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Kobayashi, Fuminori
Maeta, Eri
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Takumi, Shigeo

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Positive role of a wheat *HvABI5* ortholog in abiotic stress response of seedlings

Fuminori Kobayashi[1,2,3], Eri Maeta[1], Akihiro Terashima[1] and Shigeo Takumi[1]*

[1]Laboratory of Plant Genetics, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

[2]Research Fellow of the Japan Society for the Promotion of Science

[3]Present address: Plant Genome Research Unit, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

*Corresponding author

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

Abstract

AREB and ABF proteins, members of the bZIP-type protein family, act as major transcription factors in ABA-responsive gene expression under abiotic stress conditions in *Arabidopsis*. Barley *HvABI5* and rice *TRAB1* are homologs of *AREB/ABF* and are expressed in drought- and ABA-treated seedlings. However, no direct evidence has shown an association of an AREB/ABF-type transcription factor with stress tolerance in cereals. To understand the molecular basis of abiotic stress tolerance through a cereal AREB/ABF-type transcription factor, a wheat *HvABI5* ortholog, *Wabi5*, was isolated and characterized. *Wabi5* expression was activated by low temperature, drought and exogenous ABA treatment, and its expression pattern differed between two wheat accessions with distinct levels of stress tolerance and ABA sensitivity. *Wabi5*-expressing transgenic tobacco plants showed a significant increase in tolerance to abiotic stresses such as freezing, osmotic and salt stresses and a hypersensitivity to exogenous ABA in the seedling stage compared with wild-type plants. Expression of a *GUS* reporter gene under the control of promoters of three wheat *Cor/Lea* genes, *Wdhn13*, *Wrab18* and *Wrab19*, was enhanced by ectopic *Wabi5* expression in wheat callus and tobacco plants. These results clearly indicated that WABI5 functions as a transcriptional regulator of the *Cor/Lea* genes in multiple abiotic stress responses in common wheat.

Abbreviations

ABA, abscisic acid; ABI, ABA-insensitive; ABF, ABA-responsive element binding factor; ABRE, ABA-responsive element; AREB, ABA-responsive element binding protein; bZIP, basic region leucine zipper; CBF, C-repeat binding factor; *Cor*/*Lea*, cold-responsive/late-embryogenesis-abundant; DHN, dehydrin; DPBF, Dc3 promoter-binding factor; DREB, dehydration responsive element binding protein; *Fr*, frost resistance; GUS, β-glucuronidase; LIP, low-temperature-induced protein; OBF1, octopine synthase gene enhancer binding factor; RAB, responsive to ABA; TRAB, transcription factor responsible for ABA regulation 1; *Vrn*, vernalization requirement

Introduction

Temperate plants are capable of developing freezing tolerance when they are exposed to low but non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow 1999). In this adaptive process, plant cells undergo drastic physiological, biochemical and metabolic changes leading to the development of freezing tolerance at the cellular level. One of the mechanisms is induction of the *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) gene family, and the COR/LEA proteins promote the development of freezing tolerance by protecting cellular components (Thomashow 1999). Expression of many *Cor/Lea* genes is regulated by major transcription factors in the CBF/DREB and AREB/ABF families under abiotic stress conditions such as low temperature and osmotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006).

Arabidopsis thaliana AREB/ABF genes encode bZIP (basic region/leucine zipper)-type transcription factors, and AREB/ABF proteins are categorized into the group A bZIP subfamily together with ABI5 and AtDPBFs (Jakoby et al. 2002). Among the group A bZIPs, AREB1/ABF2, AREB2/ABF4, ABF1 and ABF3 function in ABA signaling in vegetative tissues under abiotic stress conditions (Choi et al. 2000, Uno et al. 2000). The AREBs/ABFs can bind to an ABRE *cis*-acting element and *trans*-activate downstream gene expression.

AREB/ABF-overexpressing plants show ABA hypersensitivity and enhanced abiotic stress tolerance such as to freezing, drought and salt stress (Kang et al. 2002, Kim et al. 2004, Fujita et al. 2005, Furihata et al. 2006). In cereals, many bZIP-type transcription factors have been identified. The LIP19 subfamily including rice LIP19 and OsOBF1, maize mLIP15 and OBF1 and wheat WLIP19 and TaOBF is involved as transcription factors in the low temperature signaling pathway (Aguan et al. 1993, Shimizu et al. 2005, Kusano et al. 1995, Kobayashi et al. 2008a). Rice TRAB1, barley HvABI5 and wheat TaABF are ABI5/ABF/AREB homologs and function in ABA-inducible gene expression in seeds (Hobo et al. 1999, Casaretto and Ho 2003, Johnson et al. 2002). Among them, TRAB1 and HvABI5 show high homology to AREB2/ABF4, and expression of the *TRAB1* and *HvABI5* genes is detected respectively in ABA-treated and drought-stressed seedlings (Hobo et al. 1999, Casaretto and Ho 2003, Xue and Loveridge 2004). HvABI5 binds to *cis*-elements in the promoter region of *HVA1*, which is an ABA- and stress-responsive *Cor/Lea* gene (Casaretto and Ho 2003, Xue and Loveridge 2004). *HvABI5* is assigned to the long arm of chromosome 5H and overlaps a quantitative trait locus (QTL) for drought tolerance (Tondelli et al. 2006). However, the contribution of TRAB1 and HvABI5 to abiotic stress tolerance remains unknown.

A number of *Cor*/*Lea* genes have been characterized in common wheat. Among them, 5’ upstream sequences were isolated from *Wcs120*, *Wcor15*, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Quellet et al. 1998, Takumi et al. 2003, Kobayashi et al. 2008b). *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* are responsive to low temperature, drought and ABA (Ohno et al. 2003, Kobayashi et al. 2004a, 2006, Egawa et al. 2006), and their promoter regions contain putative ABRE motifs (Kobayashi et al. 2008b). These observations suggest that the AREB/ABF-type transcription factors play roles in regulation of *Cor*/*Lea* gene expression.

The winter wheat cultivar ‘Mironovskaya 808’ (M808) develops a higher level of freezing tolerance than the spring-type cultivar ‘Chinese Spring’ (CS) after cold acclimation (Ohno et al. 2001, Kobayashi et al. 2004a, Kume et al. 2005). A fairly good correlation is observed between the cultivar-dependent levels of freezing tolerance and those of *Cor*/*Lea* family expression (Kobayashi et al. 2004a). In wheat and barley, *Vrn-1* and *Fr-1* are well known major QTLs respectively determining vernalization requirement for flowering and freezing tolerance. The two loci are closely linked on the long-arm of homoeologous group 5 chromosomes (reviewed by Cattivelli et al. 2002). It was previously supposed that winter-habit wheat should possess a winter-type *Fr-1* allele guaranteeing winter survival, but that such allele is unnecessary for spring-habit cultivars (Thomashow 1999). The allelic differences of *Vrn-1/Fr-1* intervals affect cold-responsiveness of *Cor/Lea* and transcription factor genes and result in influencing the freezing tolerance level in common wheat (Kobayashi et al. 2004b, 2005, Ishibashi et al. 2007). ABA sensitivity of M808 is higher than that of CS at the seedling stage, and M808 accumulates more *Cor*/*Lea* transcripts than CS after exogenous ABA treatment (Kobayashi et al. 2006). In this study, we isolated a wheat *HvABI5* ortholog, *Wabi5*, as a counterpart of *AREB/ABF*, and its expression profile was compared between two common wheat varieties, M808 and CS. Moreover, we produced *Wabi5*-expressing tobacco plants and studied their abiotic stress tolerance and activation of wheat *Cor*/*Lea* gene expression. Based on these results, we discuss the development of abiotic stress tolerance through WABI5-mediated *Cor*/*Lea* expression in common wheat.

Materials and methods

Isolation and sequencing of *Wabi5*

The *Wabi5* cDNA clones were isolated by RT-PCR using total RNA from ABA-treated leaves of common wheat (*Triticum aestivum* L.) cultivar CS seedlings with the primer set 5’-GAGGGGGTCATGGACTTCAG-3’ and 5’-GCCTACAGGTCAGCGGTCTC-3’. Amplified cDNAs of *Wabi5* were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and nucleotide sequences were determined by an automated fluorescent Dye Deoxy terminator cycle sequencing system using an ABI PRISM 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA).

Nucleotide sequences of the isolated cDNA clones and the predicted amino acid sequences were analyzed by DNASIS software (Hitachi, Tokyo, Japan). The cDNA sequences were deposited into the DDBJ database under these accession numbers: AB362818 (*Wabi5*-1), AB362819 (*Wabi5*-2) and AB362820 (*Wabi5*-3). Multiple sequence alignments were carried out using the ClustalW computer program (Thompson et al 1994, http://align.genome.jp/), and a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987).

Southern blot analysis

For genomic Southern blot analysis, total DNA extracted from hexaploid wheat cv. CS and M808, tetraploid emmer wheat (*T. durum*) cv. ‘Langdon’ (Ldn) and diploid wheat *T. monococcum* (mnc) was digested with the restriction enzyme *Hin*dIII. The digested DNA was fractionated by electrophoresis through an 0.8% agarose gel, transferred to Hybond N^+ nylon membrane (GE Healthcare, Piscataway, NJ, USA) and hybridized with ^{32}P-labeled *Wabi5* cDNA as a probe. Probe labeling, hybridization, washing and autoradiography were performed according to Takumi et al. (1999). For chromosome assignment of *Wabi5*, genomic Southern blot analysis was conducted using total DNA from a nulli-tetrasomic series of CS (Sears 1966). Each line of the nulli-tetrasomic series lacks a given pair of homoeologous A, B or D genome chromosomes (the nullisomic condition) that have been replaced by the corresponding homoeologous chromosome pair (the tetrasomic condition). Total DNA extracted from the nulli-tetrasomics was digested with *Hin*dIII.

Gene expression analysis

To analyze the gene expression pattern of *Wabi5*, 7-day-old seedlings of CS and M808 grown under standard conditions (25˚C) according to Kobayashi et al. (2004a) were transferred to 4˚C and kept for various time periods under standard lighting conditions. Seven-day-old seedlings were also treated with a solution containing 20 μM ABA by a foliar spray or dehydrated on dry filter paper in a desiccator. Total RNA was extracted from the seedlings, and accumulation of *Wabi5* transcript was detected by RT-PCR amplification as previously reported (Kobayashi et al. 2004b, Kobayashi et al. 2006). RT-PCR was conducted with the gene-specific primer set 5'CACCCTCAGCGCCAAGAC-3' and 5'-CTCCCATACCAACTGCCCTC-3'. The ubiquitin gene (*Ubi*) was used as an internal control (Kobayashi et al. 2005). The PCR products were separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. Intensity of the fragments was assessed by scanning the electropherograms with ImageJ 1.37v software (http://rsb.info.nih.gov/ij/), and relative values were calculated after normalization to *Ubi* transcripts. The entire experiment was conducted twice.

