crops

MDPI

Article

Overexpression of *ONAC054* Improves Drought Stress Tolerance and Grain Yield in Rice

Yasuhito Sakuraba [1,*] and Nam-Chon Paek [2,*]

[1] Plant Functional Biotechnology, Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[2] Department of Agriculture, Forestry and Bioresources, Plant Genomics and Breeding Institute, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea
* Correspondence: sakuraba0425@gmail.com (Y.S.); ncpaek@snu.ac.kr (N.-C.P.)

Abstract: Drought stress negatively affects plant growth and development, thus reducing plant productivity. Therefore, understanding the molecular mechanisms underlying drought stress responses is essential for crop improvement. The plant-specific NAM/ATAF1,2/CUC2 (NAC) transcription factors play important roles in the drought stress response. Here, we show that rice (*Oryza sativa*) ONAC054, a membrane-bound NAC transcription factor, is involved in the drought stress response. We found that *onac054* mutants were sensitive, whereas *ONAC054*-overexpressing (*ONAC054*-OX) plants were tolerant to drought stress. Under drought stress conditions, several genes associated with abscisic acid (ABA) synthesis and signaling were downregulated in *onac054* mutants but upregulated in *ONAC054*-OX plants. Among these genes, the *TRANSCRIPTION FACTOR RESPONSIBLE FOR ABA REGULATION 1* (*TRAB1*), which encodes an ABA-inducible bZIP transcription factor, was directly activated by ONAC054. On the other hand, the expression of *ONAC054* was directly activated by several ABA-responsive elements (ABRE)-binding factors (ABFs) in an ABA-dependent manner, indicating that ONAC054 acts as an enhancer of ABA-induced drought stress tolerance. Additionally, the overexpression of *ONAC054* in rice greatly improved grain yield under drought stress conditions, indicating that the overexpression of *ONAC054* could facilitate the improvement of drought stress tolerance in rice and other crops.

Keywords: rice; drought stress; NAM/ATAF1,2/CUC2 (NAC)

Citation: Sakuraba, Y.; Paek, N.-C. Overexpression of *ONAC054* Improves Drought Stress Tolerance and Grain Yield in Rice. *Crops* **2022**, *2*, 390–406. https://doi.org/10.3390/crops2040027

Academic Editor: Malgorzata Szostek

Received: 9 September 2022
Accepted: 18 October 2022
Published: 20 October 2022

1. Introduction

In an agricultural field and a natural ecosystem, environmental stresses such as salinity, extreme temperatures, drought, and nutrient deficiency seriously threaten plant health by negatively affecting plant survival, biomass production, and crop yield, thus reducing the average yield of most major crop plants by more than 50% worldwide [1,2]. Since the area of water deficiency-affected land is dramatically increasing across the world, plant breeding has emerged as one of the most effective methods for the development of drought-tolerant crop plants. Extensive research conducted to date shows that plants employ highly complex adaptation mechanisms, intricately regulated by multiple genes, proteins, and metabolites, to survive under water deficit conditions [3]. For instance, drought stress triggers the accumulation of phytohormones, such as abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA), and other metabolites, including sugar derivatives and amino acids (such as proline and glutamine) in plants, which facilitates their acclimation to drought stress [4–6]. Furthermore, drought stress modulates the expression of genes associated with drought stress responses [7], and a number of studies show that altering the expression or activity of such drought stress-responsive genes can lead to the development of drought-tolerant plants [3,8]. For instance, some ABA-inducible bZIP transcription factors (TFs), called ABA-responsive element (ABRE)-binding factors (ABFs), interact with the ABRE motif in the promoters of downstream genes to induce their expression [9].

In *Arabidopsis thaliana*, dehydration and/or ABA treatment strongly upregulated three *ABF* genes: *ABF2/ABRE-BINDING PROTEIN 1* (*AREB1*), *ABF3*, and *ABF4/AREB2* [10]. In addition, transgenic Arabidopsis plants overexpressing these *ABF* genes exhibited greater tolerance to drought stress than the wild type [11,12].

The NAM/ATAF1,2/CUC2 (NAC) proteins comprise one of the largest families of plant-specific TFs, encoded by 106 and 149 genes in the model plant species Arabidopsis and rice (*Oryza sativa*), respectively [13,14]. NAC proteins harbor a highly conserved DNA-binding domain, termed the NAC domain, in their N-terminal region [15]. However, the C-terminal region of NAC family proteins is diverse, suggesting that the C-terminal region plays a critical role in determining the *cis*-element-specificity of NACs in their respective target genes. Furthermore, some NAC TFs harbor a transmembrane domain (TMD) in their C-terminal region, which determines their subcellular localization. Under moderate growth conditions, TMD-containing NAC TFs localize to the endoplasmic reticulum (ER); however, under specific stress conditions, the TMD is cleaved off, which allows the NAC TFs to translocate to the nucleus. Thus, the NAC proteins exhibit conditional TF activity [16,17].

NAC TFs are involved in multiple developmental processes, such as shoot meristem development [18], lateral root formation [19], phytohormone signaling [19,20], and leaf senescence [21,22]. In addition, several NAC TFs in Arabidopsis and crop plants are involved in drought stress responses. Three Arabidopsis *NAC* genes, *ANAC019*, *ANAC055*, and *ANAC072*, were induced by the drought stress treatment, and transgenic plants overexpressing these *NAC* genes exhibited increased tolerance to drought stress, indicating that these three NAC TFs act as enhancers of drought stress responses [23]. The Arabidopsis NAC TF ANAC096 also positively regulates the drought stress response by specifically binding to the ABRE motif [12] in the promoters of several drought stress-responsive genes [24]. Similarly, in rice, overexpression of several *NAC* genes, including *STRESS-RESPONSIVE NAC1*, *ONAC045*, and *ONAC066*, which have been identified as positive regulators of drought stress responses, improved drought stress tolerance [25–27]. Several Arabidopsis TMD-containing NAC TFs, such as ANAC016, are also involved in drought stress responses; *anac016* knockout mutants exhibited tolerance to drought stress, whereas *ANAC016*-overexpressing transgenic plants were sensitive to drought stress [28]. Under drought stress conditions, TMD cleavage allows ANAC016 to translocate to the nucleus, where it directly represses the transcription of *AREB1* [28], which encodes a key TF involved in the stress-responsive ABA signaling pathway [12]. In Arabidopsis, other TMD-containing NAC TFs, including NAC WITH TRANSMEMBRANE MOTIF 1-LIKE4 (NTL4) and NTL6, also act as positive and negative regulators of drought stress responses [29,30]. Together, these studies indicate that TMD-containing NAC TFs play crucial roles in drought stress responses. Nevertheless, the functions of TMD-containing NAC TFs in crop plants are not yet fully understood.

We previously showed that a rice TMD-containing NAC TF, ONAC054, plays an important role in the regulation of ABA-induced leaf senescence. We reported that the nuclear import of ONAC054 requires the ABA-dependent cleavage of its C-terminal TMD [31]. Once in the nucleus, ONAC054 directly activates the transcription of *ABA INSENSITIVE5* (*OsABI5*), encoding a key TF involved in ABA-mediated leaf senescence [32], and *NON-YELLOWING COLORING1*, encoding a chlorophyll catabolic enzyme [33]. Furthermore, the *ONAC054* transcript (termed *ONAC054α*) has an alternatively spliced variant, *ONAC054β*, which carries a 7-nt insertion in-between the sequences corresponding to intron 5 and exon 6 of the *ONAC054* gene. The insertion of these 7-nt creates a premature stop codon in the deduced amino acid sequence, resulting in the production of the TMD-lacking ONAC054β protein [31]. While both *ONAC054α* and *ONAC054β* are strongly induced by ABA, the transcript level of *ONAC054β* increases at a much faster pace than that of *ONAC054α*, indicating that the two *ONAC054* isoforms form an intricate regulatory cascade to control the induction of ABA-induced leaf senescence.

In this study, we show that ONAC054 also participates in drought stress responses by modulating the expression of a number of genes associated with ABA metabolism and

signaling. Additionally, a subset of rice ABF TFs, including OsbZIP23, OsABF2, OsABF4, and TRAB1, could bind to the ABRE sequence in the *ONAC054* gene promoter and directly activate its transcription. Furthermore, overexpression of *ONAC054α* and *ONAC054β* greatly improved rice grain yield under drought stress conditions. Finally, we discuss the significance of the OsABF–ONAC054 regulatory module in the drought stress response in rice.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Plants of *onac054* T-DNA insertion knockout mutant lines (*onac054-1*, PFG_3A-07241; *onac054-2*, PFG_3A-07240), *ONAC054*-overexpressing transgenic lines (*ONAC054α*-OX and *ONAC054β*-OX), and parental wild-type (WT) *japonica* rice (*Oryza sativa*) cultivar Dongjin were grown in a growth chamber at 28 °C/25 °C day/night temperature under long-day (LD; 16 h light/8 h dark) photoperiod and cool-white fluorescent light (400 μmol m^{-2} s^{-1}). *Onac054* knockout (*onac054*-KO) mutant lines, which were obtained from the Crop Biotech Institute at Kyung Hee University (South Korea), and *ONAC054*-OX lines have been described previously [31].

2.2. Abiotic Stress Treatments

Rice seedlings were initially grown in soil (Honenagri Co., Ltd., Niigata, Japan) in a growth chamber for 14 days under LD conditions. Then, seedlings were subjected to dehydration or treated with 200 mM PEG6000 (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. During the 5 days of dehydration stress, leaves were collected on the indicated days and used for further experiments. Subsequently, the seedlings were rehydrated, and their survival rate was determined 3 days after rehydration.

2.3. Measurement of Physiological Traits

The ion leakage rate of seedlings was measured as described previously [34]. In brief, three 1 cm^2 leaf discs from each treatment were immersed in 6 mL of 0.4 M mannitol at room temperature for 3 h with gentle shaking. The initial conductivity of the solution was measured with a conductivity meter (CON6METER). Then, the samples were incubated at 85 °C for 20 min. The ion leakage rate was calculated by dividing the initial conductivity by the total conductivity and expressed as a percentage.

