Three WRKY transcription factors additively repress abscisic acid and gibberellin signaling in aleurone cells

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Abstract:

Members of the WRKY transcription factor superfamily are essential for the regulation of many plant pathways. Functional redundancy due to duplications of WRKY transcription factors, however, complicates genetic analysis by allowing single-mutant plants to maintain wild-type phenotypes. Our analyses indicate that three group I *WRKY* genes, *OsWRKY24*, -53, and -70, act in a partially redundant manner. All three showed characteristics of typical WRKY transcription factors: each localized to nuclei and yeast one-hybrid assays indicated that they all bind to W-boxes, including those present in their own promoters. Quantitative real time-PCR (qRT-PCR) analyses indicated that the expression levels of the three *WRKY* genes varied in the different tissues tested. Particle bombardment-mediated transient expression analyses indicated that all three genes repress the GA and ABA signaling in a dosage-dependent manner. Combination of all three *WRKY* genes showed additive antagonism of ABA and GA signaling. These results suggest that these WRKY proteins function as negative transcriptional regulators of GA and ABA signaling. However, different combinations of these *WRKY* genes can lead to varied strengths in suppression of their targets.

Keywords: WRKY transcription factor; Absciscic acid signaling; Gibberellin signaling; *Oryza sativa*; Rice

1. Introduction

Hormones play critical roles in regulating plant development and plant responses to abiotic stress. Seed germination is an all-or-nothing process. Through germination, the plant transforms from a hearty, desiccation-tolerant seed into a fragile seedling. As such, the timing of germination is essential to the survival of the plant. This regulation is mediated by ABA, GA, ethylene, and brassinosteroids [1]. Antagonistic effects between GA and ABA signaling are the major regulators of germination. During this process, GA moves from embryos into aleurone cells to promote the expression of hydrolytic enzymes, such as α -amylases, to break down starches in the endosperm. In contrast, seed germination is blocked by ABA, which suppresses the GA-induced expression of genes such as those encoding α -amylases [2, 3].

In rice, GA signaling is perceived by a soluble receptor encoded by *GID1* [4]. Binding of GA leads to interaction with a DELLA protein, a master negative regulator in GA signaling. The complex of GA/*GID1*/DELLA recruits an SCF E3 ubiquitin ligase, via the specific F-box protein *SLY1* (*SCF^{SLY1}*), leading to degradation of the DELLA protein through the 26S proteasome pathway [5]. Downstream positive and negative targets of DELLA proteins in the GA signaling pathway have also been characterized [6, 7]. Among these, a transcriptional activator (*GAMYB*) induces transcription of α -amylase genes in barley aleurone [8].

The ABA signaling pathway works to antagonize GA signaling to promote seed dormancy. In addition, ABA regulates adaptive responses to drought and high salinity [9].

ABA signaling comprises of several steps: perception of ABA by a soluble receptor, PYR1/PYL/RCAR, and association of these proteins with PP2Cs, leading to the inhibition of PP2C phosphatase activity. This inhibition allows activation of SnRK2 protein kinases, which phosphorylate ABI5 and related transcription factors to direct ABA-regulated responses [10]. ABA functions through a complex web of signaling networks which includes various classes of transcription factors, such as those in the MYB, AP2/EREBP, bZIP and WRKY gene families.

There are over 100 WRKY members in *indica* and *japonica* rice [11]. Members of the WRKY transcription factor family are defined by the presence of one or two WRKY domains, which contain a highly conserved 60 amino acid sequence [12]. WRKY transcription factors are grouped into three classes, group I, II, and III, based on both the number of WRKY domains and the composition of zinc finger motifs [13]. The highly conserved WRKY domain specifically recognizes and binds to the W-box consensus (TTGACY) in the promoters of target genes [14-16]. Recently, the two WRKY domains in group I WRKY proteins have been found to bind to the W-box with different binding specificities [16]. Due to gene duplication, some WRKY transcription factors have been found to play redundant or partially-redundant roles [17]. AtWRKY25, AtWRKY26, and AtWRKY33 are closely related group I WRKY transcription factors, and they function redundantly as positive regulators in resistance to heat stress [18]. In addition, AtWRKY25 and AtWRKY33 showed functional redundancy in oxidative stress and partial redundancy in salinity stresses [19].

There are 15 group I WRKY genes in *japonica* rice. Eight of these have been further studied [7, 20-24], and the functional information available suggest that most of them are involved in signaling response to GA, ABA, abiotic, or biotic stresses. Phylogenetic analysis showed that OsWRKY24, OsWRKY70 and OsWRKY53 are close paralogs. Our recent studies revealed that one member of this group, *OsWRKY24*, encodes a transcriptional repressor specifically repressing both the GA-mediated induction of low pI α -amylase *Amy32b* gene expression, and the ABA-mediated induction of *HVA22* gene expression, in barley aleurone cells [25]. Previous reports found that the expression of *OsWRKY53* was induced by treatment with pathogen-derived elicitors, and transgenic rice plants overexpressing *OsWRKY53* showed enhanced resistance to the rice blast fungus *Magnaporthe grisea* [26]. The expression of *OsWRKY70* was also induced by elicitors, such as flagellin and chitin oligosaccharide [27, 28]. These data suggest that OsWRKY53 and OsWRKY70 are transcription factors involved in basal defense responses. Here, we extended functional analysis of OsWRKY24, OsWRKY53 and OsWRKY70 and found that they function partially redundantly as repressors in regulating GA and ABA signaling pathways in aleurone cells.

