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RESEARCH PAPER

Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat

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Abstract

Cereal *lip19* genes encoding bZIP-type transcription factors are assumed to play a regulatory role in gene expression during the cold acclimation process. However, no direct evidence shows an association of LIP19-type bZIPs with stress tolerance or activation of stress-responsive *Cor*/*Lea* genes. To understand the molecular basis of development of abiotic stress tolerance through the LIP19 transcription factor, a wheat *lip19* homologue, *Wlip19*, was isolated and characterized. *Wlip19* expression was activated by low temperature in seedlings and was higher in a freezing-tolerant cultivar than in a freezing-sensitive one. *Wlip19* also responded to drought and exogenous ABA treatment. *Wlip19*-expressing transgenic tobacco showed a significant increase in abiotic stress tolerance, especially freezing tolerance. Expression of a *GUS* reporter gene under the control of promoter sequences of four wheat *Cor*/*Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19*, was enhanced by *Wlip19* expression in wheat callus and tobacco plants. These results indicate that WLIP19 acts as a transcriptional regulator of *Cor*/*Lea* genes in the development of abiotic stress tolerance. Moreover, direct protein–protein interaction between WLIP19 and a wheat OBF1 homologue TaOBF1, another bZIP-type transcription factor, was observed, suggesting that this interaction is conserved in cereals.

Key words: Abiotic stress tolerance, bZIP protein, *Cor*/*Lea* genes, transgenic plants, *Triticum aestivum* L.

Introduction

Cold and freezing stress have a significant impact on agricultural production, limiting the geographical distribution of plants and reducing crop quality and productivity. Many plants from temperate regions develop increased freezing tolerance in response to low but non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow, 1999). A large number of genes with various functions are induced during cold acclimation (Seki *et al*., 2002; Rabbani *et al*., 2003). In particular, expression of the *Cor* (cold-responsive)/*Lea* (late embryogenesis-abundant) gene family is up-regulated under abiotic stress conditions such as low temperature, drought, and high salinity, and the gene products function in stress tolerance (Thomashow, 1999; Xiong *et al*., 2002). Proteins interacting with major *cis*-acting elements of *Cor*/*Lea* promoters in the abiotic stress response include some, such as members of the CBF/DREB family, that function in abscisic acid (ABA)-independent stress signalling pathways (Stockinger *et al*., 1997; Liu *et al*., 1998) and others, such as AREB/ABF, that activate gene expression in response to stress and ABA and contain a bZIP (basic region/leucine zipper) domain (Choi *et al*., 2000; Uno *et al*., 2000).

In *Arabidopsis*, 75 bZIP protein members have been divided into 10 subgroups based on the sequence similarities of common domains (Jakoby *et al*., 2002). According to this classification, the AREB/ABF subfamily belongs to group A. Group A bZIPs generally function in ABA signalling during seed maturation or

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under stress conditions. Group S is the largest bZIP group in *Arabidopsis*, and several members of this group, including the ATB2-type bZIP proteins, are involved in stress responses (Jakoby *et al*., 2002; Satoh *et al*., 2004). In cereals, many transcription factors including CBF/DREB homologues and those of the bZIP type have been identified. Rice *lip19* (low-temperature-induced protein 19) encodes a bZIP-type protein and is strongly induced by low temperature (Aguan *et al*., 1991, 1993). Expression of maize *mlip15*, a counterpart of *lip19*, is increased by low temperature, salt stress, and exogenous ABA (Kusano *et al*., 1995). The mLIP15 protein binds to sequences that contain an ACGT core nucleotide motif as well as a promoter sequence lacking the motif (Kusano *et al*., 1995). Maize OBF1 is another low temperature-responsive bZIP-type protein recognizing ACGT motifs (Singh *et al*., 1990; Kusano *et al*., 1995). LIP19, mLIP15, and OBF1 are categorized into group S bZIPs (Shimizu *et al*., 2005). mLIP15 dimerizes with OBF1; mLIP15 and OBF1 also form homodimers, though LIP19 does not (Kusano *et al*., 1995; Shimizu *et al*., 2005). LIP19 and mLIP15 dimerize respectively with OsOBF1 and OBF1, which then bind to *cis*-elements containing ACGT core motifs (Shimizu *et al*., 2005). However, downstream target genes of LIP19 and mLIP15 and the contribution of the LIP19-type bZIPs to abiotic stress tolerance remain unknown. *Trans*-activation of some wheat *Cor/Lea* genes by WCBF2 and WDREB2, wheat CBF/DREB homologues, was observed in transgenic tobacco plants; moreover, ectopic expression of *Wcbf2* and *Wdreb2* improved freezing tolerance in transgenic tobacco plants (Kobayashi *et al*., 2008*a*; Takumi *et al*., 2008). The heterologous tobacco system clearly revealed the roles of WCBF2 and WDREB2 transcription factors in the abiotic stress response.

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*., 2008*a*). *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19* are responsive to low temperature, drought, and ABA (Ohno *et al*., 2003; Kobayashi *et al*., 2004*a*, 2006; Egawa *et al*., 2006), and their promoter regions contain ACGT core motifs (Kobayashi *et al*., 2008*a*). These observations suggest that bZIP-type transcription factors play significant roles in regulation of *Cor/Lea* gene expression. Here, a wheat *lip19* homologue, *Wlip19*, and a wheat *OBF1* homologue, *TaOBF1*, were isolated. Based on their expression profiles, protein–protein interactions, and stress tolerance of *Wlip19*-expressing tobacco plants, the development of abiotic stress tolerance through WLIP19-mediated *Cor/Lea* expression in two wheat varieties with different levels of stress tolerance is discussed.

Materials and methods

Isolation of Wlip19 *and* TaOBF1

Total RNA from cold-treated leaves of common wheat (*Triticum aestivum* L.) cultivar 'Chinese Spring' (CS) seedlings was used as a template in RT-PCR. Three homoeologous *Wlip19* homologues were isolated using the following primer sets: 5′-TTTTTCCTTGCAGTCTACTCC-3′ and 5′-GCAGCAGTAATCAGCAACTTCTTA-3′ for *Wlip19b*, 5′-TCCCTGTAGTTTGGTTTTCCT-3′ and 5′-CGCCGTGGCATGACTTGTCT-3′ for *Wlip19a*, and 5′-GTCCCTGTAGCTTCTTTTTTCCTT-3′ and 5′-TGAGCAGCAACCACATCCAT-3′ for *Wlip19d*. Based on the nucleotide sequences of *TaOBF1* (accession no. AF479057) and whr19m05, a wheat expressed sequence tag (EST) clone (BJ280934) registered in the EST database of the NBRP (National BioResource Project) KOMUGI website (http://shigen.lab.nig.ac.jp/wheat/komugi), the primer set 5′-TGATCTCATAATTGGCCCTC-3′ and 5′-ATAGCAGCAAACTACGCCTT-3′ was designed. Full-length cDNA of *TaOBF1* was isolated from leaf RNA of CS by RT-PCR with this primer set. Amplified cDNAs of *Wlip19* and *TaOBF1* were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and nucleotide sequences were determined by the automated fluorescent Dye Deoxy terminator cycle sequencing system using an ABI PRISM 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA).

The nucleotide sequences of the isolated cDNA clones and the predicted amino acid sequences were analysed by DNASIS software (Hitachi, Tokyo, Japan). The cDNA sequences were deposited in the DDBJ database under these accession numbers: AB334126 (*Wlip19b*), AB334127 (*Wlip19a*), AB334128 (*Wlip19d*), AB334129 (*TaOBF1a*), AB334130 (*TaOBF1b*), and AB334131 (*TaOBF1d*). Multiple sequence alignments were carried out using the ClustalW computer program (Thompson *et al*., 1994), and a phylogenetic tree was constructed by the Neighbor–Joining method (Saitou and Nei, 1987). Software was provided by Kyoto University Bioinformatics Center (http://align.genome.jp/).

Southern blot analysis and chromosome assignment

For genomic Southern blot analysis, total DNA extracted from hexaploid wheat cultivars CS and 'Mironovskaya 808' (M808), tetraploid emmer wheat (*T. durum*) cv. 'Langdon' (Ldn), and ancestral diploid species (*T. boeoticum*, *T. urartu*, *T. monococcum*, *Aegilops speltoides*, and *A. taushii*) was digested with the indicated restriction enzymes. The digested DNA was fractionated by electrophoresis through a 0.8% agarose gel, transferred to Hybond N^+ nylon membrane (GE Healthcare, Piscataway, NJ, USA), and hybridized with ^{32}P-labelled *Wlip19* or *TaOBF1* cDNA as a probe. Probe labelling, hybridization, washing, and autoradiography were performed according to Takumi *et al*. (1999).

