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Transcriptional activation of *Cor*/*Lea* genes and increase in abiotic stress tolerance through expression of a wheat *DREB2* homolog in transgenic tobacco

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Abstract *Wdreb2*, previously isolated as a *DREB2* homolog, is expressed in wheat seedlings under abiotic stresses, such as cold, drought, and high salinity, and following treatment with exogenous ABA. In the present study, we generated transgenic tobacco plants expressing *Wdreb2* to clarify roles of *Wdreb2* in stress tolerance and the direct trans-activation of *Cor*/*Lea* genes by WDREB2. *Wdreb2* expression significantly improved freezing and osmotic stress tolerance in tobacco plants. Several putative stress- and ABA-responsive *cis*-elements were found in the 5' upstream regions of four wheat *Cor*/*Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*. The expression level of a *gusA* reporter gene under control of *Cor*/*Lea* promoter sequences was enhanced by cold, drought and ABA treatment in transgenic tobacco plants. Moreover, the *gusA* expression level was markedly enhanced by *Wdreb2* expression under nonstressful conditions. These results clearly indicate that WDREB2 acts as a transcription factor and positively regulates *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* in the development of multiple abiotic stress tolerance in wheat.

Keywords abiotic stress tolerance, *Cor*/*Lea* genes, DREB2, transgenic plants, *Triticum aestivum* L.

Introduction

Plants rapidly respond and adapt to environmental changes through numerous physiological and biochemical changes. Over-wintering plants have the ability to develop freezing tolerance after some period of exposure to low but nonfreezing temperature. This adaptive process, called cold acclimation, is an important trait that causes marked changes in biochemical and physiological conditions (Thomashow 1999). In the cold-acclimation process, a large number of *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) genes are transcriptionally activated, and the accumulated proteins and metabolites lead to protection of integrity of cell structures and functions from freezing damage (Thomashow 1999). A number of *Cor*/*Lea* genes are not only responsive to low temperature but also to drought, high salinity and abscisic acid (ABA) (Xiong et al. 2002).

Promoter regions of abiotic stress-responsive *Cor*/*Lea* genes contain several conserved motifs as *cis*-acting elements functioning in stress-responsive transcription; such regulatory systems include both ABA-dependent and -independent signaling pathways (Yamaguchi-Shinozaki and Shinozaki 2005). A conserved motif, PyACGTGGC, was first identified as a *cis*-acting element named ABRE (ABA-responsive element) in promoter regions of ABA-responsive genes such as the wheat *Em* gene and rice *Rab16* gene (Guiltinan et al. 1990; Mundy et al. 1990). A number of ABRE-binding proteins are members of the bZIP (basic-domain leucine zipper)-type DNA-binding proteins, such as *Arabidopsis* bZIP-type ABF (ABRE-binding factor)/AREB (ABA-responsive element binding) proteins, which bind to ABRE and activate ABA-dependent stress-responsive gene expression (Choi et al. 2000; Uno et al. 2000). Another functional *cis*-acting element of the *Cor* genes, i.e., the CCGAC core

motif known as a CRT (C-repeat)/DRE (dehydration responsive element) sequence, plays a critical role in the promoter function of *Arabidopsis COR15A* and *RD29A* genes (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). *Arabidopsis* CBF (CRT binding factor)/DREB1 (DRE binding protein 1) and DREB2 recognize CRT/DRE and belong to the EREBP (ethylene-responsive element binding protein)/AP2 (APETALA2) protein family (Stockinger et al. 1997; Liu et al. 1998). *CBF*/*DREB1* and *DREB2* gene expression can be induced either by low temperature or by drought stress, and both activate the expression of genes possessing a CRT/DRE *cis*-element.

Overexpression of *Arabidopsis DREB2A* results in significant drought stress tolerance but only slight freezing tolerance in transgenic plants (Sakuma et al. 2006a, 2006b). DREB2A positively regulates expression of many abiotic stress-responsive genes through activation of stress-responsive genes possessing DRE sequences in their 5' upstream regions. Many cereal *DREB2* homologs have been identified in rice, maize, pearl millet, barley and wheat (Dubouzet et al. 2003; Qin et al. 2007; Agarwal et al. 2007; Xue and Loveridge 2004; Shen et al. 2003; Egawa et al. 2006). Although among these proteins, OsDREB2A, PgDREB2A and TaDREB1 bind to DRE sequences (Dubouzet et al. 2003; Agarwal et al. 2007; Shen et al. 2003), no direct evidence indicates which downstream target genes are controlled by these transcription factors. The barley *HvDRF1* gene also belongs to the CBF/DREB subfamily of the EREBP/AP2 family (Xue and Loveridge 2004). The HvDRF1 transcription factor binds preferentially to a CT-rich element called a DRF1E motif (T(T/A)ACCGCCTT) rather than to CRT/DRE. A wheat *DREB2* homolog, *Wdreb2*, shows quite a high level of homology to *HvDRF1* and its transcription is enhanced by low temperature, drought, salt and ABA (Egawa et al. 2006). Like *HvDRF1*, *Wdreb2* transcripts form three types of mRNA products, i.e., *Wdreb2α*, *Wdreb2β* and

Wdreb2γ, through alternative splicing (Xue and Loveridge 2004; Egawa et al. 2006). One of the splicing products, *Wdreb2β*, encodes a nonfunctional polypeptide lacking EREBP/AP2 and the transcriptional activation domain (Egawa et al. 2006).

Wheat ABA-responsive *Cor*/*Lea* genes have been characterized (Danyluk et al. 1994; Tsuda et al. 2000; Ohno et al. 2003; Kobayashi et al. 2004, 2006). Expression patterns of the four *Cor*/*Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*, correspond well to the expression profile of *Wdreb2* under low temperature, drought and exogenous ABA-treated conditions (Egawa et al. 2006). Therefore, in the present study we attempted to clarify the direct relationship between WDREB2 and the four *Cor*/*Lea* genes in development of abiotic stress tolerance using heterologous transgenic plants. Furthermore, transgenic tobacco plants expressing *Wdreb2* were analyzed to infer its function in multiple stress tolerance. We discuss the roles of *Wdreb2* in abiotic stress tolerance of common wheat.

Materials and methods

Isolation of the 5' upstream regions of wheat *Cor*/*Lea* genes

Total DNA of hexaploid wheat (*Triticum aestivum* L.) cultivar 'Chinese Spring' (CS), single-digested with a restriction enzyme, *Hin*dIII, *Nco*I or *Sac*I, and self-circularized, was used as a template for the inverse PCR (IPCR) method (Ochman et al. 1988) to obtain the 5' upstream regions of four *Cor*/*Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*. The IPCR products were cloned into the pGEM-T vector (Promega, USA) and sequenced by an automated

fluorescent BigDye® Terminator Cycle sequencing system using an ABI PRISM™ 310 Genetic Analyser (PE Applied Biosystems, USA). Nucleotide sequences were analyzed by DNASIS software (Hitachi, Tokyo, Japan). IPCR was repeated to obtain sequences of ca. 1 to 2 kbp. Based on the nucleotide sequences, primer sets for specific amplification of the 5' upstream regions were designed and used for PCR. The PCR was performed using total DNA of CS as a template and the following nucleotide sequences as primers: 5'-GAATTGATGGATAACGGTGTTGGAT-3' and 5'-CTTCTCCGTGATGCTTTC-3' for *Wdhn13*, 5'-CGAAGTCAATACATAAAGAAGTGCA-3' and 5'-GAAGTTGATGGAGTGTTGGTAC-3' for *Wrab17*, and 5'-TCTTAATTTCATCTTCAATGGCTTT-3' and 5'-CCTCGTTATCCATTTACACCAAA-3' for *Wrab18* and *Wrab19*. The PCR products were cloned and sequenced. The nucleotide sequences were deposited into the DDBJ database; and the accession numbers are AB297677, AB297678, AB297679 and AB297680 for *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*, respectively.

