˚C
	R: 5’-GCGGAATTTCGTAATCTGTA-3’		
whf1b10	F: 5’-TATGCGAGCAGTCTAGGGAA-3’	811	56˚C
	R: 5’-CGAGGAGGAATGTTCACTTG-3’		
TW1	F: 5’-CTCCAAAACCCTCGCCTCAC-3’	518	57˚C
	R: 5’-CCAATCCAGTTCATGCGGTG-3’		
TW4#18	F: 5’-ATTGAAGATGCTAGGTGTCG-3’	581	58˚C
	R: 5’-GGTGGATCTAAAACAGTGCA-3’		
TW4#21	F: 5’-ACCATTTGTTACGTCCCATC-3’	516	56˚C
	R: 5’-CTACAGCACAAATTGGCAAG-3’		
ubiquitin	F: 5’-GCATGCAGATATTTGTGAA-3’	226	50˚C
	R: 5’-GGAGCTTACTGGCCAC-3’		

Table 2 BLAST analysis of three transcripts identified by the differential display method with RAPD primers.

Name	Primer set	Length (bp)	Gene or product name (similar gene)	Accession no.
TW1	OPT-01/OPW-01	649	whh21m05 (putative acetyltransferase)	BJ259669
TW4#18	OPT-04/OPW-04	647	Rice hypothetical protein P0702E04.20-1	Q6YZD8
TW4#21	OPT-04/OPW-04	636	not detected	

Figure legends

Figure 1. ABA sensitivity evaluated by bioassay and *Cor*/*Lea* transcript accumulation in CS and M808. Lengths of shoots (a) and primary roots (b) were measured after 6 days. The relative growth (growth with ABA/growth without ABA) was calculated to evaluate the magnitude of inhibition by ABA treatment (c). Seeds (n=10) were germinated with and without 20 μM ABA at 20˚C for 6 days under the darkness. The experiments were performed four times. The small bars represent standard errors. One and two asterisks indicate stational significance at the 5% and 1% level (Student's *t*-test), respectively. (d) Time-course of *Wdhn13* transcript accumulation after ABA treatment in CS and M808. (e) Comparison of the *Wrab17*, *Wrab18* and *Wrab19* transcript accumulation in CS and M808. Seedlings were treated with 20 μM ABA and RNA extracted at the indicated time points. Northern blots were probed with ^{32}P-labelled cDNAs of the *Cor*/*Lea* genes. rRNAs are used as a control.

Figure 2. ABA sensitivity evaluated by bioassay and transcript accumulations of ABA-responsive genes in Kitakei and EH47-1. Comparison of shoot length (a), root length (b) and magnitude of ABA inhibition of seedling growth (c) between Kitakei and EH47-1. (d) Comparison of the *Wdhn13* and *Wrab17* transcripts accumulation after 20 μM ABA treatment in seedlings of Kitakei and EH47-1. (d) Time-course of the transcripts accumulation of *Cor*/*Lea*, TF (*Wdreb2*, *Wlip19* and *Wabi5*) and PP2C-like (whyd2h10 and whf1b10) genes in Kitakei and EH47-1. Northern blots (d) were probed with ^{32}P-labelled cDNAs of the *Cor*/*Lea* genes. RT-PCR (e) was performed using the gene-specific primer sets. Cycle numbers of PCR are indicated at the right sides of electrophoregrams. rRNAs and ubiquitin gene (*Ubi*) are used as controls for northern blot and RT-PCR, respectively.

Figure 3. Developmental time-course of freezing tolerance after cold acclimation in Kitakei and EH47-1. Bioassay was performed under the standard assay conditions; 4˚C for the indicated period for cold acclimation and –15˚C for 6 h for freezing. Data are represented as means±standard errors (n=3-4 per time point). An asterisk indicates statistical significance at the 5% level (Student's

t-test). Open and black bars indicate Kitakei and EH47-1, respectively.

Figure 4. Expression profiles of *Cor*/*Lea*, PP2C-like and *WP5CS* genes in seedling leaves of Kitakei and EH47-1 under the LT condition. Transcript accumulation was monitored for 10 days by northern blot (a) or RT-PCR analysis (b), showing in the left and center panels. Comparison of the transcript accumulation in non-acclimated seedlings of Kitakei and EH47-1 is shown in the right panels by RT-PCR. Northern blots were probed with ^{32}P-labelled cDNAs of the *Cor*/*Lea* genes. RT-PCR was performed using the gene-specific primers. Cycle numbers of PCR are indicated at the right sides of electrophoregrams. rRNAs and the ubiquitin gene (*Ubi*) are used as controls for northern blot and RT-PCR, respectively.