The malondialdehyde (MDA) content of seedlings was determined as described previously [35].

2.4. RNA Extraction and Gene Expression Analysis

Total RNA was isolated from rice seedlings using the ISOSPIN Plant RNA Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript^{TM} II reverse transcriptase and oligo$(dT)_{15}$ primers (Invitrogen, Waltham, MA, USA) in a 20 μL reaction mixture. Then, quantitative PCR with reverse transcription (RT-qPCR) was performed on the StepOnePlus System (Applied Biosystems, Waltham, MA, USA), with each reaction containing 1 μL of reverse transcriptase, 5 μL of 2× KAPA SYBR Fast qPCR mixture (KAPA biosystems, Waltham, MA, USA), and 0.25 mM of forward and reverse gene-specific primers (Table S1). Transcript levels of each gene were normalized relative to those of *UBIQUITIN 5* (*OsUBQ5*).

2.5. Chromatin Immunoprecipitation (ChIP) Assays

The *35S:GFP-ONAC054α* and *35S:GFP-ONAC054β* plasmids, previously constructed in the pMDC43 binary vector [36], were transfected into rice protoplasts, as described previously [37]. The transfected protoplasts were then subjected to crosslinking for 20 min in 1% (*v*/*v*) formaldehyde under a vacuum. The chromatin complexes were isolated and sonicated, as previously described [38], with slight modifications. After crosslinking and nuclei isolation/lysis, DNA was subjected to ten 30 s sonication pulses using BIORUPTOR

II (Cosmo Bio, Tokyo, Japan) at high power mode, with a 30 s interval between two pulses. Protein G agarose conjugated with anti-Myc polyclonal antibody (Millipore, Waltham, MA, USA) was used for immunoprecipitation. DNA recovered from agarose beads was purified using the DNeasy Plant Mini Kit (Qiagen, Hilden, The Netherlands). Then, quantitative PCR (qPCR) was performed using sequence-specific primers (Table S1), as described above.

2.6. Protoplast Transient Expression Assay

To construct reporter plasmids, the promoter fragment of *TRAB1* (−2000 to −1 bp, relative to the translation start site) was cloned into the pJD301 vector upstream of the *luciferase* (*LUC*) reporter gene [39]. To construct effector plasmids, the cDNAs of *OsbZIP23*, *OsABF2*, *OsABF4*, *TRAB1*, and *OsABI5* were first individually cloned into the pCR8/GW/TOPO Gateway vector (Invitrogen, Waltham, MA, USA), and then cloned upstream of a sequence encoding four copies of the MYC epitope tag in the pGWB17 binary vector [40]. The reporter (4 μg) and effector plasmids (8 μg) were co-transfected into 5×10^4 rice protoplasts by the polyethylene glycol (PEG)-mediated transfection method [41]. The transfected protoplasts were suspended in a protoplast culture medium (0.4 mM mannitol, 4 mM MES buffer, and 15 mM $MgCl_2$ [pH 5.8]), and incubated in the dark for 16 h. Then, LUC activity in each cell lysate was determined using the luciferase assay system kit (Promega, Madison, WI, USA).

2.7. Quantification of Chlorophyll Pigment

Chlorophyll pigments were extracted (using 80% [*v*/*v*] ice-cold acetone) from leaf discs homogenized with zirconia beads (Nikkato, Osaka, Japan). The absorbance of extracts was measured at 647 and 664 nm, and chlorophyll contents were calculated as described previously [42].

2.8. Measurements of Agronomic Traits

To measure the agronomic traits of rice plants under normal growth conditions, plants were grown in soil (Honenagri Co., Ltd., Niigata, Japan) in a growth chamber under LD conditions for 150 days. To measure the agronomic traits of plants under drought stress conditions, plants were initially grown in soil in a growth chamber under LD conditions for 90 days, then dehydrated for 7 days, and subsequently rehydrated for 3 days. These processes were repeated six times before measuring the agronomic traits.

2.9. Accession Numbers

Sequence data used in this study can be found in the National Center for Biotechnology Information (NCBI) under the following accession numbers: *ONAC054*, Os03g0119966; *OsABA1*, Os04g0448900; *OsABA3*, Os06g0670000; *OsABF1*, Os01g0867300; *OsABF2*, Os06g0211200; *OsABF4*, Os09g0456200; *TRAB1*, Os08g0472000; *OsCYP707A6*, Os08g0472800; *OsCYP707A7*, Os09g0457100; *OsLEA3*, Os05g0542500; *OsABI1*, Os05g0572700; *OsABI2*, Os01g0513100; *OsABI3*, Os01g0911700; *OsABI4*, Os05g0351200; *OsABI5*, Os01g0859300; *OsbZIP23*, Os02g0766700; *OsRAB16a*, Os11g0454300; *OsUBQ5*, Os01g0328400.

3. Results

3.1. Overexpression of ONAC054 Enhanced Tolerance to Drought Stress in Rice Seedlings

Recently, we showed that rice *onac054*-KO mutants exhibited accelerated leaf yellowing, while the leaves of *ONAC054*-OX lines retained greenness during ABA-induced leaf senescence [31]. Since ABA is one of the phytohormones that enhances the tolerance to drought stress [6], it is probable that ONAC054 also functions in drought stress responses. To examine this possibility, we first examined and monitored the expression levels of *ONAC054α* and *ONAC054β* in seedling leaves sampled during the dehydration stress treatment. The transcript levels of both *ONAC054α* and *ONAC054β* were significantly increased during dehydration stress. However, their expression patterns during dehydration stress were somewhat different: *ONAC054β* was induced much faster than *ONAC054α*, while

the expression of *ONAC054α* was much higher than that of *ONAC054β* in the later stage of dehydration (Figure 1A), consistent with their expression patterns during the ABA treatment [31]. The application of high-molecular-weight PEG is known to disrupt the absorption of water by plant roots, causing drought stress in shoots [43]. Similar to their expression patterns during dehydration stress, *ONAC054α* and *ONAC054β* were strongly induced by the PEG treatment (Figure 1B), indicating that both ONAC054 isoforms are associated with drought stress responses.

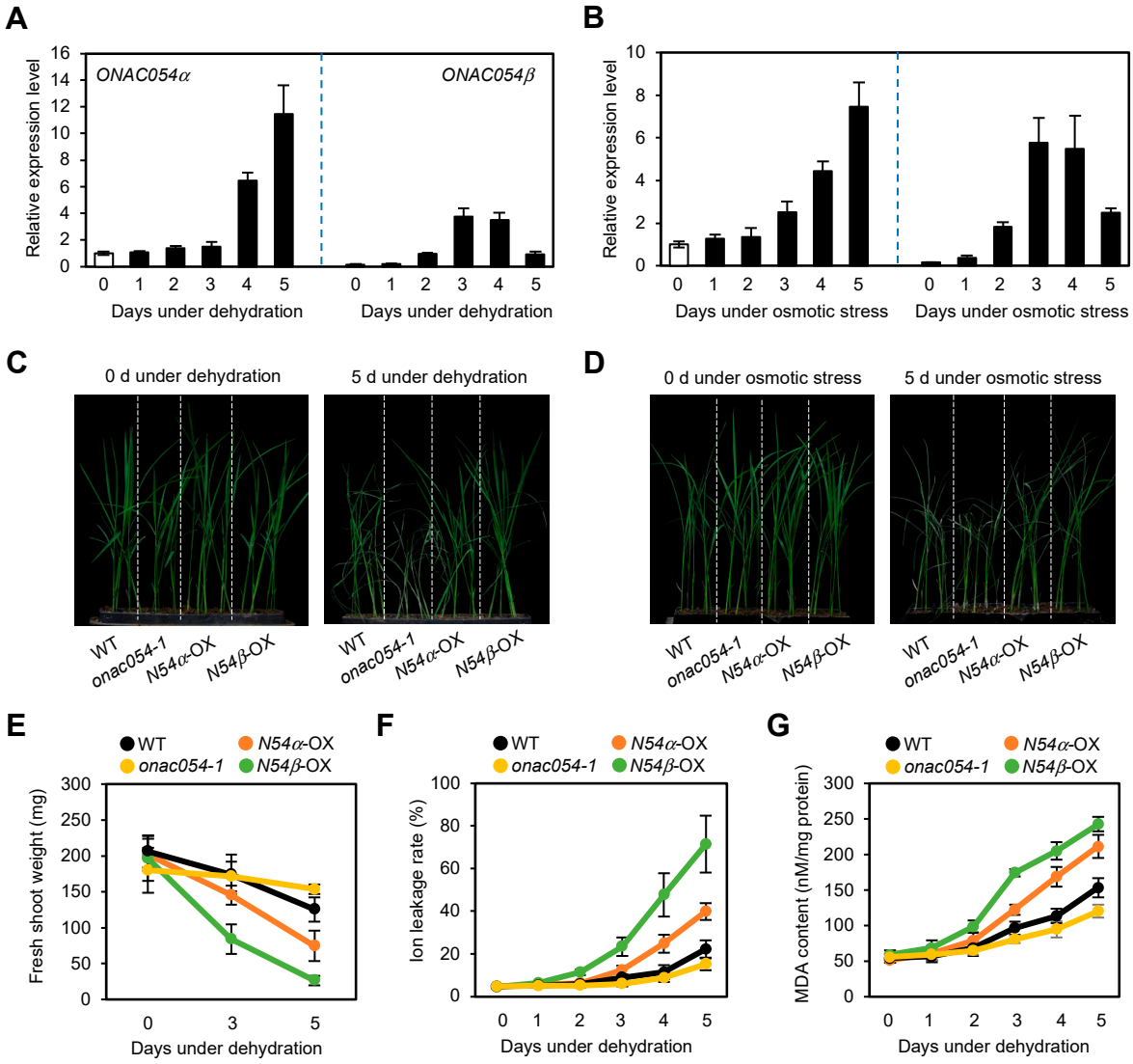

Figure 1. Overexpression of *ONAC054α* and *ONAC054β* enhanced the tolerance to drought stress. (**A**,**B**) Expression patterns of *ONAC054α* and *ONAC054β* in the leaves of Dongjin (WT) seedlings under dehydration (**A**) and osmotic (**B**) stress. Gene expression levels were examined every day for 5 days after the start of each treatment. Transcript levels of *ONAC054α* and *ONAC054β* were normalized first against *OsUBQ5* transcript levels, and then against the values obtained at time zero. (**C**,**D**) Phenotype of Dongjin (WT), *onac054-1*, *ONAC054α*-OX (*N54α*-OX), and *ONAC054β*-OX (*N54β*-OX) seedlings under normal growth conditions (**C**) and drought stress conditions (**D**). Seedlings were initially grown in soil for 14 days under normal conditions. Then, drought stress was induced for 5 days by either withholding watering or treating the seedlings with 200 mM PEG. (**E**–**G**) Changes in fresh shoot weight (**E**), ion leakage rate (**F**), and MDA content (**G**) of Dongjin (WT), *onac054-1*, *ONAC054α*-OX (*N54α*-OX), and *ONAC054β*-OX (*N54β*-OX) seedlings under dehydration stress for 5 days. Data represent the mean ± standard deviation (SD) of four biological replicates (**A**,**B**) or six biological replicates (**E**–**G**).