Construction of wheat *Cor*/*Lea* promoter-*gusA* chimeric genes and tobacco transformation

The 5' upstream regions of *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* were amplified with the following promoter-specific primer sets containing *Hin*dIII and *Xba*I linkers: 5'-CCAAGCTTCAGTCGGGGAGAGATGTTAT-3' and 5'-CCTCTAGACTGACACTGGTTCTGTTGCT-3' for *Wdhn13*, 5'-CCAAGCTTGGTTTCTTCAGCTACTAGGC-3' and 5'-CCTCTAGAGTGTTGGTACCTACTAATTTGTGTTC-3' for *Wrab17*, and

5’-CCAAGCTTTTAATTTCATCTTCAATGGCTTTC-3’ and 5’-CCTCTAGACTCTTGTCTCTCACCAACAA-3’ for *Wrab18* and *Wrab19*. The PCR-amplified fragments were digested with *Hin*dIII and *Xba*I and inserted into plasmid pBI101 (Clontech, CA, USA) to produce *Wdhn13 pro::gusA*, *Wrab17 pro::gusA*, *Wrab18 pro::gusA* and *Wrab19 pro::gusA* constructs. These *gusA* constructs were introduced into leaf discs of *Nicotiana tabacum* cv. ‘Petit Havana’ using *Agrobacterium tumefaciens* LBA4404. Transformants were recovered on MS medium (Murashige and Skoog 1962) containing 0.1 mg l^{-1} alpha-naphthalene acetic acid, 1.0 mg l^{-1} 6-benzyl aminopurine, 250 mg l^{-1} kanamycin and 125 mg l^{-1} carbenicillin. The transformants (T_0 generation) were regenerated on hormone-free MS medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} carbenicillin.

Abiotic stress treatment and GUS assays of *Cor*/*Lea pro::gusA* tobacco

T_2 progeny of *Cor/Lea pro::gusA* plants were grown on MS medium containing 50 mg l^{-1} kanamycin at 27˚C under a 16 h photoperiod for 7 d. The plants were then cold acclimated for 7 d at 4 ± 0.5˚C under the same photointensity and photoperiod conditions. A different set of seven-day-old seedlings were dehydrated on dry filter paper in a desiccator for 4 h. For exogenous ABA treatment, seven-day-old seedlings were grown on MS medium containing 50 mg l^{-1} kanamycin and 20 μM ABA.

GUS activity was assessed histochemically using kanamycin-resistant F_1 tobacco plants and homozygous T_2 progeny of the *Cor*/*Lea pro::gusA* plants. The staining solution for the GUS assay contained the following components: 1.9 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-gluc), 0.5 mM potassium ferricyanide, 0.5

mM potassium ferrocyanide, and 0.3% Triton X-100. The transgenic tobacco plants were incubated with the staining solution at 37˚C for 24 h. GUS activity was quantified according to Jefferson (1987). The means with standard error were calculated based on three independent experiments, and the data were statistically analyzed by Student's *t*-test.

Production of transgenic tobacco plants expressing *Wdreb2*

Out of three spliced products of the *Wdreb2* transcripts, *Wdreb2γ* cDNA sequence (accession number AB193608) was amplified with the following primer set containing a *Bam*HI linker: 5'-CGGGATCCGACAAGATTGCGAACGCTAGA-3' and 5'-CGGGATCCCCGACCAAACACCATAGACA-3'. The PCR fragment was digested with *Bam*HI and inserted into the *Bam*HI site between the *CaMV35S* promoter and the *Nos* terminator in plasmid pROK1a (Baulcombe et al. 1986). The chimeric *Wdreb2* construct was named *35S::Wdreb2*. Transgenic tobacco plants were produced by the same method described above.

To check *Wdreb2* expression in *35S::Wdreb2* transgenic tobacco plants, total RNA was extracted by guanidine thiocyanate from leaves of transgenic plants. For RT-PCR, first-strand cDNA was synthesized from DNaseI-treated RNA samples with oligo-dT primers using ReverTra Ace® (TOYOBO, Osaka, Japan). RT-PCR was conducted with the following primers: 5'-CCCAAGCTTTCACTTTTCCTAGCATC-3' and 5'-CGGGATCCTCGCCTGTTTTCTTCGCT-3', and the annealing temperature was 60˚C. An actin gene (*Act*) was used as an internal control and amplified with the following primers: 5'-GGCTGGTTTTGCTGGTGACGAT-3' and 5'-AATGAAGGAAGGCTGGAAGAGGA-3'.

RT-PCR products were separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide.

Bioassays for ABA sensitivity and abiotic stress tolerance in *35S::Wdreb2* tobacco

Seed germination was studied in three replicates of 100 seeds of wild type and *35S::Wdreb2* T_2 progeny. The seeds were placed on MS medium with or without 1 μM ABA, and incubated at 27˚C under a 16 h photoperiod. Germination was scored up to 10 d after planting. In the bioassay for ABA sensitivity, ten 5-day-old seedlings of wild-type and *35S::Wdreb2* plants were placed in a glass petri dish (60 mm in diameter and 15 mm in depth) containing filter paper (55 mm in diameter) wetted with 3 ml of distilled water or 1 μM ABA solution, and incubated at 27˚C under a 16 h photoperiod. After 8 d, the length of primary roots was recorded. The whole experiment was repeated three times and the data were statistically analyzed.

To assay freezing tolerance, twenty 2-week-old seedlings of wild type and *35S::Wdreb2* T_2 progeny were frozen at –25 ± 1˚C for 45 min in the dark. The frozen seedlings were thawed overnight at 4˚C and transferred back to normal temperature conditions (27˚C). At 2 weeks after transfer, the number of surviving seedlings was recorded. To assay osmotic stress tolerance, one-week-old seedlings of wild-type and *35S::Wdreb2* tobacco plants were placed on two sheets of filter paper (55 mm in diameter) wetted with 3 ml of 0.5 M mannitol solution or 0.2 M NaCl solution in a glass petri dish (60 mm in diameter and 15 mm in depth) at normal temperature conditions (27˚C). At 6 d after treatment with mannitol, or 3 d after treatment with NaCl, the number of plants with green cotyledons was scored. The experiments were repeated three times and the data were statistically analyzed.

Crossing of transgenic plants and GUS assays

35S::Wdreb2 transformants were used as the pollen parent in crosses with transgenic tobacco plants expressing a chimeric *gusA* gene under the control of the *Cor*/*Lea* promoter. F_1 transgenic tobacco plants were selected on hormone-free MS medium containing 50 mg l^{-1} kanamycin. To check *Wdreb2* and *gusA* expression in the F_1 transgenic tobacco, RT-PCR was conducted as described above. The *gusA* transcript was amplified with the following primers: 5'-GATCAGCGTTGGTGGGAAAG-3' and 5'-CGGCGTGGTGTAGAGCATTA-3', and the annealing temperature was 53˚C. GUS activity in the kanamycin-resistant F_1 tobacco plants was examined as described above.

