Members of TALE and WUS subfamilies of homeodomain proteins with potentially important functions in development form dimers within each subfamily in rice

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Transacting factors often form homo- and heterodimers and regulate various targets, the type of regulation depending on the dimeric combination. The WUS and TALE subfamilies are two atypical homeodomains in plants. A homeodomain mediates sequence-specific binding to its target DNA and usually consists of 60 amino acid residues, whereas atypical homeodomains have extra amino acid residues in the well-conserved region. The genes \(\text{OsWUS}\) and \(\text{OsPRS}\), which encode atypical homeodomain proteins from the WUS subfamily, and \(\text{OsBEL}\) and \(\text{OSH15}\), which encode those from the TALE subfamily, were isolated from rice and tested for their interactions by yeast two-hybrid analysis. \(\text{OsWUS}\) and \(\text{OsPRS}\) formed homodimers and formed heterodimers with each other but did not form dimers with the TALE family homeodomain proteins \(\text{OSH15}\) or \(\text{OsBEL}\). Likewise, \(\text{OSH15}\) and \(\text{OsBEL}\) formed homodimers and heterodimers but did not form dimers with the WUS family homeodomain proteins \(\text{OsWUS}\) and \(\text{OsPRS}\). These findings suggest that the combinations of dimers are well correlated with the classification of these proteins on the basis of sequence similarity. RT-PCR analysis revealed that expression of \(\text{OsWUS}\) and \(\text{OsPRS}\) was detected in the same organs, namely floral buds, roots, and suspension cells. Therefore, it is possible that the proteins encoded by both of these genes function as homo- and heterodimers \textit{in planta}. These results suggest that, during the evolution of these subfamilies, various combinations of dimers within proteins encoded by paralogous genes were formed and generated independent regulatory networks that enabled complex patterns of plant development.

\textbf{Key words:} heterodimer, homeodomain, homodimer, rice, yeast two-hybrid analysis

\textbf{INTRODUCTION}

Homeodomain (HD) proteins are key regulators that determine cell differentiation and cell fate in both plants and animals. The HD protein is a transcriptional regulator with a DNA-binding motif called a homeodomain. The typical structure of an HD consists of 60 highly conserved amino acids that encode three a helices connected by a loop and turn. In higher plants, \textit{KNOTTED1-like} homeobox genes have been isolated from various plant species, and they constitute a gene family called KNOX (Reiser et al., 2000). KNOX proteins have atypical HDs of 63 amino acids. These types of atypical HDs are designated TALE (three amino-acid loop extension) HD proteins because their unique feature of three extra amino acids stretches between the first and second helices. TALE atypical HDs not only exist in plants; they are also widespread in other organisms, including animals, and constitute a large gene family (Bürglin, 1997). Recently, it has been reported that, in barley, rice, \textit{Arabidopsis}, and maize, KNOX proteins can interact with proteins in the KNOX family and/or \textit{BELL1}-like HD proteins, which belong to another subclass of TALE HD proteins (Muller...
et al., 2001; Nagasaki et al., 2001; Bellaoui et al., 2001; Smith et al., 2002; Smith and Hake, 2003). KNOX protein interactions are mediated by the MEINOX domain, which is conserved among plants and animals (Bürglin, 1998). In tobacco and rice transgenic plants that over-produce KNOX proteins, the MEINOX domain is essential to induce abnormal leaf morphologies (Sakamoto et al., 1999; Nagasaki et al., 2001). These observations indicate that the interactions of KNOX proteins are important for their functions. In fact, some of the KNOX proteins in Arabidopsis, such as SHOOT MERISTEM-LESS (STM) and KNAT1, interact with BELL1 to activate the transcription of reporter genes (Bellaoui et al., 2001). Furthermore, KNOTTED1 (KN1) in maize also interacts with KNOTTED1-interacting protein (KIP), which is a BELL1-like protein, to achieve high DNA-binding affinity (Smith et al., 2002). Thus, dimer formations of KNOX proteins are necessary for transcriptional regulation.