For chromosome assignment of *Wlip19* and *TaOBF1*, Southern blot analysis was performed using total DNA from a nullitetrasomic series of CS (Sears, 1966). Each line of the nullitetrasomic 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). To distinguish the three *Wlip19* sequences, PCR analysis with the two primer sets used for *Wlip19b* and *Wlip19d* cDNA cloning was performed using total DNA of the nulli-tetrasomic series. To distinguish the three *TaOBF1* homoeologues, the following clone-specific primer sets were used: 5′-AAAATCCGCCGTCGCTAG-3′ and 5′-TGGCAGCGGAATCACTAGTACT-3′ for *TaOBF1a*, 5′-CAAATCCGCCGTTGCTAG-3′ and 5′-GGCACCGAAATTACTACTGCTT-3' for *TaOBF1b*, and 5′-CAAATCCGCCGTCGCTAG-3′ and 5′-TTGCACCGGAATCACTACTACC-3′ for *TaOBF1d*. The PCR products were

separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide.

Gene expression analyses

To analyse gene expression patterns of *Wip19* and *TaOBF1*, 7-d-old seedlings of CS and M808 grown under standard conditions (20 °C) according to Kobayashi *et al*. (2004*a*) were transferred to 4 °C and kept for various time periods under standard lighting conditions. CS and M808 were used as freezing-sensitive and freezing-tolerant cultivars, respectively (Ohno *et al*., 2001). 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 *Wlip19* and *TaOBF1* transcripts was detected by RT-PCR amplification as previously reported (Kobayashi *et al*., 2004*b*, 2006). RT-PCR was conducted with the following gene-specific primer sets: 5′-CAACATC-GACGGCGGCAG-3′ and 5′-GGCTCAGAACTGGAACGCGTC-3′ for *Wlip19*, and 5′-AAGATGTCGTCGTCGTCGCT-3′ and 5′-GTACTGGAGCATGTGCGTGG-3′ for *TaOBF1*. 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. The 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 in total.

Generation of transgenic tobacco plants expressing Wlip19

The *Wlip19a* cDNA sequence was amplified with the following primer set containing a *Bam*HI linker: 5′-CGGGATCCCGATC-CAGCCTCGTTT-3′ and 5′- CGGGATCCCGTGGCATGACTTG-TC-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} α-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::Wlip19*. To detect *Wlip19* transcripts in the *35S::Wlip19* lines, RT-PCR was conducted with the same set of primers used for construction of the chimeric plasmid. The actin gene was used as an internal control in the transgenic tobacco and was amplified with primers 5′-GGCTGGTTTTGCTGGTGACGAT-3′ and 5′-AATGAAG-GAAGGCTGGAAGAGGA-3′.

Bioassay conditions for freezing and osmotic stress tolerance

To assay freezing tolerance, wild-type and T_2 progeny of *35S::Wlip19* 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 then frozen at –15±1 °C for 1 h or 2 h 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 numbers of surviving seedlings were recorded. To assay osmotic stress tolerance, 7-d-old seedlings of wild-type and *35S::Wlip19* 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 d after treatment with mannitol and 4 d after treatment with NaCl, the number of plants with green cotyledons was scored. The experiment was performed 3–6 times and the data were analysed statistically 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::Wlip19*. 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 until 10 d after planting. In a bioassay of ABA sensitivity based on root growth, ten 5-d-old seedlings of wild-type and *35S::Wlip19* 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 d, the length of primary roots was recorded. The experiment was performed in triplicate three times and the data were analysed statistically by Student's *t*-test.

Interaction of WLIP19 with wheat Cor/Lea gene promoters

Promoter regions of four wheat *Cor/Lea* genes, *Wdhn13* (accession no. AB297677), *Wrab17* (AB297678), *Wrab18* (AB297679), and *Wrab19* (AB297680), were isolated and fused with a *GUS* reporter gene in pBI101 (Kobayashi *et al*., 2008*a*). The chimeric constructs of these *Cor/Lea* promoters were named *Wdhn13pro::GUS*, *Wrab17pro::GUS*, *Wrab18pro::GUS*, and *Wrab19pro::GUS*. The four *GUS* chimeric gene constructs were used to demonstrate induction of *GUS* gene expression by low temperature, drought, and ABA under the control of 5′ upstream sequences containing core motifs of putative stress responsive *cis*-elements such as CRT/DRE and ABRE (Kobayashi *et al*., 2008*a*). The four *Cor/Lea promoter::GUS* and *35S::Wlip19* constructs were purified using the 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 CaMV *35S* promoter into wheat callus line HY-1 by particle bombardment according to Takumi *et al*. (1999). GUS activity was quantified according to Jefferson (1987) and normalized by the luciferase activity estimated using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

The *35S::Wlip19* transformants were used as the pollen parent in crosses with the transgenic tobacco plants having introduced the *Cor/Lea promoter::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 promtoer::GUS* plants was assessed according to Takumi *et al*. (2003) and Jefferson (1987).

Yeast two-hybrid assay

A HybriZAP-2.1 two-hybrid undigested vector kit (Stratagene, La Jolla, CA, USA) was used to study the interaction between WLIP19 and TaOBF1 proteins. The entire open reading frame (ORF) sequences of *Wlip19d* and *TaOBF1d* cDNA were amplified with the following primer sets containing *Eco*RI and *Sal*I linkers: 5′-GGGAATTCCCGACCATGTCGTCGCCATC-3′ and 5′-GCGTC-GACTTGGCGGCTCTTGGCTCA-3′ for *Wlip19*, and 5′-GGGAA-TTCTCCGGTGCAAAGATGTCGTCGT-3′ and 5′-GCGTCGAC CCAATCATCACCGGATCGA-3′ for *TaOBF1*. The PCR products were digested with *Eco*RI and *Sal*I, and cloned into the *Eco*RI and *Sal*I sites of pAD-GAL4-2.1 and pBD-GAL4 Cam vectors, resulting in pAD-Wlip19, pAD-TaOBF1, pBD-Wlip19, and pBD-TaOBF1. pAD-WT and pBD-WT containing wild-type fragment C of lambda

cI were used as controls in the assay. These pAD and pBD constructs were introduced into yeast strain YRG-2 (Stratagene). The interaction was assessed on SD medium (Q-BIOgene, Irvine, CA, USA) without leucine, tryptophan, or histidine.

Results

Isolation and chromosome assignment of Wlip19 *cDNA*

Wheat EST clone WHE4110_F06_K12, containing a complete ORF, showed high homology with rice LIP19 at the amino acid sequence level. A number of wheat EST clones contained partial cDNA sequences highly homologous to WHE4110_F06_K12. Based on nucleotide sequence polymorphisms, these EST clones were divided into three groups, which presumably belong to the three homoeologous loci for *Wlip19* in common wheat. Three cDNAs of *Wlip19* with a complete ORF were isolated from leaves of cold-acclimated seedlings of CS by RT-PCR with locus-specific primer sets (see Supplementary Fig. S1 at *JXB* online), and the ORFs encoded bZIP-type proteins with 150 amino acid residues showing an amino acid identity of 76% with rice LIP19 (see Supplementary Fig. S2 at *JXB* online). A phylogenetic tree of the bZIP-type proteins belonging to group S (LIP19 subfamily) and group A (ABI5/ABF/AREB subfamily) was constructed by the Neighbor–Joining method (Fig. 1). The three WLIP19 sequences showed the highest level of identity with LIP19 and mLIP15. A multiple alignment of these bZIP-type proteins indicated that homology was especially high in the basic regions (see Supplementary Fig. S2 at *JXB* online).