Results

Isolation of promoter regions of wheat *Cor*/*Lea* genes

The 5' upstream regions of the four wheat *Cor*/*Lea* genes were isolated by IPCR. The amplified fragments of the *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* loci contained 1790, 1853, 944 and 837 bp upstream regions from their respective ORFs (Fig. 1). The nucleotide sequences of the upstream regions possessed a TATA-box and several core sequences of major stress-responsive *cis*-elements such as the CRT/DRE motif with CCGAC and the ABRE motif with ACGT (Fig. 1). DRF1E, which is a putative *cis*-element with ACCGCC recognized by HvDRF1/WDREB2

(Xue and Loveridge 2004; Egawa et al. 2006), was found in the 5' upstream regions of *Wdhn13*, *Wrab18* and *Wrab19*, but not *Wrab17* (Fig. 1). In addition to these motifs, CE1 (coupling element 1) with CCACC and an S-box with CACCT, which seems to be involved in EREBP/AP2-type ABI4-mediated ABA signaling (Niu et al. 2002; Acevedo-Hernández et al. 2005), were also found in the four genes (Fig. 1).

Abiotic stress responsiveness of the *Cor*/*Lea* promoters

To examine whether the cold-, drought- and ABA-responsiveness of *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* genes are transcriptionally controlled by the 5' upstream regions, transgenic tobacco plants were generated by introducing a chimeric *gusA* gene fused with the *Cor*/*Lea* upstream region. The four chimeric genes contained a *gusA* reporter gene under the control of the 1.2, 1.8, 0.9 and 0.8 kb upstream sequences of *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*, respectively. Kanamycin-resistant progeny (T_1 and T_2 generation) were generated from self-pollinated seeds of regenerated transgenic tobacco plants (T_0 generation), in which *Cor*/*Lea pro::gusA* integration was confirmed by Southern blot analysis (data not shown). The T_2 seedlings were treated with low temperature for 7 d, drought for 4 d or 20 μM ABA for 7 d.

Strong *gusA* expression was observed in the leaves and stems of *Wdhn13 pro::gusA* and *Wrab17 pro::gusA* transformants under abiotic stress and ABA-treated conditions (Fig. 2), and GUS activity was significantly higher under stress and ABA conditions than under normal conditions (Fig. 3A, B, C). In *Wdhn13 pro::gusA* plants, especially, GUS activity was drastically elevated by ABA treatment (Fig. 3A).

In the untreated seedlings, weak *gusA* expression was observed in the roots and vascular

system. The *gusA* expression pattern in *Wrab18 pro::gusA* transformants was similar to that in the *Wrab19 pro::gusA* lines (Fig. 2). The *gusA* expression level in these tissues of transgenic plants was increased by low temperature, drought and ABA treatment, and GUS signals were slightly detectable in the leaves (Fig. 2), although the GUS accumulation level in *Wrab18*/*19 pro::gusA* transgenic plants was lower than in *Wdhn13 pro::gusA* and *Wrab17 pro::gusA* plants (Fig. 2). The GUS activity in *Wrab18*/*19 pro::gusA* plants was significantly increased by the stress and ABA treatments (Fig. 3D, 3E). The *GUS* gene in *Wrab18*/*19 pro::gusA* tobacco was inducible to a similar level under low temperature, drought and ABA conditions (Figs. 2, 3D, 3E). GUS accumulation was observed in roots of *Wdhn13 pro::gusA* and *Wrab18*/*19 pro::gusA* plants, although no GUS signal was detected in the root systems of *Wrab17 pro::gusA* plants (Fig. 2).

ABA sensitivity in transgenic tobacco plants expressing *Wdreb2*

To analyze *Wdreb2* function, transgenic tobacco lines expressing *Wdreb2γ* were produced. Twenty-four *35S::Wdreb2* transgenic tobacco plants were recovered on selection medium, and integration of the introduced gene was confirmed by Southern blot analysis (data not shown). Expression of the transgene in these transgenic T_1 plants was revealed by RT-PCR analysis (Fig. 4A). Based on these data, three transgenic lines, *35S::Wdreb2*-#8, *35S::Wdreb2*-#11 and *35S::Wdreb2*-#17, which possessed one or two copies of the introduced gene and accumulated abundant transcripts of *Wdreb2*, were selected. Their T_2 progeny were used for the following analysis.

We compared germination rates from mature seeds under both ABA and non-ABA

conditions among the three *35S::Wdreb2* lines and wild-type tobacco plants. In the absence of exogenous ABA, *35S::Wdreb2*-#11 and #17 showed delayed germination in comparison with wild-type plants (Fig. 4B). On the other hand, germination rates of *35S::Wdreb2*-#8 seeds were similar to those of wild type throughout the tested periods (Fig. 2B). In the presence of 1 μM ABA, seed germination of *35S::Wdreb2*-#11 and #17 was more markedly delayed, whereas germination of wild type was slightly inhibited by ABA treatment (Fig. 4C). The germination rate of *35S::Wdreb2-#8* was not significantly altered by the presence or absence of ABA (Fig. 4B, C). These results indicated that *35S::Wdreb2*-#11 and #17 plants were more sensitive to ABA during seed germination than wild type, and that *35S::Wdreb2*-#8 plants were insensitive to both endogenous and exogenous ABA during seed germination.

To study ABA sensitivity during early seedling development, inhibition of seedling growth by exogenous ABA (1 μM) was compared among the three *35S::Wdreb2* and wild-type plants. The magnitude of inhibition of root growth was estimated by the relative growth rate (% growth in the presence of ABA relative to growth in the absence of ABA) and was significantly greater in the *35S::Wdreb2* lines than in wild type (Fig. 4D). Therefore, primary root elongation of the *35S::Wdreb2* plants was hypersensitive to exogenous ABA during post-germination growth.

Abiotic stress tolerance of *Wdreb2*-expressing transgenic tobacco

Overexpression of *Arabidopsis DREB2* resulted in a significant increase in tolerance of multiple stresses in transgenic plants (Sakuma et al. 2006b). To clarify the roles of *Wdreb2* in abiotic stress, the levels of freezing and osmotic stress tolerance were compared for the three *35S::Wdreb2* and wild-type plants. A bioassay for freezing tolerance was performed using

2-week-old seedlings of each line without cold acclimation. Freezing tolerance of *35S::Wdreb2*-#8 and #11 was improved and was significantly higher than that of the wild type (Fig. 5A).

Tolerance levels of *35S::Wdreb2* plants to osmotic stress were estimated by treatment with mannitol and NaCl. Under mannitol- and NaCl-treated conditions, cotyledons of the wild-type tobacco seedlings became yellowish. After 6 d of mannitol treatment, plants with healthy green cotyledons were 63%, 80% and 40% of the *35S::Wdreb2*-#8, #17 and wild-type lines, respectively (Fig. 5B). The two *35S::Wdreb2* lines exhibited improved tolerance to high mannitol concentration, although line #11 was more sensitive to mannitol stress than wild-type plants (Fig. 5B). On the other hand, under NaCl stress, plants with green cotyledons were 93%, 47%, 50% and 13% in the *35S::Wdreb2*-#8, #11, #17 and wild-type lines, respectively (Fig. 5C). The three *35S::Wdreb2* transgenic lines had significantly higher NaCl tolerance than the wild-type plants.