Near-isogenic lines (NILs) for the *Vrn-1* genes of spring-type common wheat cultivar 'Triple Dirk' (TD) (Pugsley 1971, 1972) were also used in gene expression study. A winter-type non-carrier line TD(C) was bred by eliminating all of the dominant *Vrn-1* alleles from TD with both dominant *Vrn-A1* and *Vrn-B1*. Two *Vrn-1* NILs, TD(D) and TD(B), carry dominant *Vrn-A1* and *Vrn-B1* alleles, respectively. Total RNA extraction from the NILs was performed according to Kobayashi et al. (2005).

Generation of transgenic tobacco plants expressing *Wabi5*

The *Wabi5*-1 cDNA sequence was amplified with the following primer set containing the *Bam*HI linker: 5'-CGGGATCCGGGGTCATGGACTTCA-3' and 5'-CGGGATCCACAAAGCAGGTCGACC-3'. The PCR fragment was digested with *Bam*HI and inserted into the *Bam*HI site after the cauliflower mosaic virus (*CaMV*) *35S* promoter in pROK1a (Baulcombe et al. 1986). Transgenic tobacco plants were produced by the *Agrobacterium*-infection method. The construct was introduced into leaf discs of *Nicotiana tabacum* cv. 'Petit Havana' using *Agrobacterium tumefaciens* LBA4404. Transformants were selected in Murashige-Skoog (MS) medium (Murashige and Skoog 1962) containing 0.1 mg l^{-1} alpha-naphthalene acetic acid, 1.0 mg l^{-1} 6-benzylaminopurine, 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. The transgenic tobacco plants generated were named *35S::Wabi5*. The *Wabi5* transcripts in the *35S::Wabi5* lines were detected by RT-PCR with the same set of primers used for the chimeric plasmid construction. The actin gene was used as an internal control in the transgenic tobacco and was amplified with primers 5'- GGCTGGTTTTGCTGGTGACGAT-3' and 5'-AATGAAGGAAGGCTGGAAGAGGA-3'.

Bioassay conditions for freezing and osmotic stress tolerance

To assay freezing tolerance, T_2 progeny of *35S::Wabi5* and wild-type tobacco were planted on MS agar plates for germination. Two weeks after planting, 20 seedlings from each line were transferred to a new MS agar plate and incubated at normal temperature condition (27˚C). After 2 days, the tobacco plants were frozen at –15±1˚C for 1 h or 2 h in the dark without cold acclimation using a freezer EFD-25F2 (Fukushima Kogyo, Osaka, Japan). Next, 10 two-week-old seedlings from each of the *35S::Wabi5*-#21, *35S::Wcbf2*-#4 (Takumi et al. 2008), *35S::Wdreb2*-#11 (Kobayashi et al. 2008b) and *35S::Wlip19*-#9 (Kobayashi et al. 2008a) transgenic tobacco lines were planted in one MS agar plate and then frozen at –15±1˚C for 1.5 h in the dark without cold acclimation. 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, 7-day-old seedlings of wild-type and *35S::Wabi5* 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) under normal temperature conditions. At 2 days after treatment with mannitol and 4 days after treatment with NaCl, the number of plants with green cotyledons was scored. The experiment was performed in triplicate three or four times and the data were statistically analyzed by Student's *t*-test.

Bioassay for ABA sensitivity during germination

Seed germination was studied in three sets of 100 seeds each of wild-type and T_2 progeny of *35S::Wabi5*. The seeds were placed on MS agar plates with or without 1 μM ABA, and incubated at 27˚C under a 16 h photoperiod. Germination was scored for 10 days after planting.

In a bioassay of ABA sensitivity based on root growth, ten 5-day-old seedlings of wild-type and *35S::Wabi5* plants were placed in a glass petri dish containing filter paper wetted with 3 ml of distilled water or 1 μM ABA solution, and incubated at 27˚C under a 16 h photoperiod. After 8 days, the length of primary roots was recorded. The experiment was performed in triplicate three times and the data were statistically analyzed by Student's *t*-test.

Interaction of WABI5 with wheat *Cor/Lea* gene promoters

Cor/Lea pro::GUS and *35S::Wabi5* constructs were purified using a Maxi-V500 ultrapure plasmid extraction system (Viogene, Sunnyvale, CA, USA) and introduced with a chimeric construct of the luciferase gene under the control of the *CaMV35S* promoter into wheat callus line HY-1 by particle bombardment according to Takumi et al. (1999). GUS activity was quantified by the method reported in Jefferson (1987) and normalized by the luciferase activity estimated using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

35S::Wabi5 transformants were used as pollen parent in crosses with transgenic tobacco plants having the *Cor/Lea pro::GUS* constructs. F_1 transgenic tobacco plants were selected on hormone-free MS medium containing 50 mg l^{-1} kanamycin. GUS activity in the kanamycin-resistant F_1 tobacco plants and homozygous T_2 progeny of *Cor/Lea pro::GUS* plants was assessed according to Takumi et al. (2003) and Jefferson (1987).

Results

Isolation and chromosome assignment of *Wabi5* cDNA

Two wheat EST clones, FGAS072486 (accession number CV778078) and whd17e22 (BJ228615, BJ223890), showed high homology with *HvABI5* at the nucleotide and amino acid sequence levels, but did not contain a complete ORF. Three cDNA sequences, *Wabi5*-1, *Wabi5*-2 and *Wabi5*-3, with a complete ORF were isolated from leaves of ABA-treated seedlings of CS by RT-PCR with a gene-specific primer set designed based on the EST and *HvABI5* sequences. Comparison of the three *Wabi5* cDNA sequences showed that nucleotide substitutions, insertions and deletions occurred within their ORFs and in the 3' untranslated regions (see Appendix S1 in Supplementary Material). WABI5 was a bZIP-type protein containing three N-terminal (C1, C2 and C3) and one S-terminal (C4) conserved domains among ABI5/ABF/AREB bZIPs and showed an amino acid identity of 92% with HvABI5 (see Appendix S2 in Supplementary Material). A phylogenetic tree of the bZIP-type proteins belonging to groups S, A (Jakoby et al. 2002) and other types in monocots was constructed by the neighbor-joining method (Fig. 1). The three WABI5 sequences showed the highest levels of identity with HvABI5, rice EST clone J013049N23 and TRAB1, followed by AREB/ABF-type proteins of dicots including *Arabidopsis* AREB1/ABF2, AREB2/ABF4, ABF1 and ABF3 (Fig. 1). TaABI5, which had been registered in database as a wheat homolog of *Arabidopsis* ABI5, was closely related to TaABF, HvABF1 and OsABI5 but not to WABI5, HvABI5 and TRAB1 (Fig. 1). Therefore, *TaABI5* was considered to be either homoeologous or paralogous to *TaABF* in hexaploid wheat and distinct from *Wabi5*.

To study copy number of the *Wabi5* genes in the wheat genome, Southern blot analysis was conducted using total DNA isolated from hexaploid, tetraploid and diploid wheat. Southern blots showed low copy numbers of *Wabi5* in hexaploid, tetraploid and diploid wheat genomes (Fig. 2a). To assign *Wabi5* to wheat chromosomes, aneuploid analysis was performed using a series of CS nulli-tetrasomic lines. *Wabi5*-specific bands were absent only in the nulli-tetrasomic lines of homoeologous group 5 chromosomes (Fig. 2b), indicating that the common wheat genome contains three copies of *Wabi5* and that *Wabi5* should be assigned to the three homoeologous loci on chromosomes 5A, 5B and 5D.

Expression profile of *Wabi5* during cold acclimation

Wabi5 expression was detected at a low level under non-stress conditions, and its level increased slightly within 15 min after exposure of wheat seedlings to low temperature (Fig. 3A,B). The transcript level reached a maximum by 6 h and then gradually decreased over 24 h in CS, while a high expression level was observed within 2 h and then the accumulation was fluctuated during 4-12 h in M808 (Fig. 3A,B). The temporal enhancement of *Wabi5* expression was gradual in comparison with that of other transcription factor genes such as *Wcbf2*, *Wdreb2* and *Wlip19* (Fig. 3A,B, Kume et al. 2005, Egawa et al. 2006, Kobayashi et al. 2008a). After 3 d of low temperature treatment, the *Wabi5* expression level decreased continuously over 7 d, and the accumulation was upregulated at day 10 in CS and M808 (Fig. 3C,D). During long-term low temperature treatment, the *Wabi5* transcript level increased again by 21 d of low temperature in M808 while a low level was maintained through 35 d in CS, then high-level expression was detected at days 42 and 63 in both CS and M808 (Fig. 3E,F). Although no apparent differences on *Wabi5* expression were observed between CS and M808 during long-term treatment (Fig. 3E,F), the expression level was higher in M808 than in CS over 10 d of treatment (Fig. 3A-D), indicating that observed differences in the transcript accumulation patterns of *Wabi5* between the two cultivars correlated partly with their distinct levels of freezing tolerance.

The low temperature responsibility of *Wcbf2*, *Wdreb2* and *Wlip19* transcription factor genes were at least partly regulated by the *Vrn-1*/*Fr-1* chromosomal regions, and NILs with dominant alleles of *Vrn-1* significantly reduced the transcript accumulation levels of *Wcbf2*, *Wdreb2* and *Wlip19* compared with NIL with the recessive *vrn-1* alleles (Kobayashi et al. 2004b, 2005). Therefore, effect of the chromosomal regions on the *Wabi5* expression was studied using wheat NILs for the *Vrn-1* loci. However, no obvious difference of the *Wabi5* expression levels was observed among the four NILs (Fig. 3G,H).

Wabi5 **response to drought stress and ABA treatment**

To study the effect of other stimuli on *Wabi5* expression, the time course of its expression was studied during 24 h of drought stress or ABA treatment in leaves of CS and M808 seedlings. Enhancement of *Wabi5* expression was observed within 15 min of drought and the transcript level reached a high plateau by 4 h in both CS and M808 (Fig. 4A,B); the *Wabi5* transcript then decreased over 24 h in CS, while it was maintained at a high level until 12 h in M808 (Fig. 4A,B). Comparison of *Wabi5* and *Cor*/*Lea* gene expression showed that the induction of *Wabi5* occurred more than 2 h prior to that of *Wdhn13*, *Wrab18* and *Wrab19* (Fig. 4A,B, Egawa et al. 2006).

