Interactions of Two Transcriptional Repressors and Two Transcriptional Activators in Modulating Gibberellin Signaling in Aleurone Cells

Xiaolu Zou, Dawn Neuman, and Qingxi J. Shen*
School of Life Sciences, University of Nevada, Las Vegas, Nevada 89154

Gibberellins (GAs) regulate many aspects of plant development, such as germination, growth, and flowering. The barley (*Hordeum vulgare*) *Amy32b* α-amylase promoter contains at least five cis-acting elements that govern its GA-induced expression. Our previous studies indicate that a barley WRKY gene, *HvWRKY38*, and its rice (*Oryza sativa*) ortholog, *OsWRKY71*, block GA-induced expression of *Amy32b-GUS*. In this work, we investigated the functional and physical interactions of *HvWRKY38* with another repressor and two activators in barley. *HvWRKY38* blocks the inductive activities of SAD (a DOF protein) and HvGAMYB (a R2R3 MYB protein) when either of these proteins is present individually. However, SAD and HvGAMYB together overcome the inhibitory effect of *HvWRKY38*. Yet, the combination of *HvWRKY38* and BPBF (another DOF protein) almost diminishes the synergistic effect of SAD and HvGAMYB transcriptional activators. Electrophoretic mobility shift assays indicate that *HvWRKY38* blocks the GA-induced expression of *Amy32b* by interfering with the binding of HvGAMYB to the cis-acting elements in the α-amylase promoter. The physical interaction of *HvWRKY38* and BPBF repressors is demonstrated via bimolecular fluorescence complementation assays. These data suggest that the expression of *Amy32b* is modulated by protein complexes that contain either activators (e.g. HvGAMYB and SAD) or repressors (e.g. *HvWRKY38* and BPBF). The relative amounts of the repressor or activator complexes binding to the *Amy32b* promoter regulate its expression level in barley aleurone cells.

1 This work was supported by the U.S. Department of Agriculture (grant no. 2007–53304–18297 to Q.J.S.), the National Institutes of Health Biomedical Research Infrastructure Network (grant no. P20 RR–16464 to the state of Nevada), and the National Science Foundation (EPS–0132556 to X.Z.).

* Corresponding author; e-mail jeffery.shen@ccmail.nevada.edu.

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.123653
Ca^{2+} concentrations (Gilroy and Jones, 1992) follow GA treatment, although it is not entirely clear how they are linked to upstream and downstream events. Several types of cis-acting elements for the GA responses of high-pI and low-pI α-amylase genes have been defined (Skrivér et al., 1991; Gubler and Jacobsen, 1992; Lanahan et al., 1992; Rogers and Rodgers, 1992; Rogers et al., 1994; Tanida et al., 1994). These motifs interact with various transcription factors controlling seed germination. In the low-pI α-amylase promoter, Amy32b, five elements, namely, O2S/W-box, pyrimidine box, GA response element (GARE), amylase box (Amy), and downstream amylase element, are essential for the high level of GA-induced expression (Lanahan et al., 1992; Rogers and Rodgers, 1992; Rogers et al., 1994; Gómez-Cadenas et al., 2001). Each of these elements may be bound by one or more transcription factor(s) of R2R3 MYB, R1 MYB, DOF, and zinc finger protein families (Gubler et al., 1995; Raventós et al., 1998; Diaz et al., 2002; Isabel-LaMoneda et al., 2003; Washio, 2003; Mare et al., 2004; Peng et al., 2004; Zhang et al., 2004; Rubio-Somoza et al., 2006; Xie et al., 2006; Moreno-Risueno et al., 2007). However, it still remains unclear how these repressors and activators interact with each other in regulating gene expression.

WRKY proteins mediate plant responses to biotic and abiotic stresses as well as anthocyanin biosynthesis, senescence, and trichome development (Eulgem et al., 2000; Ulker and Somssich, 2004). Our previous studies indicate that a rice WRKY gene, OsWRKY71, repressed GA-induced expression of the barley Amy32b promoter in barley aleurone cells (Zhang et al., 2004). We further showed that OsWRKY71, by forming a complex with OsWRKY51, functionally interferes with OsGAMYB, a transcriptional activator of GA signaling, hence blocking the GA-induced expression of Amy32b-GUS in barley aleurone cells (Xie et al., 2006). We studied those rice WRKY genes in barley because of the technical difficulties of using rice aleurone cells, such as the high background of the GUS reporter driven by the rice GA-inducible promoters and, hence, the low level of induction (Zhang et al., 2004). Later, we showed, unsurprisingly, that the putative barley ortholog of OsWRKY71, HvWRKY38 (Mare et al., 2004), also represses the GA-induced expression of Amy32b-GUS (Xie et al., 2007). This role was confirmed in a dosage experiment in which the reporter construct (Amy32b-GUS) concentration was kept constant, whereas the effector construct (UBI-HvWRKY38) varied from 0% to 100% (Fig. 1). When the Amy32b-GUS construct was transformed alone, treatment with 1 μM GA induced the expression of this gene construct by 37-fold. The expression of Amy32b-GUS in response to GA was reduced to 7-fold in the presence of 10% of the relative amount of effector. When the relative amounts of effector were higher than 25%, GUS expression was reduced to 2-fold or less. These data indicate that the effect of HvWRKY38 on the repression of GA induction of the Amy32b α-amylase gene expression is dosage dependent, similar to what was reported for OsWRKY71 (Xie et al., 2006).