Trans-activation of wheat *Cor*/*Lea* promoters by WDREB2

To examine direct relationships between *Wdreb2* and wheat *Cor*/*Lea* genes, *gusA* expression was monitored in F_1 progeny derived from crossing *Cor/Lea pro::gusA* plants with the *35S::Wdreb2* line (#8, #11 and #17). The *Wdreb2* expression was transmitted from the *35S::Wdreb2* lines to the F_1 plants revealed by RT-PCR analysis (Fig. 6). The *gusA* expression was confirmed in the F_1 plants, whereas the parental *Cor/Lea pro::gusA* plants also showed the *gusA* expression as backgrounds.

To clarify the trans-activation of the *Cor*/*Lea* promoters by WDREB2, GUS assay was

conducted in the F_1 progeny and their parental *Cor/Lea pro::gusA* plants. GUS activity in *Wdhn13 pro::gusA* and *Wrab17 pro::gusA* plants was significantly increased by constitutive expression of *Wdreb2* under normal conditions (Fig. 7A, B, E). GUS accumulation was clearly observed in leaves and stems of *Wdhn13 pro::gusA* x *35S::Wdreb2* and *Wrab17 pro::gusA* x *35S::Wdreb2* F_1 plants, and these patterns were similar to those of *Wdhn13 pro::gusA* and *Wrab17 pro::gusA* plants under stress-treated conditions (Figs. 2, 7A, B).

On the other hand, GUS activity in *Wrab18*/*19 pro::gusA* plants was also dramatically increased in whole plant tissues by *Wdreb2* expression (Fig. 7C, D, E). This constitutive GUS expression pattern in the F_1 plants was inconsistent with the low GUS level observed in leaves of the *Wrab18*/*19 pro::gusA* lines under stress-treated conditions (Fig. 2).

Discussion

The 5' regulatory regions of two wheat cold-responsive genes, *Wcs120* and *Wcor15*, precisely function to control reporter *gusA* gene expression in dicotyledonous plants (Quellet et al. 1998; Takumi et al. 2003). In the present study, we isolated the 5' upstream regions of four ABA-responsive *Cor*/*Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*, and generated transgenic tobacco plants expressing chimeric *Cor*/*Lea pro::gusA* genes. The GUS accumulation levels were different, but the GUS induction patterns showed quite similar among the separate transgenic lines each of the four *Cor*/*Lea pro::gusA* constructs under the abiotic stress conditions. The difference of the GUS accumulation levels was probably due to position effect of the integrated construct in the tobacco genome. These 5' upstream regions responded

efficiently to low temperature, drought and exogenous ABA in a heterologous tobacco system. The *gusA* expression patterns under the control of the four 5' upstream regions were consistent with the results obtained from RNA gel blots probed with ^{32}P-labelled cDNA clones of the four *Cor*/*Lea* genes (Kobayashi et al. 2004; Egawa et al. 2006). These results indicated that the 5' upstream regions of the four *Cor*/*Lea* genes function as promoters of their transcription under low temperature, drought and ABA treatment, and suggested that at least some of the putative *cis*-elements found in their promoters are essential for *Cor*/*Lea* gene expression. The heterologous tobacco system is useful to analyze abiotic stress responsiveness of wheat *Cor*/*Lea* promoters.

Among three spliced products of the *Wdreb2* transcripts, *Wdreb2γ* contains functional domains, retains transcriptional activity and is accumulated more abundantly under abiotic stress conditions (Egawa et al. 2006). Therefore, we produced the transgenic tobacco plants expressing *Wdreb2γ*. Expression and GUS assays in transgenic tobacco plants revealed that *Wdreb2* enhanced *gusA* expression under the control of 5' upstream sequences of *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* under nonstressful conditions (Figs. 6,7). The 5' upstream regions contained putative *cis*-elements, CRT/DRE and DRF1E, recognized by cereal DREB2-type transcription factors (Fig. 1). In heterologous plants, the wheat *DREB2* homolog efficiently activated the 5' upstream sequences of the four wheat *Cor*/*Lea* genes. These results strongly suggest that *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* are direct targets of the WDREB2 transcription factor and that expression of the four *Cor*/*Lea* genes was regulated at least by WDREB2.

DRF1E was identified as the HvDRF1 recognition sequence, and HvDRF1 activated the promoter activity of a stress- and ABA-responsive *HVA1* gene in barley leaves (Xue and

Loveridge 2004). WDREB2 is a wheat ortholog of HvDRF1 based on their high sequence homology and transcriptional characteristics (Xue and Loveridge 2004; Egawa et al. 2006). One or two DRF1E sequences were in the 5' upstream regions of *Wdhn13*, *Wrab18* and *Wrab19*, but not in *Wrab17* (Fig. 1). Therefore, WDREB2 seems to recognize DRF1E and to activate transcription of *Cor*/*Lea* genes possessing DRF1E in their promoter sequences. Although there is no complete DRF1E motif in the *Wrab17* promoter, *Wrab17* was transcriptionally activated by WDREB2 (Figs. 1, 6). Two CRT/DRE core motifs were present within a region c.a. –1 kb from the ORF of *Wrab17*. Therefore, *Wrab17* transcription may be positively regulated through the interaction of WDREB2 with CRT/DRE or other unknown *cis*-elements.

Except for the CRT/DRE and DRF1E motifs, ABRE (ACGT), CE1 (CCACC) and an S-box (CACCT) were found in all four genes (Fig. 1). *Arabidopsis* bZIP-type ABF/AREB transcription factors recognize ABRE (Choi et al. 2000; Uno et al. 2000) and EREBP/AP2-type transcription factors (maize ZmABI4 and *Arabidopsis* ABI4,) bind to CT-rich sequences such as CE1-like and the S-box (Niu et al. 2002; Acevedo-Hernández et al. 2005). Several ABRE-recognition factors such as EmBP-1, EmBP-2, TRAB1 and ZmBZ-1 have been identified in rice, maize and wheat (Guiltinan et al. 1990; Hobo et al. 1999; Nieva et al. 2005), implying that their wheat homologs might function in the ABA-dependent stress signal pathway. The presence of these stress-related *cis*-elements suggests that transcriptional regulation of the four *Cor*/*Lea* genes is mediated by the wheat ABF/AREB homologs in addition to CBF/DREB homologs such as WDREB2. Compared with *Wdhn13* and *Wrab17* expression, *Wrab18/19* transcripts accumulate more abundantly in roots than in leaves (Kobayashi et al. 2004; Fig. 2), indicating that these tissue-specific expression patterns are conserved between monocotyledonous and dicotyledonous plants. Therefore, genetic factors

controlling tissue-specific expression should be cooperatively associated with stress-inducible expression of *Cor*/*Lea*. In the F_1 plants between the *Wrab18*/*19 pro::gusA* lines and the *35S::Wdreb2* line, the GUS accumulation was observed in leaves as well as in roots (Fig. 7), which ectopic activation of the *Wrab18/19* promoters was probably due to the extremely accumulated WDREB2 in the F_1 leaves.