Next, we examined whether the *onac054*-KO mutant lines *onac054-1* and *ONAC054*-OX lines (*ONAC054α*-OX and *ONAC054β*-OX), which were used in our previous study [31], were susceptible or tolerant to drought stress. Under normal growth conditions, the growth phenotype of *onac054-1*, *ONAC054α*-OX, and *ONAC054β*-OX seedlings was similar to that of the WT (Dongjin). However, after dehydration for 5 days, the *onac054-1* seedlings exhibited severe wilting symptoms, whereas *ONAC054α*-OX and *ONAC054β*-OX seedlings exhibited strong drought tolerance compared with WT seedlings (Figure 1C). Similar susceptible and tolerant phenotypes of *onac054-1* and *ONAC054*-OX seedlings, respectively, were also observed during the PEG treatment (Figure 1D). Consistent with the growth phenotype data, the fresh shoot weight of *onac054-1* dramatically decreased, while *ONAC054α*-OX and *ONAC054β*-OX seedlings were unaffected by dehydration compared with the WT (Figure 1E). Furthermore, we measured the ion leakage rate and malondialdehyde (MDA) content, which are used as parameters of membrane damage caused by drought stress. Compared with the WT, the ion leakage rate and MDA content of *osnac054-1* seedlings were dramatically higher, while those of *ONAC054α*-OX and *ONAC054β*-OX seedlings were much lower under dehydration stress (Figure 1F,G), indicating that the cell membrane of *osnac054-1* seedlings was highly unstable. The dehydration susceptible phenotype of the *onac054*-KO mutant was also confirmed in *onac054-2*. Similar to *onac054-1*, the *onac054-2* mutant showed a significantly higher ion leakage rate and a faster decline in fresh shoot weight than the WT (Figure S1). To evaluate the effects of the KO mutation and overexpression of *ONAC054* on drought stress tolerance, we determined the survival rate of *onac054-1* and *ONAC054*-OX seedlings after 3 days of rehydration. While most of the *onac054-1* seedlings failed to recover, the *ONAC054α*-OX and *ONAC054β*-OX seedlings showed a significantly higher survival rate than WT seedlings (Figure S2). Taken together, these results suggest that both ONAC054 isoforms act as enhancers of drought stress tolerance in rice.

3.2. ONAC054 Upregulates Genes Associated with Drought Stress Responses

We previously showed that the knockout mutation of *ONAC054* modulates the expression levels of a number of genes associated with ABA metabolism and signaling, especially during dark- and ABA-induced leaf senescence [31]. To understand how ONAC054 participates in the drought stress responses, we examined the effects of the KO mutation and overexpression of *ONAC054* on the expression of four genes associated with ABA metabolism (*OsABA1*, *OsABA3*, *CYTOCHROME P450*, *FAMILY 707*, *SUBFAMILY A*, *POLYPEPTIDE 3* [*OsCYP707A6*], and *OsCYP707A7*) and twelve genes associated with ABA signaling (*OsLEA3*, *OsABI1–5*, *OsbZIP23*, *OsABF1*, *OsABF2*, *OsABF4*, *TRAB1*, and *RESPONSIVE TO ABA 16A* [*OsRAB16a*]) by real-time quantitative PCR. Most genes examined were highly upregulated after 4 days of dehydration, whereas the expression levels of *CYP707A6* and *CYP707A7*, encoding ABA catabolic enzymes [44], were not significantly changed (Figure 2). Several genes, including *OsLEA3*, *OsABI5*, *OsbZIP23*, *OsABF4*, and *TRAB1*, were strongly downregulated in *onac054-1* but upregulated in *ONAC054α*-OX and *ONAC054β*-OX seedlings after 4 days of dehydration. Moreover, even before the dehydration, these genes were significantly upregulated in *ONAC054β*-OX but not in *ONAC054α*-OX, indicating that dehydration stress controls the cleavage of TMD from ONAC054, as observed previously in the ABA treatment [31].

3.3. ONAC054 Directly Activates TRAB1 Transcription

We previously showed that ONAC054 specifically interacts with the CTTGXXXXXCA[C/A] sequence, named the mitochondrial dysfunction motif (MDM) [45], in target gene promoters to regulate their expression [31]. ONAC054 binds to the MDM sequence in the promoter of *OsABI5* and enhances its expression [31]. However, ONAC054 does not bind to the *OsABF4* promoter, which harbors an MDM variant (CTAGAACTTCAAG), indicating that ONAC054 indirectly enhances the expression of *OsABF4* [31].

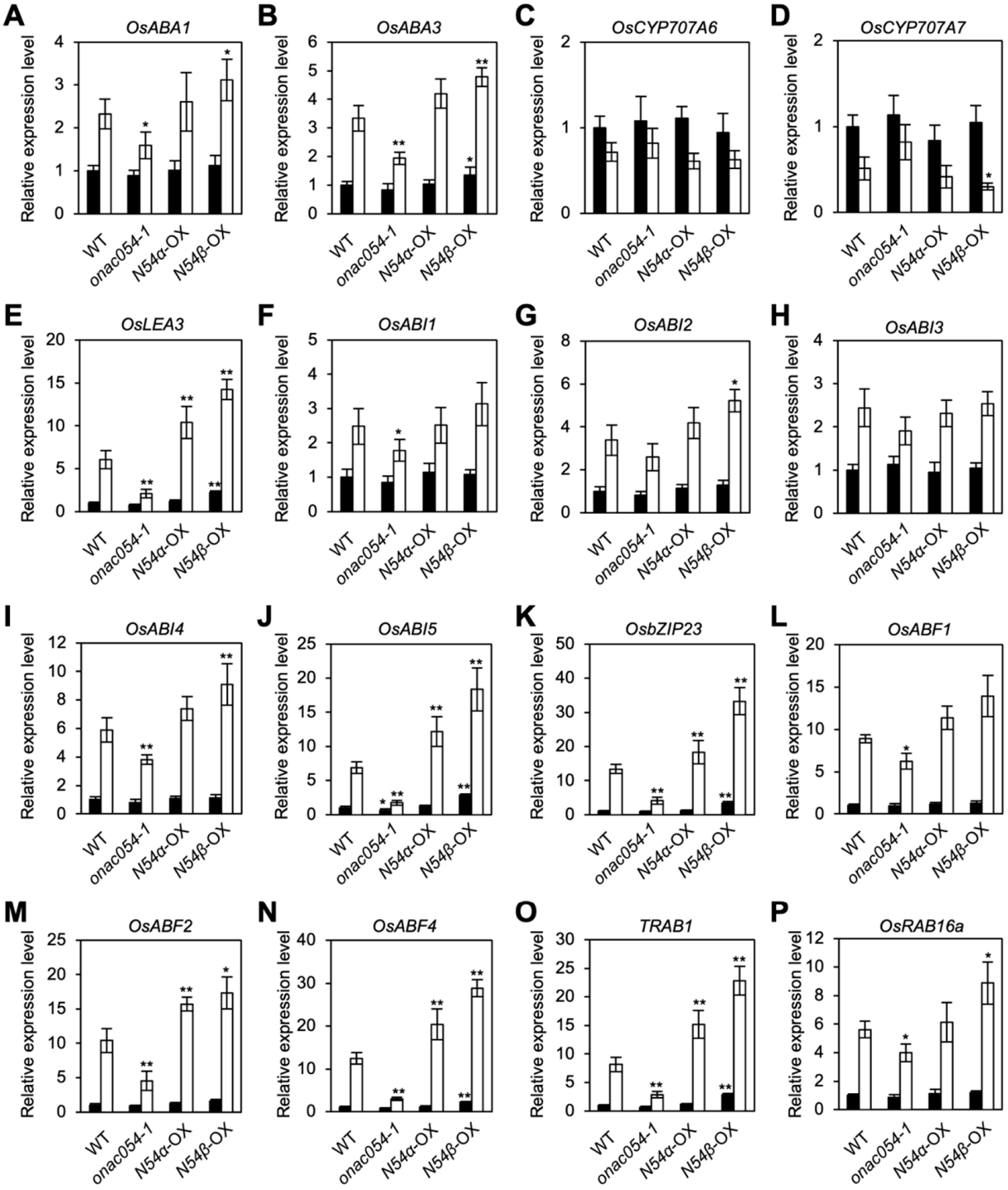

Figure 2. Effects of the KO mutation and overexpression of *ONAC054* on the transcript levels of genes associated with ABA metabolism and signaling. (**A**–**P**) Transcript levels of genes associated with ABA metabolism, including *OsABA1* (**A**), *OsABI3* (**B**), *OsCYP707A6* (**C**), and *OsCYP707A7* (**D**), and with ABA signaling, including *OsLEA3* (**E**), *OsABI1* (**F**), *OsABI2* (**G**), *OsABI3* (**H**), *OsABI4* (**I**), *OsABI5* (**J**), *OsbZIP23* (**K**), *OsABF1* (**L**), *OsABF2* (**M**), *OsABF4* (**N**), *TRAB1* (**O**), and *OsRAB16a* (**P**), in Dongjin (WT), *onac054-1*, *ONAC054α*-OX (*N54α*-OX), and *ONAC054β*-OX (*N54β*-OX) seedlings before (white bars) and after 4 days of dehydration (black bars). The transcript level of each gene was normalized first against that of *OsUBQ5*, and then against the value obtained in WT seedlings before dehydration. Data represent the mean ± SD of four biological replicates. Asterisks (* $p < 0.05$; ** $p < 0.01$) indicate significant differences identified between the WT and other genotypes by Student's *t*-test.

