2004

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Citation/Publisher Attribution
Zou, X., Seemann, J .R., Neuman, D., & Shen, Q. J. (2004). A *WRKY* Gene from Creosote Bush Encodes an Activator of the Abscisic Acid Signaling Pathway. *Journal of Biological Chemistry, 279*, 55770-55779. doi: 10.1074/jbc.M40853620
Available at: http://.dx.doi.org/10.1074/jbc.M408536200

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55770–55779, 2004
Printed in U.S.A.

A *WRKY* Gene from Creosote Bush Encodes an Activator of the Abscisic Acid Signaling Pathway*

Received for publication, July 28, 2004
Published, JBC Papers in Press, October 25, 2004, DOI 10.1074/jbc.M408536200

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The creosote bush (*Larrea tridentata*) is a xerophytic evergreen C3 shrub thriving in vast arid areas of North America. As the first step toward understanding the molecular mechanisms controlling the drought tolerance of this desert plant, we have isolated a dozen genes encoding transcription factors, including *LtWRKY21* that encodes a protein of 314 amino acid residues. Transient expression studies with the *GFP-LtWRKY21* fusion construct indicate that the LtWRKY21 protein is localized in the nucleus and is able to activate the promoter of an abscisic acid (ABA)-inducible gene, *HVA22*, in a dosage-dependent manner. The transactivating activity of LtWRKY21 relies on the C-terminal sequence containing the WRKY domain and a N-terminal motif that is essential for the repression activity of some regulators in ethylene signaling. LtWRKY21 interacts synergistically with ABA and transcriptional activators VP1 and ABI5 to control the expression of the *HVA22* promoter. Co-expression of *VP1*, *ABI5*, and *LtWRKY21* leads to a much higher expression of the *HVA22* promoter than does the ABA treatment alone. In contrast, the LtWRKY21-mediated transactivation is inhibited by two known negative regulators of ABA signaling: 1-butanol, an inhibitor of phospholipase D, and abi1-1, a dominant negative mutant protein phosphatase. Interestingly, abi1-1 does not block the synergistic effect of *LtWRKY21*, *VP1*, and *ABI5* co-expression, indicating that LtWRKY21, VP1, and ABI5 may form a complex that functions downstream of ABI1 to control ABA-regulated expression of genes.

The phytohormone abscisic acid (ABA)[1] modulates plant developmental processes such as seed formation, dormancy, and germination, as well as plant responses to environmental stresses such as drought, cold, high salinity, pathogen attack, and UV radiation (1–6). Plant responses to ABA are mediated at several molecular levels including transcription, RNA processing, post-translational modification, and metabolism of the secondary messengers (reviewed in Refs. 5–7). Recent data indicate that the ABA signaling pathways appear to be conserved among higher plant species and even bryophytes (8, 9).

Both ABA-resistant and ABA-hypersensitive mutants have been extremely valuable in helping define ABA signaling pathways. Studies of mutants in several plant species suggest that the ABA signaling is mediated by a membrane-bound metal sensor (10, 11), type 2C serine/threonine protein phosphatases (12–14), a Ser/Thr protein kinase (15, 16), a protein farnesyl transferase (17), a steroid reductase (16), an inositol polyphosphate 1-phosphatase (18), and several transcription factors (19–26). In addition, the mutant studies also suggest that RNA processing plays an important role in the regulation of ABA signaling (27) because several ABA response mutants are impaired in a double-stranded RNA-binding protein (28), a mRNA CAP-binding protein (29), or a U6-related Sm-like small ribonucleoprotein (18). In line with these reports, an ABA-induced maize glycine-rich protein can bind to uridine- and guanosine-rich RNA fragments (30).

Several types of *cis*-acting elements are involved in ABA responses, such as the 10-bp element containing an ACGT core (ACGT box, also referred as G box or ABRE), CE, RY/Sph, AT-rich elements, and Myb and Myc-binding sites (reviewed in Refs. 6 and 7). In a series of mutational analyses of two ABA-responsive barley genes, *HVA1* and *HVA22*, it was shown that in addition to the ACGT box (*A3*, GCCACGTACA, or *A2*, CCTACGTGGC), a coupling element (*CE1*, TGCCACCGG, or *CE3*, ACGCGTGTCCTC) is also necessary for the ABA response (31, 32). The combination of the ACGT box and the *CE* forms an ABA response complex, which has been shown to be the smallest ABA-responsive promoter unit (32). Recently, the ACGT box is further narrowed down to be ACGTGGC, and CE1 and CE3 are narrowed down to be CCACC and GCGTGTC, respectively (33). ABREs and CE3 are bound by bZIP proteins (24, 34–43); CE1 is bound by ABI4 (44); RY/Sph elements are bound by those containing B3 domains (19, 20, 45–55); AT-rich elements are bound by homeodomain leucine zipper proteins (56, 57); MYC sites are bound by AtMYC (58); and MYB sites are bound by AtMYB (58, 59).