DREB2 homologs contribute to increase multiple stress tolerance in several plant species (Sakuma et al. 2006b; Qin et al. 2007). *Wdreb2* expression is induced by low temperature, drought, NaCl exposure and exogenous ABA treatment (Egawa et al. 2006). In the present study, *35S::Wdreb2* transgenic tobacco lines were significantly improved tolerance to stresses such as freezing, mannitol and salt stress (Fig. 5). The results obtained in the heterologous species strongly suggest that *Wdreb2* is also associated with development of multiple stress tolerance through trans-activation of *Cor*/*Lea* genes in common wheat. In wheat and barley, major quantitative trait loci (QTLs) for winter hardiness and freezing tolerance (*Fr*) were identified on group 5 chromosomes (Cattivelli et al. 2002). Most *Cor*/*Lea* gene expression is controlled by the *Fr-1* chromosomal regions through CBF transcription factors (Kobayashi et al. 2005). The *CBF* genes are clustered on chromosomal regions containing another QTL for freezing tolerance, *Fr-2* (Vágújfalvi et al. 2003; Miller et al. 2005). Because *Wdreb2* is assigned to the long arm of group 1 chromosomes (Egawa et al. 2006), *Wdreb2* is not located at the *Fr-2* locus. Therefore, *Wdreb2* activates *Cor*/*Lea* gene expression independently of *Fr-2*. Abiotic stress-responsive expression of the wheat *Cor*/*Lea* genes is separately controlled through multiple CBF/DREB transcription factors.

Moreover, *35S::Wdreb2* transgenic plants became hypersensitive to exogenous ABA during post-germination growth compared with wild-type tobacco (Fig. 4). This result suggests

that *Wdreb2* expression affects ABA sensitivity as well as the ABA-dependent stress response in heterologous plants, although the molecular mechanisms increasing ABA sensitivity remain unknown. *Wdreb2* expression is responsive to exogenous ABA treatment in common wheat (Egawa et al. 2006), and *Wdreb2* takes part directly in ABA-mediated stress-responsive pathways. In addition, *Wdreb2* might contribute indirectly to development of abiotic stress tolerance through an increase in ABA sensitivity.

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References

Acevedo-Hernández GJ, León P, Herrera-Estrella LR (2005) Sugar and ABA responsiveness of a minimal *RBCS* light-responsive unit is mediated by direct binding of ABI4. Plant J 43:506-519

Agarwal P, Agarwal PK, Nair S, Sopory SK, Reddy MK (2007) Stress-inducible DREB2A transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA-binding activity. Mol Genet Genomics 277:189-198

Baker SS, Wilhelm KS and Thomashow MF (1994) The 5'-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene

expression. Plant Mol Biol 24:701-713

Baulcombe DC, Saunders GR, Bevan MW, Mayo MA, Harrison BD (1986) Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature 321:446-449

Cattivelli L, Baldi P, Crosatti C, Di Fronzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchioni N, Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in Triticeae. Plant Mol Biol 48:649-665

Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275:1723-1730

Danyluk J, Houde M, Rassart E, Sarhan F (1994) Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant gramineae species. FEBS Lett 344:20-24

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

Egawa C, Kobayashi F, Ishibashi M, Nakamura T, Nakamura C, Takumi S (2006) Differential regulation of transcript accumulation and alternative splicing of a *DREB2* homolog under abiotic stress conditions in common wheat. Gene Genet Syst 81:77-91

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250:267-271

Hobo T, Kowyama Y, Hattori T (1999) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proc Natl Acad Sci USA 96:15348-15353

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5:387-405

Kobayashi F, Takumi S, Nakata M, Ohno R, Nakamura T, Nakamura C (2004) Comparative study of the expression profiles of the *Cor*/*Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. Physiol Plant 120:585-594

Kobayashi F, Takumi S, Kume S, Ishibashi M, Ohno R, Murai K, Nakamura C (2005) Regulation by *Vrn-1*/*Fr-1* chromosomal intervals of CBF-mediated *Cor*/*Lea* gene expression and freezing tolerance in common wheat. J Exp Bot 56:887-895

Kobayashi F, Takumi S, Egawa C, Ishibashi M, Nakamura C (2006) Expression patterns of low temperature responsive genes in a dominant ABA-less-sensitive mutant line of common wheat. Physiol Plant 127:612-623

Liu Q, Sakuma Y, Abe H, Kasuga M, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 12:165-178

Miller AK, Galiba G, Dubcovsly J (2005) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A*^{m}*2* in *Triticum monococcum*. Mol Genet Genomics 275:193-203

Mundy J, Yamaguchi-Shinozaki K, Chua NH (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene. Proc Natl Acad Sci USA 87:1406-1410

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nieva C, Busk PK, Domínquez-Puigjaner E, Lumbreras V, Testillano PS, Risueno MC, Pagés M (2005) Isolation and functional characterization of two new bZIP maize regulators of the ABA responsive gene *rab28*. Plant Mol Biol 58:899-94

Niu X, Helentjaris T, Bate NJ (2002) Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes. Plant Cell 14:2565-2575

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of inverse polymerase chain reaction. Genetics 120:621-623

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

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Phan Tran L-S, Shinozaki K, Yamaguchi-Shinozaki K (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. Plant J 50:54-69

Quellet F, Vazquez-Tello A, Sarhan F (1998) The wheat *wcs120* promoter is cold-inducible in both monocotyledonous and dicotyledonous species. FEBS Lett 423:324-328

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

Sakuma Y, Maruyama K, Qin F, Osakabe Y, Shinozaki K, Yamaguchi-Shinozaki K (2006b) Dual function of an Arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proc Natl Acad Sci USA 103:18822-18827

Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q, Chen SY (2003) An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theor Appl Genet 106:923-930

Stockinger EJ, Gilmour SJ and Thomashow MF (1997) *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 94:1035-1040

Takumi S, Koike A, Nakata M, Kume S, Ohno R, Nakamura C (2003) Cold-specific and light-stimulated expression of a wheat (*Triticum aestivum* L.) *Cor* gene *Wcor15* encoding a chloroplast-targeted protein. J Exp Bot 54:2265-2274

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Ann Rev Plant Physiol Plant Mol Biol 50:571-599

Tsuda K, Tsvetanov S, Takumi S, Mori, N, Atanassov, A, Nakamura C (2000) New members of a cold-responsive group-3 *Lea/Rab*-related *Cor* gene family from common wheat (*Triticum aestivum* L.). Gene Genet Syst 75:179-188

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA 97:11632-11637

Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. Mol Genet Genomics 269:60-67

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress.

Plant Cell 14:S165-S183

Xue GP, Loveridge CW (2004) *HvDRF1* is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. Plant J 37:326-339

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

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

Legends of Figures

Figure 1. Genomic structures of *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*. Boxes indicate exons, and the putative start codon (ATG) was located at the +1 position. Putative *cis*-elements are represented in the 5' upstream region.

Figure 2. Expression patterns of a *gusA* reporter gene under control of *Cor*/*Lea* 5' upstream regions in transgenic tobacco plants. Histochemical GUS staining was performed after abiotic stress treatments such as cold (4˚C for 7 d), drought (27˚C for 4 h) and exogenous 20 μM ABA (27˚C for 7 d).