Wabi5 expression was increased by exogenous treatment with ABA and reached a maximum level after 10 h in M808 leaves, whereas in CS the increase was gradual and continued for at least 24 h (Fig. 4C,D). *Wabi5* expression was higher in M808 than in CS after ABA treatment, indicating that the cultivar difference in the *Wabi5* transcript level correlated with sensitivity to exogenous ABA (Kobayashi et al. 2006).

Abiotic stress tolerance of transgenic tobacco plants expressing *Wabi5*

To study the contribution of *Wabi5* to abiotic stress tolerance, *35S::Wabi5* transgenic tobacco plants were produced. Twenty-four transgenic tobacco plants were generated on kanamycin-containing medium, and integration of the introduced chimeric gene was confirmed by Southern blot analysis (data not shown). Ectopic expression of *Wabi5* was detected by RT-PCR in these transgenic T_1 plants (Fig. 5A). Based on the stability and inheritance of *Wabi5* expression, three transgenic lines, *35S::Wabi5*-#20, *35S::Wabi5*-#21 and *35S::Wabi5*-#22, were established and their T_2 progeny were used for the following analysis. No phenotypic alteration was observed in these transgenic tobacco plants.

The level of freezing stress tolerance was compared between the three *35S::Wabi5* lines and wild-type tobacco plants (Fig. 5B). Three *35S::Wabi5* lines showed more than 60% survival after 1 h of freezing, whereas wild type had only 5% survival (Fig. 5C). Freezing tolerance was improved in all the transgenic tobacco lines by *Wabi5* expression. The *35S::Wabi5*-#20 and #21 plants showed 6.7% and 3.3% survival respectively, but the wild-type and *35S::Wabi5*-#22 plants were killed when exposed to –15˚C for 2 h, showing that the levels of freezing tolerance in *35S::Wabi5*-#20 and #21 were higher than that in *35S::Wabi5*-#22. The level of freezing tolerance in *35S::Wabi5*-#21 was also compared with other transgenic tobacco plants expressing either *Wcbf2*, *Wdreb2* or *Wlip19*. The *35S::Wcbf2*-#4, *35S::Wdreb2*-#11 and *35S::Wlip19*-#9 transgenic tobacco lines showed the highest freezing tolerance levels of all transgenic lines in our studies (Takumi et al. 2008, Kobayashi et al. 2008a, 2008b). *35S::Wabi5*-#21 showed a 32.5% survival after 1.5 h of freezing, while other transgenic lines had more than 50% survival. In particular, the tolerance level in *35S::Wcbf2*-#4 was significantly higher than that in *35S::Wabi5*-#21 (Fig. 5D).

Next, tolerance to osmotic stress was estimated by treatment with mannitol and NaCl solutions. Under mannitol and NaCl stress, cotyledons of wild-type seedlings of tobacco yellowed. After 4 d of mannitol treatment, plants with healthy green cotyledons were 35%, 80%, 63.3% and 45% of wild-type, *35S::Wabi5*-#20, #21 and #22 lines, respectively (Fig. 5E), indicating that the tolerance levels of the three *35S::Wabi5* tobacco lines were higher than that of wild-type plants. Under NaCl stress, the *35S::Wabi5*-#20 and #21 lines exhibited significantly increased tolerance to high NaCl concentration, although *35S::Wabi5*-#22 was as sensitive to NaCl stress as wild type (Fig. 5F).

ABA sensitivity in transgenic tobacco expressing *Wabi5*

To study ABA sensitivity in early seedling development, inhibition of seedling growth by exogenous ABA (1 μM) was compared among the wild-type and three *35S::Wabi5* tobacco lines. Root elongation of the tobacco plants was inhibited by exogenous ABA treatment. The magnitude of inhibition of root growth, estimated by the relative root growth rate (% growth in the presence of ABA relative to growth in the absence of ABA), was greater in the *35S::Wabi5* lines than in wild type (Fig. 5G), indicating that primary root elongation of the *35S::Wabi5* plants was hypersensitive to exogenous ABA during post-germination growth. On the other hand, germination rates of mature seeds were compared under both ABA and non-ABA conditions among the wild-type and three *35S::Wabi5* lines. Under both conditions, the *35S::Wabi5* transgenic lines showed similar germination rates to that of wild type (data not shown).

***Trans*-activation of wheat *Cor*/*Lea* promoters by WABI5**

To study direct interaction between *Cor/Lea* promoters and WABI5, transient expression analysis was conducted by introducing a chimeric *35S::Wabi5* gene with each of four *Cor/Lea pro::GUS* constructs (*Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19*; Kobayashi et al. 2008b) into a wheat cell line. The *Cor/Lea pro::GUS* constructs with the exception of *Wrab17 pro::GUS* yielded higher GUS activity when co-introduced with the *35S::Wabi5* construct, although no significance of the effect of co-introduction was observed in this transient experiment using the wheat cell line (Fig. 6A).

To clarify the interaction between WABI5 and wheat *Cor/Lea* promoters, F_1 progeny was produced by crossing the *Cor/Lea pro::GUS* plants with the *35S::Wabi5* lines. A histchemical GUS staining assay showed that *Wabi5* expression enhanced GUS levels under control of the 5' upstream sequences of the *Wdhn13*, *Wrab18* and *Wrab19* at the normal growth temperature for the F_1 plants. Weak background GUS expression was detected in vascular bundle and stem tissues, and rarely observed in leaves of *Cor/Lea pro::GUS* plants, whereas *Wabi5* enhanced GUS activity in leaves of the *Wdhn13 pro::GUS*, *Wrab18 pro::GUS* and *Wrab19 pro::GUS* F_1 seedlings (Fig. 6B). However, the *Wrab17 pro::GUS* lines had no visible enhancement of GUS staining levels with the *35S::Wabi5* construct in the F_1 plants (data not shown). GUS quantification showed that GUS activity under the control of the *Wdhn13*, *Wrab18* and *Wrab19* promoters was significantly increased in F_1 seedlings compared with the parental transgenic plants, although no increase in GUS activity under control of the *Wrab17* promoter was observed in the F_1 plants (Fig. 6C).

Discussion

Expression profiles of *Wcbf2*, *Wdreb2*, *Wlip19* and *Cor/Lea* genes showed dramatic upregulation during cold acclimation and good correlation with development of freezing tolerance (Ohno et al. 2001, 2003, Takumi et al. 2003, Kume et al. 2005, Egawa et al. 2006, Kobayashi et al. 2008a). *Wabi5* expression was also enhanced by low temperature, and its expression profile corresponded to cultivar differences in freezing tolerance between freezing-tolerant M808 and freezing-sensitive CS (Fig. 3A-D). Differences in the *Wabi5* expression profiles also seemed to reflect the distinct expression patterns of the *Cor*/*Lea* family members between M808 and CS as well as other transcription factor genes such as *Wcbf2*, *Wdreb2* and *Wlip19*. Heterologous expression of *Wabi5* increased freezing tolerance of the transgenic tobacco lines (Fig. 5B,C), implying that *Wabi5* functions in cold acclimation and development of freezing tolerance. However, the enhanced expression level of *Wabi5* under low temperature was likely to be lower than that of *Wcbf2*, *Wdreb2* and *Wlip19* (Fig. 3A-D, Kume et al. 2005, Egawa et al. 2006, Kobayashi et al. 2008a), and no cultivar differences of *Wabi5* expression level between CS and M808 was observed during long-term low temperature treatment (Fig. 3E,F). The *35S::Wabi5* tobacco line was more sensitive to freezing stress than *35S::Wcbf2*, *35S::Wdreb2* and *35S::Wlip19* (Fig. 5D). These results suggested that the effect of *Wabi5* is inferior in development of freezing tolerance to that of other transcription factors such as *Wcbf2*, *Wdreb2* and *Wlip19*.

The *Vrn-1/Fr-1* chromosomal regions control *Cor*/*Lea* gene expression through CBF transcription factors (Kobayashi et al. 2005). *Wdreb2* and *Wlip19* activation under low temperature conditions is also affected by the *Fr-1* allele (Kobayashi et al. 2004b). Especially the expression level of *Wcbf2* was dramatically reduced in NILs carrying the spring-type *Vrn-1/Fr-1* even under low temperature condition, whereas the expression was upregulated in the TD(C) line with the winter-type *vrn-1/Fr-1* (Kobayashi et al. 2005). Comparison of gene expression profiles of *Wabi5* among NILs for *Vrn-1* showed no apparent differences during low temperature treatment (Fig. 3G,H), indicating that cold responsiveness of *Wabi5* was not affected by *Fr-1*. The differences of gene expression level during cold acclimation and freezing tolerance level between CS and M808 are assumed to be in large part caused by the *Fr-1* allelic difference (Kume et al. 2005). The cultivar difference of *Wabi5* expression between CS and M808, therefore, was not so large as compared with those of *Wcbf2*, *Wdreb2* and *Wlip19*. We previously reported that the low temperature-induced expression of *Wabi5* is enhanced in an ABA-hypersensitive mutant line (Kobayashi et al. 2008c). *Wabi5* expression was responsive to ABA (Fig. 4C,D), and *35S::Wabi5* transgenic tobacco lines showed ABA hypersensitivity (Fig. 5G), suggesting that *Wabi5* acts in the ABA signaling pathway during cold acclimation separately from *Fr-1*.

Wabi5 expression was also enhanced under drought stress (Fig. 4A,B), and its responsiveness was clear compared with other bZIP-type transcription factors such as *Wlip19* and *TaOBF1* (Kobayashi et al. 2008). The expression pattern of *Wabi5* showed good correlation with the expression patterns of *Cor*/*Lea* family members, especially *Wdhn13*, *Wrab18* and *Wrab19*, under drought stress (Fig. 4A,B, Egawa et al. 2006). *Wabi5* expression significantly enhanced *GUS* expression under control of the *Wdhn13*, *Wrab18* and *Wrab19* promoter regions (Fig. 6). These results prove that WABI5 functions as a transcriptional activator and positively regulates *Wdhn13*, *Wrab18* and *Wrab19* gene expression. *35S::Wabi5* transgenic tobacco became tolerant to high mannitol and salt stress compared with wild-type tobacco (Fig. 5E,F), strongly suggesting that *Wabi5* is associated with development of osmotic stress tolerance through *Cor/Lea* gene activation. In *Arabidopsis*, AREBs/ABFs play central roles as transcription factors in ABA-responsive gene expression under osmotic stress conditions (Yamaguchi-Shinozaki and Shinozaki 2006). WABI5 was classified into the group A bZIPs including AREBs/ABFs (Fig. 1) and had positive roles in development of multiple abiotic stress tolerance (Fig. 5). Thus, these results suggest that WABI5 functions as a counterpart of AREBs/ABFs under abiotic stress conditions in common wheat and that the function of the AREB/ABF family is conserved between *Arabidopsis* and wheat.