2. Materials and methods

2.1 Plant materials

Barley (*Hordeum vulgare* cv Himalaya) seeds were obtained from Washington State University (Pullman, WA). Rice (*Oryza sativa* cv Nipponbare) seeds were kindly

provided by the Dale Bumpers National Rice Research Center, USDA ARS (Stuttgart, Arkansas). Rice seeds were incubated for 24 hours in a growth chamber (12 h light 28°C/12 h dark 25°C cycles) to collect embryos and aleurone cells. Roots and shoots were harvested from 2-week-old seedlings grown in the growth chamber. Panicles before heading were prepared from the plants grown in the green house (~14 h light 30°C/~10 h dark 28°C cycles).

2.2 Subcellular localization

GFP:WRKY reporter constructs were made by inserting the appropriate *WRKY* cDNA sequence, *OsWRKY70* or *OsWRKY53*, into the *AscI* site in the *UBI-GFP* vector [29] to generate *UBI-GFP:OsWRKY70* and *UBI-GFP:OsWRKY53*. After incubation at 24°C for 24 h, the aleurone layers were peeled from barley half-seeds and soaked in a 5 µM SYTO61 solution (Molecular Probe, Eugene, OR, USA). The images of GFP fluorescence and SYTO61 staining were obtained simultaneously via the Laser Scanning Microscope LSM 510 (Carl Zeiss, Inc., Jena, Germany) with 488-nm excitation and 505- to 530-nm emission wavelengths for green fluorescence, and 633-nm excitation and 650-nm emission wavelengths for red fluorescence, in separate channels.

2.3 Constructs and yeast one-hybrid (Y1H) assays

The pAbAi vector (Clontech, Mountain View, CA) contains the *AURI-C* gene for resistance to antibiotic Aureobasidin A (AbA). Supplemental Table 1 shows the sequences of the oligonucleotides containing the W-box core sequence (TGAC) from the *Amy32b*, *OsWRKY24*, -53 and -70 promoters. Each pair of the oligonucleotides were

annealed, phosphorylated and inserted into the pAbAi vector. The resulting reporter constructs were linearized with BstB1 and integrated into the genome of the Y1HGold yeast strain (Clontech, Mountain View, CA), and selected on the synthetic defined (SD) medium lacking uracil (SD/-Ura). The effector constructs, *AD:W24*, *AD:W53* and *AD:W70*, include the coding sequence for the GAL4 activation domain (AD) and one of the OsWRKY proteins. The effector DNA was transformed into competent cells made from the Y1HGold strain (Clontech, Mountain View, CA) that had been engineered to contain one of the reporter constructs. Positive transformants were selected on the SD/-Ura/-Leu medium and then further tested on the medium containing AbA at the concentration of 100 ng/mL (SD/-Ura/-Leu/AbA).

For the dosage assays, three independent colonies for each of the five constructs grown on SD/-Ura/-Leu plates were used to inoculate 15 ml of SD/-U/-L liquid medium, respectively. After incubation for 12 h, the yeast cells are collected by centrifugation and re-suspended in 0.9% NaCl to the titer of 5×10^6 cells/ml. Four microliter of the yeast culture was dotted on the SD/-U/-L plates containing 0, 100, 200, and 250 ng/ml AbA, respectively and incubated for three days at 30 °C.

2.4 Transcriptional activation analyses

The full-length or partial sequence of *OsWRKY24*, -53 or -70 cDNA was fused in-frame with the GAL4 DNA binding domain (BD) in the pGBKT7 vector (Clontech, Mountain View, CA). The constructs were transformed into yeast strain Y2HGold containing the *AURI-C*, *ADE2*, *HIS3*, and *MEL1* reporter genes (Clontech, Mountain

View, CA). The transformed yeast cells were grown on the SD medium lacking tryptophan, and transactivation activity was tested on the SD medium containing AbA (125 ng/mL) and X- α -Gal (40 μ g/mL).

2.5 RNA extraction and qRT-PCR analyses

Total RNA was isolated from frozen tissues using an RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Turbo DNase (Life Technologies, Grand Island, NY) was used to remove genomic DNA in the RNA samples according to the manufacturer's instructions. Total RNA was used for first strand cDNA synthesis using the Omniscript RT Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. Primers were designed from the unique region in the 3'UTR of each *OsWRKY* gene and are shown in Supplemental Table 2. The qRT-PCR reaction was carried out as described by Ross and Shen [30]. The expression data were normalized by subtracting the mean *OsActin1* CT value from the *WRKY* CT value (Δ CT). The fold change value was calculated using the expression $2^{-\Delta\text{CT}}$ [31]. Statistical significance of the difference was determined using a paired Student's t-test. For each sample, three biological replicates and three technical replicates were analyzed.

2.6 Preparation of effector constructs for transient expression studies

Three types of DNA constructs were used in the transient expression experiments: reporter, effector and internal control. Plasmids containing *Amy32b-GUS* and *HVA22-GUS* were used as the reporter constructs. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by the constitutive maize ubiquitin

promoter [32], was used as an internal control construct to normalize GUS activity of the reporter construct. Full-length cDNAs of *OsWRKY70* and *OsWRKY53* were amplified from total RNA of rice leaves via RT-PCR. The fragments were then cloned into the BamH1 and AscI sites of an intermediate construct containing the *UBI* promoter and *NOS* terminator, using primers P1 and P2 for preparation of *UBI-OsWRKY53*, and P5 and P6 for *UBI-OsWRKY70* (Supplemental Table 1).

2.7 Particle bombardment and transient expression assays

The embryo was removed from Himalaya barley seeds. The cut seeds were imbibed for two days, and the pericarp and testa were removed. A DNA mixture containing 5µg each of a reporter construct, the internal control construct (*UBI-Luciferase*), and the appropriate effector constructs, was bombarded into barley half seeds (without embryos). For each bombardment, eight prepared half seeds were arranged in a small circle, about 1.8 cm in diameter, to maximize the bombarded surface area. After bombardment treatments, GUS and luciferase assays were performed as described [33]. Statistical analysis were performed using the Student's t-test when comparing two groups of data and one-way ANOVA when comparing three or more groups of data.