Figure 3. GUS activity of *Cor*/*Lea pro::GUS* transgenic tobacco plants under cold, drought and ABA conditions. Seven-day-old seedlings of *Wdhn13 pro::gusA* (A), *Wrab17 pro::gusA* (B, C), *Wrab18 pro::gusA* (D) and *Wrab19 pro::gusA* (E) plants were treated with cold (4˚C for 7 d), drought (27˚C for 4 h) or 20 μM ABA (27˚C for 7 d). Student's *t*-test was used to test for statistical significance (*$P < 0.05$, **$P < 0.01$) between the non-treatment and each stress treatment.

Figure 4. ABA sensitivity of *35S::Wdreb2* transgenic tobacco plants. (A) RT-PCR analysis of *Wdreb2* expression in three *35S::Wdreb2* transgenic tobacco lines (#8, #11 and #17). *Actin* was used as an internal control for RT-PCR. (B) Comparison of seed germination rates between *35S::Wdreb2* and wild-type plants on MS medium without ABA. The means ± standard deviations were calculated from data in 4 experiments. (C) Inhibition of

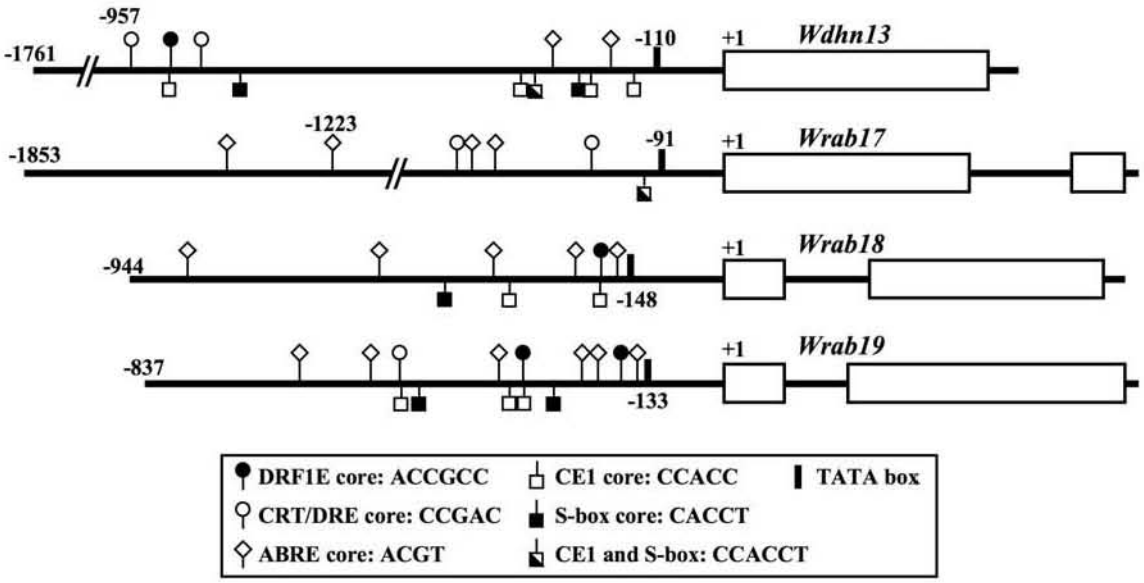
germination rates on MS medium with 1 μM ABA. (D) The magnitude of inhibition by ABA treatment. The primary root length was measured on the 8th day of treatment. Root growth was estimated as the percentage of the root length with 1 μM ABA relative to the length without ABA. Student's *t*-test was used to test for statistical significance (*$P < 0.05$, **$P < 0.01$) between wild-type plants and transgenic lines.

Figure 5. Increase in abiotic stress tolerance in *35S::Wdreb2* transgenic tobacco plants. (A) Freezing tolerance. Survival rates were compared after -25˚C treatment. (B) Osmotic stress tolerance. Percentages of plants with green cotyledons were compared after supplementation with a 0.5 M mannitol solution. (C) Salt stress tolerance. Percentages of plants with green cotyledons were compared after supplementation with a 0.2 M NaCl solution. Student's *t*-test was used to test for statistical significance (*$P < 0.05$, **$P < 0.01$) between wild-type plants and transgenic lines.

Figure 6. Expression analysis of *Wdreb2* and *gusA* genes in the F_1 transgenic tobacco. RT-PCR analysis was conducted with the gene-specific primers in the F_1 plants and their parental transgenic plants. *Actin* was used as an internal control for RT-PCR. P1, parental *35S::Wdreb2* plants; P2, parental *Cor/Lea pro::gusA* plants; F_1, F_1 plants between the P1 and P2 plants.

Figure 7. Trans-activation of *Cor/Lea pro::gusA* chimeric genes in transgenic tobacco plants. (A-D) Histochemical GUS staining in F_1 seedlings between *Cor/Lea pro::gusA* and *35S::Wdreb2* transgenic plants and the parental transgenic plants. (E) GUS activities in the

F_1 seedlings and parental *Cor/Lea pro::gusA* transgenic plants. Student's *t*-test was used to test for statistical significance (*$P < 0.05$, **$P < 0.01$) between the F_1 and parental plants.



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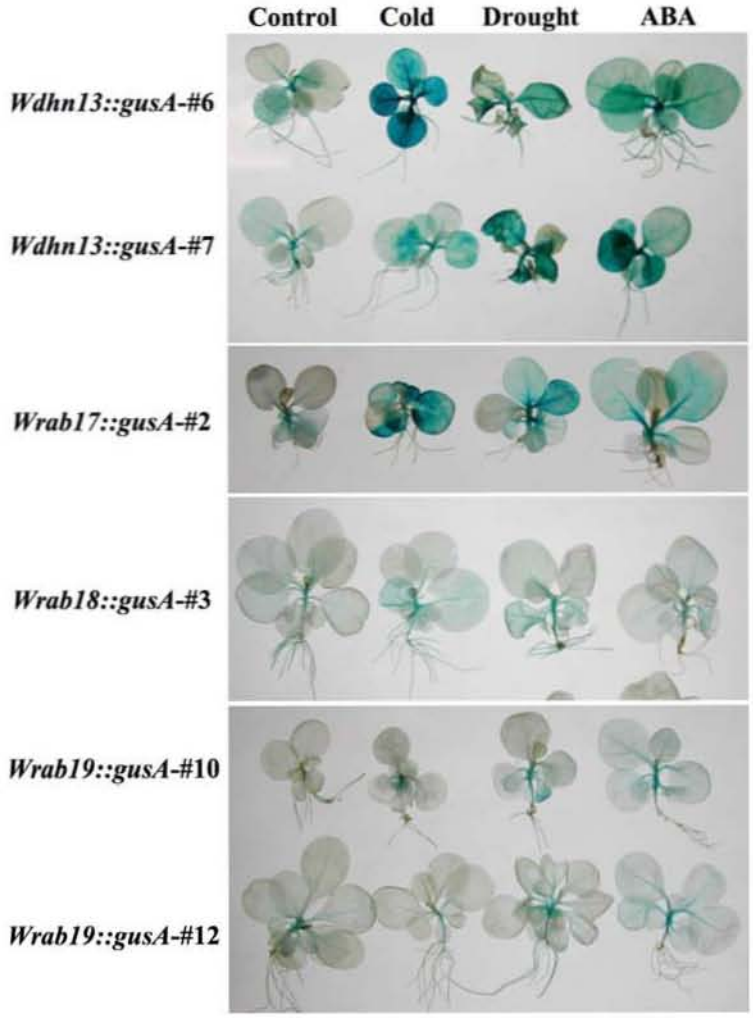