WUSCHEL (WUS), PRESSED FLOWER (PRS), and WUSCHEL-related homeobox (WOX) in Arabidopsis and PhWUS in Petunia are another class of atypical HD proteins (Haecker et al., 2004). The HDs of WUS and PhWUS proteins consist of 66 amino acids, with two extra residues between helix 1 and helix 2 and four between helix 2 and helix 3, whereas the HDs of PRS and WOX proteins consist of 65 amino acids with one extra residue between helix 1 and helix 2 and four between helix 2 and helix 3. So far, it is not known whether the proteins in this subfamily form homo- or heterodimers. To investigate this possibility, we isolated a WUS ortholog (OsWUS) and its related gene, OsPRS, which encodes an HD similar to WUS but is closest to PRS protein in Arabidopsis (Matsumoto and Okada, 2001), from rice. We used the yeast two-hybrid method to test the interactions of the HD proteins encoded by these genes with rice TALE HD proteins such as OSH15 and the rice hybridization probed with the respective cDNA fragments. The primers used for amplifications were OsWUSF3 (5’-atggcagccgaaaacctt-3’) and OsWUSR2 (5’-agaaacccegctggaaagttt-3’); OsPRSF2 (5’-tgcggcgtcctggc-3’) and OsPRSR (5’-aggagtttaagttttgagaaggttga-3’); and OsAct-U (5’-tcgctctagttcagc-3’) and OsAct-L (5’-gtacccgctgcttcgag-3’).

**Yeast two-hybrid analysis** We used the Matchmaker yeast two-hybrid system (Clontech, Palo Alto, CA). Selection for transformants with bait and prey plasmids was performed on plates lacking tryptophan and leucine. The positive clones were used in quantitative liquid β-galactosidase (LacZ) assays in accordance with the manufacturer’s instructions.

**Construction of bait and prey plasmids** Entire cDNA fragments of OsWUS, OsPRS, and OsBEL were excised with restriction enzymes and inserted into pACT2 and pGBT9 (Clontech, Palo Alto, CA). To create OsWUSAN and OsWUSAC, EcoRV and SmaI, and SalI fragments of entire OsWUS cDNA were inserted into pACT2 and pGBT9, respectively. For truncated OsPRS proteins, parts of OsPRS were amplified by PCR to introduce an SmaI site at the 5' end and a BamHI site at the 3' end of the open reading frame. These fragments were subsequently digested with SmaI and BamHI and inserted into pACT2 and pGBT9. The primers used to create truncated proteins of OsPRS were Qhyb2 (5’-cccccgggaagccggcagagatctcagc-3’) and OsPRSR (5’-aagagctctatttggaggtggta-3’) for OsPRSA1–30, Qhyb4 (5’-cccccgggctacggcagctggc-3’) and OsPRSR (5’-aagagctctatttggaggtggta-3’) for OsPRSA1–84, OsPRSF (5’-cccccgggctacggcagctggc-3’) and Qhyb5 (5’-cccccgggctacggcagctggc-3’) for OsPRSA85–203, and OsPRSF (5’-cccccgggctacggcagctggc-3’) and Qhyb5 (5’-cccccgggctacggcagctggc-3’) for OsPRSA169–203.

**RESULTS** By searching the rice genome databases we found six homologs of the WUS and WOX genes. OsWUS has already been reported as a WUS ortholog on the basis of
sequence similarity, and we designated the homologs with the greatest similarity to PRS, which is one of the WOX genes identified from a mutation called pressed flower in Arabidopsis, as OsPRS (Haecker et al., 2004; Matsumoto and Okada, 2001). We also found a BELL1 homolog and designated it OsBEL. The cDNAs of these genes were amplified from seedling and inflorescence RNA by RT-PCR. The alignments of the OsWUS, OsPRS, and OsBEL proteins with the corresponding homologs are shown in Figs. 1A and B.

To investigate the evolutionary relationship between these atypical HDs, we used the amino acid sequences of the HDs to create a neighbor-joining tree. The atypical HDs in plants could be divided into two groups (Fig. 2A): the TALE family and the WUS/WOX family. Figure 2B shows the alignments of the amino acid sequences of var-
ious plant HDs, including OsWUS, OsPRS, and OsBEL. Although the number of extra amino acids residues residing between helix 1 and helix 2 was different between OsWUS and OsPRS, it was clear that their HDs were evolutionarily related (Figs. 2A and B).