2.8 Accession numbers

The GenBank accession numbers for the *WRKY* genes discussed in this article are: NM_001051210 for *OsWRKY24*, NM_001061787 for *OsWRKY53* and NM_001062363 for *OsWRKY70*.

3. Results

3.1 OsWRKY24, OsWRKY53 and OsWRKY70 are close paralogs.

OsWRKY24, -53 and -70 fall into group I of the WRKY superfamily according to phylogenetic analyses of amino acid sequences of the predicted WRKY proteins [34]. The phylogenetic relationship of group I WRKY members in rice was analyzed by comparing their deduced protein sequences (Supplemental Figure 1). This phylogeny revealed that OsWRKY24 shows higher similarity to OsWRKY70 and OsWRKY53 than other WRKY proteins in rice, suggesting that they are close paralogs in rice.

3.2 OsWRKY70 and OsWRKY53 are localized to the nucleus.

OsWRKY24 and OsWRKY53 have been reported to be localized in the nucleus [7, 26]. OsWRKY70 contains a putative nuclear localization sequence (NLS) [34], thus we expected it to be localized within the nucleus as well. To test the subcellular distribution of OsWRKY70, *UBI-OsWRKY70:GFP* and the control construct, *UBI-GFP*, were bombarded separately into barley aleurone cell, followed by confocal microscopic observations. To visualize the nuclei, aleurone cells were stained with the red fluorescent nucleic acid stain, SYTO17. As shown in Figure 1, the GFP protein was found in both the nucleus and the cytoplasm of aleurone cells (Panel a, Figure 1). As reported, OsWRKY53-GFP was exclusively localized to the nucleus (Panel b). Similarly, OsWRKY70-GFP was targeted to the nuclei (Panel c). The nuclear localization of OsWRKY53 and OsWRKY70 was consistent with their predicted function as transcription factors.

3.3 OsWRKY24, -53 and -70 bind to the W-box in the promoters of *Amy32b* and the three *WRKY* genes.

WRKY transcription factors are known to bind to W boxes. OsWRKY24 was previously shown to bind to the O2S element of the *Amy32* promoter, which contains two classical W-box consensus sequences (TTGACY). Thus, it was expected that OsWRKY70 and -53 would also bind to the W-box. The yeast one-hybrid technique was used to test this hypothesis. To make constructs containing the W-box, three repeats of TTGACTTGACC were inserted in the *pHIS-32bWbox* plasmid. As a negative control, the P53 binding element was cloned in the *pHIS-p53b* plasmid. Both constructs contain the *AURI-C* gene for resistance to antibiotic Aureobasidin A (AbA). These reporter constructs were integrated into the genome of the yeast reporter strain. The resulting *32bWbox* and *p53b* yeast reporter strains were each transformed with *AD-W24*, *AD-W53*, and *AD-W70*. Figure 2A showed that all yeast strains grew equally well on the SD media lacking AbA. As expected, the *32bWbox* yeast strain transformed with the *AD-W24* grew well on the selection media (Figure 2B). Similarly, the *32bWbox* yeast strain transformed with *AD-W70* and *AD-W53* also grew well. However, the *p53b* yeast strain transformed with *AD-W24*, *AD-W53*, and *AD-W70* failed to grow (Figure 2B). These results demonstrated that OsWRKY53 and OsWRKY70 were capable of specifically binding to the W-box in the *Amy32b* promoter.

Many *WRKY* gene promoters contain multiple W boxes, involved in auto-regulation or cross-regulation of their expression upon hormone or stress treatments. For instance,

there are three repeats of W-boxes located in the -229 to -185 region of the *OsWRKY53* promoter, relative to the transcription start site. The sequence of this fragment is shown in Supplemental Table 1; the core sequence is 5'-TGAC-3' (forward) or 5'-GTCA-3' (reverse). These W boxes are involved in regulation of the elicitor-response of *OsWRKY53* [35]. There are also several potential W-box elements within the promoter regions of *OsWRKY24* and -70. Specifically, four W boxes are present in the -85 to -47 region of the *OsWRKY24* promoter and the -86 to -43 region of the *OsWRKY70* promoter, respectively (Supplemental Figure 2A).

We tested whether *OsWRKY24*, -53, and -70 could bind to the W-box variants in these promoters. As shown in Figure 2, the cotransformants for *OsWRKY24*, -53 and -70 grew well on the SD/-L/-U medium (Panel A) and on the SD/-L/-U medium containing AbA (100 ng/mL; Panel B). These results were confirmed with a serial dilution assay (Supplemental Figure 2B-E). This indicated that all three of these WRKY proteins have binding activities to the variants of W boxes in their promoters.

To demonstrate the binding specificity, the core of each W-box, TGAC, was mutated to CCTA, generating *mW24-AbAi*, *mW53-AbAi* and *mW70-AbAi* (see Supplemental Table 1 for the full sequences). Clearly, these mutants gave results similar to those obtained with the *p53-AbAi* negative control (Figure 2B), suggesting that *OsWRKY24*, -53, and -70 are capable of specifically binding to the W boxes in their own and each other's promoters.

3.4 *OsWRKY24*, -53, and -70 show transcriptional activation activity.