Figure 5. Transcript accumulation of TF genes in the seedling leaves of EH47-1 and Kitakei during cold acclimation. Transcript levels of the TF genes were monitored for 10 days by semi-quantitative RT-PCR analysis. The ubiquitin gene (*Ubi*) was used as a control. Cycle numbers of PCR are indicated at the right sides of electrophoregrams.

Figure 6. Expression analysis of cDNA clones isolated by differential display of RAPD. (a) Comparison of the transcript accumulation in non-acclimated seedlings of Kitakei and EH47-1. (b) Time-course of the transcript accumulation after ABA treatment. (c) Transcript levels of the genes during cold acclimation period were monitored for 10 days. RT-PCR was performed using the gene-specific primers. Cycle numbers of PCR are indicated at the right sides of electrophoregrams. The ubiquitin gene (*Ubi*) was used as a control.

Figure 7. Transcript accumulation of ABA-responsive genes in the developing caryopses of EH47-1 and Kitakei during 3-18DPA. Northern blot (a) and RT-PCR analysis (b) were conducted to monitor the transcript accumulation. rRNAs and ubiquitin gene (*Ubi*) were used as controls. Cycle numbers of PCR were indicated at the right sides of electrophoregrams.

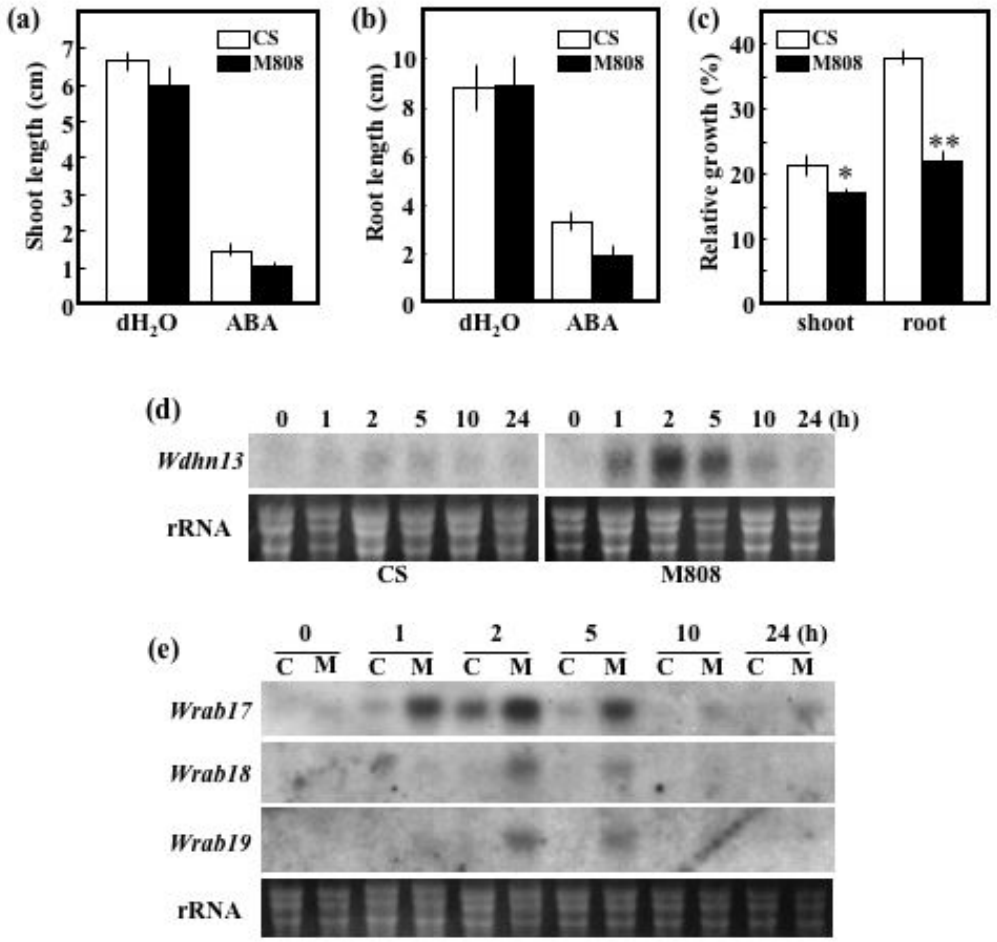
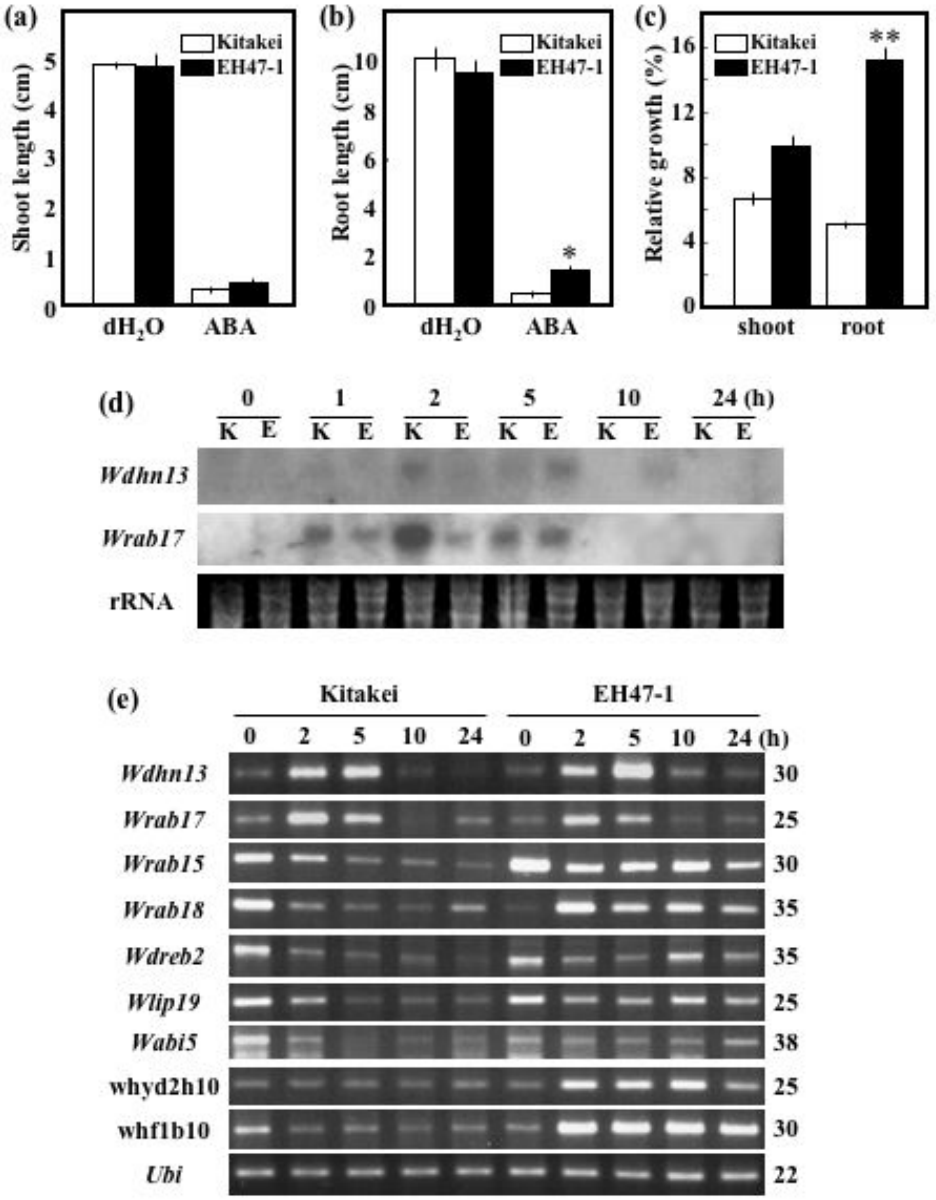


Fig. 1 (Kobayashi et al.)