Based on sequence homology and chromosome assignment (Figs. 1, 2, see Appendix S2 in Supplementary Material), *Wabi5* is a wheat ortholog of barley *HvABI5*. HvABI5 interacts with HvVP1 in the activation of a reporter gene under control of the *HVA1* promoter in barley aleurone cells (Casaretto and Ho 2003), and additionally functions in the activation of *HVA1* expression in barley leaves cooperatively with HvDRF1, which is an EREBP (ethylene-responsive element binding protein)/AP2 (APETALA2)-type transcription factor that interacts with a promoter sequence of *HVA1* (Xue and Loveridge 2004). This cooperation of HvABI5 and HvDRF1 shows a synergistic effect in the activation of *HVA1* expression. The 5' upstream sequences of *Wrab18*/*Wrab19* are also directly recognized by WDREB2 (Egawa et al. 2006, Kobayashi et al. 2008b). *Wdreb2* is considered to be a wheat ortholog of the *HvDRF1* (Egawa et al. 2006), and *Wrab18*/*Wrab19* show high homology to *HVA1* (Kobayashi et al. 2004a). Thus, WABI5 and WDREB2 act cooperatively in the activation of *Wrab18*/*Wrab19* expression, and this cooperation in the stress signal transduction pathway is conserved in wheat and barley. Expression of other *Cor/Lea* genes such as *Wdhn13* and *Wrab17* is regulated by WCBF2, WDREB2 and WLIP19 (Takumi et al. 2008, Kobayashi et al. 2008a, 2008b), implying that these transcription factors including WABI5 cooperatively activate *Cor/Lea* gene expression under abiotic stress conditions. Therefore, various stress-responsive transcription factor genes participate in the wheat abiotic stress signal network, in which *Cor/Lea* expression is finally regulated through the *Fr-1*-dependent and -independent signal transduction pathways. The cooperative interaction between transcription factors possibly has a synergistic effect on the activation of gene expression, resulting in development of abiotic stress tolerance. Thus, WABI5 acts as an important transcription factor with other transcription factor genes in abiotic stress responses and tolerance of common wheat.

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Supplementary materials

The following supplementary materials are available for this article:

Appendix S1. Alignment of nucleotide sequences of three homoeologous *Wabi5* cDNAs.

Appendix S2. Alignment of WABI5 amino acid sequences with those of the ABI5/ABF/AREB subfamily in cereals.

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Legends of Figures

Figure 1. Phylogenetic tree based on amino acid sequences, showing the relationship of WABI5 with other plant bZIP-type proteins. The deduced amino acid sequences were aligned with ClustalW. The phylogenetic tree was constructed by the neighbor-joining method based on Nei's genetic distance. The accession numbers (in parentheses) of the amino acid sequences are: wheat TaABF (AF519804), TaABI5 (AB238932), EmBP-1 (U07933), WLIP19a (AB334127) and TaOBF1a (AB334129), barley HvABI5 (AY150676), HvABF1 (DQ786408), HvABF3 (DQ786410) and HvZIP1 (AY150677), rice TRAB1 (AB023288), J013049N23 (AK065873), OsABI5 (EF199630), LIP19 (X57325), OsOBF1 (AB185280) and OSBZ8 (AY606941), maize mGBF1 (U10270), mLIP15 (D26563) and OBF1 (X62745), *Arabidopsis* AREB1/ABF2 (AB017160/AF093545), AREB2/ABF4 (AB017161/AF093547), ABF1 (AF093544), ABF3 (AF093546), AREB3 (AB017162), AtDPBF2 (AF334207), AtDPBF4 (AF334209), ABI5 (AC006921), GBF5 (AF053939), ATB2 (X99747) and AtbZIP53 (AF400620), tobacco PHI-2 (AB063648), NtbZIP (DQ073639) and TBZ17 (D63951), tomato LeAREB (AY530758), *Caragona korshinskii* CkAREB (DQ787780), *Populus trichocarpa* PtrABF2 (EF405964) and *Populus suaveolens* PsABF2 (DQ487100).

Figure 2. Copy number and chromosome assignment of *Wabi5* in the wheat genome. (A) Southern blot analysis of *Hin*dIII-digested total DNA from the indicated hexaploid, tetraploid and diploid wheat accessions. The blot was probed with ^{32}P-labelled *Wabi5* cDNA. (B) Chromosome assignment of *Wabi5* to the homoeologous group 5

chromosomes. DNA from the nulli-tetrasomic series of CS was digested with *Hin*dIII. N5AT5B, for example, represents a line nullisomic for chromosome 5A and tetrasomic for chromosome 5B.

Figure 3. Expression analyses of *Wabi5* in two wheat cultivars and near isogenic lines during low temperature conditions. (A, B) Transcript accumulation profiles in response to low temperature within one day revealed by RT-PCR (A) and quantified relative to the *Ubi* transcript as mean values with standard deviation (B). (C, D) Transcript accumulation profiles in response to low temperature from 1 to 10 d. (E, F) Transcript accumulation profiles in response to low temperature from 14 to 63 d. (G, H) Transcript accumulation in the NILs for *Vrn-1* loci treated with low temperature for 0, 7 and 14 d. TD(C), carrying recessive *vrn-1* alleles at all three *Vrn-1* homoeologous loci; TD(D), carrying a dominant *Vrn-A1* allele; TD(B) carrying a dominant *Vrn-B1* allele; TD, original line carrying dominant *Vrn-A1* and *Vrn-B1* alleles.

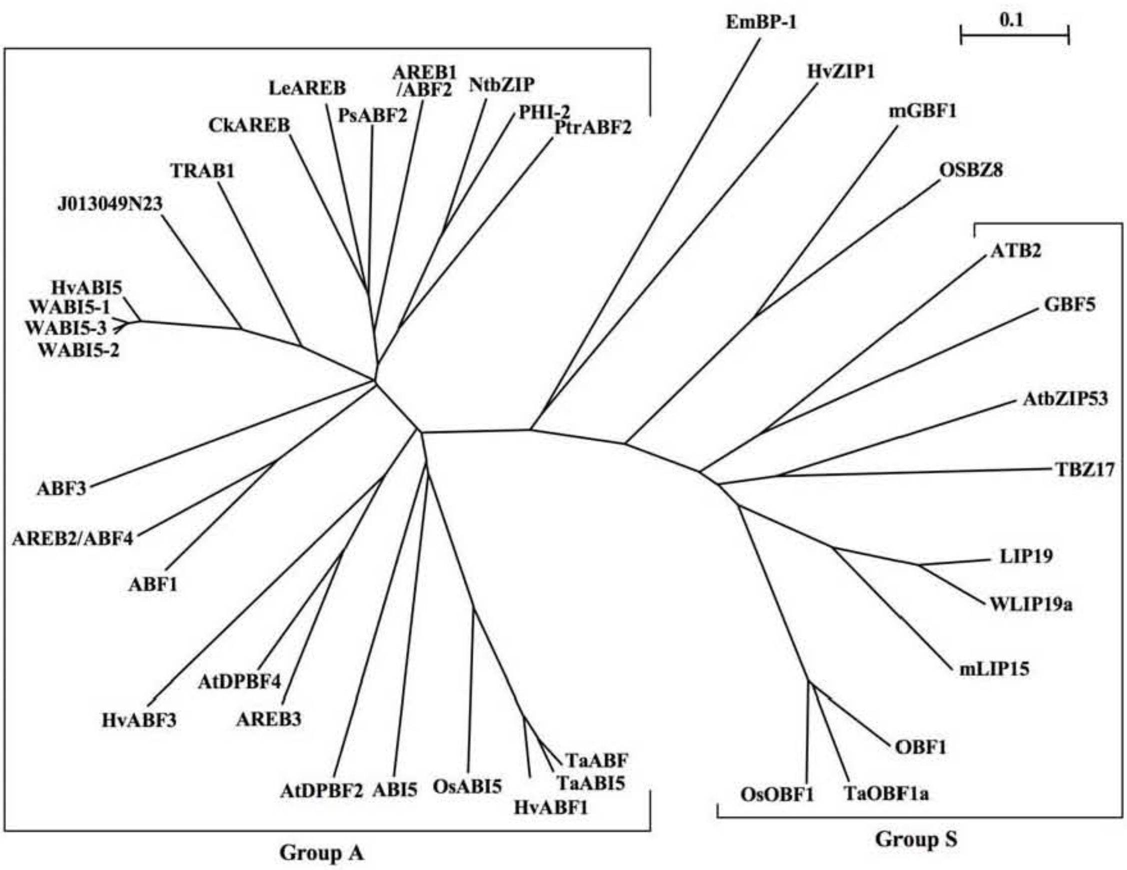
Figure 4. Expression profiles of *Wabi5* under drought and ABA treatment. (A, B) Time course of transcript accumulation during drought treatment in two wheat cultivars revealed by RT-PCR (A) and quantified relative to the *Ubi* transcript as mean values with standard deviation (B). (C, D) ABA responsiveness in two wheat cultivars. The ubiquitin gene (*Ubi*) was used as a control in RT-PCR.