Two Group I WRKY proteins, OsWRKY53 and AtWRKY63, have been reported to have transcriptional activation activity [10, 26]. To investigate whether OsWRKY24 and -70 are also transcriptional activators in yeast, we prepared effector constructs, *BD:W24*, *BD:W53* and *BD:W70*, for fusion proteins GAL4 BD:OsWRKY24, GAL4 BD:OsWRKY53 and GAL4 BD:OsWRKY70. The effector constructs were respectively introduced into the GoldY2H yeast strain, which carries the *AURI-C*, *ADE2*, *HIS3*, and *MEL1* reporter genes driven by promoters containing slightly different GAL4 binding sites. OsWRKY51, a group II WRKY protein that has been shown to be incapable of binding to the W-box [29], was included as a negative control. As shown in Figure 3A, cells containing the *BD:W24*, but not *BD:W51*, were able to grow well on the SD medium lacking tryptophan and containing AbA and X- α -gal (SD/-W/AbA/X- α -gal), with blue colonies. This was also the case for yeast containing *BD:W53* or *BD:W70*, suggesting that OsWRKY24 and -70 have transcriptional activation activity in yeast cells.

The N-terminal regions of OsWRKY24, -53, and -70 proteins contain 38-45% proline and serine, with most of these two amino acids in SP clusters, in contrast to OsWRKY51, which only contains 26% of proline and serine and much fewer SP clusters (http://www.bi.up.ac.za/cgi-bin/emboss.pl?_action=input&_app=pepstats). These proline- and serine-rich regions may be responsible for transcriptional activation of the group I WRKY proteins. Indeed, when the N-terminal 145 amino acids of OsWRKY53 were deleted, the auto-activation activity seen in the full-length genes was completely abolished. Similarly, deletion of the N-termini of OsWRKY24 and -70 greatly reduced, though did not eliminate, the auto-activation activity of these transcription factors (Figure 3B). This

result suggests that, while the amino acid sequences of these three proteins are highly similar, the protein motifs involved in transcription activation are slightly different.

3.5 The expression patterns of the three *WRKY* genes vary in different tissues.

WRKY proteins have been reported to be involved in abiotic stresses and hormone signaling. To help study the function of *OsWRKY24*, *OsWRKY53* and *OsWRKY70*, quantitative Real Time-PCR (qRT-PCR) analyses were performed to determine the expression pattern of the three genes in different tissues. The results indicated that the overall expression of *OsWRKY70* was relatively low in all five tested tissues, while expression levels of *OsWRKY24* and -53 were relatively high (Figure 4). Comparison of expression patterns of each gene showed that the expression levels of all three genes were relatively low in panicles and embryos, and relatively high in aleurone layers and leaves. Therefore, we performed transient studies in aleurone cells to investigate the functions and interactions of these genes in regulating GA and ABA signaling.

3.6 *OsWRKY70* and *OsWRKY53* suppress both GA and ABA signaling.

ABA mediates plant response to drought stress and inhibits seed dormancy and it often plays an antagonistic role in the GA signaling pathway. However, we found that *OsWRKY24* negatively regulates both GA and ABA signaling in aleurone cells [7]. Because *OsWRKY53* and -70 are close paralogs of *OsWRKY24* (Supplemental Figure 1), we wondered whether they could also repress both GA and ABA signaling. Transient expression analyses were carried out to address this question. Each of the two *WRKY*

transcription factors, under the control of a ubiquitous promoter, was co-transformed with reporter genes controlled by promoters known to be responsive to ABA or GA signaling, the *HVA22* and *Amy32b* promoters, respectively (Figure 5A and [36]).

When the ABA-signaling reporter construct *HVA22-GUS* alone was bombarded into aleurone cells, the reporter gene showed 15 fold induction of expression after ABA treatment, compared to the non-treated sample (Figure 5B). Addition of the *UBI-OsWRKY53* effector construct decreased the ABA-induced expression of the reporter gene to 5 fold. Similarly, co-bombardment of *UBI-OsWRKY70* and the reporter construct led to a mere 6 fold induction of the reporter construct under ABA treatment. The data suggest that like *OsWRKY24* [7], *OsWRKY53* and -70 are negative regulators of ABA signaling in aleurone cells. The data also show that these two genes repressed ABA signaling with similar potency.

To test the role of the two WRKY transcription factors in the GA signaling pathway, the effector constructs were co-bombarded with a *GUS* reporter gene under the control of the *Amy32b* promoter. When the *Amy32b* promoter alone was bombarded into aleurone cells and treated with exogenous GA, the reporter showed a 55-fold induction of expression compared to the control (Column 1, Figure 5C). When *UBI-OsWRKY53* was co-bombarded with the reporter construct, induction was decreased to 14 fold (Column 4), compared to the control (Column 1). *UBI-OsWRKY70* also produced suppression of GA induction, with a mere 2 fold induction of the reporter with the GA treatment (Column 6), compared to the control (Column 1). The repression potencies of *OsWRKY53* and

OsWRKY70 in the GA signaling pathway were not significantly different ($p > 0.05$, Student's t-test). These data suggest that, like OsWRKY24 [7], OsWRKY53 and -70 are also negative regulators of GA signaling in aleurone cells.

3.7 The suppression of OsWRKY24, -53 and -70 is additive in ABA and GA signaling.

Our previous work showed that OsWRKY24 repressed the GA-inducible *Amy32b* promoter and the ABA-inducible *HVA22* promoter in a dosage-dependent manner [7]. To investigate the dosage dependence of OsWRKY53 and -70, the ratio of the effector construct to the reporter construct was varied in a similar particle bombardment experiment, ranging from 0% (i.e. with no effector construct) to 100% (with equal molar concentrations of effector and reporter constructs). As shown in Figure 6A, the GUS activities under the ABA or GA treatment decreased with increasing amounts of the effector *Ubi-W53* or *Ubi-W70* construct, suggesting that OsWRKY53 and OsWRKY70 repressed the GA and the ABA signaling pathways in a dosage-dependent manner. Dosage at the range from 5% to 25% led to a linear decrease in GUS activity.