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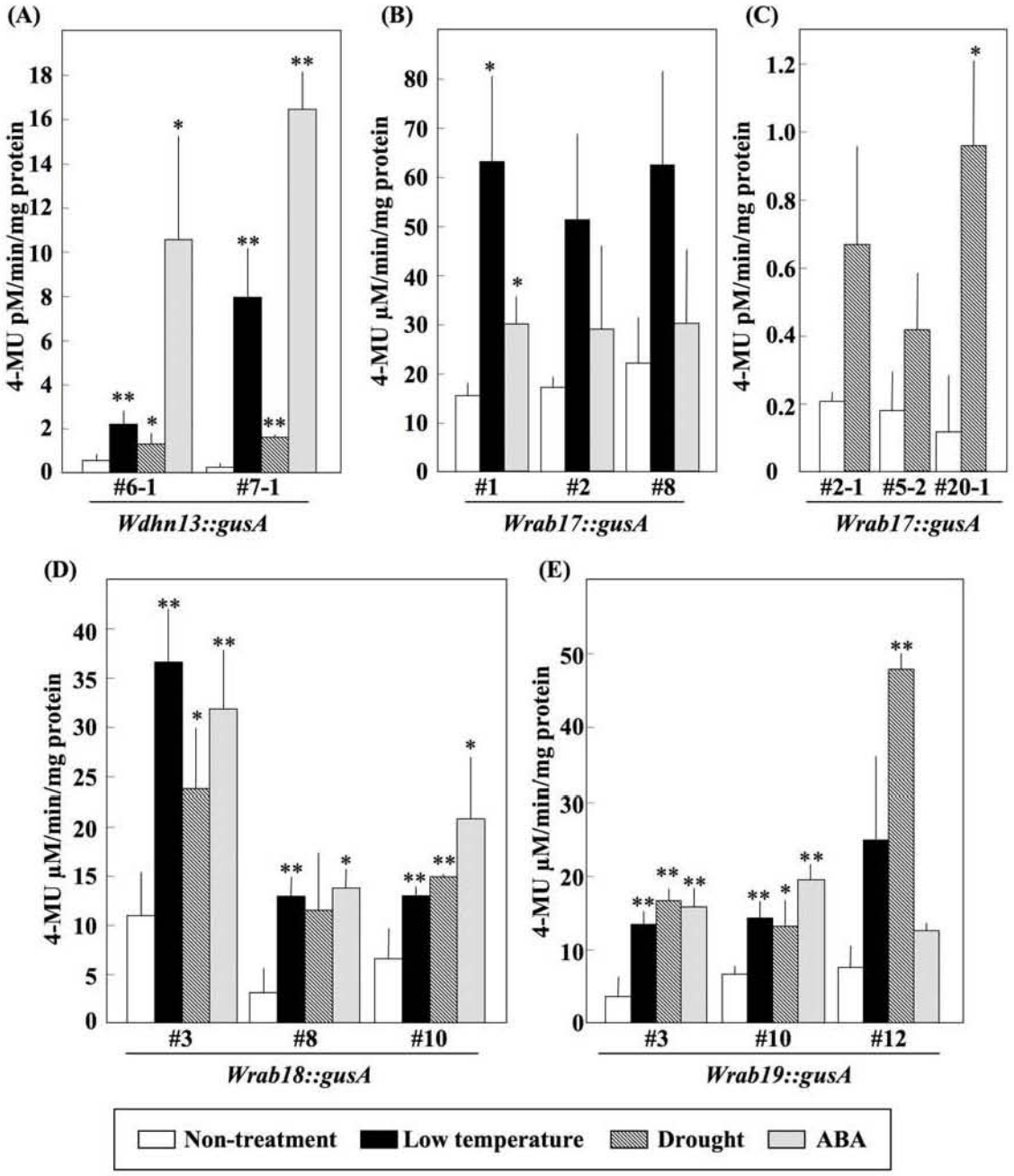

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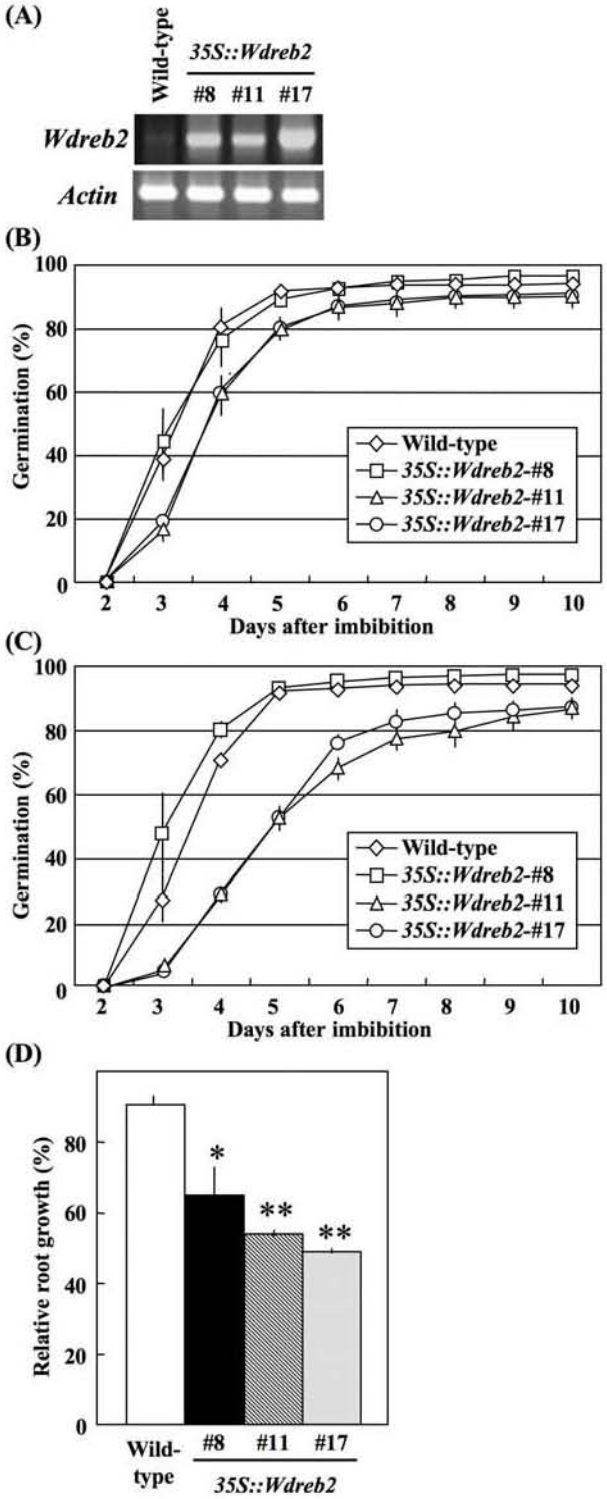