Figure 5. Abiotic stress tolerance in *35S::Wabi5* tobacco plants. (A) RT-PCR analysis of *Wabi5* expression in three *35S::Wabi5* transgenic tobacco lines (#20, #21 and #22). *Actin* was

used as an internal control for RT-PCR. (B) Increased freezing tolerance in transgenic tobacco plants expressing *Wabi5*. The introduced *Wabi5* was controlled by a CaMV35S promoter. Non-acclimated transgenic and wild-type plants were treated at freezing temperature (-15˚C) for 1 h. (C) Comparison of freezing tolerance between *35S::Wabi5* and wild-type tobacco plants. Survival rates were compared after 1 h of -15˚C treatment. (D) Comparison of freezing tolerance among four transgenic tobacco lines, *35S::Wabi5*-#21, *35S::Wcbf2*-#4, *35S::Wdreb2*-#11 and *35S::Wlip19*-#9. Survival rates were compared after 1.5 h of -15˚C treatment. (E) Comparison of osmotic stress tolerance. Percentages of plants with green cotyledons were compared after supplementation with a 0.5 M mannitol solution. (F) Comparison of salt stress tolerance. Percentages of plants with green cotyledons were compared after supplementation with a 0.2 M NaCl solution. (G) The magnitude of inhibition by ABA treatment. Relative root growth was estimated as the percentage of the length of roots treated with 1 μM ABA to those without ABA. The primary root length was measured on the 8th day of treatment. The means ± standard deviations were calculated from data in 3 or 4 experiments. Student's *t*-test was used to test the statistical significance (**P*<0.05, ***P*<0.01) between wild-type plants and transgenic lines (C, E, F, G) and between *35S::Wabi5*-#21 and other transgenic lines (D).

Figure 6. *Trans*-activation of *Cor/Lea pro::GUS* chimeric genes by WABI5. (A) Transient expression analysis in wheat cultured cell line HY-1. GUS activity was normalized as luciferase activity expressed under the control of the *CaMV35S* promoter. The *GUS* gene under the control of a *CaMV35S* promoter and the maize *Adh1* first intron (pCaMVIGN) was used as a control. (B) Comparison of histochemical GUS staining in F_1 seedlings of

Cor/Lea pro::GUS and *35S::Wabi5* transgenic plants and parental transgenic plants. (C) GUS activity in F_1 seedlings and parental *Cor/Lea pro::GUS* transgenic plants. Means ± standard deviations were calculated from data in 3 experiments. Asterisks indicate significance at the 5% (*) and 1% (**) level (Student's *t*-test).



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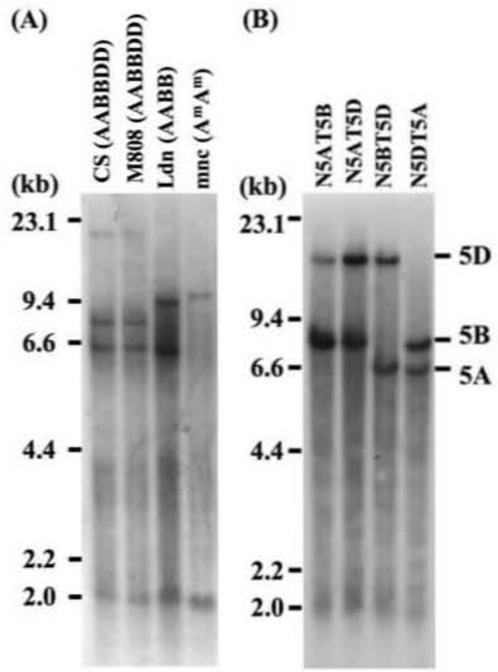

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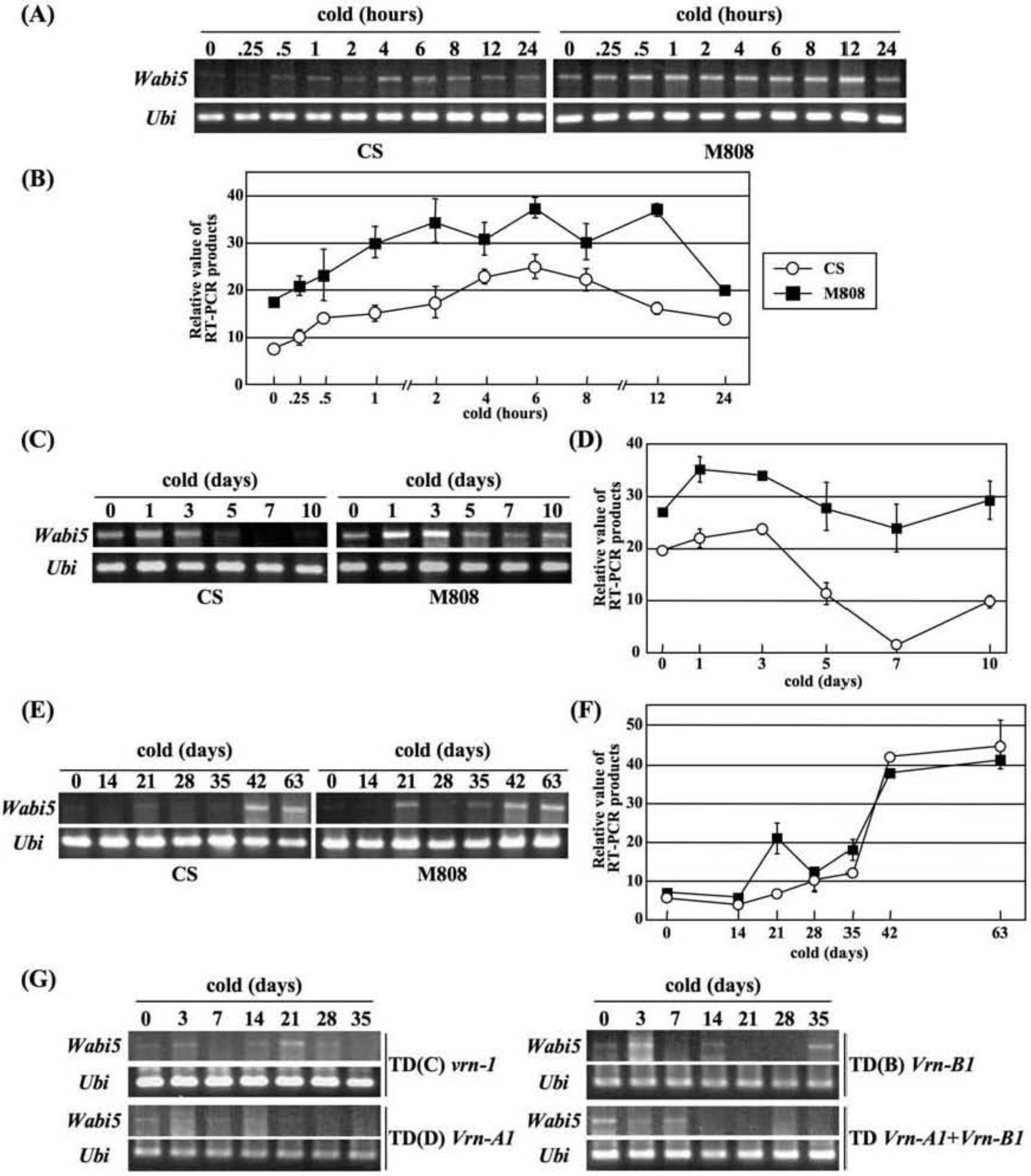

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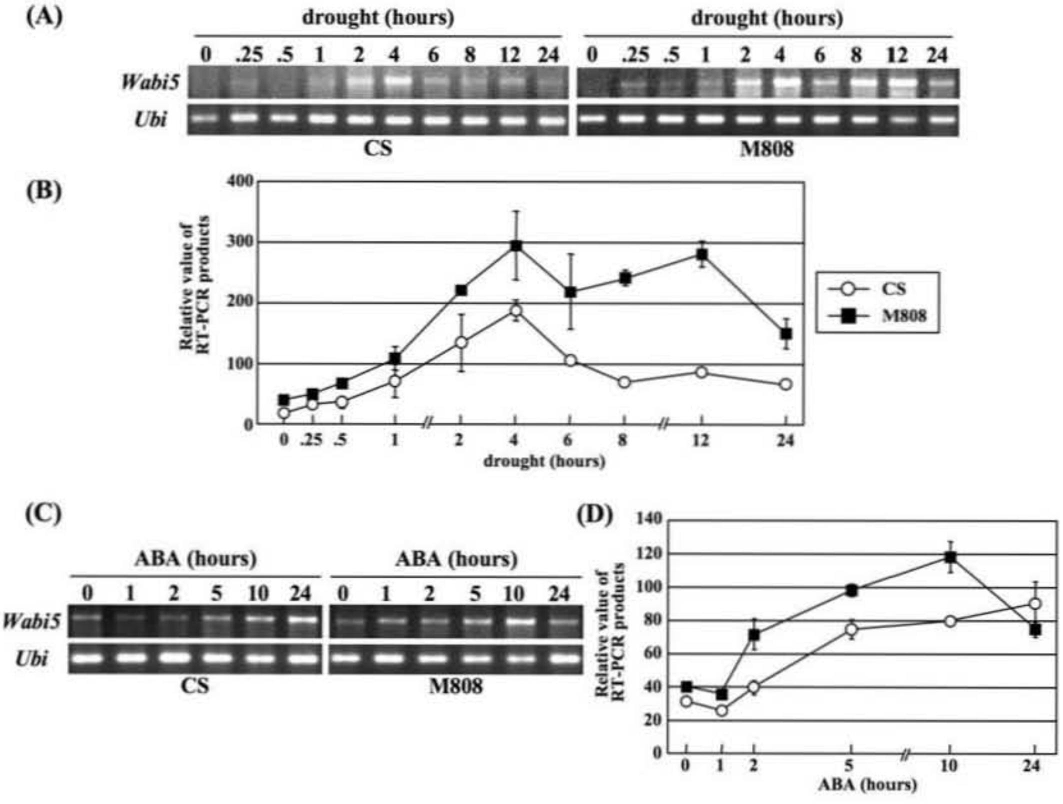