To study the combinatory effect of these repressors on ABA signaling, we compared the effect of each WRKY gene with those of combinations of two WRKY genes and the combination of all three. In the single bombardment experiment, the WRKY constructs were added at 15% of the reporter construct. In dual bombardment experiments, 7.5% of each of the two WRKYs were added relative to the reporter construct, and in the triple

bombardment experiment, 5% of each WRKY construct was used. Similar to the results in Section 3.6 (Figure 5B), OsWRKY24, -53 and -70 repressed ABA signaling with a similar potency (Columns 3 through 5, Figure 6B, $P>0.05$, one-way ANOVA). When two of these three genes were combined with the usage of 7.5% of each of the two WRKYs relative to the reporter, similar repression levels were obtained (Columns 6 through 8, $P>0.05$, one-way ANOVA). This was also true for the combination of all three WRKY genes with the addition of 5% of each WRKY construct (Column 9, $P>0.05$, one-way ANOVA).

A similar experiment was carried out for GA signaling using an equal molar amount of effector construct(s) for all samples. OsWRKY24, -53 and -70 showed a similar potency in suppressing GA signaling in aleurone cells (compare Column 5 with Columns 3 and 4, $P>0.05$, one-way ANOVA). Combinations of two of these three WRKY genes decreased the reporter gene expression to levels similar to those achieved with a single effector gene (compare Columns 6 through 8 with Columns 3 through 5, $P>0.05$, one-way ANOVA). However, the combination of all three of the WRKY genes significantly decreased *Amy32b-GUS* expression to the background level (compare Column 9 with Column 1, $P>0.05$, Student's t-test), even though the total amount of effector constructs in this sample is the same as that in others, i.e. 5% for each for a total of 15%. Therefore, the combination of all three of these WRKY genes appeared to at least have an additive effect on GA signaling. Whether they are involved in synergistic interactions remains to be investigated in the future.

4. Discussion

In this work, we studied the interactions of OsWRKY24, OsWRKY53 and -70 in regulating ABA and GA signaling in aleurone cells. All these three WRKY proteins were localized to the nucleus (Figure 1 and [7]) and have transactivation activity (Figure 3), confirming their role as transcription factors. All three are capable of binding to the canonical WRKY binding sites in the *Amy32b* promoter as well as their own and each other's promoters (Figure 2). Transient expression analyses indicated that all three antagonize the GA and ABA signaling pathways in a dosage-dependent manner (Figure 6A). When the three transcription factors were combined, they acted additively in the antagonism of both ABA and GA signaling (Figure 6B and 6C). Interestingly, while all three showed maximal expression in rice aleurone and leaves, the expression of *OsWRKY70* was much lower than that of *OsWRKY24* and -53 (Figure 4). These data suggest that OsWRKY24, -53, and -70 may function in a partially redundant manner to antagonize the ABA and GA signaling pathways.

Of the three WRKY transcription factors, OsWRKY24 and -70 were more closely related to each other, with some slight variations seen in OsWRKY53. OsWRKY53 and OsWRKY70 were both similar to OsWRKY24 in many aspects. GFP fusions with each of these three proteins localized to the nuclei of transformed aleurone cells (Figure 1 and [7, 26]). OsWRKY70, though, shared a closer relationship with OsWRKY24 than did OsWRKY53. The proteins encoded by *OsWRKY24* and *OsWRKY70* were of similar lengths, 555 and 572 amino acid residues, respectively, whereas the *OsWRKY53* gene

encodes a smaller protein, at 487 amino acid residues. This difference appeared to afford additional transcriptional activation activity to OsWRKY24 and OsWRKY70. Deletion of the N-terminus totally abolished the transcriptional activation activity of OsWRKY53, whereas deletion of the N-terminus of OsWRKY24 and OsWRKY70 greatly reduced, but did not abolish transcriptional activation. There are 4 continuous SP sites (between amino acid 65 and 120) in the N-termini of OsWRKY24, -53 and -70. Phosphorylation of OsWRKY30, another group I WRKY protein, at its N-terminal SP cluster was necessary for the performance of its transactivation activity [37]. This suggests that the N-terminal SP cluster is essential for transcriptional activation activity of OsWRKY53, whereas OsWRKY24 and -70 contain additional transcriptional activation domains. While *OsWRKY24* and *OsWRKY70* showed similar expression patterns in the tissues tested, with peak expression in leaf and aleurone tissue, the expression level of *OsWRKY70* was much lower than that of *OsWRKY24* (Figure 4). This difference in expression may indicate a subordinate role of OsWRKY70 to OsWRKY24. Alternatively, OsWRKY70 may be specific to a tissue or developmental stage that was not tested in these experiments. *OsWRKY53* showed similar expression levels to *OsWRKY24* in leaves and aleurone, but much higher levels in roots and embryos.

Three tandem W-box elements responsible for elicitor induction of *OsWRKY53* have been identified in the *OsWRKY53* promoter, and chromatin immunoprecipitation analysis revealed that OsWRKY53 can bind to the W-box elements in vivo [35]. Promoter analysis showed there are 4 tandem W-box elements in *OsWRKY24* and -70 promoters.

The yeast one-hybrid test demonstrated that OsWRKY24, -53 and -70 were able to recognize all three W-box clusters in the *OsWRKY24*, -53 and -70 promoters, and mutation of all W-boxes abolished the binding of the WRKY proteins to DNA (Figure 2). In addition the expression of *OsWRKY24* and -53 were down-regulated in the *OsWRKY70* overexpression line (data not shown). These data suggest that the three WRKY transcription factors participate in self- and cross-regulation. In a similar manner, the three Arabidopsis WRKY paralogs, AtWRKY18, -40, and -60, were recently demonstrated to have similar self- and cross-regulatory capabilities [38, 39]. These three WRKY transcription factors were found to enact an inhibitory effect on the expression of their own and the other's promoters.