WRKY genes are known to be involved in biotic (bacterial and fungal diseases) and abiotic (heat, drought, wounding, and freezing) stress responses, anthocyanin and starch biosynthesis, senescence and trichome development, and hormone responses (60–72). *WRKY* genes have either one or two WRKY domains, each containing a 60-amino acid region with a core sequence, WRKYGQK, at its N-terminal end and a novel zinc finger-like motif. The WRKY domain binds specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the

* This work was supported by a University of Nevada, Las Vegas start-up grant, National Institutes of Health Biomedical Research Infrastructure Network Seed Grant P20 RR16464, and National Science Foundation Experimental Program to Stimulate Competitive Research (EPSCoR) Seed Grant EPS-9977809 (to Q. J. S.) and National Science Foundation EPSCoR IAAS Fellowship EPS-0132556 (to X. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AY792618.

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[1] The abbreviations used are: ABA, abscisic acid; GFP, green fluorescent protein; ERF, ethylene-responsive element-binding factor; EAR, ERF-associated amphiphilic repression; PLD, phospholipase D.

W box. Despite the strong conservation of their DNA-binding domain, the overall structures of WRKY proteins are highly divergent and can be categorized into distinct groups, which might reflect their different functions (67).

In this work, we identified and characterized a WRKY family transcription factor, *LtWRKY21*, from creosote bush. We then co-expressed an ABA-regulated reporter construct with effector constructs encoding LtWKYR21 and other known ABA signaling regulators, such as ABI1, VP1, and ABI5, in barley aleurone layers to better define the signal transduction pathways mediating ABA signaling. Our results indicate that LtWRKY21 activates ABA-regulated transcription by interacting with VP1 and ABI5 and acting downstream of ABI1.

EXPERIMENTAL PROCEDURES

Construction of Creosote Bush cDNA Libraries—Total RNA was isolated from creosote bush leaves from the Nevada Desert research center (www.unlv.edu/Climate_Change_Research/) with the TRIzol® reagents (Invitrogen). The first strand cDNA was synthesized via priming of the poly(A) tail with the primer 5′-GACTAGTTCT AGATCGCGAG CGGCCGCCCT TTTTTTTTTTTTTTT-3′. This primer contains four restriction sites: SpeI, XbaI, NruI, and NotI. Once the double-stranded cDNAs were synthesized, their ends were polished with Klenow. This was followed by digestion with NotI, leaving a sticky NotI site at the 3′ end and a blunt end at the 5′ end. The digested fragments were then cloned into EcoRV and NotI cut pCMVSport6 vector (Invitrogen). Sequencing of the expression sequence tags was done using Applied Biosystems Prism 3730 DNA analyzer at the Nevada Genomics Center (www.ag.unr.edu/genomics/).

RNA Gel Blot Analysis—Total RNA was isolated from creosote bush seeds with the LiCl precipitation method as described (72). Ten μg of total RNA was separated in a 1.2% polyacrylamide gel and transferred to nylon membrane as described (73). The gene-specific fragment of *LtWRKY21* was amplified by PCR using two primers: CCTCTTAGGG CATCTAATGA AGCTTCACC and CTCTTATCGT CTTTGTCGGT TCGGACATAA TC. The gene-specific fragment of the 18 S rRNA from creosote bush was amplified by PCR using GTGGTGCATG GCCGTTCTTA GTTG and ACTCGTTGGA TACATCAGTG TAGC. The membrane was probed with digoxigenin-labeled DNA using a digoxigenin probe synthesis kit (Roche Applied Science) according to the manufacturer's instructions. After hybridization, the membrane was washed twice with 2× SSC plus 0.1% SDS for 5 min at room temperature and then twice with 0.1× SSC plus 0.1% SDS for 15 min at 50 °C. The signal was detected using a digoxigenin chemiluminescent detection kit (Roche Applied Science).

Genomic DNA Isolation—Creosote bush seeds were germinated on wet Whatman paper saturated with imbibing solution (20 mM $CaCl_2$ and 20 mM sodium succinate) in the dark at 26 °C. Genomic DNA was isolated from 10-day-old seedlings. Briefly, sterile shoots were frozen in liquid nitrogen and ground into a powder. The frozen tissue was suspended in CTAB extraction buffer (55 mM hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA) plus 2% β-mercaptoethanol. The homogenate was incubated at 55 °C for 30–45 min, cooled to room temperature, and extracted twice with an equal volume of chloroform. The DNA was precipitated with isopropanol and then redissolved in TE buffer (10 mM Tris, pH 8, 1 mM EDTA) plus RNase A (20 μg/ml). After incubation at 37 °C for 1 h, the DNA was precipitated with ammonium acetate and ethanol and then dissolved in TE buffer.