To study the copy number of *Wlip19* in the wheat genome, Southern blots were analysed using total DNA isolated from diploid, tetraploid, and hexaploid wheat. Southern blots showed low copy numbers of *Wlip19* in hexaploid and tetraploid wheat genomes (data not shown). A single major band was detected in A, S, and D diploid genomes (Fig. 2A). To assign the three *Wlip19* loci to homoeologous wheat chromosomes, aneuploid analysis was performed using a series of nulli-tetrasomic lines. *Wlip19*-specific bands were absent only in the nulli-tetrasomic lines of homoeologous group 1 chromosomes (Fig. 2B). These Southern blots indicated that *Wlip19* represented the three homoeologous loci on chromosomes 1A, 1B, and 1D in common wheat. The three cDNAs were designated *Wlip19a*, *Wlip19b*, and *Wlip19d*. PCR analysis using the primer sets specific to *Wlip19b* and *Wlip19d* showed no amplification in the corresponding nullisomic-1B and nullisomic-1D lines (Fig. 2C),

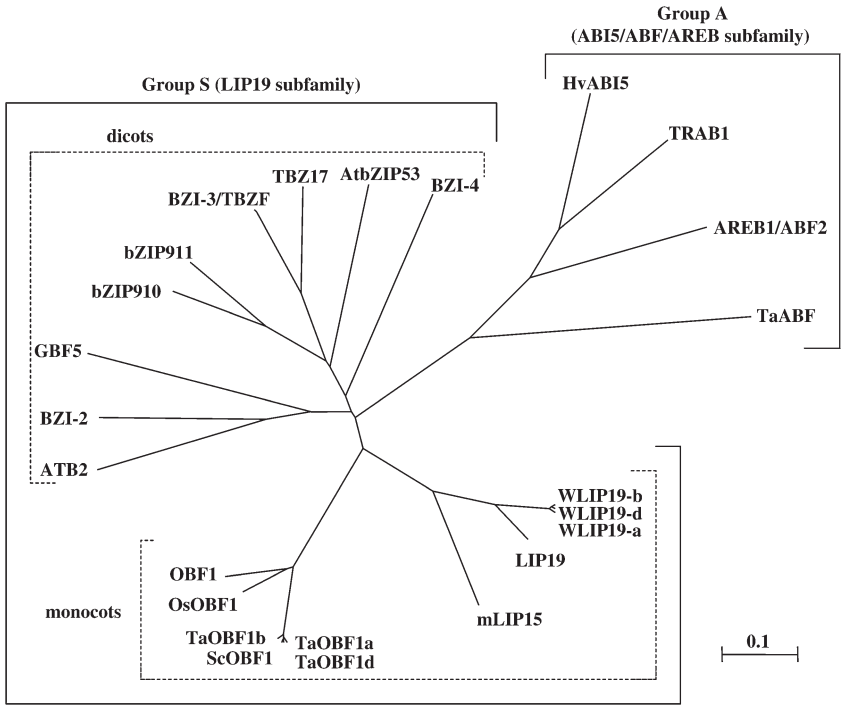


Fig. 1. Phylogenetic tree based on amino acid sequences, showing the relationship of WLIP19 and TaOBF1 to other plant bZIP-type proteins. The deduced amino acid sequences were aligned with ClustalW. The phylogenetic tree was constructed by the Neighbor–Joining method based on Nei's genetic distance. The accession numbers (in parentheses) of the amino acid sequences are: wheat TaABF (AF519804), rice LIP19 (X57325), OsOBF1 (AB185280), and TRAB1 (AB023288), barley HvABI5 (AY150676), rye ScOBF1 (AJ617794), maize mLIP15 (D26563) and OBF1 (X62745), *Arabidopsis* AREB1/ABF2 (AB017160/AF093545), GBF5 (AF053939), ATB2 (X99747), and AtbZIP53 (AF400620), snapdragon bZIP910 (Y13675) and bZIP911 (Y13676), and tobacco TBZ17 (D63951), BZI-2 (AY045570), BZI-3/TBZF (AY045571/AB032478), and BZI-4 (AY045572).

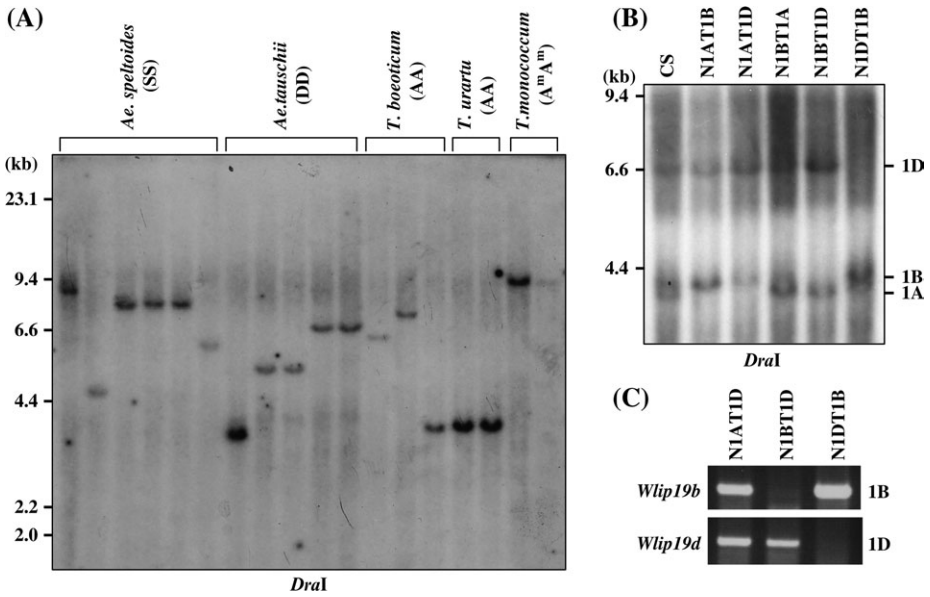


Fig. 2. Copy number and chromosome assignment of the *Wlip19* gene in the wheat genome. (A) Southern blot analysis of *Dra*I-digested total DNA from multiple accessions of the indicated diploid wheat species. The blot was probed with ^{32}P-labelled *Wlip19* cDNA. (B) Chromosome assignment of the *Wlip19* gene to the homoeologous group 1 chromosomes. DNA from the nulli-tetrasomic series of CS was digested with *Dra*I. N1AT1B, for example, represents a line nullisomic for chromosome 1A and tetrasomic for chromosome 1B. (C) PCR analysis with the homoeologue-specific primer sets of *Wlip19b* and *Wlip19d* using total DNA from the nulli-tetrasomic lines as templates.

indicating that the *Wlip19a*, *b*, and *d* loci should be assigned to chromosomes 1A, 1B, and 1D, respectively.

Expression profile of Wlip19 *during cold acclimation*

The *Wlip19* transcript was detected at a low level under non-stress conditions, and its level increased within 30 min after exposure of wheat seedlings to low temperature (Fig. 3A, B). The transcript level reached a high plateau by 6 h or 8 h and then gradually decreased over 24 h in both CS and M808 (Fig. 3A, B). Next, the *Wlip19* transcript level increased again by 3 d of low temperature and was maintained at a high level between 5 d and 10 d in M808, while the transcript decreased in CS after 1 d (Fig. 3C, D). The transcript level of *Wlip19* was higher in M808 than in CS over 10 d of treatment (Fig. 3A–D). During long-term low temperature treatment, the transcript level of *Wlip19* increased at days 21 and 42 in both CS and M808, then decreased towards day 63 in CS, while a high level was maintained in M808 (Fig. 3E, F). These expression patterns of *Wlip19* were similar to those of *Wcbf2* and *Wdreb2* (Kume *et al*., 2005; Egawa *et al*., 2006); the observed differences in the transcript level of *Wlip19* between the two cultivars appears to correlate with their freezing tolerance. Comparison of *Wlip19* and *Cor/Lea* gene expression showed that the expression patterns of *Cor/Lea* members *Wdhn13* and *Wrab17* agreed well with that of *Wlip19* in both cultivars (Fig. 3; Kume *et al*., 2005; Egawa *et al*., 2006).

Rice *lip19* expression is induced by low temperature in roots of rice seedlings (Wen *et al*., 2002). *Wlip19* expression was examined in cold-acclimated (4 °C for 3 d) leaves and roots of M808 seedlings. The *Wlip19* transcript level was increased by low temperature in leaves, while in roots the level appeared unchanged (Fig. 4A, B). However, the transcript was more abundant in roots than in leaves under both normal and low temperature conditions (Fig. 4A, B).

Wlip19 *response to drought stress and ABA treatment*

Expression of rice *lip19* is activated by low temperature and slightly stimulated by ABA but not by drought stress (Aguan *et al*., 1991; Rabbani *et al*., 2003). To study the effect of abiotic stress on *Wlip19* expression, M808 seedlings were dehydrated for 6 h or treated with 20 μM ABA solution for 2 h under the normal temperature conditions. The level of *Wlip19* transcript increased in response to both dehydration and ABA in the leaves but not in roots (Fig. 4A, B).