Like OsWRKY24 [7], OsWRKY53 and OsWRKY70 functioned as negative regulators of the GA-induced *Amy32* promoter and ABA-induced *HVA-22* promoter. Transient co-expression of the three proteins under GA and ABA treatment indicated that a combination of the three WRKY proteins functioned additively in both ABA and GA signaling. The conventional model of ABA and GA signaling crosstalk involves antagonism between the two transcription factors. The antagonism of both pathways by the three WRKY transcription factors is surprising in light of this model. There are additional hormone pathways, though, that antagonize and are antagonized by both the ABA and GA signaling pathways. The biotic stress regulating hormones, jasmonic acid (JA), and ethylene share antagonistic relationships with ABA [40, 41]. In addition, JA has been found to inhibit the biosynthesis of gibberellic acid in *Nicotiana attenuata* stems,

inhibiting stem elongation [42]. A role of OsWRKY53 in biotic stress is supported by recent reports demonstrating that phosphorylation of OsWRKY53 at the SP cluster via an MAPK cascade enhanced disease resistance to rice blast fungus [43]. On the other hand, it has been found that JA inhibits, and ethylene promotes, seed germination [44]. Further investigation is required to confirm the role, if any, of OsWRKY24, 53, and 70 in the crosstalk between ABA, GA, and biotic stress response in rice knockout mutants, followed by the study of detailed mechanism that involved in WRKY interaction proteins such as mitogen-activated protein kinases and VQ proteins, which have recently been shown to interact with group I WRKYs [45].

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6. References

- [1] R.R. Finkelstein, The role of hormones during seed development and germination, in: P.J. Davis (Ed.) *Plant Hormones*, Springer, New York City, 2010, pp. 549-573.
- [2] A. Lovegrove, R. Hooley, Gibberellin and abscisic acid signalling in aleurone, *Trends Plant Sci.*, 5 (2000) 102-110.
- [3] A. Gómez-Cadenas, R. Zentella, T.D.H. Sutliff, T.H.D. Ho, Involvement of multiple *cis*-elements in the regulation of GA responsive promoters: Definition of a new *cis*-element in the *Amy32b* gene promoter of barley (*Hordeum vulgare*), *Physiol. Plant.*, 112 (2001) 211-216.
- [4] M. Ueguchi-Tanaka, M. Nakajima, E. Katoh, H. Ohmiya, K. Asano, S. Saji, X. Hongyu, M. Ashikari, H. Kitano, I. Yamaguchi, M. Matsuoka, Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin, *Plant Cell*, 19 (2007) 2140-2155.
- [5] C. Jiang, X. Gao, L. Liao, N.P. Harberd, X. Fu, Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in *Arabidopsis*, *Plant Physiol*, 145 (2007) 1460-1470.
- [6] R. Zentella, Z.L. Zhang, M. Park, S.G. Thomas, A. Endo, K. Murase, C.M. Fleet, Y. Jikumaru, E. Nambara, Y. Kamiya, T.P. Sun, Global analysis of della direct targets in early gibberellin signaling in *Arabidopsis*, *Plant Cell*, 19 (2007) 3037-3057.
- [7] Z.L. Zhang, M. Shin, X. Zou, J. Huang, T.H. Ho, Q.J. Shen, A negative regulator encoded by a rice WRKY gene represses both abscisic acid and gibberellins signaling in

aleurone cells, *Plant Mol Biol*, 70 (2009) 139-151.

[8] F. Gubler, R. Kalla, J.K. Roberts, J.V. Jacobsen, Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pI alpha-amylase gene promoter, *Plant Cell*, 7 (1995) 1879-1891.

[9] S.R. Cutler, P.L. Rodriguez, R.R. Finkelstein, S.R. Abrams, Absciscic acid: emergence of a core signaling network, *Annu Rev Plant Biol*, 61 (2010) 651-679.

[10] A.S. Raghavendra, V.K. Gonugunta, A. Christmann, E. Grill, ABA perception and signalling, *Trends Plant Sci.*, 15 (2010) 395-401.

[11] P.J. Rushton, I.E. Somssich, P. Ringler, Q.J. Shen, WRKY transcription factors, *Trends Plant Sci*, 15 (2010) 247-258.

[12] T. Eulgem, P.J. Rushton, S. Robatzek, I.E. Somssich, The WRKY superfamily of plant transcription factors, *Trends Plant Sci.*, 5 (2000) 199-206.

[13] C.A. Ross, Y. Liu, Q.J. Shen, The *WRKY* gene family in rice (*Oryza sativa*), *Journal of Integrative Plant Biology*, 49 (2007) 827-842.

[14] K. Yamasaki, T. Kigawa, M. Inoue, M. Tateno, T. Yamasaki, T. Yabuki, M. Aoki, E. Seki, T. Matsuda, Y. Tomo, N. Hayami, T. Terada, M. Shirouzu, A. Tanaka, M. Seki, K. Shinozaki, S. Yokoyama, Solution Structure of an Arabidopsis WRKY DNA Binding Domain, *Plant Cell*, 17 (2005) 944-956.

[15] K. Yamasaki, T. Kigawa, S. Watanabe, M. Inoue, T. Yamasaki, M. Seki, K. Shinozaki, S. Yokoyama, Structural basis for sequence-specific DNA recognition by an Arabidopsis WRKY transcription factor, *J Biol Chem*, 287 (2012) 7683-7691.

