LIP19, a Basic Region Leucine Zipper Protein, is a Fos-like Molecular Switch in the Cold Signaling of Rice Plants

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The rice low-temperature-induced lip19 gene encodes a 148-amino-acid basic region/leucine zipper (bZIP) protein, termed LIP19. In this study we characterized LIP19 and showed that it lacks the usual ability of bZIP proteins to homodimerize and to bind DNA, as does the Fos protein in mammals. Using a yeast two-hybrid system, the cDNA clones whose products interact with LIP19 were screened. This search revealed a clone termed OsOBF1 (Oryza sativa OBF1) that encodes a new bZIP protein (OsOBF1). This protein forms a homodimer and binds to the hexamer motif sequence (5′-ACGTCA-3′). The protein–protein interaction in homo- and hetero-combinations between LIP19 and OsOBF1 was confirmed in vitro and in planta. LIP19 and OsOBF1 most likely interact with each other more strongly than OsOBF1 interacts with itself, and the resulting heterodimer binds to the C/G hybrid sequence but not to the hexamer sequence. Whereas the expression patterns of lip19 and OsOBF1 in response to low temperatures were totally opposite, the locations of their expression were almost identical. Based upon the presented data, we propose a model describing the low-temperature signal switching mediated by LIP19 in rice.

Keywords: bZIP protein — Cold signaling — DNA binding — Heterodimer — Molecular switch — Rice

Abbreviations: BF, binding form; BiFC, bimolecular fluorescence complementation; bZIP, basic region/leucine zipper; DIG, digoxigenin; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactoside; OBF1, ocs-element Binding Factor 1; ocs, actinopeptide synthase; ONPG, o-nitrophenyl-β-D-galactopyranoside; ORF, open reading frame; OsOBF1, Oryza sativa OBF1; PMSF, phenylmethylsulfonyl fluoride; SD, synthetic drop-out; TF, transcription factor; YFP, yellow fluorescent protein.

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Introduction

The mechanisms that regulate gene expression in eukaryotes, including higher plants, have been extensively investigated. Gene expression control at the level of transcription is primarily governed by highly specific DNA (cis-elements found in the promoter regions)—protein (transcription factor [TF]) interactions (Meshi and Iwabuchi 1995, Yanagisawa 1998, Liu et al. 1999, Riechmann and Ratcliffe 2000). The classification of TFs depends on their structural features, such as a homeodomain or the presence of a basic region/leucine zipper (bZIP), a zinc-finger, or an EREBP/AP2 domain. Of these, the bZIP family of proteins is characterized by the presence of a basic region followed by a leucine zipper region. The basic region is a subdomain that is rich in basic amino acids and is involved in DNA binding, while the leucine zipper contains at least 3–4 repeats of Leu or other hydrophobic amino acids at every 7th position that mediate dimer formation through hydrophobic bonding (Landschulz et al. 1988, Hurst 1994). Most bZIP proteins form homodimers and are able to bind to DNA bearing the AC/CT core sequence. However, there are a few exceptions. An example is the Fos oncoprotein, which cannot form homodimers and cannot bind DNA (Kouzarides and Ziff 1988, Ziff 1990). However, this bZIP can interact with Jun, another bZIP protein, and the resulting heterodimer binds to the AP-1 binding site sequence (Ziff 1990, Hai and Curran 1991).

Abiotic stresses, including low temperatures, are major limiting factors for plant growth and productivity. To understand the underlying molecular mechanisms and/or to yield cold/freeze-tolerant plants, many intensive studies on cold stress have been performed. As a result, parts of the cold-induced signaling pathways have been elucidated (Thomashow 1999, Thomashow 2001, Xiong et al. 2002, Chinnusamy et al. 2003, Shinozaki et al. 2003, and the references therein). In Arabidopsis thaliana, it is known that CBF/DREB1 proteins are the key players that switch on the expression of the downstream target genes in the cold-induced signaling pathway (Stockinger et al. 1997, Jaglo-Ottosen et al. 1998, Liu et al. 1998, Kasuga et al. 1999). Recent research has demonstrated that CBF/DREB-like TFs function in monocotyledons as well as in dicotyledons (Doubzet et al. 2003). However, microarray data have suggested the presence of multiple cold-signaling pathways in A. thaliana since more than a dozen genes encoding TFs are up-regulated during cold stress (Fowler and...
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Thomashow 2002, Seki et al. 2002). The situation seems to be the same in rice since cDNA microarray and Northern blot analyses showed that rice genes encoding Cys2His2-type zinc-finger-, Myb-type- and NAC-type- TFs are up-regulated during cold stress (Rabbani et al. 2003). In addition, we have previously reported that lip19, a bZIP-encoding gene, is strongly induced by low temperature in rice (Aguan et al. 1991, Aguan et al. 1993). However, its physiological role is totally unknown.