**RESULTS**

**HvWRKY38 Represses GA Induction of the Amy32b α-Amylase Promoter in Aleurone Cells**

HvWRKY38 is a member of the group II WRKY transcription factors, which contain only one WRKY domain (Mare et al., 2004). It is capable of binding to W-boxes/Box2 in the GA-responsive Amy32b promoter (Mare et al., 2004). We showed that HvWRKY38 suppressed the GA-induced expression of Amy32b-GUS (Xie et al., 2007). This role was confirmed in a dosage experiment in which the reporter construct (Amy32b-GUS) concentration was kept constant, whereas the effector construct (UBI-HvWRKY38) varied from 0% to 100% (Fig. 1). When the Amy32b-GUS construct was transformed alone, treatment with 1 μM GA induced the expression of this gene construct by 37-fold. The expression of Amy32b-GUS in response to GA was reduced to 7-fold in the presence of 10% of the relative amount of effector. When the relative amounts of effector were higher than 25%, GUS expression was reduced to 2-fold or less. These data indicate that the effect of HvWRKY38 on the repression of GA induction of the Amy32b α-amylase gene expression is dosage dependent, similar to what was reported for OsWRKY71 (Xie et al., 2006).

**Loss of HvWRKY38 Activity Leads to Increased Expression of Amy32b in the Absence of GA**

If HvWRKY38 indeed encodes a negative regulator of the GA pathway controlling the expression of Amy32b in aleurone cells, knocking down its expression by double-stranded RNA interference (RNAi) should enhance the expression of the Amy32b promoter in the absence of GA. To test this hypothesis, UBI-Null and UBI-HvW38(RNAi) constructs were prepared (Fig. 2A). The UBI-Null control contains the second intron (1,273 bp) of OsGAMYB, which is flanked by the UBI promoter and the NOS terminator (Zhang et al., 2004). The OsGAMYB intron functions as a loop in the RNAi construct. The UBI-HvW38(RNAi) construct contains two HvWRKY38 fragments in opposite orientations. The Amy32b-GUS reporter construct was cobombarded into aleurone cells along with the UBI-Null or the UBI-HvW38(RNAi) construct. As shown in Figure 2B, coexpression of UBI-Null had little effect on Amy32b-GUS expression at 24, 48, and 72 h after bombardment. In contrast, coexpression of UBI-HvW38(RNAi) led to a 3-fold increase in Amy32b-GUS expression without GA at the 72-h time point. The relative low induction might be due to the presence of WRKY genes with redundant functions, a phenomenon commonly observed for supergene families. Alternatively, the HvWRKY38 protein might be stable in...
the absence of GA, similar to what we observed for the OsWRKY71-GFP fusion (Zou et al., 2004).

Physical Interactions of HvWRKY38 and HvBPBF Proteins in the Nuclei of Aleurone Cells

To understand how HvWRKY38 modulates the expression level of Amy32b, we studied whether HvWRKY38 proteins physically interact with each other in aleurone cells. The HvWRKY38 coding sequences were fused in-frame with sequences encoding the N-terminal fragment of yellow fluorescent protein (YFP, YN) or the C-terminal fragment (YC), respectively. The complementation between different combinations of fusion proteins was tested by introducing the corresponding constructs into barley aleurone cells. Confocal microscopic studies showed that YFP fluorescence was detected in the entire cells bombarded with the UBI-YFP construct (Fig. 3B, I). Consistent with the nuclear localization data in Figure 3A, YFP fluorescence was only observed in the nuclei of the aleurone cells bombarded with UBI-YFP:HvWRKY38 (Fig. 3B, II). No fluorescence was detected when both YN and YC fusion constructs were introduced into aleurone cells (Fig. 3B, III). However, yellow fluorescence was detected in the nuclei of aleurone cells bombarded with UBI-YN:HvWRKY38.