According to the results of RT-qPCR analysis (Figure 2), in addition to *OsABI5* and *OsABF4*, three genes associated with ABA signaling (*OsLEA3*, *OsbZIP23*, and *TRAB1*) were strongly downregulated in *onac054-1* seedlings but upregulated in *ONAC054*-OX lines (Figure 2). To explain this result, we examined the promoter sequences of *OsLEA3*, *OsbZIP23*, and *TRAB1*. An MDM variant (CTAGGCCGCCACG) was found in the promoter of *TRAB1* (Os08g0472000; −546 to −534 bp, relative to the translation start site) (Figure 3A); however, *OsLEA3* and *OsbZIP23* promoters showed neither an MDM nor its variant.

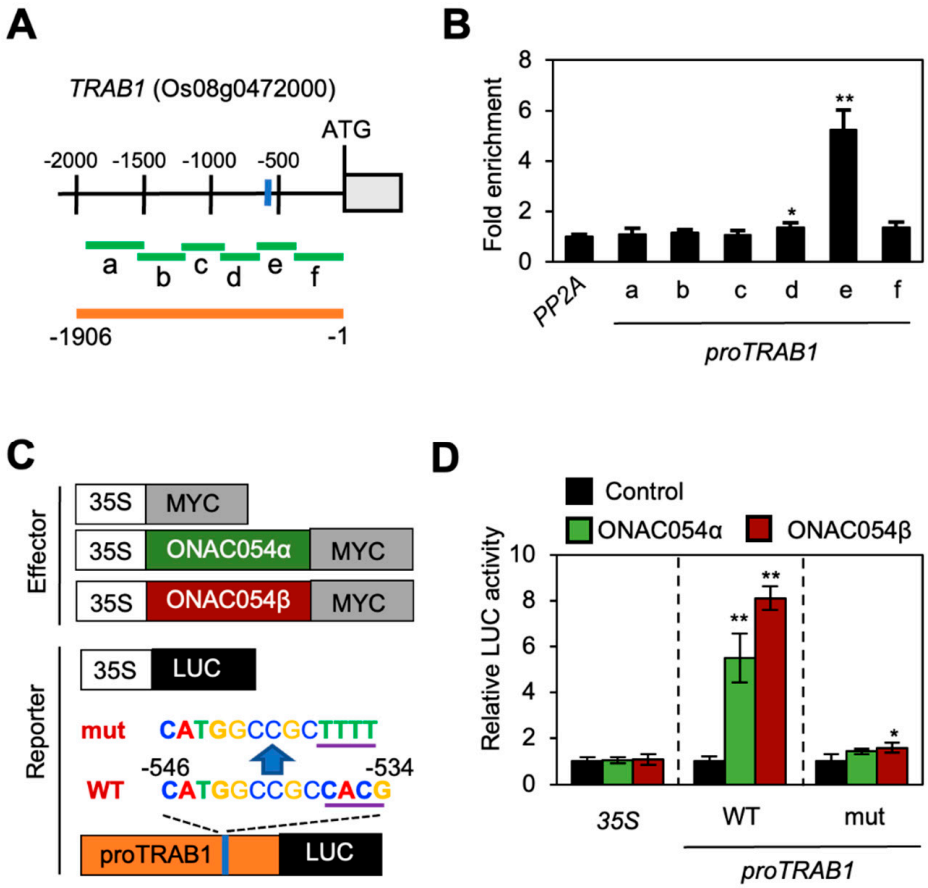

Figure 3. ONAC054 directly activates *TRAB1* expression. (**A**) Structure of the *TRAB1* promoter. Regions amplified in the ChIP assay and used for the protoplast co-transfection assay are indicated using green and orange horizontal bars, respectively. The MDM variant is indicated by a blue vertical bar. (**B**) Analysis of the binding of ONAC054 (ONAC054β) to the *TRAB1* promoter in planta using the ChIP assay. GFP-ONAC054β was transiently expressed in protoplasts isolated from 10-day-old WT seedlings. Fold enrichment of promoter fragments was measured by immunoprecipitation with an anti-GFP antibody (see Materials and Methods). Enrichment of the promoter region of an unrelated gene, *PP2A*, served as a negative control. Asterisks above each bar indicate significant differences between *PP2A* and other samples (* $p < 0.05$; ** $p < 0.01$; Student's *t*-test). (**C**) Effector and reporter constructs were used to perform the protoplast transient expression assay. Each construct also contained the *NOS* terminator (not shown). (**D**) Activation of WT and mutant versions of the *TRAB1* promoter (*proTRAB1*; −1906 to −1 bp, relative to the translation start site) by ONAC054α and ONAC054β in protoplasts. The *35S* promoter and empty expression vector (*35S:MYC*) served as negative controls. Asterisks indicate significant differences compared with the *35S:MYC* samples (* $p < 0.05$; ** $p < 0.01$; Student's *t*-test). Data in (**B**,**D**) represent the mean ± SD of four reaction mixtures.

To examine whether ONAC054 binds to the *TRAB1* promoter, we performed a ChIP assay using rice protoplasts transiently expressing *GFP-ONAC054α*. Region **e** of the *TRAB1* promoter, which contained the MDM variant (Figure 3A), was highly enriched in the immunoprecipitate, while other regions showed no enrichment (Figure 3B).

Next, to determine the effect of ONAC054 on *TRAB1* expression, we performed a co-transfection assay using rice protoplasts. Both *GFP-ONAC054α* and *GFP-ONAC054β* fusions were used to generate the effector constructs. On the other hand, the WT *TRAB1* promoter and its mutant version (in which the MDM variant sequence, CTAGGCCGC-CACG, was changed to CTAGGCCGCTTTT) were individually fused to the *LUC* reporter gene to generate the reporter constructs (Figure 3C). Upon protoplast co-transfection, both ONAC054α and ONAC054β activated the WT *TRAB1* promoter sequence, although ONAC054β showed a stronger effect (Figure 3D). However, the mutated *TRAB1* promoter was hardly activated by *GFP-ONAC054α* and *GFP-ONAC054β* co-transfection, indicating that ONAC054 activates the *TRAB1* genes by directly binding to the MDM variant in their promoter.

3.4. ONAC054 Expression Is Regulated by Multiple ABA bZIP TFs

In previous studies and the current study, we showed that ONAC054 modulates the expression of several genes associated with ABA metabolism and signaling as well as those involved in drought stress responses, especially ABA-induced leaf senescence and dehydration [31] (Figure 2). Nonetheless, it remains unclear how the expression of *ONAC054* (both *ONAC054α* and *ONAC054β*) is induced by ABA and dehydration stress treatments. To understand the regulation of *ONAC054* expression, we first checked the promoter sequence of *ONAC054* (−2000 to −1 bp, relative to the translation start site). The results revealed the presence of an ABRE core sequence, ACGTG, in the *ONAC054* promoter (Figure 4A).

Some ABA-inducible bZIP TFs, including ABFs, interact with the ABRE motif in the promoters of ABA-inducible genes to modulate their expression [9]. Moreover, five rice bZIP TFs, OsbZIP23, OsABF2, OsABF4, TRAB1/OsABF5, and OsABI5, are phylogenetically close to Arabidopsis ABF TFs [46] and are involved in ABA-mediated stress responses [46–49]. To examine whether these bZIP TFs interact with the *ONAC054* promoter, we performed a ChIP assay using rice protoplasts transiently expressing *OsbZIP23-MYC*, *OsABF2-MYC*, *OsABF4-MYC*, *TRAB1-MYC*, or *OsABI5-MYC*. The results indicated that OsbZIP23, OsABF2, OsABF4, and TRAB1 bind to the region **e** of the *ONAC054* promoter, which contains an ABRE motif (−724 to −720 bp, relative to the translation start site), but OsABI5 does not (Figure 3B).

We subsequently performed co-transfection experiments using protoplasts isolated from ABA-treated or -untreated WT seedlings, expressing various combinations of reporter and effector plasmids (Figure 3C). OsbZIP23, OsABF2, OsABF4, and TRAB1 enhanced the activity of the *ONAC054* promoter, whereas OsABI5 did not (Figure 3D). Notably, the activation of the *ONAC054* promoter by these bZIP TFs was much stronger in protoplasts treated with ABA than in those not subjected to the ABA treatment (Figure 3D). Because four bZIP TFs could bind to the ABRE motif-containing region e of the *ONAC054* promoter (Figure 3B), we performed protoplast transient assays to examine whether these bZIP TFs could activate the mutated *ONAC054* promoter, in which the ABRE sequence (ACGTG) was changed to AAAAA (Figure 3E). We found that the mutated *ONAC054* promoter was not strongly activated by co-transfection with *OsbZIP23-MYC*, *OsABF2-MYC*, *OsABF4-MYC*, or *TRAB1-MYC* (Figure 3F). Taken together, these results suggest that *ONAC054* expression is upregulated by several ABFs.