Here we report the characterization of LIP19, the product of the lip19 gene. LIP19 is distinct from most bZIP proteins since it is unable to bind DNA or form homodimers. Thus, it resembles the mammalian Fos bZIP protein mentioned above. A search for LIP19-interacting proteins identified a novel bZIP protein, OsOBF1. This protein is able to bind DNA and forms homodimers but prefers to partner with LIP19. While the two bZIP genes are expressed in an opposite manner in response to

Fig. 1 Identification of OsOBF1 as a clone encoding a LIP19-interacting protein. (A) Alignment of the OsOBF1 amino acid sequence with those of the lip19 subfamily. The identical and conserved residues are highlighted with black and gray backgrounds, respectively. The basic region is underlined and the heptad Leu or hydrophobic residues are indicated by asterisks. The family members have long leucine-zipper motifs.

(B) Phylogenetic tree of the LIP19 subfamily members, including OsOBF1. (C) Cellular localization of the LIP19 and OsOBF1 proteins. Onion bulbs were transformed with pLIP19-GFP2, pOsOBF1-GFP2 and pGFP2 by particle bombardment. After overnight incubation at 22°C in complete darkness, the epidermal layers were peeled off and observed by fluorescence microscopy. Epifluorescence derived from GFP was observed (top row). The same cells were stained with DAPI to determine the positions of the nucleus (bottom row).
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cold stress, their localizations of expression overlap. On the basis of these observations, a model explaining the role LIP19 plays in a cold-signaling pathway in rice is proposed.

Results

Isolation of OsOBF1 as a clone encoding a LIP19-interacting protein

Rice lip19 encodes a 148-amino-acid bZIP protein and is strongly induced by low temperatures (5°C) (Aguan et al. 1993, Rabbani et al. 2003), but its biological function is unclear. It is known that some bZIP proteins, like Fos in mammals (Kouzarides and Ziff 1988) and GBF4 in A. thaliana (Menkens and Cashmore 1994), neither form a homodimer nor bind to DNA. Our previous observations suggested that this may also be the case for LIP19 (Kusano et al. 1995). Thus, we performed a yeast two-hybrid screening using intact LIP19 as bait to identify clone(s) that encode LIP19-interacting proteins. We confirmed that native LIP19 does not have transactivation activity in yeast cells (data not shown). Among the positive clones obtained, we focused on clone #25 because it appeared to encode a TF of bZIP type. Its product showed highest identity to maize ocs-element Binding Factor 1 (OBF1) (Singh et al. 1990) (Fig. 1 A, B) and thus the cDNA was termed OsOBF1 (Oryza sativa OBF1) (Accession number, AB185280). Genomic Southern blot analysis showed that OsOBF1 as well as lip19 is present as a single copy gene per haploid genome (data not shown). A database search on the whole rice genome sequence also supports this notion. The longest OsOBF1 cDNA obtained was 1,277 bp and the main open reading frame (ORF) encoded a 145 amino acid bZIP protein denoted OsOBF1. OsOBF1 and OBF1 share 82% identity (Fig. 1A). There is a small ORF in the 5' upstream region of the main ORF. It encodes a 28-amino-acid-peptide and is very much conserved in the genes of the lip19 subfamily (not shown, Wiese et al. 2004). The cellular localization of LIP19 and OsOBF1 proteins was then investigated by fusing them to green fluorescent proteins (GFPs) and transforming them into onion epidermal cells. The data revealed that both LIP19 and OsOBF1 localized in the nuclei (Fig. 1C).