Next, the time-course of *Wlip19* expression was studied during 24 h of drought stress or ABA treatment in leaves of CS and M808 seedlings. The *Wlip19* transcript level in M808 increased during 6–12 h of drought, then decreased at 24 h, whereas in CS the increase was delayed until 12 h of drought and continued until at least 24 h (Fig. 4C, D). *Wlip19* expression was slightly increased by exogenous treatment with ABA and reached a maximum level after 10 h in M808 leaves (Fig. 4E, F). In CS, however, ABA responsiveness of *Wlip19* was not apparent (Fig. 4E, F). These results showed that *Wlip19* was responsive not only to cold and ABA but also to drought, indicating that the

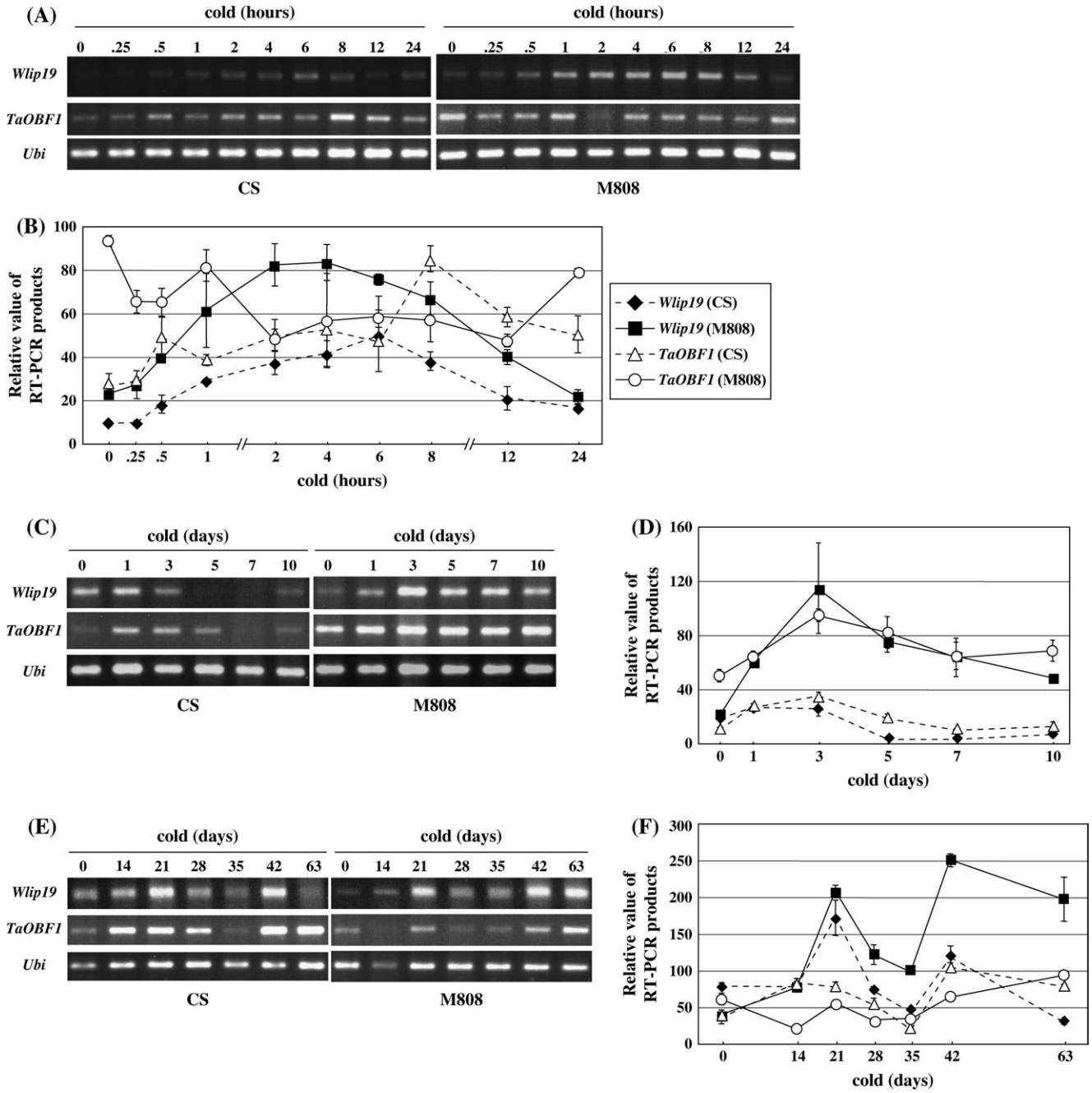


Fig. 3. Expression analyses of *Wlip19* and *TaOBF1* in two wheat cultivars during low temperature conditions. (A, B) Transcript accumulation profiles in response to low temperature within 1 d revealed by RT-PCR (A) and quantified as mean values with standard deviations relative to the *Ubi* transcript (B). (C, D) Transcript accumulation profiles in response to low temperature from 1 d to 10 d. (E, F) Transcript accumulation profiles in response to low temperature from 14 d to 63 d. The ubiquitin gene (*Ubi*) was used as a control in RT-PCR.

expression patterns of *Wlip19* differ from those of rice *lip19*.

Abiotic stress tolerance of transgenic tobacco plants expressing Wlip19

To study the contribution of *Wlip19* to the abiotic stress response, *35S::Wlip19* transgenic tobacco plants were generated, and their stress tolerance was analysed. Twenty-four transgenic tobacco plants were generated, and integration of the introduced chimeric gene was confirmed by Southern blot analysis (data not shown). Ectopic expression of the transgene was observed by RT-PCR in these transgenic T_1 plants (Fig. 5A). Based on these data, two transgenic lines, *35S::Wlip19*-#9 and *35S::Wlip19*-#15, were established and their T_2 progeny used for the following analysis. No phenotypic alteration