3.5. A Sequence Downstream of the ONAC054 Coding Region Is Not Required for ABA Induction

While we showed that both *ONAC054α* and *ONAC054β* were strongly upregulated by dehydration, osmotic stress, and ABA treatment (Figure 1A,B) [31], the timing of their induction was somewhat different; *ONAC054β* was induced much faster than *ONAC054α* (Figure 1A,B). This may be because the 3′-untranslated region (3′-UTR) of *ONAC054β* is different from that of *ONAC054α*, since *ONAC054β* carries a 7-nt insertion between intron 5 and exon 6, leading to a frameshift mutation and consequently the insertion of a premature stop codon [31] (Figure 5A). Moreover, the *cis* elements responsible for gene

expression could be located downstream of the coding sequence of *ONAC054β*, as in the case of Arabidopsis *NITRATE REDUCTASE1* (*NIA1*), which contains nitrate-responsive *cis* elements downstream of its stop codon [50]. To examine this possibility, we performed protoplast transient expression assays using reporter plasmids in which the 3′-end of *ONAC054α* or *ONAC054β* was fused downstream of the *LUC* gene under the control of the *ONAC054* promoter (−2000 to −1, relative to the translation start site) (Figure 5B). While LUC activity was induced in protoplasts transformed with both *proONAC054-LUC-3′-ONAC054α* and *proONAC054-LUC-3′-ONAC054β* after the ABA treatment, as in protoplasts transformed with *proONAC054-LUC*, the LUC activity showed no significant differences among *proONAC054-LUC*, *proONAC054-LUC-3′-ONAC054α*, and *proONAC054-LUC-3′-ONAC054β* protoplasts (Figure 5C), indicating that the difference between the 3′-UTRs of *ONAC054α* and *ONAC054β* is not related to the differential expression patterns of the two *OsNAC054s* under dehydration stress and ABA treatments.

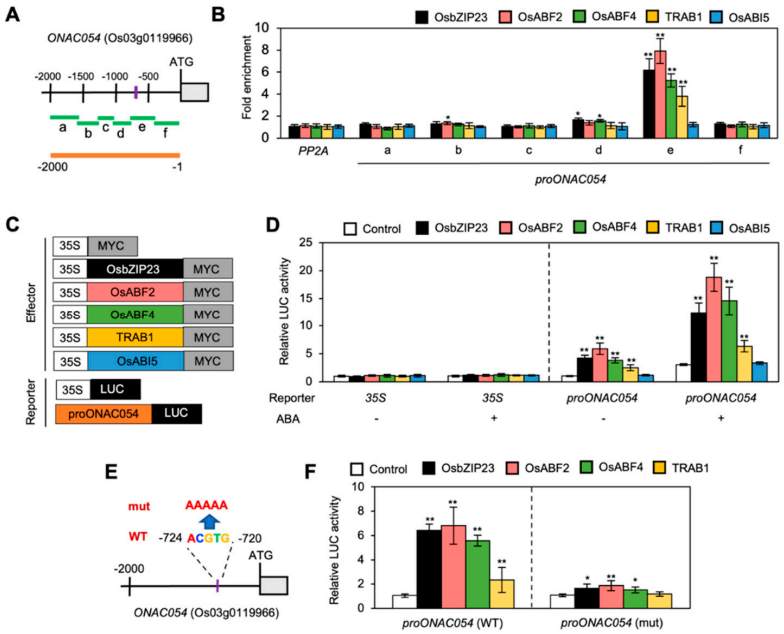

Figure 4. ABF transcription factors directly activate *ONAC054*. (**A**) Structure of the *ONAC054* promoter. Regions amplified in the ChIP assay (regions a, b, c, d, e, and f) and used for the protoplast co-transfection assay are indicated using green and orange horizontal bars, respectively. The ABRE motif is indicated by a purple vertical bar. (**B**) Analysis of the binding of OsbZIP23, OsABF2, OsABF4, TRAB1, and OsABI5 to *ONAC054* promoter in planta using ChIP assay. OsbZIP23-MYC, OsABF2-MYC, OsABF4-MYC, TRAB1-MYC, and OsABI5-MYC were transiently expressed in protoplasts isolated from 10-day-old WT seedlings. Fold enrichment of promoter fragments was measured by immunoprecipitation with an anti-MYC antibody (see Materials and Methods). Enrichment of the promoter region of an unrelated gene, *PP2A*, served as a negative control. Asterisks above each bar indicate significant differences between *PP2A* and other promoter fragments of *ONAC054* (* $p < 0.05$; ** $p < 0.01$; Student's *t*-test). (**C**) Effector and reporter constructs are used in the protoplast transient expression assay. Each construct also contained the *NOS* terminator (not shown). (**D**) Activation of the *ONAC054* promoter (−2000 to −1 bp, relative to the translation start site) by OsbZIP23, OsABF2, OsABF4, TRAB1, and OsABI5 in protoplasts treated with or without 10 μM ABA. The *35S* promoter and empty expression vector (*35S:MYC*) served as negative controls. Asterisks indicate significant differences compared with *35S:MYC* samples (** $p < 0.01$; Student's *t*-test). (**E**) Structure of a mutated *ONAC054* promoter. In *proNAC054* (mut), the ABRE motif ACGTG (−724 to −1 bp, relative to the translation start site) was changed to AAAAA. (**F**) Activation of WT and mutated *ONAC054* promoters by OsbZIP23, OsABF2, OsABF4, and TRAB1 in protoplasts. Asterisks indicate significant differences compared with *35S:MYC* samples (* $p < 0.05$; ** $p < 0.01$; Student's *t*-test). Data in (**B**,**D**,**F**) represent the mean ± SD values of four biological replicates.

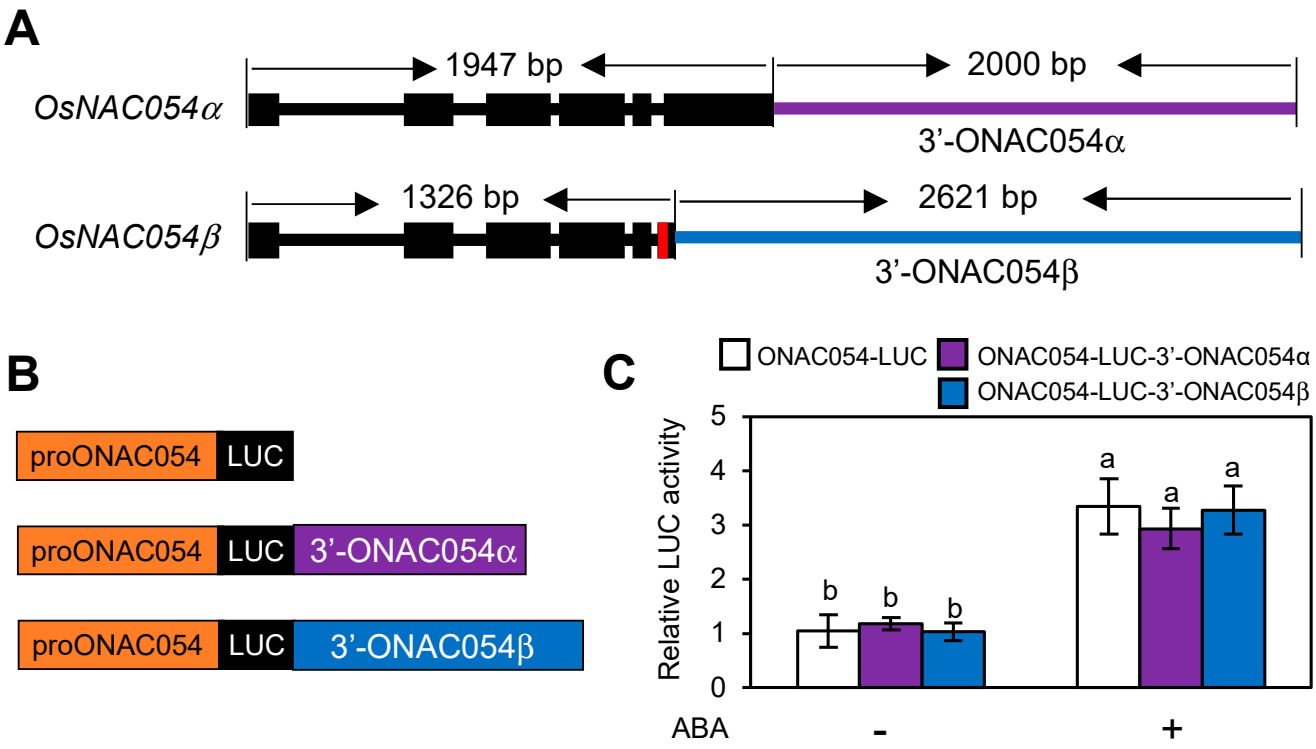

Figure 5. The 3′-UTRs of *ONAC054α* and *ONAC054β* do not affect their expression patterns. (**A**) Gene structure of *ONAC054α* and *ONAC054β*. The insertion of 7 bp in the fifth intron of *ONAC054β* created an alternative 3′ splice site and a premature stop codon, leading to a difference in the length of the 3′-UTRs of *ONAC054α* (purple horizontal bar) and *ONAC054β* (blue horizontal bar). (**B**) Reporter constructs are used in the protoplast transient expression assay. (**C**) Effects of 3′-flanking sequences of *ONAC054α* and *ONAC054β* on the activity of the *ONAC054* promoter (−2000 to −1 bp, relative to the translation initiation site) in protoplasts treated with or without ABA. Data represent the mean ± SD of four biological replicates, and different lowercase letters indicate significant differences ($p < 0.05$; Tukey's multiple comparison test).

3.6. Overexpression of ONAC054 Increases Grain Yield under Drought Stress Conditions

Drought stress greatly and negatively affects rice grain yield under drought stress conditions [51]. To examine whether the KO mutation and overexpression of *ONAC054* affect grain yield under drought stress conditions, we evaluated the agronomic traits of WT, *onac054-1*, *ONAC054α*-OX, and *ONAC054β*-OX plants, including plant height, panicle number per plant, spikelet number per panicle, seed fertility, and grain yield per plant, after sequential drought stress treatments. We previously showed that the KO mutation of *ONAC054* did not affect grain yield but slightly decreased seed fertility [31]. Under non-stress conditions, the grain yield and all other traits of *onac054-1*, *ONAC054α*-OX, and *ONAC054β*-OX plants were similar to those of the WT (Figure 6A–G). However, under drought stress conditions, the grain yield of *onac054-1* was significantly reduced, while that of *ONAC054α*-OX and *ONAC054β*-OX was highly retained compared with the grain yield of the WT (Figure 6F,G). The dramatic decline in the grain yield of *onac054-1* was mainly caused by the decrease in two traits: spikelet number per panicle and seed fertility, both of which were highly retained in the two *ONAC054*-OX lines compared with the WT (Figure 6D,E,G). Overall, *ONAC054* overexpression improved the grain yield of rice without causing any growth defects.