Homo- and hetero-dimerization between the LIP19 and OsOBF1 proteins in yeast cells

The present study investigated whether LIP19 and OsOBF1 form homo- and hetero-dimers using a yeast GAL4 two-hybrid assay system. As mentioned, no interaction was observed between the LIP19 bait and the LIP19 prey construct (Fig. 2A). Unlike LIP19, OsOBF1 can interact with itself and...
also with LIP19 in yeast cells (Fig. 2 B, C). Next, we compared the relative intensities of the interactions of OsOBF1 with itself and with LIP19 in yeast cells. The LIP19-OsOBFI heterodimer appears to interact more strongly than the OsOBFI homodimer since the growth of *Saccharomyces cerevisiae* cells carrying pBD-LIP19 and pAD-OsOBFI on synthetic dropout (SD) medium lacking histidine was faster than that of the yeast cells carrying pBD-OsOBFI and pAD-OsOBFI (data not shown). To verify this observation quantitatively, the β-galactosidase activity of the yeast cells was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. This revealed that LIP19 homodimers formed very infrequently since the relative β-galactosidase activity (0.093 units) of the yeast cells was almost at background level (0.046 units) (Fig. 2D). In addition, the relative β-galactosidase activity (0.473 units) of the yeast cells bearing the OsOBFI homodimer form was less than one-fifth of that of yeast cells harboring the LIP19/OsOBFI heterodimer, independent of the respective cloning vectors used for expression of the single proteins (Fig. 2D, 4D). Thus, we conclude that OsOBFI binds to the Hex sequence but not to OCS.

**OsOBFI binds to DNA**

We then tested whether OsOBFI binds to DNA. To do this, OsOBFI was produced as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* JM109 cells (Fig. 3A) and subjected to an electrophoretic mobility shift assay (EMSA). This revealed that OsOBFI homodimers formed very infrequently since the relative β-galactosidase activity (0.093 units) of the yeast cells was almost at background level (0.046 units) (Fig. 2D). In addition, the relative β-galactosidase activity (0.473 units) of the yeast cells bearing the OsOBFI homodimer form was less than one-fifth of that of yeast cells harboring the LIP19/OsOBFI heterodimer, independent of the respective cloning vectors used for expression of the single proteins (Fig. 2D, 4D). Thus, interaction between OsOBFI and LIP19 is likely to be stronger than that between two OsOBFI molecules. To conclude this notion more solidly, kinetic analysis of dimer formation between LIP19 and OsOBFI or OsOBFI with itself will be required.

**The leucine zipper region of LIP19 is responsible for its inability to form homodimers**

To clarify why LIP19 does not form homodimers, the two domains of LIP19 and OsOBFI, namely the basic region and the leucine zipper region, were swapped. As depicted in Fig. 4A, two domain-swapped constructs were generated. b-L/z-O is a chimera bearing the amino-distal half of LIP19 containing the basic region and the carboxy-distal half of OsOBFI containing the leucine zipper. b-O/z-L has the opposite structure. The b-L/z-O molecule appears to form homodimers; in contrast, b-O/z-L, like LIP19, cannot form homodimers (Fig. 4 B, D). Thus, the leucine zipper portion of LIP19 is the cause of its inability to homodimerize. Furthermore, using the LIP19/OsOBFI chimeric molecules, the hetero-combination of b-L/z-O and b-O/z-L showed strong activity qualitatively (Fig. 4C) and quantitatively (Fig. 4D), even when the cloning vectors were exchanged. Helical wheel display of the LIP19 leucine zipper revealed that positions e (e') and g (g'), in which the electrostatic interactions occur between charged residues, were occupied by acidic ones, but not basic ones, which probably prevented homodimer formation. In contrast, OsOBFI contained one basic residue in the g position (Fig. 4E).

**Confirmation of the interaction between LIP19 and OsOBFI by in vitro pull-down and in planta bimolecular fluorescence complementation (BiFC) assays**

We first tested the interaction between LIP19 and OsOBFI proteins in vitro. As expected, an in vitro pull-down
assay revealed that LIP19 does not bind to itself but binds to OsOBF1 (Fig. 5A). After coupled transcription and translation in rabbit reticulocyte lysate, we detected two proteins as lip19 products by SDS-PAGE. At the moment, we can not distinguish whether one of them is a truncated form of the nascent protein or a degradation product. In contrast to LIP19, OsOBF1 could bind to both itself and LIP19 (Fig. 5B). These results further confirm the inability of LIP19 to homodimerize. Thus, the in vitro pull-down assay data perfectly mirror the yeast two-hybrid experiment data. We then examined the interaction between LIP19 and OsOBF1 in planta using the BiFC assay (the whole assay system was kindly provided by Dr. T. Tzfira). In our experimental design, the OsOBF1-encoded sequence was fused in frame with the sequence corresponding to the amino-terminal half of yellow fluorescent protein (YFP) while the LIP19-encoded sequence was fused similarly to the sequence corresponding to the carboxy-terminal half of YFP, or vice versa (Fig. 6A). As with the in vitro pull-down assay and yeast-two hybrid experiment results, OsOBF1 was found to interact with both itself and LIP19. The reconstituted YFP fluorescence observed in these combinations was located in the nuclei of the onion epidermal cells (Fig. 6B).
The LIP19/OsOBF1 heterodimer, but not the OsOBF1 homodimer, binds to the C/G hybrid sequence