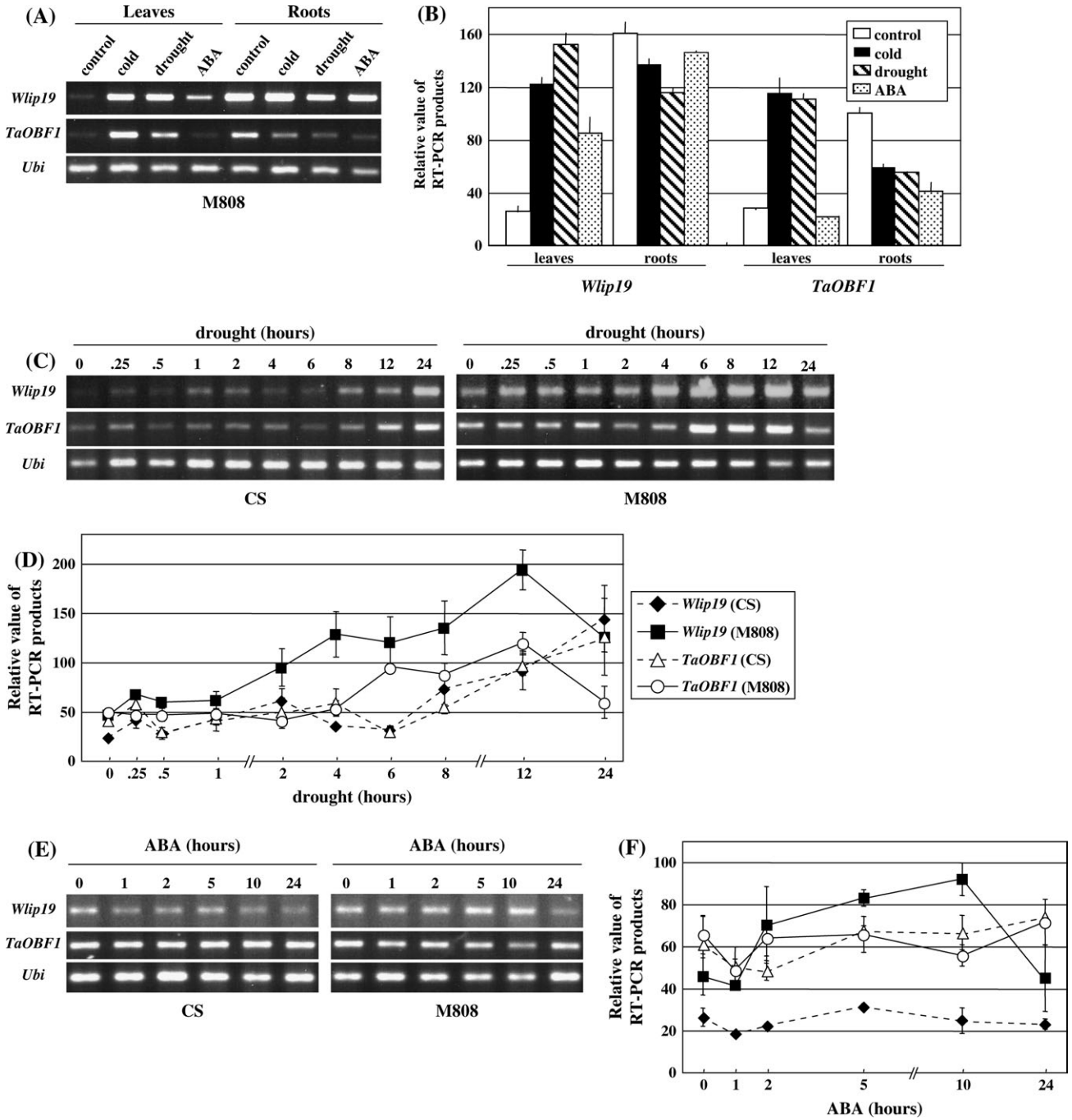
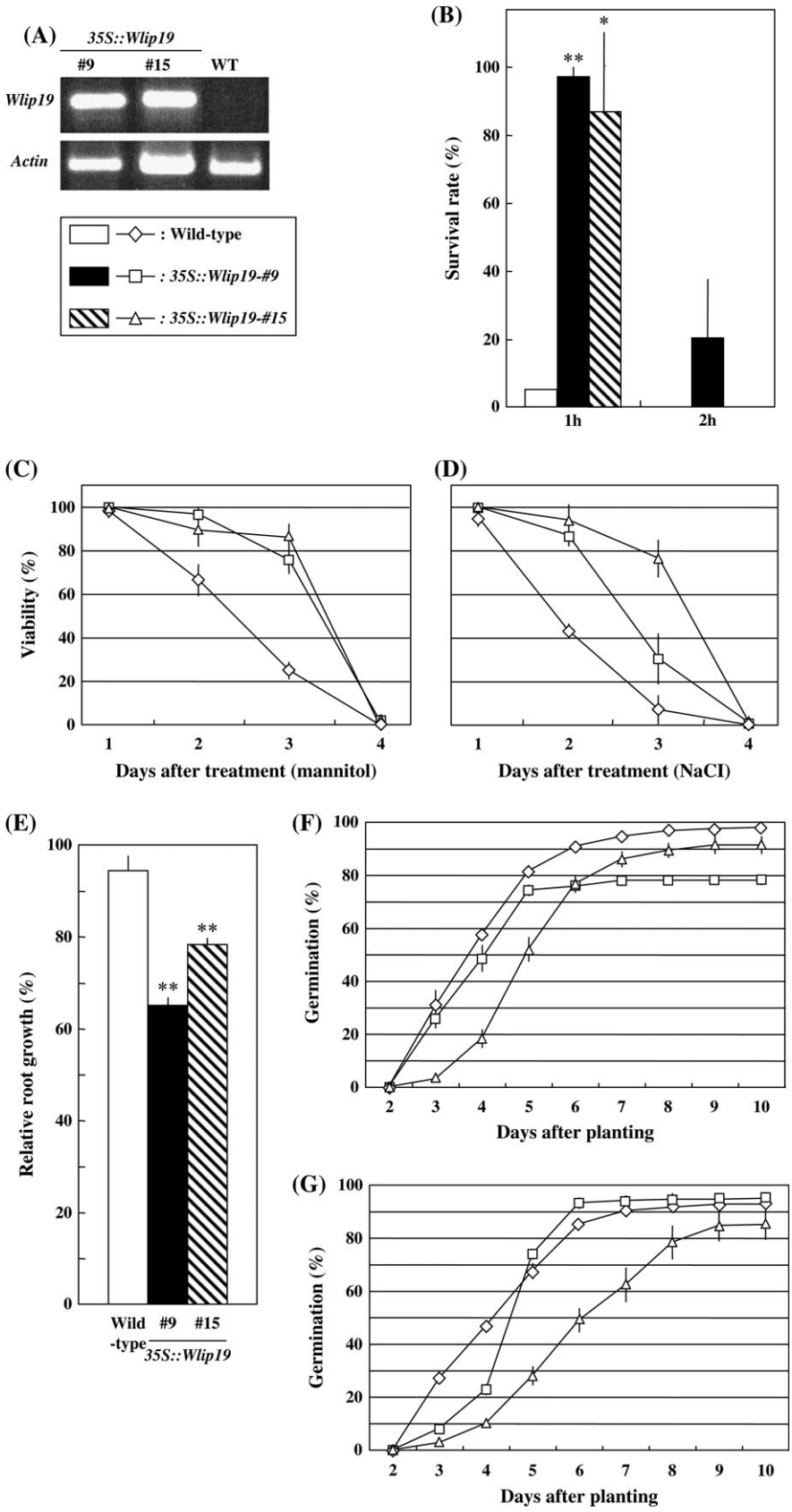


Fig. 4. Expression profiles of *Wlip19* and *TaOBF1* under drought and ABA treatment. (A, B) Comparison of transcript accumulation levels in leaves and roots of M808 seedlings revealed by RT-PCR (A) and quantified as mean values with standard deviations relative to the *Ubi* transcript (B). Control, normal temperature condition (20 °C); cold, 4 °C for 3 d; drought, dehydration for 6 h; ABA, 2 h after spraying with 20 μM ABA solution. (C, D) Time-course of transcript accumulation during drought treatment in two wheat cultivars. (E, F) ABA responsiveness of *Wlip19* and *TaOBF1* in two wheat cultivars. The ubiquitin gene (*Ubi*) was used as a control in RT-PCR.

was observed in these transgenic tobacco plants under non-stressed conditions.

The level of freezing stress tolerance was compared between the two *35S::Wlip19* lines and wild-type tobacco plants. Both *35S::Wlip19* lines showed >80% survival after 1 h of freezing, while the wild type had only 5% survival (Fig. 5B). Freezing tolerance was dramatically improved in the transgenic tobacco lines by *Wlip19* expression. Several *35S::Wlip19*-#9 plants survived under –15 °C for 2 h, whereas all wild-type and *35S::Wlip19*-#15



plants were killed by the freezing treatment (Fig. 5B), showing that the level of freezing tolerance in *35S::Wlip19*-#9 was higher than that in *35S::Wlip19*-#15.

Next, tolerance to osmotic stress was estimated by treatment with mannitol and NaCl solutions. Under mannitol and NaCl stress, cotyledons of the tobacco seedlings yellowed and then died. The percentage of plants with healthy green cotyledons decreased more rapidly in the wild-type plants than in *35S::Wlip19*-#9 and *35S::Wlip19*-#15 plants during 4 d of mannitol treatment (Fig. 5C), indicating that the tolerance of *35S::Wlip19* transgenic tobacco plants was higher than that of wild-type plants. Under NaCl stress conditions, both *35S::Wlip19* transgenic tobacco lines showed a significantly higher tolerance than the wild type (Fig. 5D). These results indicate that *Wlip19* expression contributes to development of osmotic stress tolerance in tobacco plants.

ABA sensitivity in transgenic tobacco expressing Wlip19

To study ABA sensitivity during early seedling development, inhibition of seedling growth by exogenous ABA (1 μM) was compared among the wild-type, *35S::Wlip19*-#9 and *35S::Wlip19*-#15 tobacco lines. Root elongation of the tobacco plants was inhibited by exogenous ABA treatment. The magnitude of inhibition in root growth, estimated by the relative growth rate (percentage growth in the presence of ABA relative to growth in the absence of ABA), was greater in the *35S::Wlip19* lines than in the wild type (Fig. 5E), indicating that primary root elongation of the *35S::Wlip19* plants was hypersensitive to exogenous ABA during post-germination growth.