Using yeast two-hybrid analysis, we investigated the interactions among OsWUS, OsPRS, OsBEL, and one of the rice KNOX proteins, OSH15 (Fig. 3). cDNAs for each protein were fused translationally to a GAL4 DNA binding domain (GDBD) or GAL4 activation domain (GAD).

We verified that none of the bait constructs activated the reporter gene when introduced with a prey construct with only a GAD (Fig. 3, lanes 1, 6, 11, and 16). When OSH15 was used as both bait and prey, high LacZ activity was observed (Fig. 3, lane 2). This is consistent with our previous finding that OSH15 forms a homodimer, and/or a heterodimer with other KNOX proteins (Nagasaki et al., 2001). High LacZ activity was also observed when OsBEL was used as both bait and prey (Fig. 3, lane 8), and when OSH15 and OsBEL were used as bait and prey together (Fig. 3, lanes 3 and 7). These results suggest that OSH15 and OsBEL form homodimers and/or form heterodimers together. On the other hand, when OsWUS or OsPRS was used as prey with the combinations of OSH15 or OsBEL as bait, LacZ activity was at the background level (Fig. 3, lanes 4, 5, 9, and 10). The same results were obtained when bait and prey constructs were switched (Fig. 3, lanes 12, 13, 17, and 18). These results suggest that neither OsWUS nor OsPRS interacts with OSH15 or OsBEL. Next, we tested homo- and/or heterodimer formation by OsWUS and OsPRS; high LacZ activity was observed (Fig. 3, lanes 14, 15, 19, and 20). These results suggest that OsWUS and OsPRS form homodimers and/or form heterodimers together.

To determine the domains required for homo- and/or heterodimer formation of OsWUS or OsPRS, we created several deletion constructs and subjected them to yeast two-hybrid analysis (Fig. 4A). OsWUSΔN had a deletion of the N terminal side including the HD, and OsWUSΔC was a truncated form of OsWUS that did not contain conserved motifs such as the WUS box and SLELXL. When the intact OsWUS was tested for interaction with
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OsWUSΔN, high LacZ activity was observed (Fig. 4B, lanes 3 and 5). Next, OsWUSΔN was used as both bait and prey, and LacZ activity was still observed (Fig. 4B, lane 6). The increase in LacZ activity observed when OsWUSΔN was used as prey may be due to the repressive effect of the N terminal portion of OsWUS on GAD (compare Fig. 4B, lane 2 with 3 and 5 with 6). On the other hand, when OsWUSΔC was used as either prey or bait, LacZ activity was notably decreased (Fig. 4B, lanes 4, 7, and 8). These observations suggest that homodimer formation by OsWUS does not require the N terminal side but does require the C terminal side.

We then investigated the domain required for homodimer formation by OsPRS. OsPRS Δ1–30 has a deletion of the N terminal end to helix 1 of the HD. OsPRSΔ1–84 lacks the entire HD from the N terminal. OsPRSΔ169–203 lacks the C terminal end, including the conserved WUS box, and OsPRSΔ85–203 is a truncated form of OsPRS that contains the HD and the N terminal side of the HD. When interaction of OsPRS and OsPRSΔ1–30 was tested, LacZ activity was observed at levels similar to those with homodimer formation of intact OsPRS (Fig. 4C, lanes 2 and 3). Next, OsPRSΔ1–84 was used as prey, and LacZ activity decreased to the background level (Fig. 4C, lane 4). When OsPRSΔ169–203 was used as prey, LacZ activity was observed at half the level found with homodimer formation by OsPRS (Fig. 4C, lane 5). When OsPRSΔ85–203 was used as prey, LacZ activity decreased to the background level (Fig. 4C, lane 6). In summary, formation of homodimers of OsPRS requires the regions between amino acid residues 31 and 168.