We then used EMSA to evaluate whether the LIP19/OsOBF1 heterodimer had DNA-binding ability. Before doing this, we noticed that the LIP19 protein, whether produced in E. coli or in rabbit reticulocyte lysate, is quite unstable, even when prepared at 4°C with maximal care. Therefore, we used the E. coli extracts containing the LIP19 derivatives immediately after their preparation. In the EMSA assays, with different probes having the same base-pair length, we found that OsOBF1 bound to Hex but did not bind to the C/G hybrid sequence (Fig. 3B, 7B left). In contrast, the LIP19/OsOBF1 heterodimer bound primarily to this C/G hybrid sequence (Fig. 3B, 7B right). It should be emphasized that the binding form (BF) of the C/G hybrid sequence and the LIP19/OsOBF1 heterodimer migrated a little faster in EMSA gel than the BF of...
the Hex sequence and OsOBF1. These separately displayed results (Fig. 7B) were obtained from one electrophoresis gel. As LIP19 is unstable after purification, we changed the molar ratio of LIP19 and OsOBF1. As expected, the greater the amount of LIP19 that was added to the binding mixtures, the more intensely the BFs were detected (Fig. 7B right, lanes 4–6). The coexistence of LIP19 and OsOBF1 in the presence of the Hex probe caused no difference in the migration of the BF compared with that observed with OsOBF1 alone (Fig. 7B, left, second and third lanes from the left). This indicates that LIP19/OsOBF1 cannot bind to the Hex sequence. Importantly, the above result tells us two things. First, LIP19 gains DNA-binding ability after forming a heterodimer with OsOBF1. Second, the preferred DNA sequence of the heterodimer is different from the one recognized by the OsOBF1 homodimer. There are two replacements between the two Hex and C/G hybrid probe sequences (Fig. 7A), namely, T at −5 (C/G hybrid) to G (Hex), and C at +3 (C/G hybrid) to A (Hex), if we count the positions of the ACGT core sequence as A (−1), C (−0), G (+0) and T (+1) (see asterisks in Fig. 7A). The −5 and/or +3 positions thus may be responsible for the discriminating DNA recognition of the LIP19/OsOBF1 heterodimer and the OsOBF1 homodimer.

OsOBF1 and lip19 expression in rice seedlings show inverse low-temperature responses but their localization sites in leaf blades overlap

The effect of low temperature on lip19 and OsOBF1 expression in rice plants was investigated, initially by Northern blot hybridization. As reported, exposure to low temperatures induced an elevation in lip19 transcript amounts that were maintained at high levels. In contrast, OsOBF1 transcripts were abundant in plants grown at normal temperatures, e.g. 25°C, but at 5°C their levels gradually decreased over time (Fig. 8A). Thus, in the temperature range of 5°C to 25°C, the expression of the lip19 and OsOBF1 genes correlated inversely. Next, we performed in situ hybridization to examine the spatial expression patterns of lip19 and OsOBF1. In leaf blades of rice plants exposed to 5°C for 24 h, lip19 transcripts were abundantly and primarily detected in mesophyll cells surrounding vascular bundles; expression of OsOBF1 was sparse (Fig. 8B a, c). In
25°C-grown rice leaf blades, however, lip19 transcript levels were low while OsOBF1 transcripts were abundant in the mesophyll cells surrounding vascular bundles (Fig. 8B b, d). Thus, the lip19 and OsOBF1 transcripts localize in almost identical places within rice leaf blades.