The reporter construct

A Reporter construct

Amy32b promoter

GUS

Amy32b-T

Effector construct

UBI promoter

HvWRKY38

NOS-T

B

Figure 1. HvWRKY38 specifically represses GA induction of the Amy32b α-amylase promoter in aleurone cells. A, Schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. B, The effector construct, UBI-HvWRKY38, was co-bombarded into barley aleurone cells along with the reporter construct, Amy32b-GUS, and the internal control construct, UBI-Luciferase. The amount of reporter and internal control plasmid DNA was always constant, whereas that of the effector varied with respect to the reporter, as shown on the x axis. One hundred percent means that the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the same number of luciferase activity units (Lanahan et al., 1992). The lines indicate GUS activities ± se after 24 h of incubation of the bombarded aleurone cells with (+) or without (−) 1 μM GA. Data are means ± se of four replicates.

Figure 2. Loss of HvWRKY38 activity leads to increased expression of Amy32b in the absence of GA. A, Schematic diagrams of gene, reporter construct, and internal control construct, UBI-HvWRKY38 and UBI-Luciferase. The amount of effector and reporter plasmid DNA was always constant, whereas that of the reporter varied with respect to the effector, as shown on the x axis. Numbers below the effector construct represent the size (in base pairs) of each segment (not drawn to scale). B, The reporter construct, Amy32b-GUS, and the internal control construct, UBI-Luciferase, were co-bombarded into barley aleurone cells with the effector construct, UBI-Null or UBI-HvW38(RNAi), using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Lines indicate GUS activities ± se after 24, 48, or 72 h of incubation of the bombarded aleurone cells without GA. Data are means ± se of four replicates.

To examine the subcellular localization of the HvWRKY38 protein, the HvWRKY38 gene was fused in frame to the 3' end of a GFP gene that is driven by the constitutive maize (Zea mays) UBI promoter. The UBI-GFP control or the UBI-GFP:HvWRKY38 plasmid was introduced into barley aleurone cells by particle bombardment, and GFP fluorescence was visualized with confocal microscopy. In the control, GFP fluorescence was observed throughout the cells (Fig. 3A, I). In contrast, GFP:HvWRKY38 fusion proteins were localized exclusively in the nuclei (Fig. 3A, III), as confirmed by staining with the red fluorescent nucleic acid stain SYTO 17 (Fig. 3A, II and IV). These results suggest that HvWRKY38 is targeted to the nuclei of aleurone cells.

Figure 2. Loss of HvWRKY38 activity leads to increased expression of Amy32b in the absence of GA. A, Schematic diagrams of gene constructs. Arrowheads indicate the orientations of the gene fragments. Numbers below the effector constructs represent the size (in base pairs) of every segment (not drawn to scale). B, The reporter construct, Amy32b-GUS, and the internal control construct, UBI-Luciferase, were co-bombarded into barley aleurone cells with the effector construct, UBI-Null or UBI-HvW38(RNAi), using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Lines indicate GUS activities ± se after 24, 48, or 72 h of incubation of the bombarded aleurone cells without GA. Data are means ± se of four replicates.

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and UBI-YC:HvWRKY38 constructs (Fig. 3B, IV), suggesting that HvWRKY38 proteins interact in the nuclei of aleurone cells.

We further studied whether the WRKY domain of HvWRKY38 is essential for the interaction. The N-terminal and C-terminal regions of HvWRKY38 were fused in-frame with UBI-YN to produce UBI-YN:N-HvWRKY38 and UBI-YN:C-HvWRKY38, respectively. The N-terminal region includes the first 199 amino acid residues. The WRKY domain is present in the C-terminal domain, which is 154 amino acids in length. Yellow fluorescence was detected in the nuclei of aleurone cells bombarded with UBI-YN:N-HvWRKY38 and UBI-YN:C-HvWRKY38 (Fig. 3B, V). However, no fluorescence was detected when UBI-YN:C-HvWRKY38 and UBI-YN:HvWRKY38 (Fig. 3B, VI) were introduced into aleurone cells. These data suggest that the interaction domain is located in the N terminus.