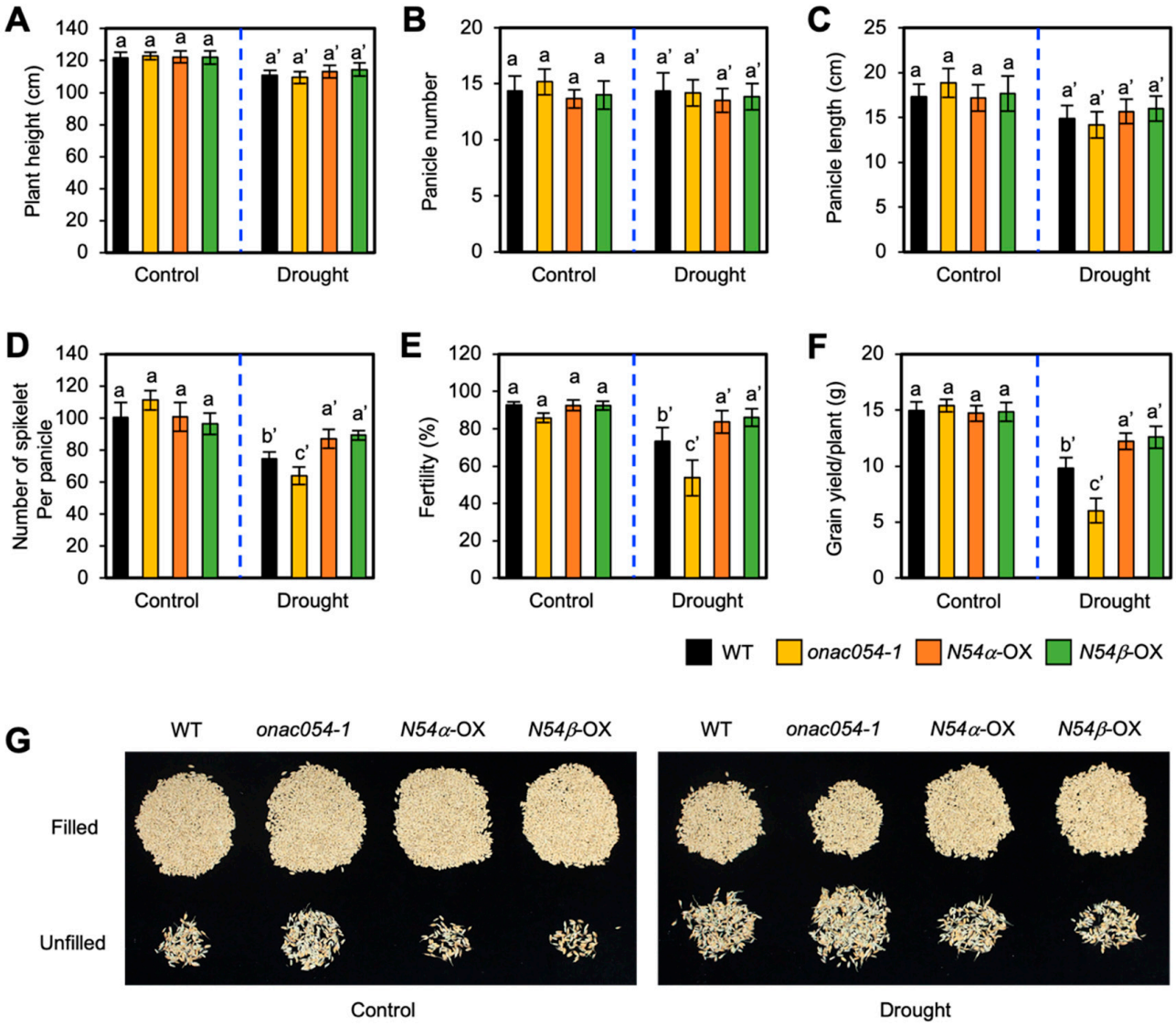

Figure 6. Agronomic traits of *onac054-1* and *ONAC054*-OX plants under drought stress conditions. (**A**–**F**) Agronomic traits, including plant height (**A**), panicle number per plant (**B**), panicle length (**C**), spikelet number per panicle (**D**), seed fertility (**E**), and grain yield per plant (**F**), of WT, *onac054-1*, *ONAC054α*-OX (*N054α*-OX), and *ONAC054β*-OX (*N054β*-OX) plants grown under normal growth conditions or drought stress conditions (see Materials and Methods). Data represent the mean ± SD (n = 6 plants), and different lowercase letters (letters with ′ for drought stress conditions) indicate significant differences ($p < 0.05$; Tukey's multiple comparison test). (**G**) Relative abundance of fertile and sterile spikelets in different genotypes.

4. Discussion

4.1. Regulatory Mechanism Underlying the ONAC054-Mediated Drought Stress Responses

In this study, we showed that ONAC054 acts as a key regulator of drought stress responses in rice. After 5 days of dehydration, *onac054*-KO seedlings exhibited severe wilting, while *ONAC054*-OX plants showed a tolerant phenotype (Figure 1). These differences in phenotypes may be caused by altered expression of genes associated with ABA metabolism and signaling (Figure 2). Furthermore, we showed that ONAC054 directly or indirectly activates the transcription of several genes associated with ABA signaling, including *OsABI5*, *OsLEA3*, *OsbZIP23*, *OsABF4*, and *TRAB1* [31] (Figures 2 and 3), which have previously been functionally characterized. Overexpression of *OsLEA3*, *OsbZIP23*, or *OsABF4* enhanced drought tolerance in rice [37,49,52]. The role of TRAB1 in drought

stress responses has not yet been elucidated. However, given that TRAB1 is required for the induction of ABA-responsive genes by interacting with the ABRE sequence in their promoters [53], it is likely that TRAB1 plays an important role in the drought stress response. Thus, the upregulation of ABA signaling genes may contribute to the drought tolerance phenotype of *ONAC054*-OX plants. On the other hand, *OsABI5* antisense transgenic rice plants were reported to be tolerant to drought and high salinity stresses [49]. Thus, the role of the ONAC054–OsABI5 regulatory module, which is important for ABA-induced leaf senescence [31], in drought stress responses needs to be elucidated.

In this study, four ABFs, including OsbZIP23, OsABF2, OsABF4, and TRAB1, activated the transcription of *ONAC054* (Figure 4). On the other hand, ONAC054 also directly or indirectly activated the transcription of *OsbZIP23*, *OsABF4*, and *TRAB1* (Figure 3), as shown previously [31]. Thus, our findings suggest that ONAC054 and the four ABFs form a feedforward transcriptional loop for the enhancement of ABA signaling responses (Figure S3). Similar feedforward transcriptional regulatory mechanisms have been previously reported. In Arabidopsis, Dof2.1 directly activates the transcription of *MYC2*, and the encoded MYC2 TF, a central regulator of the JA response [54], directly activates the transcription of *Dof2.1*, thus forming a feedforward loop for the enhancement of the JA response [55]. In rice, PHYTOCHROME-INTERACTING FACTOR-LIKE1 (OsPIL1), a basic helix-loop-helix (bHLH) transcription factor, activates chlorophyll biosynthesis genes, *OsPORB* and *OsCAO* [56,57], and GARP-type TF genes, *OsGLK1* and *OsGLK2* [58], while OsGLK1 and OsGLK2 directly activate the transcription of *OsPORB* and *OsCAO*, thus forming a feedforward transcriptional loop for the promotion of chlorophyll biosynthesis [59]. Such coherent feedforward loops may increase the robustness of biological signaling processes [60].

Additionally, both *ONAC054α* and *ONAC054β* were strongly induced under dehydration and osmotic stress conditions (Figure 1A,B), similar to their expression patterns in ABA-treated plants [31]. However, *ONAC054β* was induced much faster than *ONAC054α* (Figure 1) [31]. In this study, we examined whether the difference in the 3′-UTRs of *ONAC054α* and *ONAC054β* was responsible for the difference in their expression patterns in the protoplast transient expression assay. However, the 3′-UTRs of *ONAC054α* and *ONAC054β* did not affect LUC activity in response to ABA (Figure 5). Thus, the cause of the differential expression patterns of *ONAC054α* and *ONAC054β* in response to dehydration and ABA treatments remains unclear. The 3′-UTR is sometimes associated with the regulation of mRNA stability as it contains several sequence elements required for the destabilization of mRNA, which are enriched among short- and long-lived transcripts [61]. Thus, it is necessary to examine whether the stability of *ONAC054α* and *ONAC054β* mRNAs is regulated at the post-translational level.

4.2. Advantages of Membrane-Bound NAC TFs in Crop Biotechnology Research

Altering the expression or activity of stress-responsive genes is one of the most useful approaches for generating crops with enhanced environmental stress tolerance [8], although this technique sometimes has a negative impact on plant growth and development. A number of stress-tolerant plants generated by the overexpression of stress-responsive genes exhibit a dwarf phenotype. For instance, the overexpression of *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1A* (*DREB1A*), one of the most extensively studied drought stress-responsive genes, greatly improved drought stress tolerance in Arabidopsis and other crops, including peanut (*Arachis hypogaea* L.) [62] and wheat (*Triticum aestivum* L.) [63], but caused severe dwarfism and late flowering in plants [64]. On the other hand, a knockdown mutation of *BRASSINOSTEROID INSENSITIVE1* (*BRI1*) increased tolerance to drought stress but caused severe dwarfism in *Brachypodium distachyon* [65]. These negative effects on plant growth and development may be caused by the constitutive overexpression or knockout of stress-responsive genes.