Discussion

Failure of LIP19 to bind to DNA is due to its inability to homodimerize

The lip19 gene was isolated as a low temperature-induced gene from rice (Aguan et al. 1991, Aguan et al. 1993). Its product, LIP19, is a 148 amino acid bZIP protein, but it does not seem to have DNA-binding ability (Kusano et al. 1995, unpublished data). The basic region of LIP19 is identical to that of its maize counterpart protein, mLIP15, which binds to the G-box and Hex sequences (Kusano et al. 1995). This suggests two possible reasons why LIP19 is unable to bind to DNA. First, the amino-distal peptide outside the basic region of LIP19 somehow inhibits its DNA binding. Second, the structure of LIP19 blocks dimer formation. In this study we characterized the LIP19 protein and found that this bZIP has similarities to Fos and A. thaliana GBF4 (Menkens and Cashmore 1994, Ziff 1990) in that it is unable to homodimerize and bind DNA. A ‘domain-swap’ experiment (Fig. 4) then showed that the second of the two possibilities noted above is true, since it indicated that the leucine-zipper portion of LIP19 is responsible for its inability to dimerize. O’Shea et al. (1989) have shown that the residues in the e and g positions within the leucine zipper, which lie adjacent to the hydrophobic interface provided by the heptad Leu repeats, are critical for the dimer formation of bZIP proteins (Fig. 4E). In the case of Fos or the A. thaliana GBF4 protein, seven or six of the ten residues are occupied by Glu, respectively; thus, basic residues are not present within those positions (Menkens and Cashmore 1994, Ziff 1990). In addition, it was demonstrated that Arabidopsis GBF4 mutated at a single amino acid of the leucine zipper (Glu223Arg) was able to dimerize and bind DNA. In LIP19, six acidic (five Glu and one Asp) and no basic residues are present in the above positions, whereas in OsOBF1, four acidic (three Glu and one Asp) and one basic (Arg) residues are present. Since the hydrophobic interface of the leucine-zipper region of LIP19 is highly negatively charged, it is likely that a strong electrical repulsion exists between two LIP19 molecules, thus preventing LIP19/ LIP19 homodimer formation.

OsOBF1 favors LIP19 as a partner

Through yeast two-hybrid screening, we identified the OsOBF1 protein as a LIP19-interacting protein. Unlike LIP19, OsOBF1 can homodimerize and bind to the Hex sequence. While its maize counterpart protein, OBF1, was originally isolated as an OCS sequence-Binding Factor by a Southwestern method (Singh et al. 1990), the rice homologue OsOBF1 cannot bind to the OCS sequence. OBF and OsOBF1 share 82% identity and their basic regions are almost identical, so the reason behind the disparate affinities of the two proteins for the OCS sequence remains unclear.

In A. thaliana, there are 75 members of the bZIP protein family; these are divided into ten subgroups on the basis of the length of the peptides, common motifs, and the position of the bZIP domain, among other criteria (Jakoby et al. 2002). In this categorization, LIP19 and OsOBF1 both belong to group S. The group S proteins tend to heterodimerize within the subgroup or with group C bZIP proteins (Jakoby et al. 2002, Wiese et al. 2004). Examples of the former type are A. majus 910 and 911 (Martínez-Garcia et al. 1998) and maize mLIP15 and OBF1 (Kusano et al. 1995). Examples of the latter type have been reported in parsley, where CPRF6 heterodimerizes with CPRF2 (Rugner et al. 2001), in barley, where BLZ1 and BLZ2 heterodimerize (Onate et al. 1999), in maize, where OHP1 interacts with opaque2 (Pysh et al. 1993), and in tobacco, where BZI-1 forms dimers with BZI-2, BZI-3 (TBZF) and BZI-4 (Strathmann et al. 2001). We found that OsOBF1 interacts with LIP19 more strongly than with itself. In general, the heterodimerization of bZIP proteins alters their DNA-binding specificity (Hai and Curran 1991). For example, in A. majus, while both the 910 and 911 homodimers preferred a C-box/G-box hybrid, the 910/911 heterodimer showed a relaxed specificity, although it still bound the hybrid motif (Martínez-Garcia et al. 1998). We found this was also true for the LIP19/ OsOBF1 heterodimer, as it prefers the C/G hybrid sequence but does not bind the Hex sequence that is recognized by the OsOBF1 homodimer. In contrast, the OsOBF1 homodimer does not bind to the C/G hybrid sequence. Thus, after dimerizing with OsOBF1, LIP19 gains a distinct DNA-binding ability, similar to the TFs Fox/Jun and ATF/CREB, whose heterodimerization alters their DNA-binding specificity (Hai and Curran 1991). On the above evidence, we conclude that LIP19 is a Fos-like bZIP-type TF functioning in response to cold stress.