A putative LZ motif from residues Leu-63 to Leu-91 was identified in this N-terminal fragment. To determine whether this putative LZ motif is involved in protein-protein interaction, a double mutant construct was created in which Leu-63 and Leu-77 were changed to Arg and His residues, respectively. The LZ motif mutation of HvWRKY38 was then fused in-frame with UBI-YN to produce UBI-YN:LZ-HvWRKY38. Interestingly, no fluorescence was detected when UBI-YN:LZ-HvWRKY38 and UBI-YN:HvWRKY38 were introduced into aleurone cells in the bimolecular fluorescence complementation (BiFC) experiment (Fig. 3B, VII). These data further suggest that the putative

Figure 3. Visualization of interactions between transcription factors of GA signaling in aleurone cells by BiFC. A, Barley half-seeds were bombarded with either UBI-GFP (I and II) or UBI-GFP:HvWRKY38 (III and IV). After incubation for 24 h, the aleurone cells were stained with SYTO17 to localize nuclei (II and IV), followed by examination of GFP fluorescence (I and III). Arrows point to the same cell. Bars = 20 μm. B, Barley aleurone cells were bombarded with UBI-YFP, UBI-YFP: HvWRKY38, or a combination of constructs encoding the indicated fusion proteins. YN is the fragment containing amino acid residues 1 to 154 of YFP; and YC is the fragment containing amino acid residues 155 to 238 of YFP. After incubation at 24°C for 24 h, yellow fluorescence was observed through a confocal microscope.
LZ domain is necessary for the interaction of HvWRKY38.

HvBPBF is also a negative regulator of the GA response in aleurone cells (Mena et al., 2002). We hypothesized that HvBPBF and HvWRKY38 physically interact in a repression complex, because they bind to cis-acting elements that are only 14 bp apart. To test this hypothesis, the HvWRKY38 and HvBPBF coding sequences were fused in-frame with sequences coding the YN or the YC, respectively. Yellow fluorescence was detected in the nuclei of aleurone cells bombardted with UBI-YN:HvWRKY38 and UBI-YC:HvBPBF (Fig. 3B, VIII), indicating that HvWRKY38 interacts with HvBPBF in the nuclei of aleurone cells.

HvWRKY38 Interferes with the Binding of HvGAMYB to the Amy32b Promoter

HvGAMYB is a positive regulator mediating the pathway between GA and α-amylase in aleurone cells (Gubler et al., 1995). We showed previously that OsWRKY71 (the putative rice ortholog of HvWRKY38) functionally interferes with the activity of OsGAMYB (Xie et al., 2006). HvWRKY38 behaved similarly to OsWRKY71 in its functional interaction with GAMYB (Supplemental Fig. S1). To understand the biochemical basis of these two regulators in modulating the expression level of Amy32b, we studied whether HvWRKY38 and HvGAMYB bind to the promoter of Amy32b simultaneously or compete in binding to the promoter. EMSAs were performed with glutathione-S-transferase (GST)-tagged HvWRKY38 and HvGAMYB. A DNA fragment containing the Amy32b promoter region from −171 to −111 was used as a probe (Fig. 4A). No binding signal was detected in reactions without protein (Fig. 4B, lane 1) or with GST only (lane 2). Both HvWRKY38 and HvGAMYB bound specifically to the Amy32b promoter (lanes 3 and 6), and excess amounts of unlabeled promoter fragment competed off the in vitro binding signal (lanes 4, 5, 7, and 8).

It has been demonstrated that the dimerization of HvWRKY38 is essential for high-affinity binding to the W-boxes in the Amy32b promoter (Mare et al., 2004); we also found that HvWRKY38 proteins physically interact in nuclei (Fig. 3B, IV). Here, we show that the GST-HvWRKY38 complex was shifted higher than that of GST-HvGAMYB, although the size of the GST-HvGAMYB protein (approximately 80 kD) is greater than that of the GST-HvWRKY38 protein (approximately 60 kD; Fig. 4B, compare lane 3 with lane 6).

These data suggest that GST-HvWRKY38 binds to DNA as a dimer while GST-HvGAMYB binds as a monomer. However, the mobility of the protein-DNA complex in the gel retardation assay depends on both the size and the charge of a protein. The pI values of HvWRKY38 and HvGAMYB were predicted to be 8.2 and 4.7, respectively, using the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/protein_iep.html). Therefore, it is possible that under the electrophoresis condition (pH 8), the HvGAMYB is more negatively charged and, hence, mobilized faster than HvWRKY38.

Interestingly, HvWRKY38 and HvGAMYB bind to two different cis-acting elements that are 24 bp apart. If both of the transcription factors could bind to the DNA simultaneously, one would see a third band corresponding to the protein-DNA complex that mobilized at a rate slower than those of the HvWRKY38-DNA and HvGAMYB-DNA complexes. However, this was not what we observed; when both HvWRKY38 and HvGAMYB were present in the reactions, HvGAMYB binding to the Amy32b promoter was inhibited (Fig. 4B, lane 9). As HvWRKY38 binding was competed off by an excess amount of the competitor 1, which contains two W-boxes (the WRKY binding site), HvGAMYB was able to bind to the promoter (lanes 10 and 11). As expected, competitor 2, which contains GARE only (the HvGAMYB binding site), did not compete with the binding of HvWRKY38 (lanes 12 and 13). When both competitors 1 and 2 were added, the binding signals of both proteins were decreased (lanes 14 and 15).