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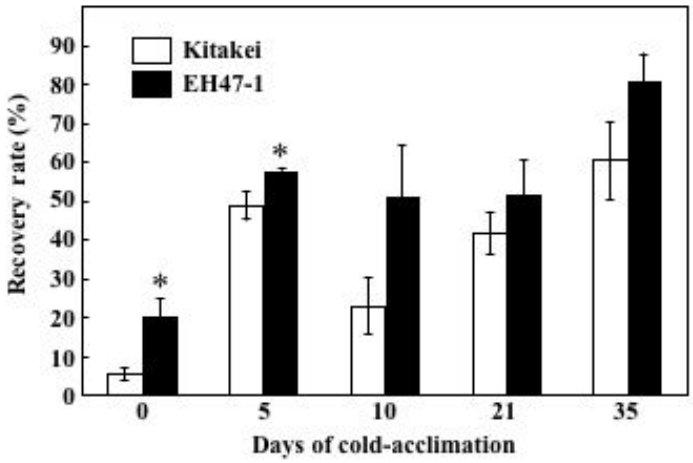

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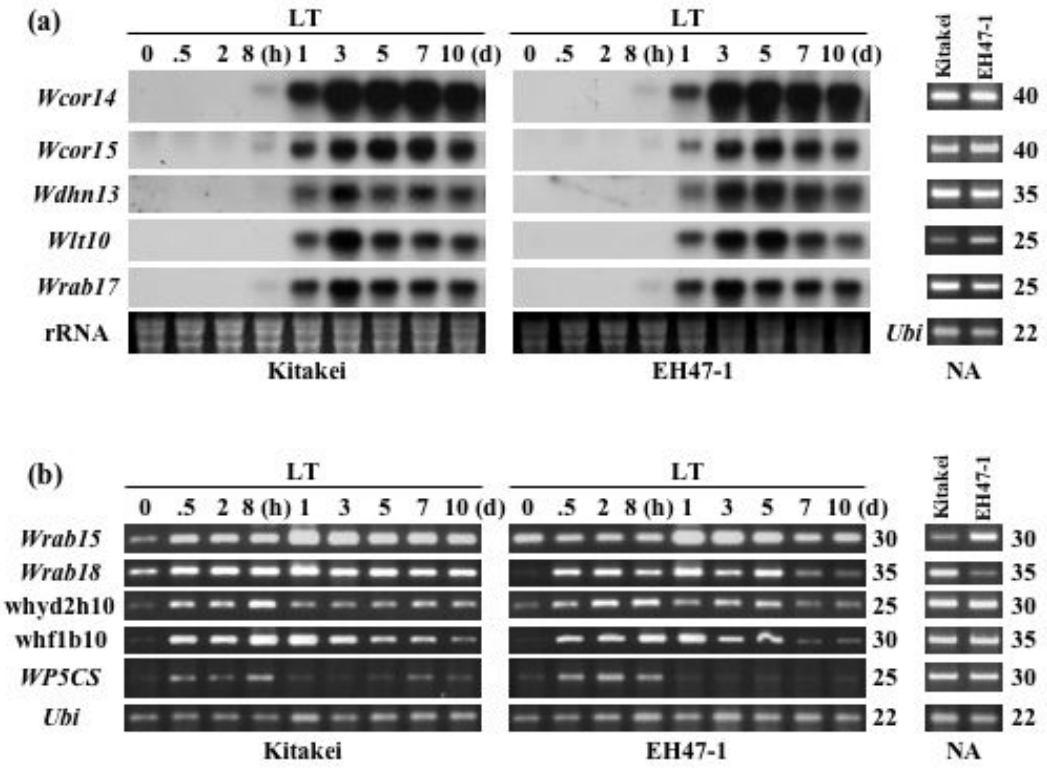