- [16] L.H. Brand, N.M. Fischer, K. Harter, O. Kohlbacher, D. Wanke, Elucidating the evolutionary conserved DNA-binding specificities of WRKY transcription factors by molecular dynamics and in vitro binding assays, *Nucleic Acids Res*, 41 (2013) 9764-9778.
- [17] N. Journot-Catalino, I.E. Somssich, D. Roby, T. Kroj, The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*, *Plant Cell*, 18 (2006) 3289-3302.
- [18] S. Li, Q. Fu, L. Chen, W. Huang, D. Yu, *Arabidopsis thaliana* WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance, *Planta*, 233 (2011) 1237-1252.
- [19] Y. Jiang, M.K. Deyholos, Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses, *Plant Mol Biol*, 69 (2009) 91-105.
- [20] R. Ramamoorthy, S.Y. Jiang, N. Kumar, P.N. Venkatesh, S. Ramachandran, A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments, *Plant Cell Physiol*, 49 (2008) 865-879.
- [21] S. Berri, P. Abbruscato, O. Faivre-Rampant, A.C. Brasileiro, I. Fumasoni, K. Satoh, S. Kikuchi, L. Mizzi, P. Morandini, M.E. Pe, P. Piffanelli, Characterization of WRKY co-regulatory networks in rice and *Arabidopsis*, *BMC Plant Biol*, 9 (2009) 120.
- [22] D. Qiu, J. Xiao, W. Xie, H. Cheng, X. Li, S. Wang, Exploring transcriptional signalling mediated by OsWRKY13, a potential regulator of multiple physiological

processes in rice, *BMC Plant Biol*, 9 (2009) 74.

[23] T. Chujo, T. Kato, K. Yamada, R. Takai, C. Akimoto-Tomiyama, E. Minami, Y. Nagamura, N. Shibuya, M. Yasuda, H. Nakashita, K. Umemura, A. Okada, K. Okada, H. Nojiri, H. Yamane, Characterization of an elicitor-induced rice *WRKY* gene, *OsWRKY71*, *Biosci Biotechnol Biochem*, 72 (2008) 240-245.

[24] C.Y. Kim, S.H. Lee, H.C. Park, C.G. Bae, Y.H. Cheong, Y.J. Choi, C. Han, S.Y. Lee, C.O. Lim, M.J. Cho, Identification of rice blast fungal elicitor-responsive genes by differential display analysis, *Mol. Plant Microbe Interact.*, 13 (2000) 470-474.

[25] Z.L. Zhang, Z. Xie, X. Zou, J. Casaretto, T.H. Ho, Q.J. Shen, A rice *WRKY* gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells, *Plant Physiol*, 134 (2004) 1500-1513.

[26] T. Chujo, R. Takai, C. Akimoto-Tomiyama, S. Ando, E. Minami, Y. Nagamura, H. Kaku, N. Shibuya, M. Yasuda, H. Nakashita, K. Umemura, A. Okada, K. Okada, H. Nojiri, H. Yamane, Involvement of the elicitor-induced gene *OsWRKY53* in the expression of defense-related genes in rice, *Biochim Biophys Acta*, 1769 (2007) 497-505.

[27] R. Takai, A. Isogai, S. Takayama, F.S. Che, Analysis of flagellin perception mediated by flg22 receptor *OsFLS2* in rice, *Mol. Plant Microbe Interact.*, 21 (2008) 1635-1642.

[28] R. Takai, T. Kaneda, A. Isogai, S. Takayama, F.-S. Che, A new method of defense response analysis using a transient expression system in rice protoplasts, *Biosci. Biotechnol. Biochem.*, 71 (2007) 590-593.

[29] Z. Xie, Z.L. Zhang, X. Zou, G. Yang, S. Komatsu, Q.J. Shen, Interactions of two

abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells, Plant J, 46 (2006) 231-242.

[30] C. Ross, Q.J. Shen, Computational prediction and experimental verification of HVA1-like abscisic acid responsive promoters in rice (*Oryza sativa*), Plant Mol Biol, 62 (2006) 233-246.

[31] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res, 29 (2001) e45.

[32] W.B. Bruce, P.H. Quail, *cis*-Acting elements involved in photoregulation of an oat phytochrome promoter in rice., Plant Cell, 2 (1990) 1081-1089.

[33] Q. Shen, P. Zhang, T.H. Ho, Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley, Plant Cell, 8 (1996) 1107-1119.

[34] Z. Xie, Z.L. Zhang, X. Zou, J. Huang, P. Ruas, D. Thompson, Q.J. Shen, Annotations and functional analyses of the rice *WRKY* gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells, Plant Physiol, 137 (2005) 176-189.

[35] T. Chujo, N. Sugioka, Y. Masuda, N. Shibuya, T. Takemura, K. Okada, H. Nojiri, H. Yamane, Promoter analysis of the elicitor-induced WRKY gene OsWRKY53, which is involved in defense responses in rice, Biosci Biotechnol Biochem, 73 (2009) 1901-1904.

[36] Q. Shen, S.J. Uknes, T.H. Ho, Hormone response complex in a novel abscisic acid and cycloheximide-inducible barley gene, The Journal of biological chemistry, 268 (1993)

23652-23660.

[37] H. Shen, C. Liu, Y. Zhang, X. Meng, X. Zhou, C. Chu, X. Wang, OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice, *Plant Mol Biol*, 80 (2012) 241-253.

[38] H. Chen, Z. Lai, J. Shi, Y. Xiao, Z. Chen, X. Xu, Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress, *BMC plant biology*, 10 (2010) 281.

[39] L. Yan, Z.Q. Liu, Y.H. Xu, K. Lu, X.F. Wang, D.P. Zhang, Auto-and cross-repression of three Arabidopsis WRKY transcription factors WRKY18, WRKY40, and WRKY60 negatively involved in ABA signaling, *J. Plant Growth Regul.*, 32 (2013) 399-416.

