<table>
<thead>
<tr>
<th>タイトル (Title)</th>
<th>Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>著者 (Author(s))</td>
<td>Kobayashi, Fuminori / Maeta, Eri / Terashima, Akihiro / Kawaura, Kanako / Ogihara, Yasunari / Takumi, Shigeo</td>
</tr>
<tr>
<td>掲載誌・巻号・ページ (Citation)</td>
<td>Journal of Experimental Botany, 59(4): 891-905</td>
</tr>
<tr>
<td>刊行日 (Issue date)</td>
<td>2008-03-07</td>
</tr>
<tr>
<td>資源タイプ (Resource Type)</td>
<td>Journal Article / 学術雑誌論文</td>
</tr>
<tr>
<td>版区分 (Resource Version)</td>
<td>publisher</td>
</tr>
<tr>
<td>権利 (Rights)</td>
<td></td>
</tr>
<tr>
<td>DOI</td>
<td>10.1093/jxb/ern014</td>
</tr>
<tr>
<td>JaLCDOI</td>
<td></td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/90000836">http://www.lib.kobe-u.ac.jp/handle_kernel/90000836</a></td>
</tr>
</tbody>
</table>

PDF issue: 2022-05-31
Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat

Fuminori Kobayashi1,*, Eri Maeta1, Akihiro Terashima1, Kanako Kawaura2, Yasunari Ogihara2 and Shigeo Takumi1,†

1 Laboratory of Plant Genetics, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
2 Kihara Institute for Biological Research, Graduate School of Integrated Science, Yokohama City University, Totsuka-ku, Yokohama 244-0813, Japan

Received 12 November 2007; Revised 3 January 2008; Accepted 7 January 2008

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 sub-family belongs to group A. Group A bZIPs generally function in ABA signalling during seed maturation or
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 OBFI is another low temperature-responsive bZIP-type protein recognizing ACGT motifs (Singh et al., 1990; Kusano et al., 1995). LIP19, mLIP15, and OBFI are categorized into group S bZIPs (Shimizu et al., 2005). mLIP15 dimerizes with OBFI; mLIP15 and OBFI also form homodimers, though LIP19 does not (Kusano et al., 1995; Shimizu et al., 2005). LIP19 and mLIP15 dimerize respectively with OsOBFI and OBFI1, 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., 2008a; 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, Wраб17, Wrab18, and Wrab19 (Quellet et al., 1998; Takumi et al., 2003; Kobayashi et al., 2008a), 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 ACGT core motifs (Kobayashi et al., 2008a). 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 OBFI homologue, TaOBFI1, 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 TaOBFI1

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'-TTTTTTCTTG-GACTGACCTCC-3' and 5'-GCACTGAAATCGAAGCTTTCT-TTA-3' for Wlip19b, 5'-TCCCTGATTTGTTGTTTCC-3' and 5'-CGCCGTGCGCATGTTGCTT-3' for Wlip19a, and 5'-GTCGACCTGACCTTTCC-3' and 5'-TGGACAGACACCATCCTACCT-3' for Wlip19d. Based on the nucleotide sequences of TaOBFI1 (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'-TGAACACTAATACTGCTC-3' and 5'-ATAGCAGAAAATGCTTC-3' was designed. Full-length cDNA of TaOBFI1 was isolated from leaf RNA of CS by RT-PCR with this primer set. Amplified cDNAs of Wlip19 and TaOBFI1 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 DNAnas software (Hitachi, Tokyo, Japan). The cDNA sequences were deposited in the DDBJ database under these accession numbers: AB334126 (Wlip19b), AB334127 (Wlip19a), AB334128 (Wlip19d), AB334129 (TaOBFI1a), AB334130 (TaOBFI1b), and AB334131 (TaOBFI1d). 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’ (Ldh), 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 32P-labelled Wlip19 or TaOBFI1 cDNA as a probe. Probe labelling, hybridization, washing, and autoradiography were performed according to Takumi et al. (1999).

For chromosome assignment of Wlip19 and TaOBFI1, 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 nullitetrasomic series. To distinguish the three TaOBFI1 homologues, the following clone-specific primer sets were used: 5'-AAAAATCCGCCGCTGCTAC-3' and 5'-TGCCAGCGGAATATCAGTAGTAC-3' for TaOBFI1a, 5'-CAATCCGCCGCTGCTAC-3' and 5'-GCCAGCAGAAATCTACTGCT-3' for TaOBFI1b, and 5'-CAATCCGCCGCTGCTAC-3' and 5'-TGGACAGACACCATCCTAC-3' for TaOBFI1d. 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. (2004a) 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 (Öhno 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 Wip19 and TaOBF1 transcripts was detected by RT-PCR amplification as previously reported (Kobayashi et al., 2004b, 2006). RT-PCR was conducted with the following gene-specific primer sets: 5'-CAACATC-GACGCGGCGAG-3' and 5'-GGTCGAATTCTGCGACGTC-3' for Wip19, and 5'-AACGATGCTGTCGTCGCTG-3' and 5'-GACTAGCATGTGCTGG-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 electrophorograms 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 Wip19
The Wip19a cDNA sequence was amplified with the following primer set containing a BamHI linker: 5'-CCGGATCCCGATCCAGCCTCGTTT-3' and 5'-GGTACCATGTCGTCGCTGTC-3'. The PCR fragment was digested with BamHI and inserted into the BamHI site of 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 (T₀ 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::Wip19. To detect Wip19 transcripts in the 35S::Wip19 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'-GGCTGGTTTTGCTGGTGACG-3' and 5'-AATGAAAG-GAAGCTGGAGAGAAG-3'.