HvWRKY38 and HvGAMYB Functionally Compete with Each Other to Regulate the Expression of Amy32b

EMSA data suggested that binding of HvWRKY38 to the W-boxes prevented HvGAMYB from binding to the Amy32b promoter. Dosage experiments were performed to study the functional interactions of these two regulators. Varied amounts of UBI-HvWRKY38 (from 0% to 100% relative to the reporter construct) were introduced into aleurone cells along with a constant amount of UBI-HvGAMYB (100%) and Amy32b-GUS (100%). The HvGAMYB induction of Amy32b was gradually suppressed by increasing the amounts of UBI-HvWRKY38 (Supplemental Fig. S1B). It also showed that 10% of UBI-HvWRKY38 is sufficient to repress the HvGAMYB transactivating activity on the Amy32 promoter. We further performed a similar dosage experiment with a fixed amount of UBI-HvWRKY38 (10%) and Amy32b-GUS (100%) and varied amounts of UBI-HvGAMYB (from 0% to 200%). The GUS activity was gradually increased as the amount of UBI-HvGAMYB was augmented in this experiment (Supplemental Fig. S1C).

We then performed EMSA to investigate the biochemical basis of the dosage effect observed in the functional assays. Varied amounts of GST-HvWRKY38 (from 0% to 100% relative to GST-HvGAMYB) with a fixed amount of GST-HvGAMYB (100%) were used in the competition study. Increasing amounts of HvWRKY38 protein gradually decreased the binding of HvGAMYB to the Amy32b promoter (Fig. 5A, lanes 4–9). On the other hand, increasing amounts of HvGAMYB enhanced the binding signal of HvGAMYB to the Amy32b promoter (Fig. 5B, lanes 6–11). Together, these data suggest that the relative amount of a repressor or activator binding to the Amy32b promoter determines its expression level.
Interactions of HvWRKY38, BPBF, SAD, and HvGAMYB in the Expression of the Amy32b Promoter

The five cis-acting elements involved in GA responses of Amy32b could be bound by either a transcriptional activator or a repressor of the same or a related gene family. For example, we have shown that the W-box (O2S) is essential for the activity of the Amy32b promoter (Zhang et al., 2004). However, this element is bound by the OsWRKY71/HvWRKY38 repressor (Mare et al., 2004; Zhang et al., 2004) and possibly by another WRKY or other zinc finger proteins that could function as activators (Zhang et al., 2004). Similarly, SAD (an activator) and BPBF (a repressor) can bind to the same cis-acting element (i.e. the pyrimidine box; Mena et al., 2002; Isabel-LaMoneda et al., 2003). The HvGAMYB transcriptional activator interacts with the third element, GARE (Gubler et al., 1995). Here, we tested the functional interactions among HvWRKY38, BPBF, SAD, and HvGAMYB on regulating the expression of the Amy32b promoter. As expected, little expression of Amy32b-GUS was detected when HvWRKY38 and BPBF were overexpressed in the absence of GA (Fig. 6B, columns 1, 3, and 4). In contrast, cotransformation of UBI-SAD or UBI-HvGAMYB alone resulted in 71-fold or 85-fold induction of the Amy32b promoter, respectively (columns 5 and 6). The inductive activity of SAD and HvGAMYB is repressed by HvWRKY38 (columns 7 and 8). Coexpression of SAD and HvGAMYB dramatically increased the expression of the Amy32b promoter to 201-fold induction (column 9). Interestingly, this combined effect of SAD and HvGAMYB was not suppressed by HvWRKY38 (column 10). However, HvWRKY38 and BPBF together dramatically reduced the combined effect of SAD and HvGAMYB on the induction of the Amy32b promoter (column 11). Taken together, these data suggest that the expression level of Amy32b is governed by ratios of a set of transcriptional repressors and a set of transcriptional activators. Because each of the cis elements in the Amy32b promoter could be bound by a repressor or an activator of the same or a related family, it is easy to envision their competition in regulating the Amy32b promoter. Our data also indicated the competition of transcriptional regulators whose binding sites are different (e.g. HvWRKY38 and HvGAMYB).