Possible role of the interaction between LIP19 and OsOBF1 in a cold signaling pathway in rice

Both LIP19 and OsOBF1 did not show transactivation activity in yeast cells (data not shown). A preliminary assay, however, showed that both LIP19 and OsOBF1 had transactivation activity in plant cells, even though the latter had weaker activity compared to the former (data not shown). We are currently investigating whether the LIP19/OsOBF1 heterodimer has transactivation activity in plant cells.

The expression of the lip19 and OsOBF1 genes is controlled in an inverse manner at temperatures ranging from 25°C to low non-freezing temperatures (Fig. 8A). However, the two genes appear to be expressed in the same location in rice plants (Fig. 8B). On the basis of all these data, the following model was proposed to elucidate the role LIP19 plays in temperature-signaling pathways. In this model, at the normal growth temperature of 25°C, the OsOBF1 gene is actively transcribed and its mRNA probably efficiently translated, whereas lip19 is
poorly expressed. The OsOBF1 protein binds to the Hex sequence and may activate the expression of target genes. At 5°C, in contrast, the lip19 gene is significantly up-regulated, whereas the OsOBF1 gene is down-regulated. While the product of the lip19 gene, LIP19, is unable to homodimerize, this bZIP protein heterodimerizes with the remaining OsOBF1 protein. The interaction between OsOBF1 and LIP19 seems to be stronger than that of OsOBF1 homodimerization. As a result, upon decreasing temperature, the normal-temperature-signaling (in which OsOBF1 is involved) is attenuated at the transcriptional level of OsOBF1; this signaling is also blocked at the OsOBF1 protein level since the presence of LIP19 dissociates the OsOBF1 homodimer, resulting in the formation of the LIP19/OsOBF1 heterodimer. The heterodimer, which recognizes the C/G hybrid promoter sequence, unlike the Hex-recognizing OsOBF1 homodimer, then possibly switches on the expression of unidentified target genes bearing the C/G hybrid sequence in their promoters. Once the temperature returns to 25°C, the instability of LIP19 may switch off this low-temperature signaling. Analysis at the LIP19 and OsOBF1 protein level has to be performed in planta to test this model and to determine the stability of the pLIP19 and OsOBF1 proteins in rice plants. Moreover, it is important to identify the gene(s) targeted by the OsOBF1 homodimer and the LIP19/OsOBF1 heterodimer. To address these issues, we are currently using an RNA-interference method (Miki and Shimamoto 2004) to generate transgenic rice plants in which the lip19 gene is not induced by low temperatures.

Materials and Methods

Plant materials and low-temperature treatment

Rice plants (Oryza sativa L. cv. Yukihikari and Akitakomachi) were used in this study. They were germinated on Murashige and Skoog agar medium, incubated for 7 days, then transplanted into soil. Low-temperature treatment, rice seedlings were initially grown hydroponically in Murashige and Skoog liquid medium in a growth cabinet set at 25°C with a 16 h light/8 h dark photocycle. To minimize the light effect, plants were kept under continuous dim light 48 h prior to the temperature shift to 5°C.

Construction of GFP-fusion plasmids and observation by fluorescence microscopy

The ORFs of lip19 and OsOBF1 were amplified by PCR with the appropriate primer pairs: lip19 forward-1 5'-CTTCTCGAGATGTGC-GTCGCTTCGCG-3' and lip19 reverse-1 5'-ATGGTACC-CTCTCGAG-3' and lip19 reverse-2 5'-AACCTGAAGTCG-ACGTGAAGGCTCGG-3' and lip19 reverse-2 5'-TTGAGATTGCT- GTGTCTCGTTCGTCG-3' and OsOBF1 reverse-2 5'-GCCGACTGACTGTGGACATGTGC-3'. The underlined sequences were introduced for cloning purposes. The plasmids carrying the correct inserts were digested with EcoRI and Xhol or SalI, and the lip19 and OsOBF1 fragments were subcloned into the yeast vector pBD-GAL4 Cam phagemid vector (Stratagene, La Jolla, CA, USA), resulting in pBD-LIP19 and pBD-OsOBF1. The constructs were introduced into S. cerevisiae strain YRG2 (Stratagene) or Y190 (BD Biosciences Clontech, Tokyo, Japan). The transformants were tested for the transcriptional activation of the two reporter HIS3 and LacZ genes. Yeast transformation, histidine autotrophic growth assays, and β-galactosidase activity assays were carried out according to the manufacturer's instructions.