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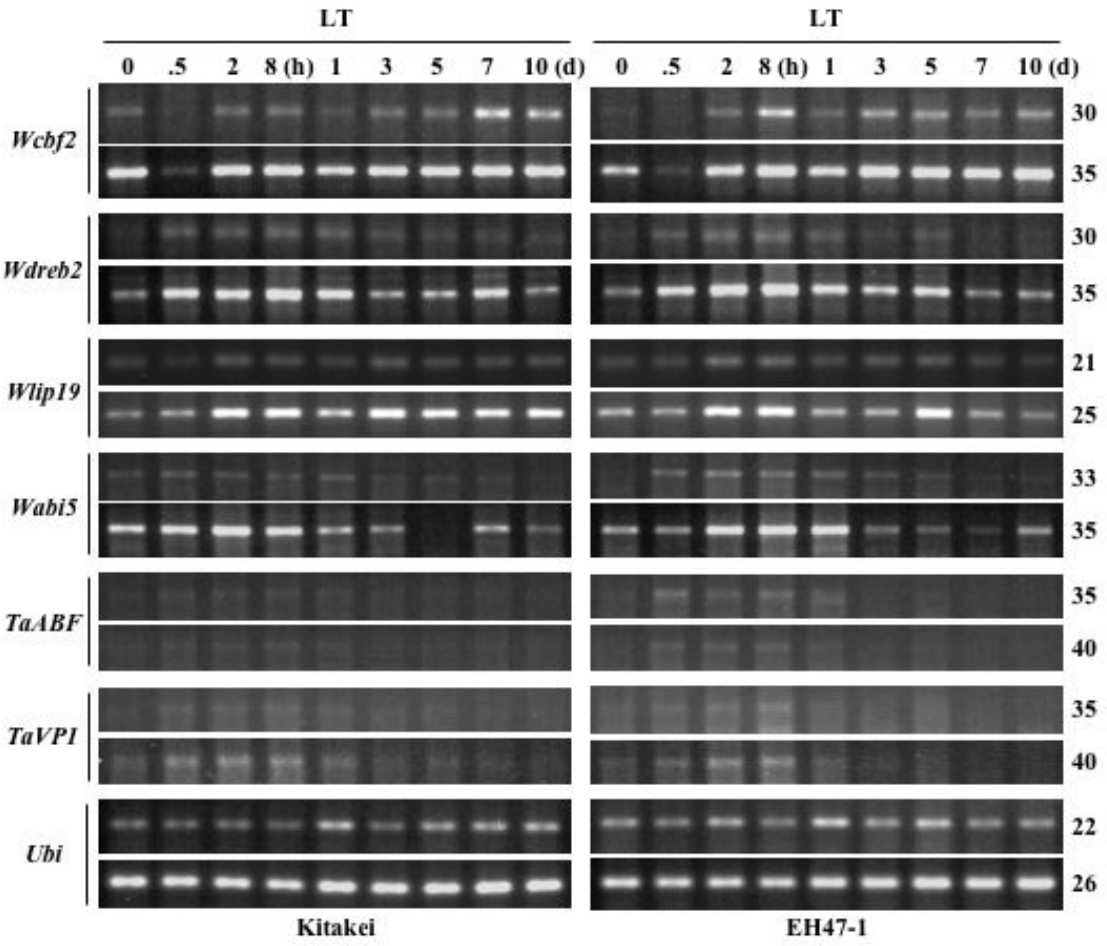

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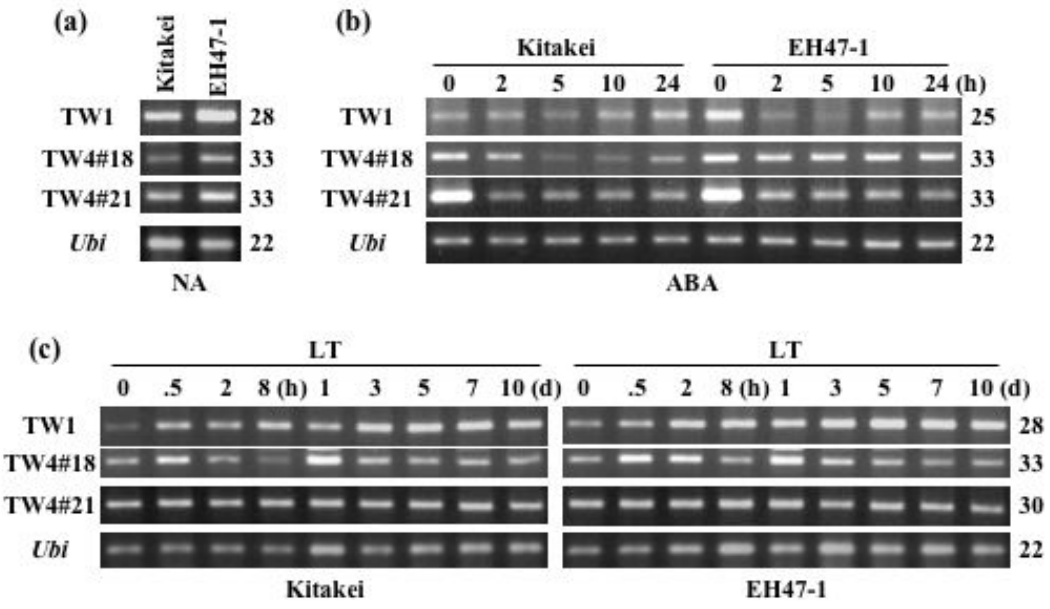

Fig. 6 (Kobayashi et al.)