DISCUSSION

In this work, we confirmed that HvWRKY38 repressed the GA-induced expression of Amy32b-GUS in barley aleurone cells (Fig. 1). The role of HvWRKY38 as a transcriptional repressor in the GA response was further demonstrated by the RNAi experiment (Fig. 2). We also found that HvWRKY38 repressed the transactivating activity of HvGAMYB (Supplemental Fig. S1) by competing with the binding of HvGAMYB to the Amy32b α-amylase promoter (Figs. 4 and 5). This inhibitory effect of HvWRKY38 was overcome by the SAD and HvGAMYB activators in combination (Fig. 6). However, combination of WRKY38 and BPBF, which appeared to physically interact (Fig. 3), suppressed the combined effect of SAD and HvGAMYB on inducing the Amy32b promoter in the absence of GA (Fig. 6).

HvWRKY38 is inducible by cold and drought treatments in barley leaves and roots (Mare et al., 2004). It is also induced by abscisic acid and salicylic acid but suppressed by GA in aleurone cells (Mare et al., 2004; Xie et al., 2007). HvWRKY38 represses GA induction of Amy32b (Fig. 1; Xie et al., 2007). Consistent with its function as a repressor, coexpression of a UBI-HvW38(RNAi) construct derepressed the expression of the Amy32b promoter, leading to increased expression of Amy32b in the absence of GA (Fig. 2). The effect of
UBI-HvW38(RNAi) was significant, although it was lower than that of UBI-HvSLN1 RNAi (Zentella et al., 2002). There are two possibilities accounting for the lower effect of UBI-HvW38(RNAi). The preexisting HvWRKY38 protein might be more stable than the SLN1 protein; HvWRKY38 RNAi might only knock out HvWRKY38 but not other WRKY genes. The WRKY family has 74 members (Eulgem et al., 2000) in Arabidopsis and about 100 members in rice (Zhang and Wang, 2005; Ross et al., 2007). As many as 45 WRKY genes have been identified in barley; among these genes, HvWRKY38 (also called HvWRKY1) is closely related to HvWRKY2, -3, and -23 (Mangelsen et al., 2008). Like OsWRKY71 and AtWRKY40, these four barley genes belong to group 2a of the WRKY gene superfamily (Eulgem et al., 2000). Several members could be involved in the same processes. For example, Arabidopsis WRKY18, WRKY40, and WRKY60 have partially redundant roles in plant responses to pathogens (Xu et al., 2006). Rice WRKY24, WRKY51, and WRKY71 blocked GA induction of the Amy32b promoter (Zhang et al., 2004; Xie et al., 2006). It will be interesting to study the function of other barley WRKY family members in GA signaling.

Mounting evidence suggests that each of the five cis-acting elements essential for GA induction of Amy32b can be bound by both transcriptional repressors and activators in barley aleurone cells. The pyrimidine box can be bound by the SAD activator and the BPBF and HvDOF19 repressors, which are DOF proteins (Diaz et al., 2002, 2005; Isabel-LaMoneda et al., 2003; Moreno-Risueno et al., 2007). In addition to the transcriptional activator GAMYB, the repressor HRT, a zinc finger protein, can also bind to GARE (Raventós et al., 1998). The Amy box can interact with the HvMCB1 repressor and the HvMYBS3 activator (Rubio-Somoza et al., 2006). We show here that HvWRKY38 interacts with the W-boxes (Fig. 6). An activator for this element has not been reported, although RAMY, another zinc finger protein, also binds to GARE (Raventós et al., 1998). The Amy box can interact with the HvMCB1 repressor and the HvMYBS3 activator (Rubio-Somoza et al., 2006). 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such as HvWRKY38, BPBF, HRT, and HvMCB1 bind to corresponding cis-acting elements and form a “repressosome,” which diminishes the binding or transactivating activities of positive regulators to the promoter, thereby preventing Amy32b transcription. In the presence of GA, positive regulators such as RAMY, SAD, HvGAMYB, and HvMYBS3 bind to their respective DNA sequences and form an “enhanceosome,” leading to a high level of Amy32b gene expression.