Assay of protein–protein interaction in yeast cells

The interaction between two proteins was assayed using a yeast GAL4 two-hybrid system (Stratagene). To construct the necessary expression plasmids, the lip19 and OsOBF1 ORFs sandwiched between EcoRI and Xhol or EcoRI and SalI sites, respectively, were inserted into the respective sites of the PAD-GAL4-2.1 phagemid vector (Stratagene), resulting in pAD-LIP19 and pAD-OsOBF1. Combinations of the pBD- and pAD-constructs (see text) were introduced into the yeast cells. The HIS3 reporter assay and the filter assay for detecting β-galactosidase activity were carried out according to the manufacturer’s instructions.

Screening of the clone(s) encoding a LIP19-interacting protein

Seedlings of O. sativa cv. Yukihikari were exposed to a low temperature (5°C) for 6 h and 12 h and then harvested. Total RNA was extracted by the hot phenol-SDS method (Shirzadegan et al. 1991) and the mRNAs were purified with an mRNA purification kit (Amersham Biosciences). These were converted into cDNAs. The double-stranded cDNAs were then cloned into the HybriZAP-2.1 vector (Stratagene) and transformed into E. coli XL-1 Blue MRF. The E. coli cells were subjected to a mass excision to obtain a phagemid cDNA library. S. cerevisiae YRG2 cells carrying pBD-LIP19 as a bait construct were transformed with the cDNA library. The clones that were positive in both the HIS3 and lacZ assays were selected.

DNA sequencing, amino acid sequence alignment and phylogenetic analysis

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method. cDNA sequences were edited by the SDC-GENETYX genetic information program (Software Development Co., Tokyo, Japan). Amino acid sequences were aligned by using the Clustal W program, and the phylogenetic tree was constructed by using the TREEVIEW program (Page 1996).

Genomic DNA blot hybridization

Rice genome DNA was extracted from seedlings of O. sativa cv. Akitakomachi by a CTAB method (Dellaporta et al. 1983). Ten µg each of the genome DNA were completely digested with BamHI, EcoRI or HindIII and loaded onto a 0.8% agarose gel for electro-
phoresis, then blotted onto a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ, USA). The blot was hybridized with the corresponding 3′-UTR region of lip19 or OsOBF1 cDNA, and washed once with 2× SSC/0.1% SDS for 20 min at room temperature, followed by a single wash with 0.1× SSC/0.1% SDS for 10 min at 65°C before autoradiography.

**Northern blot hybridization**

Total RNA was isolated according to the method (SDS-phenol) of Nagy et al. (1988). Aliquots (15 µg each) were size-separated by electrophoresis in a formaldehyde-1.2% (w/v) agarose gel and blotted onto Hybond-N+ membrane (Amersham Biosciences) in 20× SSC. Hybridization was carried out as described (Berberich et al. 1999) using the 32P-labeled 3′-UTRs of the lip19 and OsOBF1 cDNAs.

**Construction of GST fusion-plasmids and production of the fusion proteins in E. coli**

The lip19 and OsOBF1 ORF′s sandwiched between the EcoRI and XhoI or EcoRI and SalI sites, respectively, were subcloned into the corresponding sites of the pGEX-4T-1 vector (Amersham Biosciences) and transformed into E. coli BL21 or BL21(DE3)pLysS. The transformants were cultured and isopropyl-thio-galactoside (IPTG) was added to induce protein production. The harvested cells were resuspended in an extraction buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM diethiothreitol (DTT), 20% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM pepstatin), sonicated on ice, and then cell debris were removed by centrifugation. The clear lysate containing the Lip19 derivative was used immediately while the preparation containing the OsOBF1 derivative was stored at −30°C until use.

**Electrophoretic Mobility Shift Assay (EMSA)**

An EMSA was performed as described (Kusano et al. 1995) using the following double-stranded DNA probes: the OCS element (OCS, 5′-TGAGTAACGCTTACGTTA-3′) (Singh et al. 1990), the hexamer (Hex, 5′-TCGCGGCACTCAACAAATCGG-3′×3) (Nakayama et al. 1992) and the C/G-hybrid box (C/G-hybrid, 5′-TCTGCGGCACTCCCAATCGG-3′×3) (Yang et al. 2001). The ACGT core sequence is underlined in the above probes.