Germination rates of mature seeds were compared under both ABA and non-ABA conditions among the wild-type, *35S::Wlip19*-#9, and *35S::Wlip19*-#15 lines. In the absence of exogenous ABA, *35S::Wlip19*-#15 showed delayed germination in comparison with the wild-type plants (Fig. 5F). On the other hand, *35S::Wlip19*-#9 showed similar germination rates to that of the wild type between days 2 and 5, whereas germination scarcely increased after day 5 (Fig. 5F). In the presence of 1 μM ABA, seed germination of *35S::Wlip19*-#15 was more markedly delayed, whereas germination of the wild type was slightly inhibited by ABA treatment (Fig. 5G). The germination of *35S::Wlip19*-#9 following ABA treatment was also delayed between days 3 and 4 (Fig. 5G). However, the germination rate of *35S::Wlip19*-#9 increased drastically on day 5 and reached a plateau on day 6 (Fig. 5G). These results indicate that the *35S::Wlip19*-#15 plants were more sensitive to both endogenous and exogenous ABA during seed germination than the wild type, while ABA sensitivity in *35S::Wlip19*-#9 during seed germination was not clear because seed germination was inconsistent under the experimental conditions used.

Trans*-activation of wheat* Cor/Lea promoters by WLIP19

To study direct interaction between *Cor/Lea* promoters and WLIP19, transient expression analysis was conducted by introducing a chimeric *35S::Wlip19* gene with one of four *Cor/Lea promoter::GUS* constructs (*Wdhn13*, *Wrab17* *Wrab18*, and *Wrab19*; Kobayashi *et al*., 2008*a*) into a wheat cell line. For all four *Cor/Lea promoter::GUS* constructs, co-introduction with *35S::Wlip19* constructs yielded higher GUS activity (Fig. 6A). In particular, *GUS* expression under control of the *Wdhn13* and *Wrab17* promoters was significantly increased by *Wlip19*.

The interaction between WLIP19 and wheat *Cor/Lea* promoters was also confirmed in the F_1 progeny derived from crossing *Cor/Lea promoter::GUS* tobacco plants with the *35S::Wlip19* tobacco lines. A histochemical GUS staining assay showed that *Wlip19* expression enhanced GUS levels under control of the 5' upstream sequences of the four *Cor/Lea* genes at the normal growth temperature for the F_1 plants (Fig. 6B). GUS expression was rarely observed in leaves of *Cor/Lea promoter::GUS* plants, whereas *Wlip19* strongly induced GUS activity in leaves of the F_1 seedlings. GUS quantification showed that GUS activity significantly increased in F_1 seedlings compared with parental transgenic plants (Fig. 6C). These *in vivo* and heterologous tobacco systems used for monitoring interaction between the WLIP19 and *Cor/Lea* promoters clearly indicated that WLIP19 functions as a transcriptional activator and positively regulates *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19* gene expression.

Isolation and chromosome assignment of the OBF1 *homologue in wheat*

The rice LIP19 protein interacts with OsOBF1 and the resulting heterodimer binds to DNA (Shimizu *et al*.,

Fig. 5. Abiotic stress tolerance and ABA sensitivity in *35S::Wlip19* transgenic and wild-type tobacco plants. (A) RT-PCR analysis of *Wlip19* expression in two *35S::Wlip19* transgenic tobacco lines (#9 and #15). *Actin* was used as an internal control for RT-PCR. (B) Comparison of freezing tolerance. Survival rates were compared after 1 h or 2 h of –15 °C treatment. (C) Comparison of osmotic stress tolerance. The percentages of plants with green cotyledons were compared after supplementation with a 0.5 M mannitol solution. (D) Comparison of salt stress tolerance. The percentages of plants with green cotyledons were compared after supplementation with a 0.2 M NaCl solution. (E) 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 relative to that without ABA. The primary root length was measured on the eighth day of treatment. (F) Comparison of seed germination rates on MS medium without ABA. (G) Inhibition of germination on MS medium with 1 μM ABA. The means ±SDs were calculated from data from 3–6 experiments. Student's *t*-test was used to test for statistical significance (*$P < 0.05$, **$P < 0.01$) between the wild-type plant and transgenic lines.

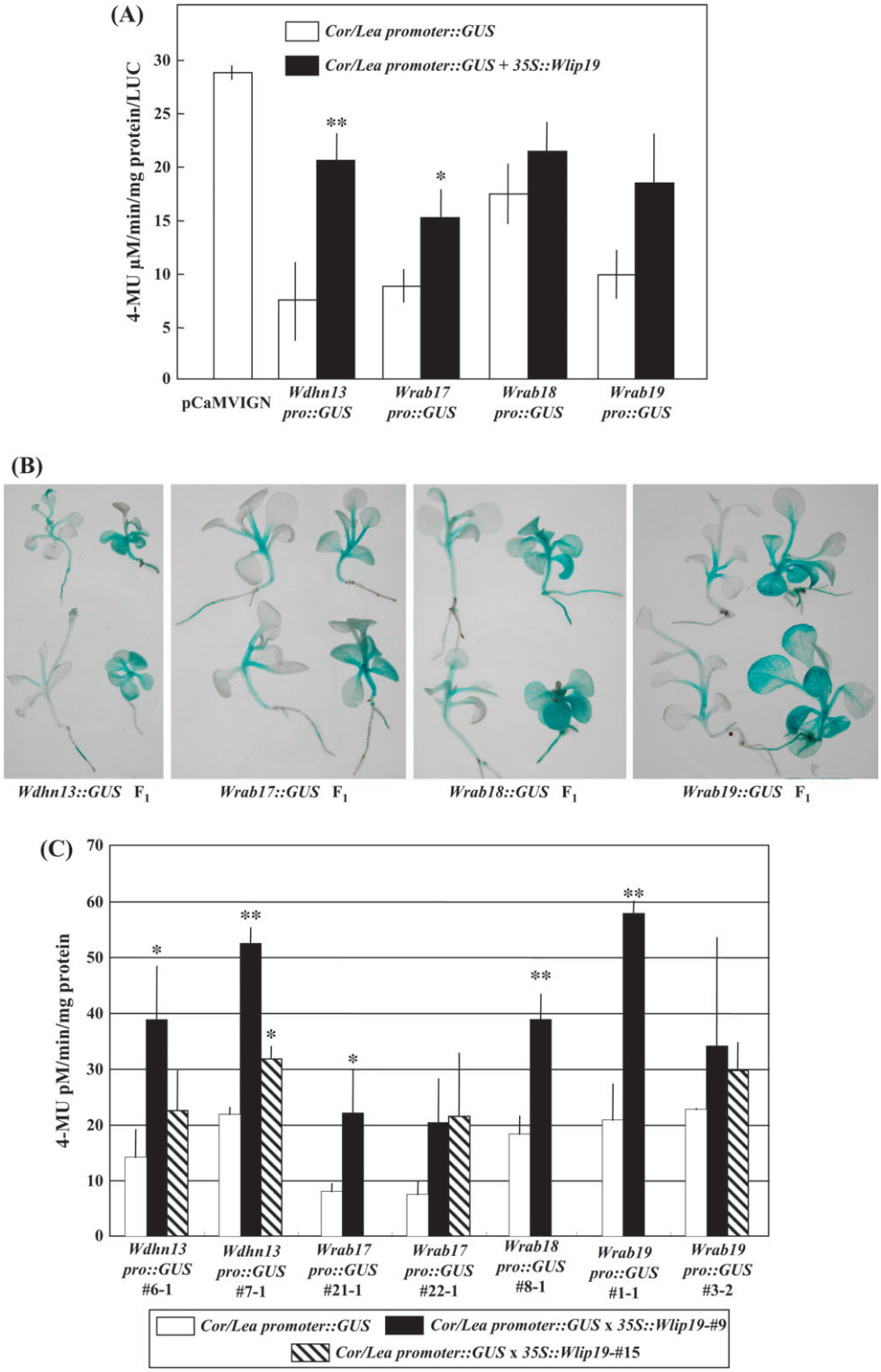


Fig. 6. *Trans*-activation of *Cor/Lea pro::GUS* chimeric genes by WLIP19. (A) Transient expression analysis in the wheat cultured cell line HY-1. GUS activity was normalized as luciferase activity expressed under the control of the CaMV *35S* promoter. The *GUS* gene under the control of a CaMV *35S* promoter and 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::Wlip19* transgenic plants and parental transgenic plants. (C) GUS activity in F_1 seedlings and parental *Cor/Lea pro::GUS* transgenic plants. Means ±SDs were calculated from data in three experiments. Asterisks indicate significance at the 5% (*) and 1% (**) level (Student's *t*-test).