Bioassay conditions for freezing and osmotic stress tolerance
To assay freezing tolerance, wild-type and T₂ progeny of 35S::Wip19 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::Wip19 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 T₂ progeny of 35S::Wip19. 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::Wip19 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 pBI1101 (Kobayashi et al., 2008a). 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., 2008a). The four Cor/Lea promoters::GUS and 35S::Wip19 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::Wip19 transformants were tested as the pollen parent in crosses with the transgenic tobacco plants having introduced the Cor/Lea promoter::GUS constructs. F₁ transgenic tobacco plants were selected on hormone-free MS medium containing 50 mg l⁻¹ kanamycin. GUS activity in the kanamycin-resistant F₁ tobacco plants and homozygous T₂ progeny of Cor/Lea promoter::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 Wip19a and TaOBF1α cDNA were amplified with the following primer sets containing EcoRI and SalI linkers: 5’-GGGAATTTCCGACCAATGTGCCTGGCATC-3’ and 5’-GGTACCACTGCTGTCGCTGTC-3’ for Wip19, and 5’-GGGACTTCCGAGCAAAAGATGTGCCTGTC-3’ and 5’-GGCTGACCAATACTACGCCGATGACG-3’ for TaOBF1. The PCR products were digested with EcoRI and SalI, and cloned into the EcoRI and SalI sites of pD-GAL4-2.1 and pBD-GAL4 Cam vectors, resulting in pAD-Wip19, pAD-TaOBF1, pBD-Wip19, 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 (AB15/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 HvAB5 (AY150676), rye ScOBF1 (AJ617794), maize mLIP15 (D26563) and OBF1 (X62745), Arabidopsis AREB1/ABF2 (AB017160/AF093545), GBF5 (AF053939), ATB2 (X99747), and AthZIP53 (AF400620), snapdragon bZIP910 (Y13675) and bZIP911 (Y13676), and tobacco TBZ17 (D63951), BZI-2 (AY045570), BZI-3/TBZF (AY045571/AB032478), and BZI-4 (AY045572).
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 3 d of low temperature and was maintained at a high level between 5 d and 10 d of treatment 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 appear 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
expression patterns of \( \text{Wlip19} \) differ from those of \( \text{rice lip19} \).

Abiotic stress tolerance of transgenic tobacco plants expressing \( \text{Wlip19} \)

To study the contribution of \( \text{Wlip19} \) to the abiotic stress response, \( 35S::\text{Wlip19} \) 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, \( 35S::\text{Wlip19-#9} \) and \( 35S::\text{Wlip19-#15} \), were established and their \( T_2 \) progeny used for the following analysis. No phenotypic alteration

Fig. 3. Expression analyses of \( \text{Wlip19} \) and \( \text{TaOBF1} \) 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 \( \text{Ubi} \) 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 (\( \text{Ubi} \)) 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 WLP19

To study direct interaction between Cor/Lea promoters and WLP19, 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., 2008a) 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 WLP19 and wheat Cor/Lea promoters was also confirmed in the F1 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 F1 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 F1 seedlings. GUS quantification showed that GUS activity significantly increased in F1 seedlings compared with parental transgenic plants (Fig. 6C). These in vivo and heterologous tobacco systems used for monitoring interaction between the WLP19 and Cor/Lea promoters clearly indicated that WLP19 functions as a transcriptional activator and positively regulates Wdhn13, Wrab17, Wrab18, and Wrab19 gene expression.

Isolation and chromosome assignment of the OBFW homologue in wheat

The rice LIP19 protein interacts with OsOBFW1 and the resulting heterodimer binds to DNA (Shimizu et al.,
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 F1 seedlings of Cor/Lea pro::GUS and 35S::WLIP19 transgenic plants and parental transgenic plants. (C) GUS activity in F1 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).
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,
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 increased 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., 2004a; 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.
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 ACCT 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., 2004b). 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 abil 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, 2008b). 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 Wvip19. 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.

Acknowledgements

We are grateful to Dr T Shimada for providing us with the HY-1 callus line. We also thank Dr C Nakamura for use of his facilities. Seeds used in this study were supplied by the National BioResource Project-Wheat (Japan, www.nbrp.jp). This work was supported by a Grant-in-Aid for Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists to FK and from the Ministry of Education, Culture, Sports, Science and Technology of Japan to ST (No. 17780005). FK is a Research Fellow of the Japan Society for the Promotion of Science (JSPS).
References


Takumi S, Murai K, Mori N, Nakamura C. 1999. Variations in the maize Ac transposable transcript level and the Ds excision