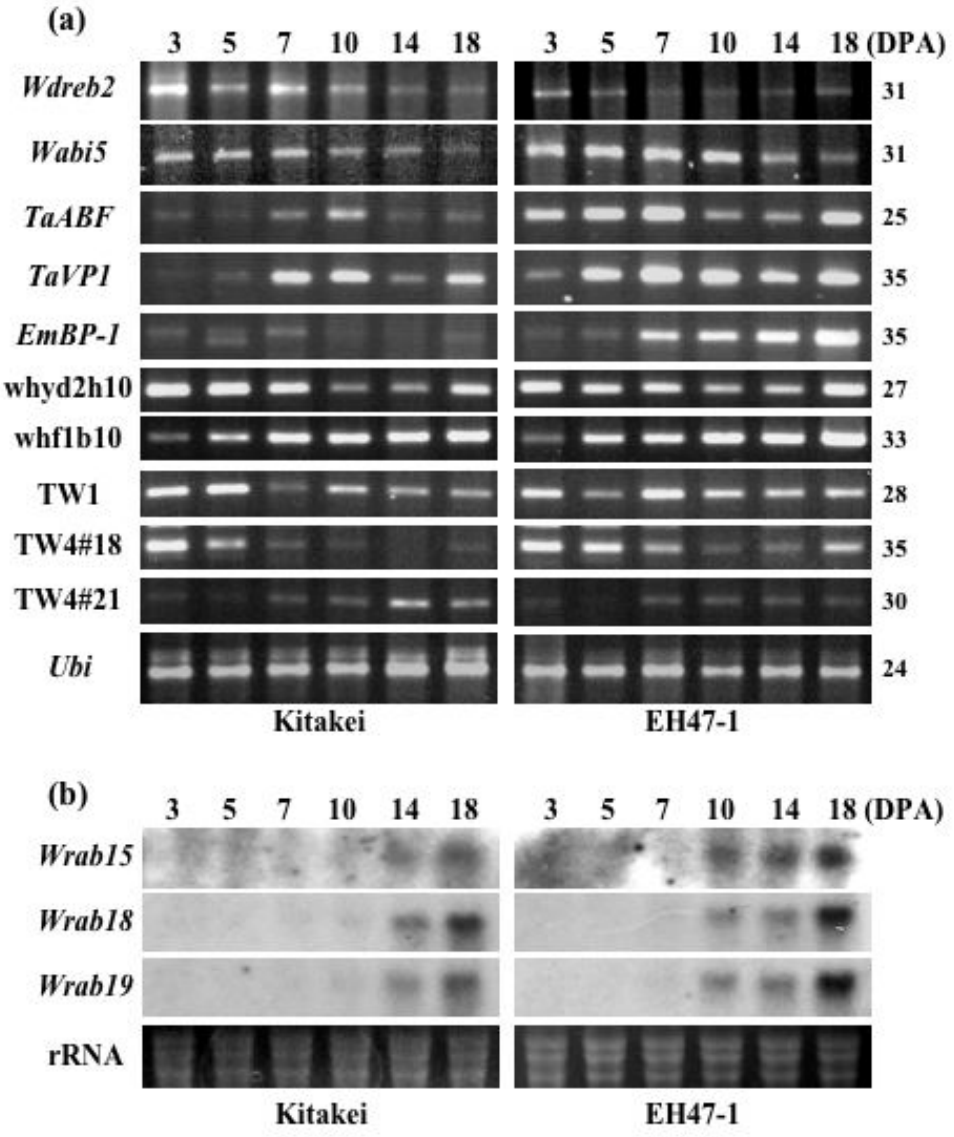

Fig. 7 (Kobayashi et al.)