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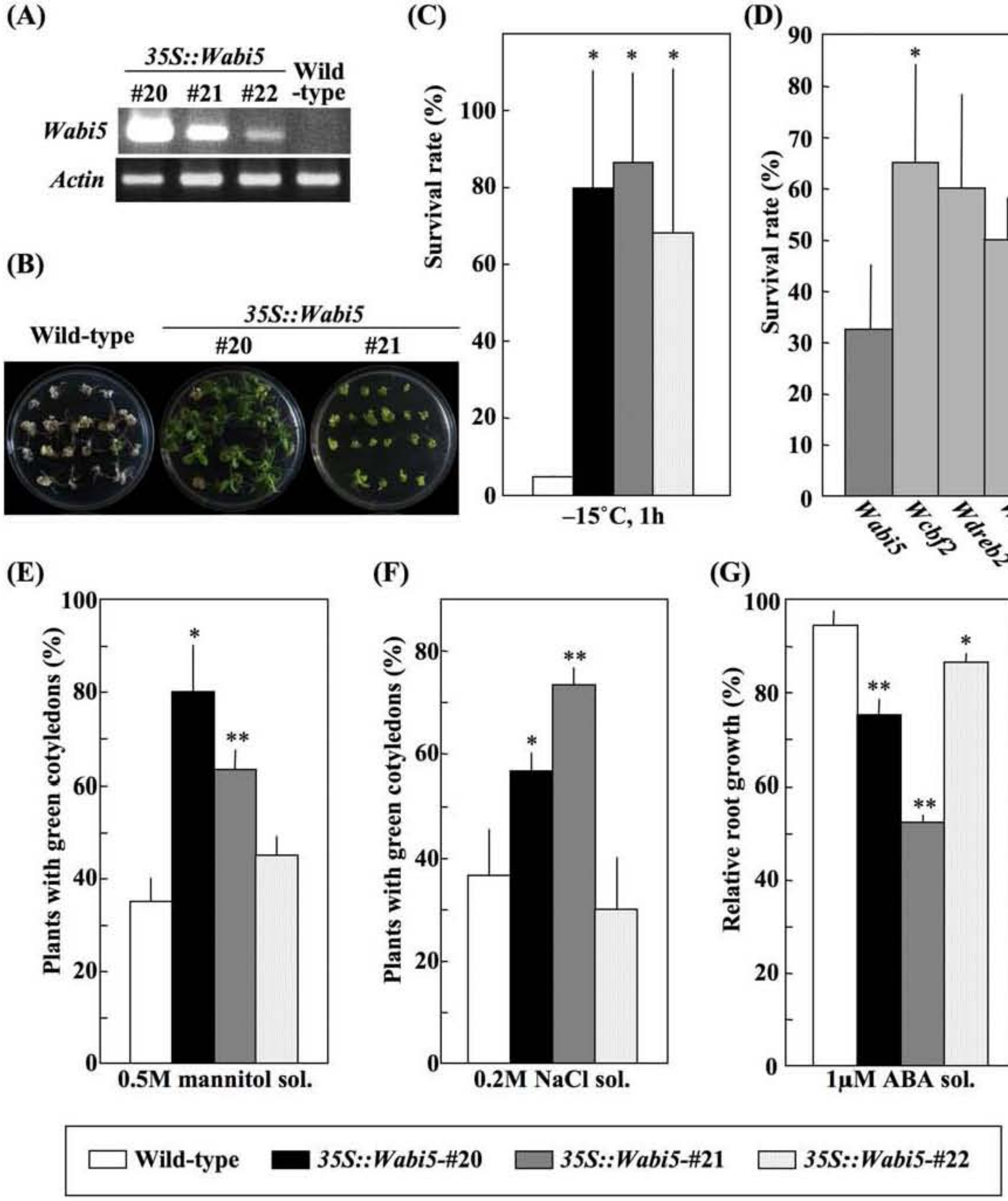

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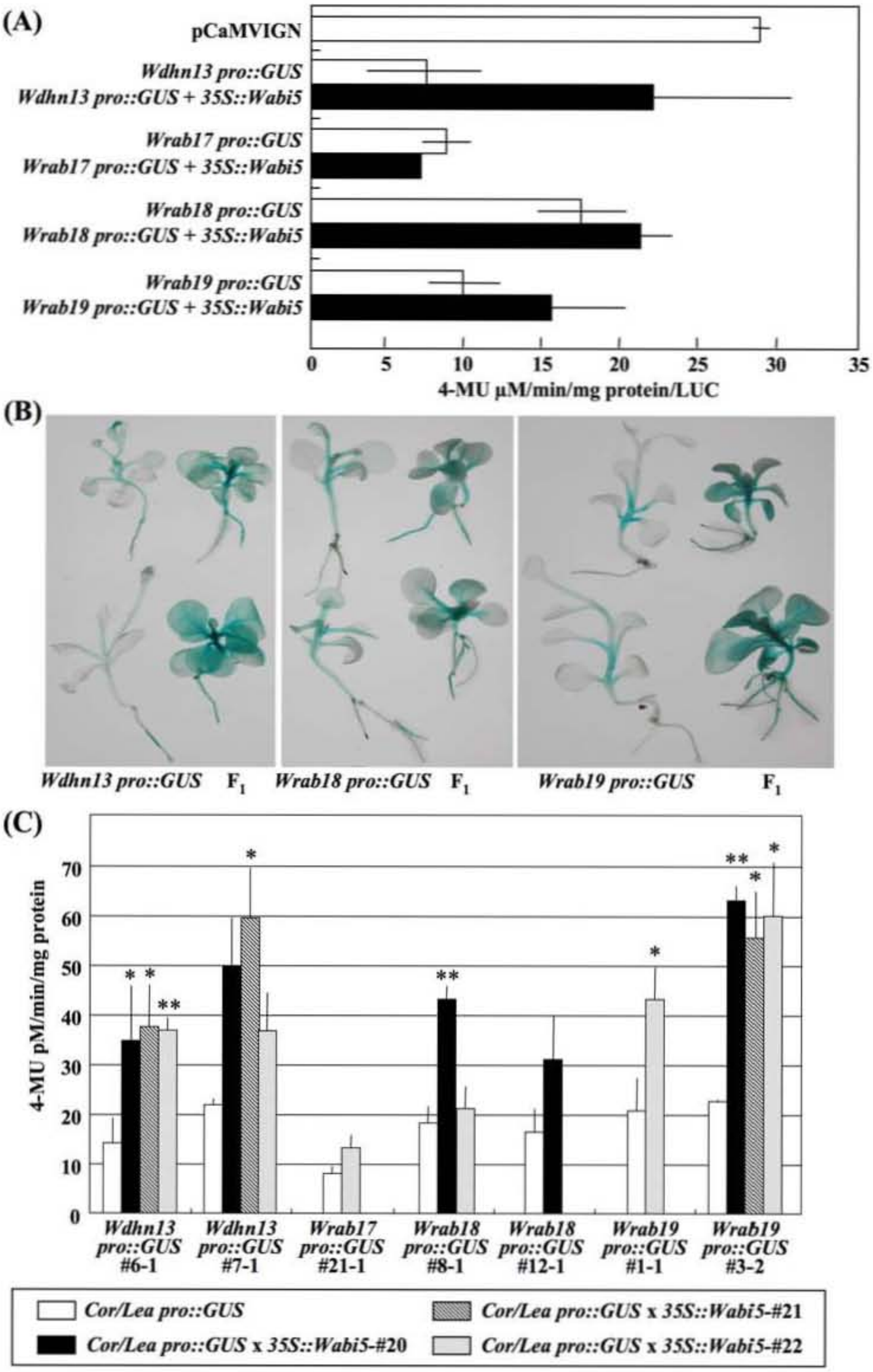

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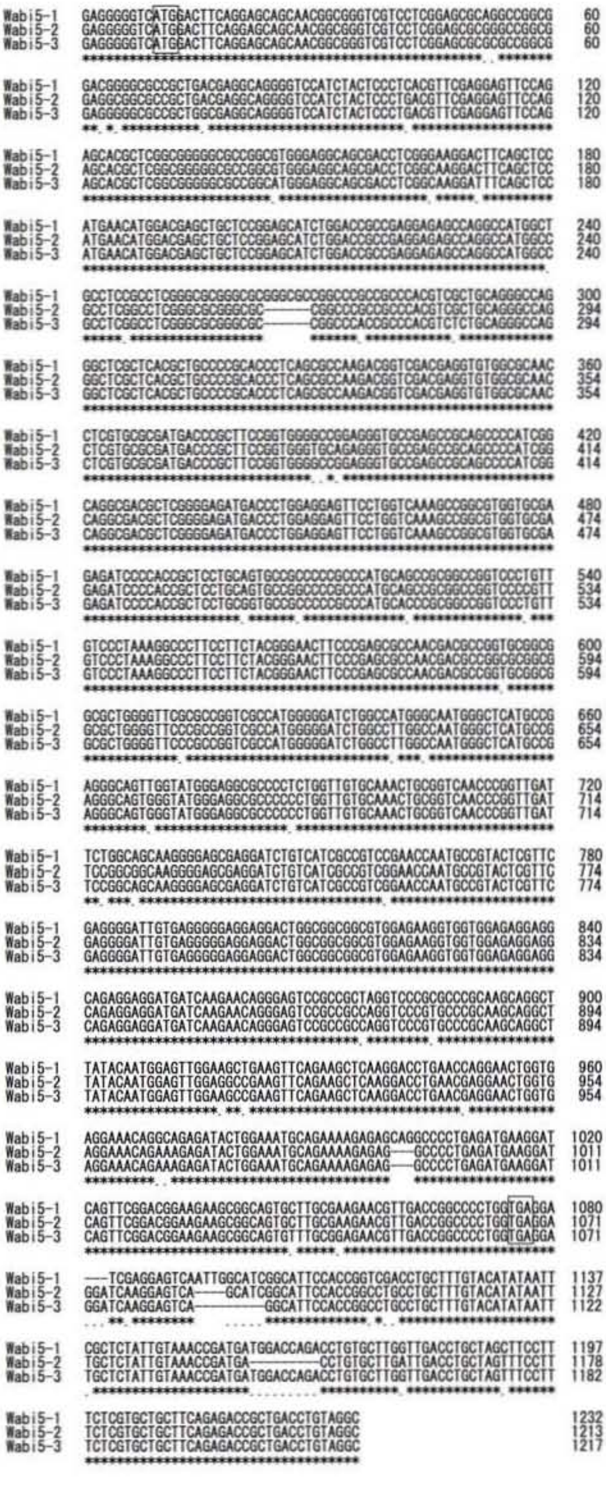

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Wabi5-1    GAGGGGGTCATGGACTTCAGGAGCAGCAACGGCGGGTCGTCCTCGGAGCGCAGGCCGGCG  60
Wabi5-2    GAGGGGGTCATGGACTTCAGGAGCAGCAACGGCGGGTCGTCCTCGGAGCGCGGGCCGGCG  60
Wabi5-3    GAGGGGGTCATGGACTTCAGGAGCAGCAACGGCGGGTCGTCCTCGGAGCGCGCGCCGGCG  60
           ***************************************************..*******

Wabi5-1    GACGGGGCGCCGCTGACGAGGCAGGGGTCCATCTACTCCCTCACGTTCGAGGAGTTCCAG  120
Wabi5-2    GAGGCGGCGCCGCTGACGAGGCAGGGGTCCATCTACTCCCTGACGTTCGAGGAGTTCCAG  120
Wabi5-3    GAGGGGGCGCCGCTGGCGAGGCAGGGGTCCATCTACTCCCTGACGTTCGAGGAGTTCCAG  120
           **.*.**********.*************************.******************

Wabi5-1    AGCACGCTCGGCGGGGGCGCCGGCGTGGGAGGCAGCGACCTCGGGAAGGACTTCAGCTCC  180
Wabi5-2    AGCACGCTCGGCGGGGGCGCCGGCGTGGGAGGCAGCGACCTCGGCAAGGACTTCAGCTCC  180
Wabi5-3    AGCACGCTCGGCGGGGGCGCCGGCATGGGAGGCAGCGACCTCGGCAAGGATTTCAGCTCC  180
           ************************.*******************.*****.*********

Wabi5-1    ATGAACATGGACGAGCTGCTCCGGAGCATCTGGACCGCCGAGGAGAGCCAGGCCATGGCT  240
Wabi5-2    ATGAACATGGACGAGCTGCTCCGGAGCATCTGGACCGCCGAGGAGAGCCAGGCCATGGCC  240
Wabi5-3    ATGAACATGGACGAGCTGCTCCGGAGCATCTGGACCGCCGAGGAGAGCCAGGCCATGGCC  240
           ***********************************************************.