The intriguing question is how the repressosome is replaced by the enhanceosome during the transition of Amy32b from the repressed state to the activated state or vice versa. Two possible mechanisms can be envisioned. (1) GA promotes the degradation of repressors, as reported for OsWRKY71 (Zhang et al., 2004), allowing transcriptional activators to occupy the cis-acting elements. (2) GA induces the expression of activators such as GAMYB (Gubler et al., 1995). These activators physically interact with repressors, eventually leading to the dissociation of repressors from and association of activators to the corresponding cis-acting elements cooperatively. Interactions of repressors and activators have been observed in mammalian systems. For example, B-Myb, a cell cycle-regulated transcriptional repressor, physically interacts with the transcriptional activators specificity protein 1 and CCAAT-binding factor and interferes with their binding to the promoter (Cicchillitti et al., 2004). As a result, COL1A1 (for type I collagen) transcription in human scleroderma fibroblasts is decreased. The barley repressors BPBF, HvDOF17, and HvDOF19 physically interact with HvGAMYB, as demonstrated in the nucleus of onion (Allium cepa) cells using the BiFC approach (Diaz et al., 2005; Moreno-Risueno et al., 2007). HvDOF17 represses the expression of A121, a thiol protease gene, probably by decreasing the binding affinity of HvGAMYB to GARE in the A121 promoter (Moreno-Risueno et al., 2007). Our data showed that the binding of HvGAMYB to the Amy32b promoter was decreased in the presence of increasing amounts of HvWRKY38 (Fig. 5), suggesting that HvWRKY38 interferes with the binding of HvGAMYB to the Amy32b promoter. Accordingly, the induction of Amy32b by HvGAMYB was reduced gradually with increasing amounts of HvWRKY38 (Supplemental Fig. S1). The repression effect of HvWRKY38 was overcome by the coexpression of two transcriptional activators, SAD and HvGAMYB. WRKY38 and BPBF together block the combined effect of SAD and HvGAMYB on inducing the Amy32b promoter in the absence of GA (Fig. 6). Therefore, the ratios of repressors to activators, and more importantly, the cooperative binding of repressors or activators to the Amy32b promoter, determine the levels of Amy32b expression (Fig. 7).

In summary, the data presented in this study support the hypothesis that the GA induction of Amy32b is modulated by two protein complexes, one for activation and the other for repression. Further work is needed to isolate these endogenous complexes using...
imunoprecipitation with antibodies against different regulators or the technique that combines tandem affinity purification with mass spectrometry (Dziembowski and Seraphin, 2004; Van Leene et al., 2007). With these methods, we will be able to further dissect the interactions among transcription factors in regulating GA-controlled gene expression.

MATERIALS AND METHODS

RNA Isolation and RT-PCR

Total RNA was isolated from barley (Hordeum vulgare) aleurone cells with the LiCl precipitation method as described (Zhang et al., 2004). The first-strand cDNAs were synthesized using ImProm-II reverse transcriptase in a 50-μL reaction containing 2.5 μM oligo(dT) primers, 2.5 μM random hexamer, and 2.5 μg of total RNA, according to the manufacturer’s instructions. Five microliters of each reaction mixture was used as a template for PCR amplification in a 25-μL mixture containing 1.5 μM MgCl2, 200 μM dNTPs, 5% dimethyl sulfoxide, 2.5 units of Taq DNA polymerase, and 0.4 μM primers.

Effector Construct Preparations

Three types of DNA constructs were used in the transient expression experiments: reporter, effector, and internal control. Plasmid Amy2b-GUS (Lanahan et al., 1992), HVA1-GUS, and HVA22-GUS (Shen et al., 1993) were used as the reporter constructs. Plasmid pAHCl8 (UBI-Luciferase), which contains the luciferase reporter gene driven by the constitutive maize (Zea mays) ubiquitin promoter (Bruce et al., 1989), was used as an internal control construct to normalize GUS activities of the reporter construct. The full-length cDNA of HvWRKY38 and HvGAMYB was amplified from total RNA of barley aleurone cells by reverse transcription-PCR and cloned into the Ascl site of the intermediate construct containing the Ubi promoter and the NOS terminator (Zhang et al., 2004) using primers P1 and P2 for the preparation of UBI-HvGAMYB (Supplemental Table S1). The full-length cDNAs of BPBF and SAD were amplified and cloned into the Ascl site of the expression vector using the following primers: P5 and P6 for the preparation of UBI-BPBF and P7 and P8 for the preparation of UBI-SAD. To construct UBI-HvWB38 (RNAi), a 331-bp gene-specific fragment was obtained using primers P9 and P10 and cloned into Bpi and BsoM1 sites, respectively, using the symmetrical directional cloning method (Zhang et al., 2004). The substitution mutants were prepared by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Single-stranded DNA from plasmid UBI-HvWRKY38 was used as a template. Primers P11 and P12 were used to mutate the putative LZ motif.

Particle Bombardment and Transient Expression Assays

Detailed descriptions of the transient expression procedure with the barley aleurone system and the particle bombardment technique have been published before (Shen et al., 1993). Briefly, deembryonated half-seeds of ‘Himalaya’ barley were soaked for 2.5 to 3 d, and then the pericarp and testa were removed. The DNA mixture (in a 1:1 molar ratio) of HVA1-GUS and UBI-Luciferase or Amy2b-GUS and UBI-Luciferase, along with or without an effector construct, was bombarded into barley embryonic half-seeds (four replicates per test construct). After incubation for 24 h with various treatments, GUS assays and luciferase assays were performed as described previously (Shen et al., 1996).