Under non-stress conditions, most TMD-containing TFs localize to the ER. Upon stress stimulation, TMD is cleaved through ER-specific proteolytic mechanisms, such as regulated

intramembrane proteolysis (RIP) [66], and regulated ubiquitin/proteasome-dependent processing (RUP) [67], which leads to the translocation of TFs to the nucleus, where they induce the expression of target genes [16]. Therefore, the overexpression of TMD-containing TF genes does not affect plant growth and development when plants are grown under moderate growth conditions. Thus, TMD-containing TF genes could be utilized for the development of ideal stress-tolerant crops.

For instance, transgenic Arabidopsis plants overexpressing full-length *NTL4*, encoding an Arabidopsis TMD-containing NAC TF, were indistinguishable from WT Col-0 plants, while the overexpression of *NTL4* lacking the C-terminal TMD caused abnormal growth phenotypes, such as reduced growth and curled leaves with asymmetric leaf axis and serrated margins [30]. Similarly, transgenic Arabidopsis plants overexpressing full-length *NAC WITH TRANSMEMBRANE MOTIF1* (*NTM1*) were indistinguishable from WT plants, while transgenic plants overexpressing a TMD-truncated *NTM1* exhibited apparent growth defects such as reduced cell numbers [68]. In the current study, we showed that the overexpression of *ONAC054* did not cause any growth defects; rather, *ONAC054* overexpression greatly improved the grain yield under drought-stress conditions (Figure 6). Thus, the TMD-containing TF genes show great potential for the development of stress-tolerant crops, although our knowledge of the roles of TMD-containing TFs in crops is still considerably limited.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/crops2040027/s1, Figure S1: Drought stress sensitive phenotypes of two rice *onac054* mutants; Figure S2: Survival rate of wild-type (WT), *onac054-1* mutants, and *ONAC054*-OX seedlings after dehydration; Figure S3: Model depicting the ONAC054-mediated drought stress responses in rice; Table S1: Primers used in this study.

Author Contributions: Conceptualization, Y.S. and N.-C.P.; methodology, Y.S.; formal analysis, Y.S.; investigation, Y.S.; resources, Y.S. and N.-C.P.; data curation, Y.S.; writing—original draft preparation, Y.S.; writing—review and editing, Y.S. and N.-C.P.; supervision, Y.S. and N.-C.P.; project administration, Y.S. and N.-C.P.; funding acquisition, Y.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Japan Society for the Promotion of Science KAKENHI (22K05368).

Data Availability Statement: Not applicable.

Acknowledgments: We thank Gynheung An at Kyung Hee University for donating the seeds of rice T-DNA insertion mutant lines, *onac054-1* and *onac054-2*.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of this study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Boyer, J.S. Plant productivity and environment. *Science* **1982**, *218*, 443–448. [CrossRef] [PubMed]
2. Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: Towards genetic erngineering for stress tolerance. *Planta* **2003**, *218*, 1–14. [CrossRef] [PubMed]
3. Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **2007**, *58*, 221–227. [CrossRef] [PubMed]
4. Streeter, J.G.; Lohnes, D.G.; Fioritto, R.J. Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. *Plant Cell Environ.* **2001**, *24*, 429–438. [CrossRef]
5. Choudhary, M.K.; Basu, D.; Datta, A.; Chakraborty, N.; Chakraborty, S. Dehydration-desponsive nuclear proteome of rice (*Oryza Sativa* L.) illustrates protein network, novel regulators of cellular adaptation, and evolutionary perspective. *Mol. Cell Proteomics* **2009**, *8*, 1579–1598. [CrossRef]
6. Ullah, A.; Manghwar, H.; Shaban, M.; Khan, A.H.; Akbar, A.; Ali, U.; Ali, E.; Fahad, S. Phytohormones enhanced drought tolerance in plants: A coping strategy. *Environ. Sci. Pollut. Res. Int.* **2018**, *25*, 33103–33118. [CrossRef]

7. Seki, M.; Narusaka, M.; Ishida, J.; Nanjo, T.; Fujita, M.; Oono, Y.; Kamiya, A.; Nakajima, M.; Enju, A.; Sakurai, T.; et al. Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* **2002**, *31*, 279–292. [CrossRef]
8. Todaka, D.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Front. Plant Sci.* **2015**, *6*, 84. [CrossRef]
9. Yamaguchi-Shinozaki, K.; Shinozaki, K. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.* **2005**, *10*, 88–94. [CrossRef]
10. Choi, H.I.; Hong, J.H.; Ha, J.O.; Kang, J.Y.; Kim, S.Y. ABFs, a Family of ABA-responsive element binding factors. *J. Biol. Chem.* **2000**, *275*, 1723–1730. [CrossRef]
11. Kang, J.Y.; Choi, H.I.; Im, M.Y.; Soo, Y.K. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* **2002**, *14*, 343–357. [CrossRef]
12. Fujita, Y.; Fujita, M.; Satoh, R.; Maruyama, K.; Parvez, M.M.; Seki, M.; Hiratsu, K.; Ohme-Takagi, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* **2005**, *17*, 3470–3488. [CrossRef]
13. Gong, W.; Shen, Y.P.; Ma, L.G.; Pan, Y.; Du, Y.L.; Wang, D.H.; Yang, J.Y.; Hu, L.D.; Liu, X.F.; Dong, C.X.; et al. Genome-wide ORFeome cloning and analysis of *Arabidopsis* transcription factor genes. *Plant Physiol.* **2004**, *135*, 773–782. [CrossRef]
14. Nuruzzaman, M.; Manimekalai, R.; Sharoni, A.M.; Satoh, K.; Kondoh, H.; Ooka, H.; Kikuchi, S. Genome-wide analysis of NAC transcription factor family in rice. *Gene* **2010**, *465*, 30–44. [CrossRef]
15. Aida, M.; Ishida, T.; Fukaki, H.; Fujisawa, H.; Tasaka, M. Genes involved in organ separation in Arabidopsis: An analysis of the cup-shaped cotyledon mutant. *Plant Cell* **1997**, *9*, 841–857. [CrossRef]
16. Seo, P.J.; Kim, S.G.; Park, C.M. Membrane-bound transcription factors in plants. *Trends Plant Sci.* **2008**, *13*, 550–556. [CrossRef]
17. Ng, S.; Ivanova, A.; Duncan, O.; Law, S.R.; van Aken, O.; de Clercq, I.; Wang, Y.; Carrie, C.; Xu, L.; Kmiec, B.; et al. A membrane-bound NAC transcription factor, ANAC017, mediates mitochondrial retrograde signaling in *Arabidopsis*. *Plant Cell* **2013**, *25*, 3450–3471. [CrossRef]
18. Souer, E.; van Houwelingen, A.; Kloos, D.; Mol, J.; Koes, R. The no apical meristem gene of petunia Is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* **1996**, *85*, 159–170. [CrossRef]
19. Xie, Q.; Frugis, G.; Colgan, D.; Chua, N.H. *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev.* **2000**, *14*, 3024–3036. [CrossRef]
20. Zhu, X.; Chen, J.; Xie, Z.; Gao, J.; Ren, G.; Gao, S.; Zhou, X.; Kuai, B. Jasmonic acid promotes degreening via MYC2/3/4- and ANAC019/055/072-mediated regulation of major chlorophyll catabolic genes. *Plant J.* **2015**, *84*, 597–610. [CrossRef]
21. Sakuraba, Y.; Piao, W.; Lim, J.H.; Han, S.H.; Kim, Y.S.; An, G.; Paek, N.C. Rice ONAC106 inhibits leaf senescence and increases salt tolerance and tiller angle. *Plant Cell Physiol.* **2015**, *56*, 2325–2339. [CrossRef] [PubMed]
22. Kim, H.J.; Nam, H.G.; Lim, P.O. Regulatory network of NAC transcription factors in leaf senescence. *Curr. Opin. Plant Biol.* **2016**, *33*, 48–56. [CrossRef] [PubMed]
23. Tran, L.S.P.; Nakashima, K.; Sakuma, Y.; Simpson, S.D.; Fujita, Y.; Maruyama, K.; Fujita, M.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. *Plant Cell* **2004**, *16*, 2481–2498. [CrossRef] [PubMed]
24. Xu, Z.Y.; Kim, S.Y.; Hyeon, D.Y.; Kim, D.H.; Dong, T.; Park, Y.; Jin, J.B.; Joo, S.H.; Kim, S.K.; Hong, J.C.; et al. The *Arabidopsis* NAC transcription factor ANAC096 cooperates with BZIP-Type transcription factors in dehydration and osmotic stress responses. *Plant Cell* **2013**, *25*, 4708–4724. [CrossRef] [PubMed]
25. Hu, H.; Dai, M.; Yao, J.; Xiao, B.; Li, X.; Zhang, Q.; Xiong, L. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in Rice. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12987–12992. [CrossRef]
26. Zheng, X.; Chen, B.; Lu, G.; Han, B. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 985–989. [CrossRef]
27. Yuan, X.; Wang, H.; Cai, J.; Bi, Y.; Li, D.; Song, F. Rice NAC transcription factor ONAC066 functions as a positive regulator of drought and oxidative stress response. *BMC Plant Biol.* **2019**, *19*, 278. [CrossRef]
28. Sakuraba, Y.; Kim, Y.S.; Han, S.H.; Lee, B.D.; Paek, N.C. The Arabidopsis transcription factor NAC016 promotes drought stress responses by repressing AREB1 transcription through a trifurcate feed-forward regulatory loop involving NAP. *Plant Cell* **2015**, *27*, 1771–1787. [CrossRef]
29. Kim, M.J.; Park, M.J.; Seo, P.J.; Song, J.S.; Kim, H.J.; Park, C.M. Controlled nuclear import of the transcription factor NTL6 reveals a cytoplasmic role of SnRK2.8 in the drought-stress response. *Biochem. J.* **2012**, *448*, 353–363. [CrossRef]
30. Lee, S.; Seo, P.J.; Lee, H.J.; Park, C.M. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. *Plant J.* **2012**, *70*, 831–844. [CrossRef]
31. Sakuraba, Y.; Kim, D.; Han, S.H.; Kim, S.H.; Piao, W.; Yanagisawa, S.; An, G.; Paek, N.C. Multilayered regulation of membrane-bound ONAC054 is essential for abscisic acid-induced leaf senescence in rice. *Plant Cell* **2020**, *32*, 630–649. [CrossRef]
32. Sakuraba, Y.; Jeong, J.; Kang, M.Y.; Kim, J.; Paek, N.C.; Choi, G. Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in *Arabidopsis*. *Nat. Commun.* **2014**, *5*, 4636. [CrossRef]