2005). A wheat *OBF1* homologue (*TaOBF1*) has an amino acid identity of 75% with OsOBF1, but its characteristics have not been reported. Therefore, three homoeologous cDNAs of *TaOBF1* were isolated in order to attempt its molecular characterization. *TaOBF1* cDNA clones with a complete ORF were isolated from CS by RT-PCR. The 31 cDNA clones isolated were divided into three groups (see Supplementary Fig. S3 at *JXB* online). The three *TaOBF1* sequences encoded a bZIP-type protein with 157 amino acid residues (see Supplementary Fig. S2 at *JXB* online) that showed the highest levels of identity with rye ScOBF1, maize OBF1, and OsOBF1 (see Supplementary Fig. S2 at *JXB* online), and could be classified into group S of the bZIP-type proteins (Fig. 1).

Southern blots probed with *TaOBF1* cDNA showed that two or three bands were detected in hexaploid and tetraploid wheat genomes (Fig. 7A, data not shown), and that a single major band was detected in wheat diploid genomes (Fig. 7B). Aneuploid analysis using a series of nulli-tetrasomic lines showed that disappearance of a *TaOBF1*-specific signal occurred in the nulli-tetrasomic lines of homoeologous group 5 chromosomes (Fig. 7C), indicating that the *TaOBF1* loci represent the three homoeologous loci on chromosomes 5A, 5B, and 5D. PCR analysis with *TaOBF1a* and *TaOBF1d* homoeologue-specific primer sets showed the absence of amplification in nullisomic 5A and 5D lines (Fig. 7D). Therefore, the three *TaOBF1* loci were designated *TaOBF1a*,

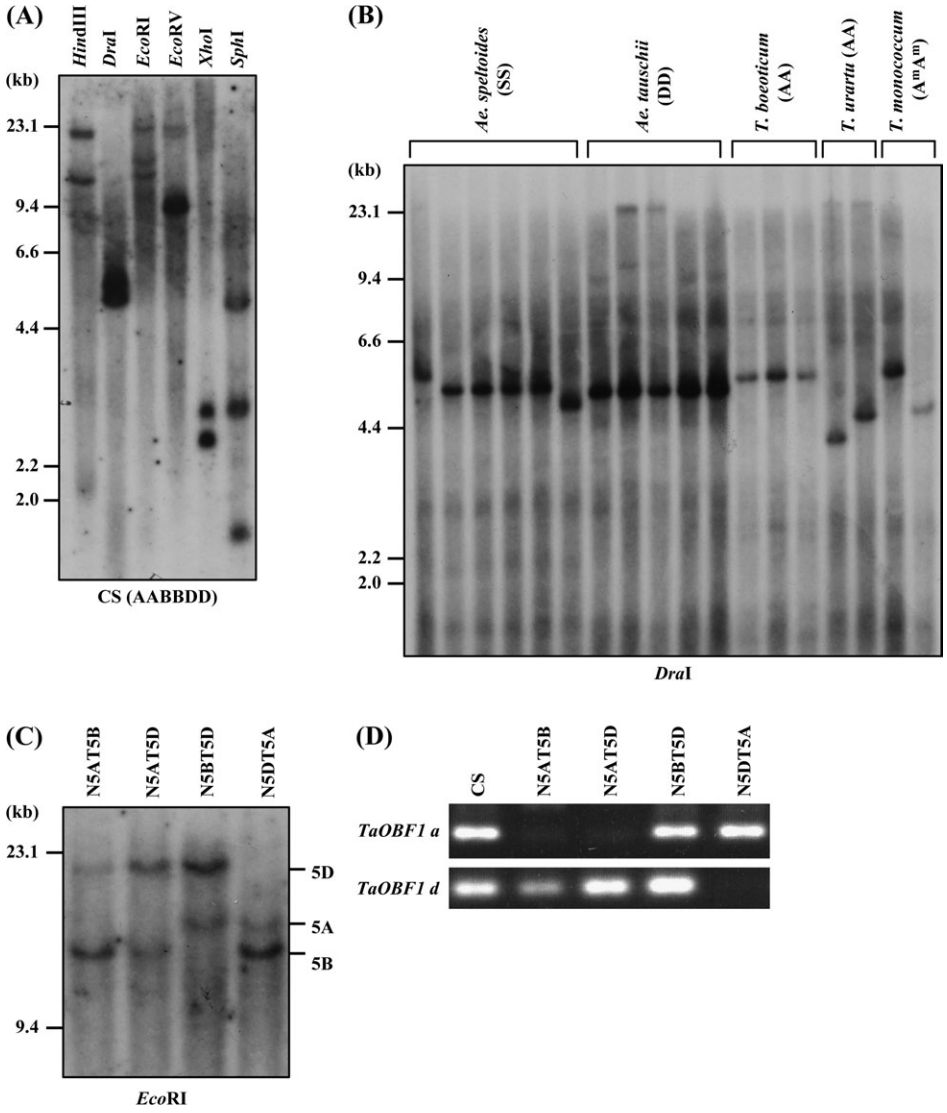


Fig. 7. Copy number and chromosome assignment of the *TaOBF1* gene in the wheat genome. (A) Estimation of the copy number in CS revealed by Southern blot analysis. Total DNA was digested with *Hin*dIII, *Dra*I, *Eco*RI, *Eco*RV, *Xho*I, and *Sph*I. The blot was probed with ^{32}P-labelled *TaOBF1* cDNA. (B) Southern blot analysis of *Dra*I-digested total DNA from multiple accessions of the indicated diploid wheat species. (C) Chromosome assignment of the *TaOBF1* gene to homoeologous group 5 chromosomes. Total DNA from the CS nulli-tetrasomic series was digested with *Eco*RI. (D) PCR analysis with homoeologue-specific primer sets of *TaOBF1a* and *TaOBF1d* using total DNA from the nulli-tetrasomic lines as templates.

TaOBF1b, and *TaOBF1d*, located on chromosomes 5A, 5B, and 5D, respectively.

Expression profiles of TaOBF1 *under cold, drought, and ABA treatment conditions*

Accumulation of maize *OBF1* transcript is positively regulated by low temperature (Kusano *et al*., 1995). *OsOBF1* transcripts are abundant in rice seedlings under normal temperature conditions, but decrease gradually during low temperature treatment (Shimizu *et al*., 2005). The time-course of *TaOBF1* expression during cold acclimation was studied in CS and M808 similarly to *Wlip19*. *TaOBF1* transcript was detected under normal temperature conditions in leaves of both CS and M808 seedlings (Fig. 3A, B). The accumulation of *TaOBF1* transcript increased with time and reached a high plateau after 8 h of low temperature in CS, but this transient increase was not clearly observed in M808 (Fig. 3A, B). The level of *TaOBF1* transcript reached a plateau by day 3 of low temperature in both CS and M808, and was maintained at a high level between days 5 and 10 in M808 (Fig. 3C, D). During long-term low temperature treatment, transcript accumulation of *TaOBF1* fluctuated in CS and M808 similarly to that of *Wlip19* (Fig. 3E, F).

The drought and ABA responsiveness of *TaOBF1* was also studied and compared for CS and M808. The *TaOBF1* transcript accumulated abundantly in the roots of M808 seedlings under non-stress conditions (Fig. 4A, B). After exposure to cold, drought, or ABA, the transcript level of *TaOBF1* decreased in the roots, whereas it increased in response to cold and drought in the leaves (Fig. 4A, B). *TaOBF1* clearly responded to drought and the response increased over time in leaves of both CS and M808 seedlings (Fig. 4C, D). However, *TaOBF1* showed no response to exogenous ABA treatment in either CS or M808 (Fig. 4E, F).

Protein–protein interaction between WLIP19 *and* TaOBF1

LIP19 forms a heterodimer with OsOBF1 but does not self-interact, while OsOBF1 can self-interact (Shimizu *et al*., 2005). To examine protein–protein interactions in wheat homologues, dimerization between WLIP19 and TaOBF1 proteins was analysed by a yeast two-hybrid assay. No interaction was observed between the control polypeptide and either the WLIP19 or TaOBF1 construct (Fig. 8, sectors D and E). Similarly to previous results in rice, TaOBF1 interacted with both WLIP19 and itself (Fig. 8, sectors B and C). Unlike rice LIP19, WLIP19 interacted with itself (Fig. 8, sector A). These results indicate that WLIP19 can form both a heterodimer with TaOBF1 and a homodimer.