Subcellular Localization and BiFC

The HvWRKY38 gene was inserted into the Ascl site in UBI-GFP (Zhang et al., 2004), UBI-YFP, UBI-YN, and UBI-YC (Xie et al., 2006) to generate UBI-GFP:HvWRKY38, UBI-YFP:HvWRKY38, UBI-YN:HvWRKY38, and UBI-YC: HvWB38. The N-terminal region of HvWRKY38 was produced by introducing a stop codon at amino acid 200, which is upstream from the WRKY domain. The rest (i.e. the C-terminal region) was amplified using primers P13 and P14 (Supplemental Table S1). The N-terminal and C-terminal regions of HvWRKY38 were inserted into the Ascl site in UBI-YN to produce UBI-YN: N-HvWRKY38 and UBI-YN-C-HvWRKY38. The LZ motif-mutated HvWRKY38 was inserted into the AscI site of UBI-YN to produce UBI-YN-LZ-HvWRKY38. The HvBPBF gene was amplified and inserted into the AscI site in UBI-YC to generate UBI-YC:HvBPBF. After incubation at 24°C for 24 h, the aleurone layers were peeled from barley half-seeds and soaked in a 5 μM SYTOI7 solution (Molecular Probes). The stained samples were observed, and images of GFP fluorescence and SYTOI7 staining were obtained simultaneously through a LSM 510 laser scanning microscope (Carl Zeiss), with 488-nm excitation and 505- to 530-nm emission wavelengths for the green fluorescence and 633-nm excitation and 650-nm emission wavelengths for the red fluorescence in separate channels. The yellow fluorescence was observed with 514-nm excitation and 530-nm emission wavelengths. At least 8,000 cells were checked in each sample, and fluorescence was observed in more than 5% of total cells in positive results. The acquired images were processed using Paint Shop Pro 7 (Jasc Software).

EMSA

The full-length cDNA of HvWRKY38 was cloned into the AscI site of the modified pGEX-KG (Zhang et al., 2004) to generate GST:HvWRKY38. The fusion constructs were then introduced into Escherichia coli strain Origami B DE3 (Novagen). The full-length cDNA of HvGAMYB was cloned into the AscI site of the modified pGEX-KG (Zhang et al., 2004) to generate GST:HvGAMYB. The fusion constructs were then introduced into E. coli BL-21 (DE3) pLYS S (Novagen). Overexpression of the fusion proteins was induced by 1 mM isopropylthiogalactoside at 25°C in 2× YT medium for 3 h. The cell suspension was passed three times through an SLM-Amino French pressure cell press at 1,600 psi. The GST fusion proteins were purified using glutathione-agarose beads (Zhang et al., 2004).

A 61-bp fragment of the Amy2b promoter (Lanahan et al., 1992) that spans positions –171 to –111 was used as a probe. The DNA probe was labeled with [α-32P]dATP by a Klenow fill-in reaction. Unless otherwise indicated, binding reactions (20 μL) contained 1.5 ng of probe, 1 μg of poly(dIdC), 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.5 mM EDTA, 5 μM ZnCl2, 0.07% (w/v) gelatin CA-630, and 10% glycerol. Equal amounts of recombinant proteins (0.5 μg for each) were added into reactions and incubated at 4°C for 20 min with labeled DNA probes in the absence or presence of competitors. After incubation, the reactions were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8) for 2 h. The signals were scanned with a Typhoon 9400 phosphorimager (Amersham Biosciences).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Functional interactions between HvWRKY38 and HvGAMYB.

Supplemental Table S1. Primers used for construct preparation.

ACKNOWLEDGMENTS

We thank Dr. Pilar Carbonero for kindly providing the BPBF and DOF DNA clones. We also thank Dr. Russell R. Johnson and members of our laboratory for advice or contributions to the improvement of this paper.

Received May 29, 2008; accepted June 18, 2008; published July 11, 2008.

LITERATURE CITED

Diaz I, Martinez M, Isabel-LaMoneda I, Rubio-Somoza I, Carbonero P (2005) The DOF protein, SAD, interacts with GAMYB in plant nuclei and
activates transcription of endosperm-specific genes during barley seed development. Plant J 42: 652–662


Rubio-Somoza I, Martinez M, Diaz I, Carbonero P (2006) HvMCBB1, a RIMYB transcription factor from barley with antagonistic regulatory functions during seed development and germination. Plant J 45: 17–30


Washio K (2003) Functional dissections between GAMYB and DOF transcription factors suggest a role for protein-protein associations in the...
gibberellin-mediated expression of the RAmy1A gene in the rice aleurone. Plant Physiol 133: 850–863