**Domain-swap constructs between LIP19 and OsOBF1**

The ‘domain-swap’ constructs between LIP19 and OsOBF1 were generated by recombinant PCR. To make OsOBF1′BR (basic region)/OsOBF1′C (C terminal) or vice versa, the proteins in E. coli BL21 or BL21(DE3)pLysS. The transformants were cultured and isopropyl-thio-galactoside (IPTG) was added to induce protein production. The harvested cells were resuspended in an extraction buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM diethiothreitol (DTT), 20% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM pepstatin), sonicated on ice, and then cell debris were removed by centrifugation. The clear lysate containing the Lip19 derivative was used immediately while the preparation containing the OsOBF1 derivative was stored at −30°C until use.

**Quantitative assay of protein–protein interaction in yeast cells**

Co-transformants of yeast bearing a combination of the pBD and pAD constructs described above were assayed for β-galactosidase activity to quantitate the strength of the physical interaction between the proteins. β-Galactosidase assays were carried out according to the manufacturer’s instructions, and the relative β-galactosidase units were given as a mean value of three independent experiments. The pBD and pAD domain-swapped constructs, in which the basic region of LIP19 was followed by the leucine zipper region of OsOBF1 or vice versa, were also introduced into yeast cells and the relative strength of the interaction was quantified.

**In vitro pull down assay**

LIP19 and OsOBF1 as GST fusion proteins were individually expressed in E. coli. Approximately 1 µg of the GST-LIP19- or GST-OsOBF1 fusion protein or GST alone as the control were adsorbed to Glutathione Sepharose 4B (Amersham Biosciences) and then used to pull down [35S]-methionine-labeled in vitro translated-(T174)nEYFP-N1 and pSAT1-c(175-end)EYFP-N1] were kindly provided by Dr. T. Tzfira. Their transfection into onion bulbs was performed by a procedure described by Ishiyama et al. (1998). The amount of protein present in each input lane equaled one third of the total protein amount used in each assay and the amount of GST fusion protein loaded in each assay was checked by Coomassie blue staining. The binding complex was analyzed by SDS-PAGE and the radioactivity was detected by fluorography. All assays were repeated twice.

**Visualization of in vivo protein–protein interactions using BiFC**

In our BiFC analysis (Hu et al. 2002, Tzfira et al. 2004), the lip19 and OsOBF1 ORF′s sandwiched between the EcoRI and KpnI or EcoRI and SalI sites, respectively, were amplified by PCR with the following primer pairs: lip19 forward-2 and lip19 reverse-3 (5′-GGGCTGCGGAAGATGCGCTG-3′) on OsOBF1 forward and lip19 reverse-3 (5′-GGGCTGACATGACGGACGG-3′) to quantitate the strength of the physical interaction between the proteins. β-Galactosidase assays were carried out according to the manufacturer’s instructions, and the relative β-galactosidase units were given as a mean value of three independent experiments. The pBD and pAD domain-swapped constructs, in which the basic region of LIP19 was followed by the leucine zipper region of OsOBF1 or vice versa, were also introduced into yeast cells and the relative strength of the interaction was quantified.

In situ hybridization

Rice plants growing in a growth chamber at 25°C for 1 month were exposed to low temperatures. Leaf blades from cold (5°C)-stressed and control (25°C) plants were cut into approximately 1 cm lengths, fixed in 0.25% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde in 0.05M sodium-sulfate buffer at 4°C for 5 h, then dehydrated by a series of mixed butanol/ethanol solutions and embedded in paraffin as described previously (Jackson 1991). The embedded leaf samples were sectioned into 10 µm thicknesses, deparaffinized with xylene, and rehydrated as described (Jackson 1991). Hybridization was performed by a procedure described by Ishiyama et al. (1998). The hybridization probes were prepared as follows. The 3′-UTRs of lip19 and OsOBF1 were amplified by PCR with the following respective primer pairs: lip19, 5′-TGAGTCACCCAATGGAGCGG-3′ and 5′-TGCT-GCAAAACATTATAGC-3′; OsOBF1, 5′-TCCAGTACTGAACGG-CAGG-3′ and 5′-ATGTGACATCACAAGAGG-3′. The PCR products were cloned into the pGEM-T Easy vector (Promega) and the recombinants were transformed into E. coli. After confirming the
DNA sequence and the orientation to the vector, we synthesized the digoxigenin (DIG)-labeled sense and antisense probes using a DIG RNA labeling kit (Roche Diagnostics, Co., Ltd., Tokyo, Japan).

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References


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