33. Kusaba, M.; Ito, H.; Morita, R.; Iida, S.; Sato, Y.; Fujimoto, M.; Kawasaki, S.; Tanaka, R.; Hirochika, H.; Nishimura, M.; et al. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *Plant Cell* **2007**, *19*, 1362–1375. [CrossRef]
34. Lee, S.H.; Sakuraba, Y.; Lee, T.; Kim, K.W.; An, G.; Lee, H.Y.; Paek, N.C. Mutation of *Oryza sativa CORONATINE INSENSITIVE 1b* (*OsCOI1b*) delays leaf senescence. *J. Integr. Plant Biol.* **2015**, *57*, 562–576. [CrossRef]
35. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198. [CrossRef]
36. Earley, K.W.; Haag, J.R.; Pontes, O.; Opper, K.; Juehne, T.; Song, K.; Pikaard, C.S. Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **2006**, *45*, 616–629. [CrossRef]
37. Zhang, Y.; Su, J.; Duan, S.; Ao, Y.; Dai, J.; Liu, J.; Wang, P.; Li, Y.; Liu, B.; Feng, D.; et al. A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods* **2011**, *7*, 30. [CrossRef]
38. Saleh, A.; Alvarez-Venegas, R.; Avramova, Z. An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. *Nat. Protoc.* **2008**, *3*, 1018–1025. [CrossRef]
39. Luehrsen, K.R.; de Wet, J.R.; Walbot, V. Transient expression analysis in plants using firefly luciferase reporter gene. *Methods Enzymol.* **1992**, *216*, 397–414.
40. Nakagawa, T.; Kurose, T.; Hino, T.; Tanaka, K.; Kawamukai, M.; Niwa, Y.; Toyooka, K.; Matsuoka, K.; Jinbo, T.; Kimura, T. Development of series of gateway binary vectors, PGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* **2007**, *104*, 34–41. [CrossRef]
41. Yoo, S.D.; Cho, Y.H.; Sheen, J. *Arabidopsis* Mesophyll Protoplasts: A Versatile Cell System for Transient Gene Expression Analysis. *Nat. Protoc.* **2007**, *2*, 1565–1572. [CrossRef]
42. Porra, R.J.; Thompson, W.A.; Kriedemann, P.E. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta. Bioenerg.* **1989**, *975*, 384–394. [CrossRef]
43. Hohl, M.; Schopfer, P. Water relations of growing maize coleoptiles comparison between mannitol and polyethylene glycol 6000 as external osmotica for adjusting turgor pressure. *Plant Physiol.* **1991**, *95*, 716–722. [CrossRef] [PubMed]
44. Yang, S.H.; Choi, D. Characterization of genes encoding ABA 8′-hydroxylase in ethylene-induced stem growth of deepwater rice. *Biochem. Biophys. Res. Commun.* **2006**, *350*, 685–690. [CrossRef] [PubMed]
45. de Clercq, I.; Vermeirssen, V.; van Aken, O.; Vandepoele, K.; Murcha, M.W.; Law, S.R.; Inzé, A.; Ng, S.; Ivanova, A.; Rombaut, D.; et al. The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. *Plant Cell* **2013**, *25*, 3472–3490. [CrossRef] [PubMed]
46. Hossain, M.A.; Cho, J.I.; Han, M.; Ahn, C.H.; Jeon, J.S.; An, G.; Park, P.B. The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. *J. Plant Physiol.* **2010**, *167*, 1512–1520. [CrossRef]
47. Xiang, Y.; Tang, N.; Du, H.; Ye, H.; Xiong, L. Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant Physiol.* **2008**, *148*, 1938–1952. [CrossRef]
48. Lu, G.; Gao, C.; Zheng, X.; Han, B. Identification of OsbZIP72 as a positive regulator of ABA response and drought tolerance in rice. *Planta* **2009**, *229*, 605–615. [CrossRef]
49. Zou, M.; Guan, Y.; Ren, H.; Zhang, F.; Chen, F. A BZIP Transcription factor, OsABI5, is involved in rice fertility and stress tolerance. *Plant Mol. Biol.* **2008**, *66*, 675–683. [CrossRef]
50. Konishi, M.; Yanagisawa, S. The regulatory region controlling the nitrate-responsive expression of a nitrate reductase gene, *NIA1*, in Arabidopsis. *Plant Cell Physiol.* **2011**, *52*, 824–836. [CrossRef]
51. Venuprasad, R.; Lafitte, H.R.; Atlin, G.N. Response to direct selection for grain yield under drought stress in rice. *Crop Sci.* **2007**, *47*, 285–293. [CrossRef]
52. Duan, J.; Cai, W. OsLEA3-2, an abiotic stress induced gene of rice plays a key role in salt and drought tolerance. *PLoS ONE* **2012**, *7*, e45117. [CrossRef]
53. Kagaya, Y.; Hobo, T.; Murata, M.; Ban, A.; Hattori, T. Abscisic acid–induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. *Plant Cell* **2002**, *14*, 3177–3189. [CrossRef]
54. Dombrecht, B.; Gang, P.X.; Sprague, S.J.; Kirkegaard, J.A.; Ross, J.J.; Reid, J.B.; Fitt, G.P.; Sewelam, N.; Schenk, P.M.; Manners, J.M.; et al. MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* **2007**, *19*, 2225–2245. [CrossRef]
55. Zhuo, M.; Sakuraba, Y.; Yanagisawa, S. A jasmonate-activated MYC2–Dof2.1–MYC2 transcriptional loop promotes leaf senescence in Arabidopsis. *Plant Cell* **2020**, *32*, 242–262. [CrossRef]
56. Sakuraba, Y.; Rahman, M.L.; Cho, S.H.; Kim, Y.S.; Koh, H.J.; Yoo, S.C.; Paek, N.C. The rice *faded green leaf* locus encodes protochlorophyllide oxidoreductase B and is essential for chlorophyll synthesis under high light conditions. *Plant J.* **2013**, *74*, 122–133. [CrossRef]
57. Lee, S.; Kim, J.H.; Eun, S.Y.; Lee, C.H.; Hirochika, H.; An, G. Differential regulation of chlorophyll *a* oxygenase genes in rice. *Plant Mol. Biol.* **2005**, *57*, 805–818. [CrossRef]

58. Waters, M.T.; Wang, P.; Korkaric, M.; Capper, R.G.; Saunders, N.J.; Langdale, J.A. GLK transcription factors coordinate expression of the photosynthetic apparatus in *Arabidopsis*. *Plant Cell* **2009**, *21*, 1109–1128. [CrossRef]
59. Sakuraba, Y.; Kim, E.Y.; Han, S.H.; Piao, W.; An, G.; Todaka, D.; Yamaguchi-Shinozaki, K.; Paek, N.C. Rice phytochrome-interacting factor-like1 (OsPIL1) is involved in the promotion of chlorophyll biosynthesis through feed-forward regulatory loops. *J. Exp. Bot.* **2017**, *68*, 4103–4114. [CrossRef]
60. Mangan, S.; Alon, U. Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11980–11985. [CrossRef]
61. Narsai, R.; Howell, K.A.; Millar, A.H.; O'Toole, N.; Small, I.; Whelan, J. Genome-wide analysis of mRNA decay rates and their determinants in *Arabidopsis Thaliana*. *Plant Cell* **2007**, *19*, 3418–3436. [CrossRef]
62. Bhatnagar-Mathur, P.; Devi, M.J.; Reddy, D.S.; Lavanya, M.; Vadez, V.; Serraj, R.; Yamaguchi-Shinozaki, K.; Sharma, K.K. Stress-inducible expression of at DREB1A in transgenic peanut (*Arachis Hypogaea* L.) increases transpiration efficiency under water-limiting conditions. *Plant Cell Rep.* **2007**, *26*, 2071–2082. [CrossRef]
63. Pellegrineschi, A.; Reynolds, M.; Pacheco, M.; Brito, R.M.; Almeraya, R.; Yamaguchi-Shinozaki, K.; Hoisington, D. Stress-induced expression in wheat of the Arabidopsis Thaliana DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* **2011**, *47*, 493–500. [CrossRef]
64. Liu, Q.; Kasuga, M.; Sakuma, Y.; Abe, H.; Miura, S.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **1998**, *10*, 1391–1406. [CrossRef]
65. Feng, Y.; Yin, Y.; Fei, S. Down-regulation of *BdBRI1*, a putative brassinosteroid receptor gene produces a dwarf phenotype with enhanced drought tolerance in *Brachypodium Distachyon*. *Plant Sci.* **2015**, *234*, 163–173. [CrossRef]
66. Bengoechea-Alonso, M.T.; Ericsson, J. SREBP in signal transduction: Cholesterol metabolism and beyond. *Curr. Opin. Cell Biol.* **2007**, *19*, 215–222. [CrossRef]
67. Hoppe, T.; Matuschewski, K.; Rape, M.; Schlenker, S.; Ulrich, H.D.; Jentsch, S. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* **2000**, *102*, 577–586. [CrossRef]
68. Kim, Y.S.; Kim, S.G.; Park, J.E.; Park, H.Y.; Lim, M.H.; Chua, N.H.; Park, C.M. A membrane-bound NAC transcription factor regulates cell division in *Arabidopsis*. *Plant Cell* **2006**, *18*, 3132–3144. [CrossRef] [PubMed]