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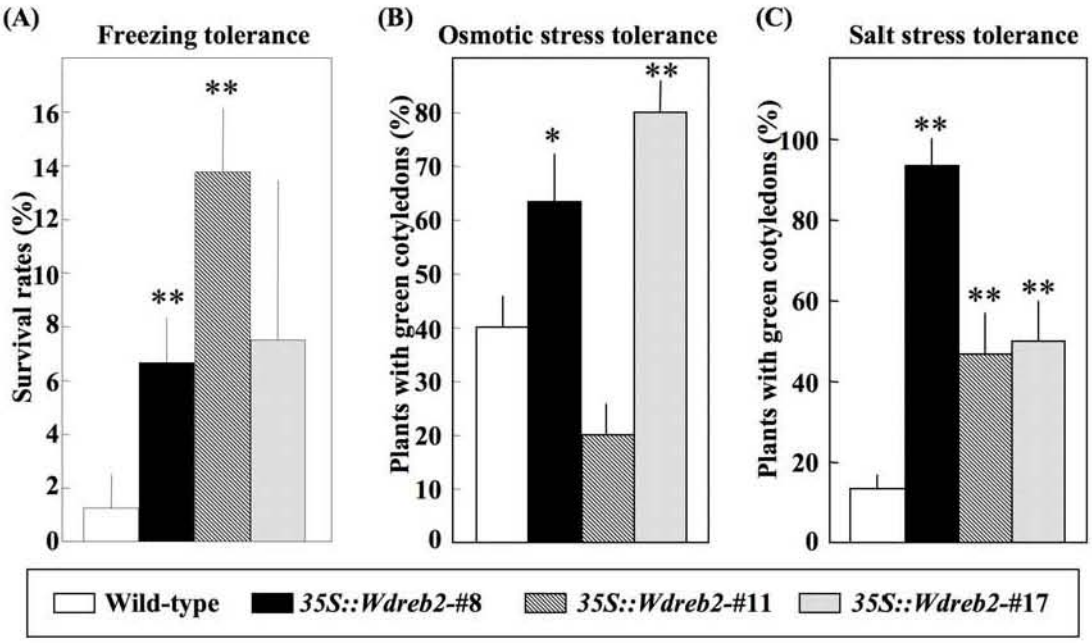

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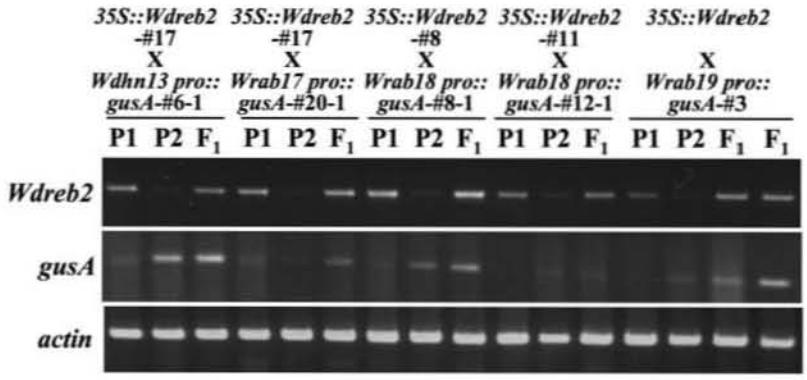

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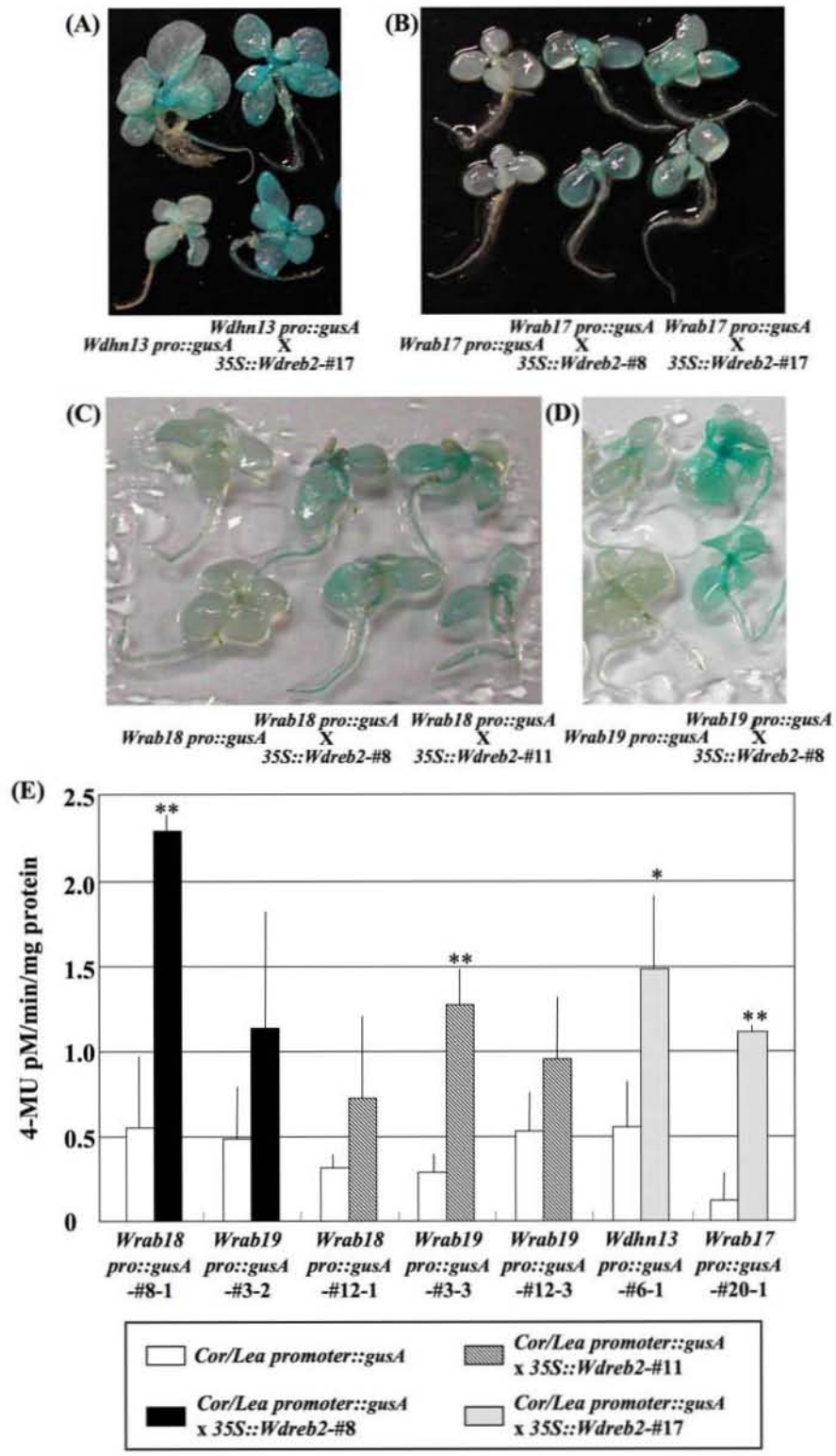

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