Discussion

Transcripts of *Cor/Lea* genes and their transcription factor genes *Wcbf2* and *Wdreb2* accumulate more abundantly in freezing-tolerant M808 than in freezing-sensitive CS during cold acclimation, and the expression profiles show good correlation with development of freezing tolerance (Ohno *et al*., 2001, 2003; Takumi *et al*., 2003; Kobayashi *et al*., 2004*a*; Kume *et al*., 2005; Egawa *et al*., 2006). Transcript accumulation of *Wlip19* was also higher in M808 than in CS, and the *Wlip19* expression profiles were similar to those of *Wcbf2* and *Wdreb2* in low temperature conditions (Fig. 3; Kume *et al*., 2005; Egawa *et al*., 2006), implying that *Wlip19* functions in cold acclimation and development of freezing tolerance in common wheat.

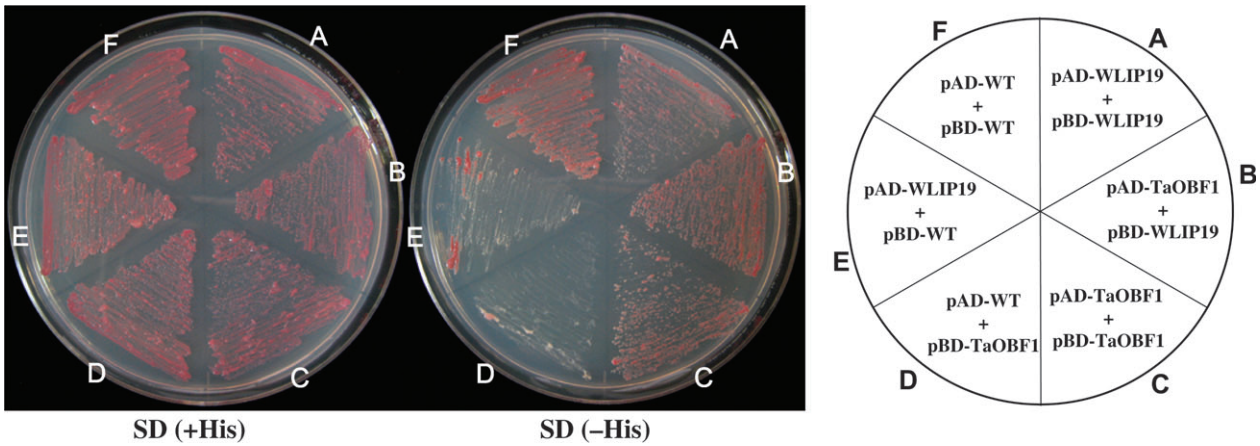


Fig. 8. Protein–protein interactions between WLIP19 and TaOBF1 proteins revealed by yeast two-hybrid analysis. Yeast transformants were grown both on SD medium lacking Leu and Trp (+His) and on SD medium lacking Leu, Trp, and His (–His). Transformed plasmid combinations are indicated on the right. The combination of pAD-WT and pBD-WT control plasmids (Stratagene) was used as a positive control for protein–protein interaction.

Wlip19 significantly enhanced *GUS* expression under control of the 5' upstream sequences of *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19* in cultured wheat cells and transgenic tobacco plants (Fig. 6). The four *Cor/Lea* promoter regions contain core ACGT motifs, which can be recognized by mLIP15 (Kusano *et al*., 1995). These results prove that *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19* are direct target genes of the WLIP19 transcription factor.

Stress-responsive transcription factors such as CBF/DREB and AREB/ABF increase abiotic stress tolerance in transgenic plants (Zhang *et al*., 2004; Umezawa *et al*., 2006). The heterologous expression of *Wlip19* significantly increased freezing tolerance of the transgenic tobacco lines (Fig. 5), strongly suggesting that *Wlip19* is associated with development of freezing tolerance through *Cor/Lea* gene activation. In wheat and barley, major quantitative trait loci for winter hardiness and freezing tolerance (*Fr*) have been identified on homoeologous group 5 chromosomes (reviewed by Cattivelli *et al*., 2002). The *Fr-1* chromosomal regions control *Cor/Lea* gene expression through CBF transcription factors (Kobayashi *et al*., 2005). *Wlip19* activation under low temperature conditions is also affected by the *Fr-1* allele (Kobayashi *et al*., 2004*b*). Therefore, the cultivar differences in *Wlip19* expression patterns under low temperature might originate from allelic differences in the *Fr-1* loci between M808 and CS. In addition to the CBF factors, *Wlip19* is certainly a downstream target transcription factor of *Fr-1*.

In *Arabidopsis*, group A bZIP-type proteins such as AREB/ABF proteins are a major factor in ABA-responsive gene expression under osmotic stress conditions (Yamaguchi-Shinozaki and Shinozaki, 2006). Their activities are reduced in both an ABA-deficient *aba2* mutant and an ABA-insensitive *abi1* mutant, but are enhanced in an ABA-hypersensitive *era1* mutant (Uno *et al*., 2000). *Wlip19* expression was also responsive to drought and ABA (Fig. 4). *35S::Wlip19* transgenic tobacco became tolerant to high mannitol and salt stress, and hypersensitive to ABA compared with wild-type tobacco (Fig. 5), suggesting that *Wlip19* at least partly functions in the ABA signalling pathway under abiotic stress conditions. However, low temperature-induced expression of *Wlip19* is not affected by mutations in some wheat mutant lines (Kobayashi *et al*., 2006, 2008*b*). The critical roles of *Wlip19* in the wheat ABA signalling pathway should be clarified in future studies.

TaOBF1 cDNAs were isolated as wheat counterparts of rice *OsOBF1* and maize *OBF1* (Fig. 1, and Supplementary Fig. S2 at *JXB* online). Although *OsOBF1* transcript accumulation decreased during low temperature treatment (Shimizu *et al*., 2005), *TaOBF1* transcript accumulation was weakly enhanced by low temperature in seedling leaves, similarly to *OBF1* (Kusano *et al*., 1995; Fig. 3). Moreover, *TaOBF1* positively responded to drought stress (Fig. 4). These expression profiles revealed that *TaOBF1* may act in abiotic stress responses, but its significance is unclear compared with other transcription factors such as *Wcbf2*, *Wdreb2*, and *Wlip19*. A transgenic approach may clarify the function of *TaOBF1* under abiotic stress.

LIP19 is unable to self-dimerize and has no DNA-binding activity, but a OsOBF1/LIP19 heterodimer binds to DNA (Shimizu *et al*., 2005). This heterodimerization seems to switch on the expression of target genes during exposure to low temperature in rice. In maize, mLIP15 also dimerizes with OBF1 *in vitro* (Kusano *et al*., 1995). TaOBF1 interacts with WLIP19 (Fig. 8), implying that the heterodimerization between LIP19-type and OBF1-type proteins is well conserved at least in cereals and that the heterodimer may regulate downstream gene expression under abiotic stress conditions. Because of WLIP19 homodimer formation (Fig. 8), it can be postulated that WLIP19 binds to DNA both as a homodimer and as a heterodimer with TaOBF1. Transcription factor dimerization can increase the selectivity of protein–DNA interactions and generate a large amount of DNA-binding diversity from a relatively small number of proteins (Wolberger, 1999). Dimerization also leads to the establishment of complex regulatory networks (Hai and Hartman, 2001). Because of no direct evidence showing DNA-binding activity of WLIP19 and TaOBF1, the co-existence of the three bZIP-dimers, WLIP19/WLIP19, TaOBF1/TaOBF1, and WLIP19/TaOBF1, and association of these dimers with expression of multiple genes including *Cor/Lea* genes under stress conditions should be confirmed in future studies. Cereal LIP19-type bZIP proteins act as an important transcriptional regulator in abiotic stress responses, especially in the cold and freezing stress signal pathway.

Supplementary material

Supplementary material is available at *JXB* online. Figure S1 shows the alignment of nucleotide sequences of three homoeologous *Wlip19* cDNAs. Figure S2 provides the alignment of WLIP19 and TaOBF1 amino acid sequences with those of the LIP19 subfamily. Figure S3 also shows the alignment of nucleotide sequences of the three homoeologous *TaOBF1* cDNAs.

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