[40] M. Fujita, Y. Fujita, Y. Noutoshi, F. Takahashi, Y. Narusaka, K. Yamaguchi-Shinozaki, K. Shinozaki, Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks, *Curr. Opin. Plant. Biol.*, 9 (2006) 436-442.

[41] J.P. Anderson, E. Badruzsaufari, P.M. Schenk, J.M. Manners, O.J. Desmond, C. Ehlert, D.J. Maclean, P.R. Ebert, K. Kazan, Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis, *Plant Cell*, 16 (2004) 3460-3479.

[42] M. Heinrich, C. Hettenhausen, T. Lange, H. Wünsche, J. Fang, I.T. Baldwin, J. Wu, High levels of jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of *Nicotiana attenuata* stems, *Plant J.*, 73 (2013) 591-606.

- [43] T. Chujo, K. Miyamoto, S. Ogawa, Y. Masuda, T. Shimizu, M. Kishi-Kaboshi, A. Takahashi, Y. Nishizawa, E. Minami, H. Nojiri, Overexpression of phosphomimic mutated OsWRKY53 leads to enhanced blast resistance in rice, *PLoS One*, 9 (2014) e98737.
- [44] A. Linkies, G. Leubner-Metzger, Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination, *Plant Cell Rep.*, 31 (2012) 253-270.
- [45] M. Weyhe, L. Eschen-Lippold, P. Pecher, D. Scheel, J. Lee, Ménage à trois: The complex relationships between mitogen-activated protein kinases, WRKY transcription factors and VQ-motif-containing proteins., *Plant Signal Behav*, 9 (2014) e29519.

7. Figure Legends

Figure 1. OsWRKY53 and -70 were localized to the nucleus.

Subcellular localization of OsWRKY53 and -70. Aleurone cells were transformed with UBI-GFP (panels A and D), UBI-GFP:OsWRKY53 (panels B and E), or UBI-GFP:OsWRKY70 (panels C and F). After incubation for 24 h, the aleurone cells were stained with SYTO61 for nuclear localization (panels D, E and F), followed by examination of GFP expression (panels A, B and C). Yellow arrows in panels A and E point to the same cell, as do those in panels B and E, and panels C and F. The bars represent 50 μ m.

Figure 2. OsWRKY24, -53 and -70 can bind to the W-box in the promoters of *Amy32b* and the three *WRKY* genes.

(A) The growth of cotransformants in media containing SD/-U/-L. (B) Detection of binding of OsWRKY24, -53, and -70 to the W boxes regions in different promoters. The growth of yeast colonies in selection media containing SD/-U/-L/+Aba indicates positive bindings. The names on the top represent different yeast strains containing different W boxes, and those on the left stand for OsWRKY24, -53, and -70 fused with BD domain.

Figure 3. OsWRKY24, -53, and -70 functioned as transcriptional activators in yeasts.

The GAL4 DNA binding domain(BD) was fused with full length OsWRKY24, -51, -53, -70, or their deletion derivatives, Δ 177W24 (deletion of 177 amino acids at the

N-terminus of OsWRKY24), $\Delta 145W53$, and $\Delta 181W70$. The constructs were transformed into yeast strain Y2Hgold containing the *Trp*, *AUR1-C* and *MEL1* reporter genes. Three independent experiments were performed and each showed similar patterns.

Figure 4. *WRKY24*, -53 and -70 were expressed most abundantly in aleurone layers and leaves.

Quantitative RT-PCR analysis of expression of *OsWRKY24*, -53 and -70 in aleurone, leaves, roots, embryos and panicles. The fold changes in gene expression were normalized to that of the *OsActin1* gene in the same sample using the $2^{-\Delta Ct}$ method. The error bars indicate the standard error of three replicates of each tissue/organ.

Figure 5. OsWRKY53 and OsWRKY70 suppressed ABA induction of *HVA22-GUS* and GA induction of *Amy32b-GUS*. (A) Schematic diagrams of the reporter and effector constructs used in the particle bombardment experiments. The grey box is the W-box region from -152 to -141 in the *Amy32b* promoter. (B) and (C) The effector constructs were co-bombarded into barley aleurone cells along with the internal control and the reporter constructs, *HVA22-GUS* (B) and *Amy32b-GUS* (C). GUS activity was normalized in each independent transformation relative to the luciferase activity. Bars indicate GUS activities $\pm$ standard error after 24 h of incubation of the bombarded half-seeds with or without 20 μ M ABA (B) or 1 μ M GA3 (C). Data are the mean $\pm$ standard error of four replicates. Different letters designate significant differences

between the pairs tested (Student's t-test, $P \leq 0.05$).

Figure 6. The combinatory suppression effect of OsWRKY24, -53 and -70 was additive in ABA and GA signaling. (A) Dosage effect of OsWRKY53, and -70 with a constant amount of the reporter construct and varying amounts of the effector constructs, as shown on the x-axis, in the presence of 1 μ M GA3 or 20 μ M ABA for 24 h. The ratio of 100% indicates the same molar ratio of effector and reporter DNA was used. (B) Different combinations of 15% effectors were co-bombarded into barley aleurone cells along with the reporter construct *Amy32b-GUS* (B) and *HVA22-GUS* (C), and the internal control construct *UBI-Luciferase*. GUS activity was measured after 24h incubation with or without 20 μ M ABA (B) or 1 μ M GA3 (C). Data are mean $\pm$ standard error of four replicates. Different letters (a, b and c) designate significant differences among groups tested (one-way ANOVA or Student's t-test, $P \leq 0.05$).

Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Figure 6