Finally, we analyzed the domains required for heterodimer formation by OsWUS and OsPRS. When we tested the interaction of OsWUS and OsPRSΔ1–30, LacZ activity was observed (Fig. 4D, lane 3). When OsPRSΔ1–84 was used as prey, LacZ activity was decreased to the background level (Fig. 4D, lane 4). Deletion of the C-terminal region of OsPRS did not affect the interaction between OsWUS and OsPRS, because high LacZ activity was observed with OsPRSΔ169–203 (Fig. 4D, lane 5). Interestingly, OsPRSΔ85–203 still gave LacZ activity in interacting with OsWUS (Fig. 4D, lane 6). In summary, heterodimer formation between OsWUS and OsPRS requires the region from helix 2 to helix 3 of the OsPRS HD. To determine the domain of OsWUS required for heterodimer formation with OsPRS, we tested the interactions of OsWUSΔN or OsWUSΔC with OsPRS. LacZ activity was observed when OsWUSΔN was used as bait but not when OsWUSΔC was used (Fig. 4D, lanes 7 and 8). This finding suggests that the heterodimer of OsWUS and OsPRS requires the C-terminal region of OsWUS.
In the yeast two-hybrid analysis, OsWUS and OsPRS formed homodimers or heterodimers with each other or both. If heterodimer formation between OsWUS and OsPRS occurs in vivo, we can expect the expression of these genes to overlap. To verify this hypothesis, we used RT-PCR to investigate the organ-specific expression of these genes (Fig. 5). Expression of both genes was observed in the floral buds, roots, and suspension cells, although OsPRS also was expressed in some other organs. In light of the fact that the expression of both genes overlapped, it is plausible that OsWUS and OsPRS form heterodimers in vivo.

**DISCUSSION**

Dimer formation by transcriptional regulators enables precise control of gene expression by various combinations. We showed that OsWUS and OsPRS form homodimers and heterodimers with each other (Fig. 3). The results of RT-PCR suggest that both OsWUS and OsPRS are expressed in the same organs (Fig. 5), and it is possible that they interact in planta.

OsWUS has the SLELXL sequence motif, which is conserved in WUS and PhWUS but not in WOX proteins including OsPRS, at the C-terminal end. This motif is similar to repressor sequences such as the EAR motif (L/FDLNLF/F, Ohta et al., 2001) or the EAR-motif-like sequence (LDLE/SL, Hiratsu et al., 2002). On the other hand, OsPRS has a homopolymeric repeat of glutamate residues, unlike OsWUS. The homopolymeric repeat of glutamate residues or glutamate-rich regions functions as an activation domain in mammalian cells (Gerber et al., 1994). Therefore, the WUS family proteins may have different types of transcriptional regulation, depending on how they are combined.

The HD region between helix 2 and helix 3 of OsPRS functions as a domain of interaction with OsWUS. Interestingly, the amino acid sequence of helix 3 of OsPRS is exactly the same as that of OsWUS. However, OsWUS does not form a homodimer through its own homeodomain. This finding suggests that helix 2 and the adjacent turn of OsPRS may give specificity to the heterodimer between OsWUS and OsPRS.

We showed here that OsWUS and OsPRS interact with each other but not with OsBEL or OSH15. OSH15 belongs to the TALE subfamily, and OsWUS and OsPRS belong to the WUS subfamilies of atypical HD proteins. These results indicate that dimer formation by these proteins occurs with proteins in the same subfamily. On the basis of these observations, we propose the following model for the evolution of these genes and their functions in development. There was a common ancestor of the TALE and WUS proteins, as indicated in the phylogenetic analysis, and it was able to form homodimers. After the divergence of the TALE and WUS subfamilies, the genes in each family were duplicated to produce paralogs. Then, various combinations of dimers within proteins from paralogous genes were formed and generated independent regulatory networks that enabled a complexed pattern of development. Both STM and WUS are required for the function of SAM (shoot apical meristem), but their actions are independent (Lenhard et al., 2002). Our result that OSH15 and OsWUS do not interact is consistent with this report. Various combinations of dimers within the TALE and WUS subfamilies may regulate the function of SAM through independent pathways.

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