Wabi5-1    GCCTCCGCCTCGGGCGCGGGCGCGGGCGCCGGCCCGCCGCCCACGTCGCTGCAGGGCCAG  300
Wabi5-2    GCCTCGGCCTCGGGCGCGGGCGC------CGGCCCGCCGCCCACGTCGCTGCAGGGCCAG  294
Wabi5-3    GCCTCGGCCTCGGGCGCGGGCGC------CGGCCCACCGCCCACGTCTCTGCAGGGCCAG  294
           *****.*****************      ******.***********.************

Wabi5-1    GGCTCGCTCACGCTGCCCCGCACCCTCAGCGCCAAGACGGTCGACGAGGTGTGGCGCAAC  360
Wabi5-2    GGCTCGCTCACGCTGCCCCGCACCCTCAGCGCCAAGACGGTCGACGAGGTGTGGCGCAAC  354
Wabi5-3    GGCTCGCTCACGCTGCCCCGCACCCTCAGCGCCAAGACGGTCGACGAGGTGTGGCGCAAC  354
           ************************************************************

Wabi5-1    CTCGTGCGCGATGACCCGCTTCCGGTGGGGCCGGAGGGTGCCGAGCCGCAGCCCCATCGG  420
Wabi5-2    CTCGTGCGCGATGACCCGCTTCCGGTGGGTGCAGAGGGTGCCGAGCCGCAGCCCCATCGG  414
Wabi5-3    CTCGTGCGCGATGACCCGCTTCCGGTGGGGCCGGAGGGTGCCGAGCCGCAGCCCCATCGG  414
           *****************************..*.***************************

Wabi5-1    CAGGCGACGCTCGGGGAGATGACCCTGGAGGAGTTCCTGGTCAAAGCCGGCGTGGTGCGA  480
Wabi5-2    CAGGCGACGCTCGGGGAGATGACCCTGGAGGAGTTCCTGGTCAAAGCCGGCGTGGTGCGA  474
Wabi5-3    CAGGCGACGCTCGGGGAGATGACCCTGGAGGAGTTCCTGGTCAAAGCCGGCGTGGTGCGA  474
           ************************************************************

Wabi5-1    GAGATCCCCACCGCTCCTGCAGTGCCGCCCCCGCCCATGCAGCCGCGGCCGGTCCCTGTT  540
Wabi5-2    GAGATCCCCACCGCTCCTGCAGTGCCGGCCCCGCCCATGCAGCCGCGGCCGGTCCCCGTT  534
Wabi5-3    GAGATCCCCACCGCTCCTGCGGTGCCGCCCCCGCCCATGCACCCGCGGCCGGTCCCTGTT  534
           ********************.******.*************.**************.***

Wabi5-1    GTCCCTAAAGGCCCTTCCTTCTACGGGAACTTCCCGAGCGCCAACGACGCCGGTGCGGCG  600
Wabi5-2    GTCCCTAAAGGCCCTTCCTTCTACGGGAACTTCCCGAGCGCCAACGACGCCGGCGCGGCG  594
Wabi5-3    GTCCCTAAAGGCCCTTCCTTCTACGGGAACTTCCCGAGCGCCAACGACGCCGGTGCGGCG  594
           *****************************************************.******

Wabi5-1    GCGCTGGGGTTCGCGCCGGTCGCCATGGGGGATCTGGCCATGGGCAATGGGCTCATGCCG  660
Wabi5-2    GCGCTGGGGTTCCCGCCGGTCGCCATGGGGGATCTGGCCTTGGCCAATGGGCTCATGCCG  654
Wabi5-3    GCGCTGGGGTTCCCGCCGGTCGCCATGGGGGATCTGGCCTTGGCCAATGGGCTCATGCCG  654
           ************.**************************.***.****************

Wabi5-1    AGGGCAGTTGGTATGGGAGGCGCCCCTCTGGTTGTGCAAACTGCGGTCAACCCGGTTGAT  720
Wabi5-2    AGGGCAGTGGGTATGGGAGGCGCCCCCCTGGTTGTGCAAACTGCGGTCAACCCGGTTGAT  714
Wabi5-3    AGGGCAGTGGGTATGGGAGGCGCCCCCCTGGTTGTGCAAACTGCGGTCAACCCGGTTGAT  714
           ********.*****************.*********************************

Wabi5-1    TCTGGCAGCAAGGGGAGCGAGGATCTGTCATCGCCGTCCGAACCAATGCCGTACTCGTTC  780
Wabi5-2    TCCGGCGGCAAGGGGAGCGAGGATCTGTCATCGCCGTCGGAACCAATGCCGTACTCGTTC  774
Wabi5-3    TCCGGCAGCAAGGGGAGCGAGGATCTGTCATCGCCGTCGGAACCAATGCCGTACTCGTTC  774
           **.***.*******************************.*********************

Wabi5-1    GAGGGGATTGTGAGGGGGAGGAGGACTGGCGGCGGCGTGGAGAAGGTGGTGGAGAGGAGG  840
Wabi5-2    GAGGGGATTGTGAGGGGGAGGAGGACTGGCGGCGGCGTGGAGAAGGTGGTGGAGAGGAGG  834
Wabi5-3    GAGGGGATTGTGAGGGGGAGGAGGACTGGCGGCGGCGTGGAGAAGGTGGTGGAGAGGAGG  834
           ************************************************************

Wabi5-1    CAGAGGAGGATGATCAAGAACAGGGAGTCCGCCGCTAGGTCCCGCGCCCGCAAGCAGGCT  900
Wabi5-2    CAGAGGAGGATGATCAAGAACAGGGAGTCCGCCGCCAGGTCCCGTGCCCGCAAGCAGGCT  894
Wabi5-3    CAGAGGAGGATGATCAAGAACAGGGAGTCCGCCGCCAGGTCCCGTGCCCGCAAGCAGGCT  894
           ***********************************.********.***************

Wabi5-1    TATACAATGGAGTTGGAAGCTGAAGTTCAGAAGCTCAAGGACCTGAACCAGGAACTGGTG  960
Wabi5-2    TATACAATGGAGTTGGAGGCCGAAGTTCAGAAGCTCAAGGACCTGAACGAGGAACTGGTG  954
Wabi5-3    TATACAATGGAGTTGGAAGCCGAAGTTCAGAAGCTCAAGGACCTGAACGAGGAACTGGTG  954
           *****************.**.***************************.***********

Wabi5-1    AGGAAACAGGCAGAGATACTGGAAATGCAGAAAAGAGAGCAGGCCCCTGAGATGAAGGAT  1020
Wabi5-2    AGGAAACAGAAAGAGATACTGGAAATGCAGAAAAGAGAG---GCCCCTGAGATGAAGGAT  1011
Wabi5-3    AGGAAACAGAAAGAGATACTGGAAATGCAGAAAAGAGAG---GCCCCTGAGATGAAGGAT  1011
           *********..****************************   ******************

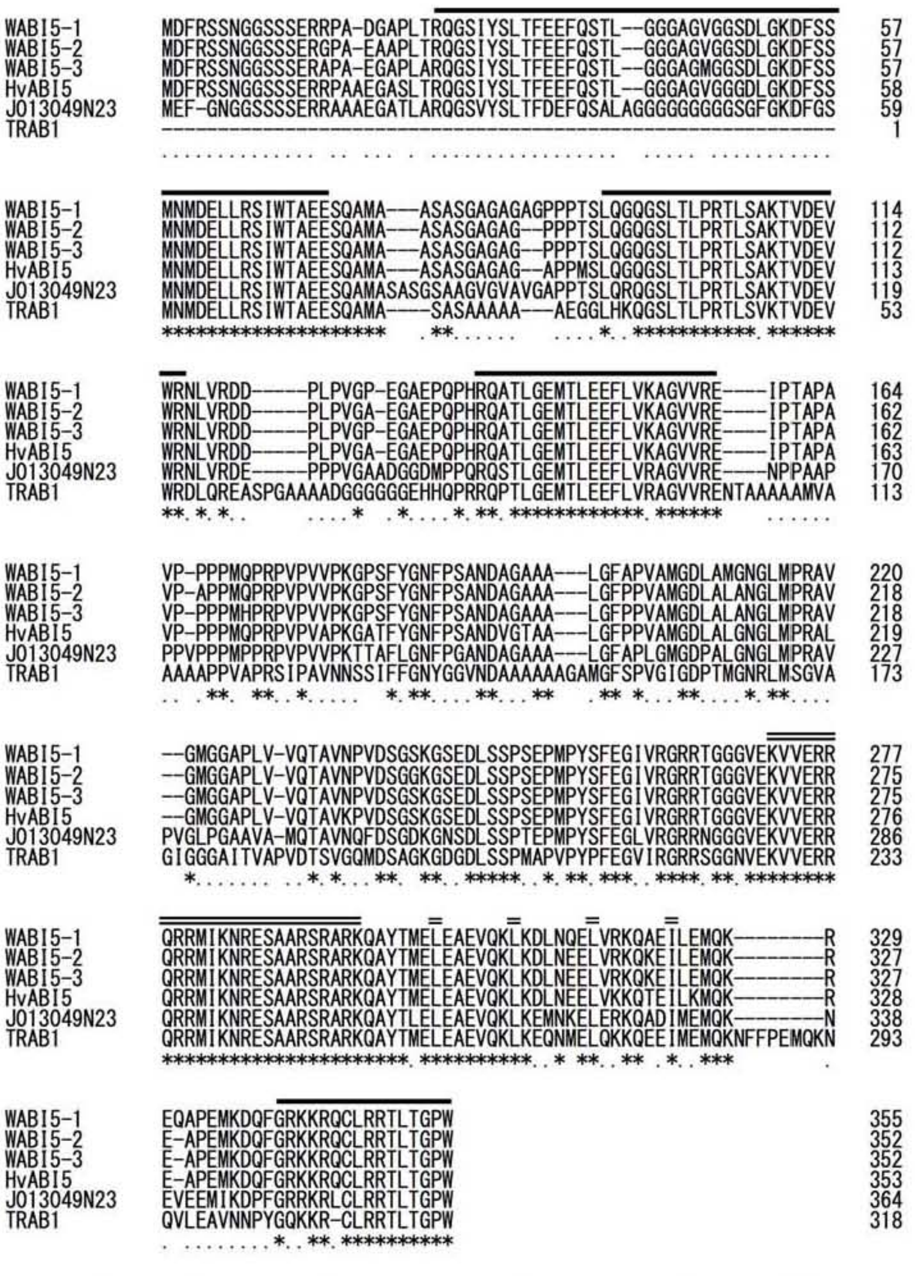
Wabi5-1    CAGTTCGGACGGAAGAAGCGGCAGTGCTTGCGAAGAACGTTGACCGGCCCCTGGTGAGGA  1080
Wabi5-2    CAGTTCGGACGGAAGAAGCGGCAGTGCTTGCGAAGAACGTTGACCGGCCCCTGGTGAGGA  1071
Wabi5-3    CAGTTCGGACGGAAGAAGCGGCAGTGTTTGCGGAGAACGTTGACCGGCCCCTGGTGAGGA  1071
           **************************.*****.***************************

Wabi5-1    ---TCGAGGAGTCAATTGGCATCGGCATTCCACCGGTCGACCTGCTTTGTACATATAATT  1137
Wabi5-2    GGATCAAGGAGTCA----GCATCGGCATTCCACCGGCCTGCCTGCTTTGTACATATAATT  1127
Wabi5-3    GGATCAAGGAGTCA---------GGCATTCCACCGGCCTGCCTGCTTTGTACATATAATT  1122
           ...**.********    .....*************.*..********************

Wabi5-1    CGCTCTATTGTAAACCGATGATGGACCAGACCTGTGCTTGGTTGACCTGCTAGCTTCCTT  1197
Wabi5-2    TGCTCTATTGTAAACCGATGA---------CCTGTGCTTGATTGACCTGCTAGTTTCCTT  1178
Wabi5-3    TGCTCTATTGTAAACCGATGATGGACCAGACCTGTGCTTGGTTGACCTGCTAGTTTCCTT  1182
           .********************.........**********.************.******

Wabi5-1    TCTCGTGCTGCTTCAGAGACCGCTGACCTGTAGGC                          1232
Wabi5-2    TCTCGTGCTGCTTCAGAGACCGCTGACCTGTAGGC                          1213
Wabi5-3    TCTCGTGCTGCTTCAGAGACCGCTGACCTGTAGGC                          1217
           ***********************************
```

Appendix S1. Alignment of nucleotide sequences of three homoeologous *Wabi5* cDNAs. Initiation and stop codons are boxed. Asterisks and dots respectively mark identical nucleotides for three and two cDNAs.



```
WABI5-1         MDFRSSNGGSSSERRPA-DGAPLTRQGSIYSLTFEEFQSTL--GGGAGVGGSDLGKDFSS     57
WABI5-2         MDFRSSNGGSSSERGPA-EAAPLTRQGSIYSLTFEEFQSTL--GGGAGVGGSDLGKDFSS     57
WABI5-3         MDFRSSNGGSSSERAPA-EGAPLARQGSIYSLTFEEFQSTL--GGGAGMGGSDLGKDFSS     57
HvABI5          MDFRSSNGGSSSERRPAAEGASLTRQGSIYSLTFEEFQSTL--GGGAGVGGGDLGKDFSS     58
J013049N23      MEF-GNGGSSSSERRAAAEGATLARQGSVYSLTFDEFQSALAGGGGGGGGGSGFGKDFGS     59
TRAB1           ------------------------------------------------------------      1
                .............. .. ... . .................  ..... ...........

WABI5-1         MNMDELLRSIWTAEESQAMA---ASASGAGAGAGPPPTSLQGQGSLTLPRTLSAKTVDEV    114
WABI5-2         MNMDELLRSIWTAEESQAMA---ASASGAGAG--PPPTSLQGQGSLTLPRTLSAKTVDEV    112
WABI5-3         MNMDELLRSIWTAEESQAMA---ASASGAGAG--PPPTSLQGQGSLTLPRTLSAKTVDEV    112
HvABI5          MNMDELLRSIWTAEESQAMA---ASASGAGAG--APPMSLQGQGSLTLPRTLSAKTVDEV    113
J013049N23      MNMDELLRSIWTAEESQAMASASGSAAGVGVAVGAPPTSLQRQGSLTLPRTLSAKTVDEV    119
TRAB1           MNMDELLRSIWTAEESQAMA----SASAAAAA---AEGGLHKQGSLTLPRTLSVKTVDEV     53
                ********************   .**......   ....*..***********.******

WABI5-1         WRNLVRDD-----PLPVGP-EGAEPQPHRQATLGEMTLEEFLVKAGVVRE----IPTAPA    164
WABI5-2         WRNLVRDD-----PLPVGA-EGAEPQPHRQATLGEMTLEEFLVKAGVVRE----IPTAPA    162
WABI5-3         WRNLVRDD-----PLPVGP-EGAEPQPHRQATLGEMTLEEFLVKAGVVRE----IPTAPA    162
HvABI5          WRNLVRDD-----PLPVGA-EGAEPQPHRQATLGEMTLEEFLVKAGVVRE----IPTAPA    163
J013049N23      WRNLVRDE-----PPPVGAADGGDMPPQRQSTLGEMTLEEFLVRAGVVRE----NPPAAP    170
TRAB1           WRDLQREASPGAAAADGGGGGGEHHQPRRQPTLGEMTLEEFLVRAGVVRENTAAAAAMVA    113
                **.*.*..     ....*  .*....*.**.************.******    ......

WABI5-1         VP-PPPMQPRPVPVVPKGPSFYGNFPSANDAGAAA---LGFAPVAMGDLAMGNGLMPRAV    220
WABI5-2         VP-APPMQPRPVPVVPKGPSFYGNFPSANDAGAAA---LGFPPVAMGDLALANGLMPRAV    218
WABI5-3         VP-PPPMHPRPVPVVPKGPSFYGNFPSANDAGAAA---LGFPPVAMGDLALANGLMPRAV    218
HvABI5          VP-PPPMQPRPVPVAPKGATFYGNFPSANDVGTAA---LGFPPVAMGDLALGNGLMPRAL    219
J013049N23      PPVPPPMPPRPVPVVPKTTAFLGNFPGANDAGAAA---LGFAPLGMGDPALGNGLMPRAV    227
TRAB1           AAAAPPVAPRSIPAVNNSSIFFGNYGGVNDAAAAAAGAMGFSPVGIGDPTMGNRLMSGVA    173
                .. .**. **..*.....  *.**....**...**   .** *...**....*.**....

WABI5-1         --GMGGAPLV-VQTAVNPVDSGSKGSEDLSSPSEPMPYSFEGIVRGRRTGGGVEKVVERR    277
WABI5-2         --GMGGAPLV-VQTAVNPVDSGGKGSEDLSSPSEPMPYSFEGIVRGRRTGGGVEKVVERR    275
WABI5-3         --GMGGAPLV-VQTAVNPVDSGSKGSEDLSSPSEPMPYSFEGIVRGRRTGGGVEKVVERR    275
HvABI5          --GMGGAPLV-VQTAVKPVDSGSKGSEDLSSPSEPMPYSFEGIVRGRRTGGGVEKVVERR    276
J013049N23      PVGLPGAAVA-MQTAVNQFDSGDKGNSDLSSPTEPMPYSFEGLVRGRRNGGGVEKVVERR    286
TRAB1           GIGGGAITVAPVDTSVGQMDSAGKGDGDLSSPMAPVPYPFEGVIRGRRSGGNVEKVVERR    233
                  *....... ..*.*...**. **..*****..*.**.***..****.**.********

                                        =      =      =      =
WABI5-1         QRRMIKNRESAARSRARKQAYTMELEAEVQKLKDLNQELVRKQAEILEMQK--------R    329
WABI5-2         QRRMIKNRESAARSRARKQAYTMELEAEVQKLKDLNEELVRKQKEILEMQK--------R    327
WABI5-3         QRRMIKNRESAARSRARKQAYTMELEAEVQKLKDLNEELVRKQKEILEMQK--------R    327
HvABI5          QRRMIKNRESAARSRARKQAYTMELEAEVQKLKDLNEELVKKQTEILKMQK--------R    328
J013049N23      QRRMIKNRESAARSRARKQAYTLELEAEVQKLKEMNKELERKQADIMEMQK--------N    338
TRAB1           QRRMIKNRESAARSRARKQAYTMELEAEVQKLKEQNMELQKKQEEIMEMQKNFFPEMQKN    293
                **********************.**********..* **..** .*..***        .

WABI5-1         EQAPEMKDQFGRKKRQCLRRTLTGPW                                      355
WABI5-2         E-APEMKDQFGRKKRQCLRRTLTGPW                                      352
WABI5-3         E-APEMKDQFGRKKRQCLRRTLTGPW                                      352
HvABI5          E-APEMKDQFGRKKRQCLRRTLTGPW                                      353
J013049N23      EVEEMIKDPFGRRKRLCLRRTLTGPW                                      364
TRAB1           QVLEAVNNPYGQKKR-CLRRTLTGPW                                      318
                . ........*..**.**********
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Appendix S2. Alignment of WABI5 amino acid sequences with those of the ABI5/ABF/AREB subfamily in cereals. Asterisks and dots mark identical and conserved residues, respectively. The basic region and heptad Leu or hydrophobic residues are indicated by double lines according to Shimizu et al. (2005). The four conserved regions are indicated by lines according to Casaretto and Ho (2002).