Effector Construct Preparation—Three types of DNA constructs were used in the transient expression experiments: reporter, effector, and internal control (32). Plasmid *HVA22-GUS* (73) was used as the reporter construct. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by the constitutive maize ubiquitin promoter (74), was used as an internal control construct to normalize GUS activities of the reporter construct. *LtWRKY21* effector construct was prepared as follows: The *LtWRKY21* effector gene was amplified from a cDNA clone by PCR using two primers: TTAGGCGCGC CATGGCATAT CCTTCTTGG and TTAGGCGCGC CTCACCAATT TCCTCCAGG, which contain an AscI site to facilitate cloning. The PCR product was confirmed by sequencing and then cloned into the AscI site of the intermediate construct containing the *UBI* promoter and *NOS* terminator (72), generating *UBI-LtWRKY21*. The deletion and substitution mutants were prepared by oligonucleotide-directed mutagenesis with the method of Kunkel *et al.* (75). Single-stranded DNA from plasmid *UBI-LtWRKY21* was used as template. The primer CTGGCCATAT TTTCTCTAG GATATCCATCT TTAAC was used to introduce a stop codon (TAG) upstream from the WRKY domain; the primer GCCCTAAGAG GATTAAGGAC TCGAGCCAAT GACGTATCTA CCC was used to mutate the EAR motif of LtWRKY21. The *UBI-ABI5*, *35S-VP1*, and *35S-abi1-1* effector constructs have been described (19, 43, 76).

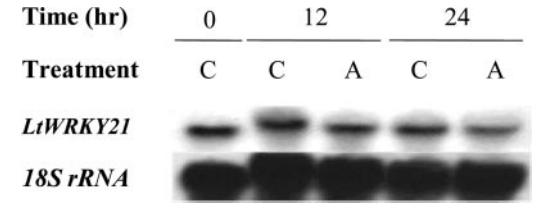

FIG. 1. **Northern analysis of the *LtWRKY21* gene expression in the seeds.** Total RNA was isolated from creosote bush seeds treated with ABA for 12 and 24 h, respectively. The RNA blot was probed with either *LtWRKY21* or *18 S rRNA* as a control. *C*, control; *A*, ABA treatment.

Particle Bombardment and Transient Expression Assays—Transformation of barley (*Hordeum vulgare* L.) aleurone cells by particle bombardment was carried out as described previously (73). Briefly, deembryonated half-seeds of Himalaya barley were imbibed for 2.5–3 days before the pericarp and testa were removed. The DNA mixture (in 1:1 molar ratio) of *HVA22-GUS* and *UBI-Luciferase*, along with or without an effector construct, was bombarded into barley embryoless half-seeds (four replicates/test construct). 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 published before (32).

Preparation of GFP Fusion Constructs and Confocal Microscopy—The coding region of *LtWRKY21* was inserted into the AscI site of *UBI-GFP* (72) to generate *UBI-GFP-LtWRKK21*. Barley aleurone cells were bombarded with *UBI-GFP-LtWRKY21* fusion constructs. After incubation at 24 °C for 24 h, the aleurone layers were peeled from barley half-seeds and soaked in a 5 μM SYTO17 solution (Molecular Probes, Eugene, OR). The stained samples were observed and images of GFP fluorescence and SYTO17 staining were obtained simultaneously through a laser scanning microscope (LSM 510; Carl Zeiss, Inc.) with 488-nm excitation and 505–530-nm emission wavelengths for the green fluorescence and 633-nm excitation and 650-nm emission wavelengths for the red fluorescence in separate channels. The acquired images were processed using Paint Shop Pro 7.

RESULTS

Northern Blot Analysis of the LtWRKY21 Gene Expression in Response to ABA Treatment—In an effort to identify creosote bush stress response genes, 43 *Arabidopsis* stress-inducible genes (77) were collected and searched against the creosote bush expression sequence tag database. Ten creosote bush genes encoding putative stress-inducible transcription factors of different families were identified (data not shown). We focused on WRKY proteins because they regulate plant response to various stresses (60–70), hence likely also mediating ABA responses. One of these genes, *LtWRKY21*, was studied in more detail.

To study the expression pattern of the *LtWRKY21* gene, RNA was isolated from creosote bush seeds without or with different ABA treatments for Northern analyses. The mRNA level of *LtWRKY21* was abundant in the seeds at onset of the ABA treatment. ABA treatments for 12 or 24 h had little effect on the abundance of the *LtWRKY21* mRNA level (Fig. 1), suggesting that ABA has little effect on regulating *LtWRKY21* at the transcriptional or post-transcriptional levels.

Protein Sequence Alignment of LtWRKY21 with Its Homologues—To identify the open reading frame of *LtWRKY21*, both strands of the cDNA clone was sequenced. The full-length of this cDNA is 945-bp, encoding a protein of 314 amino acid residues with a hydrophilic N terminus. The deduced amino acid sequence of LtWRKY21 and its homologues, including *Arabidopsis* WRKY40 (accession number At1g80840) (67), parsley WRKY4 (accession number AF204925.1) (78), cotton

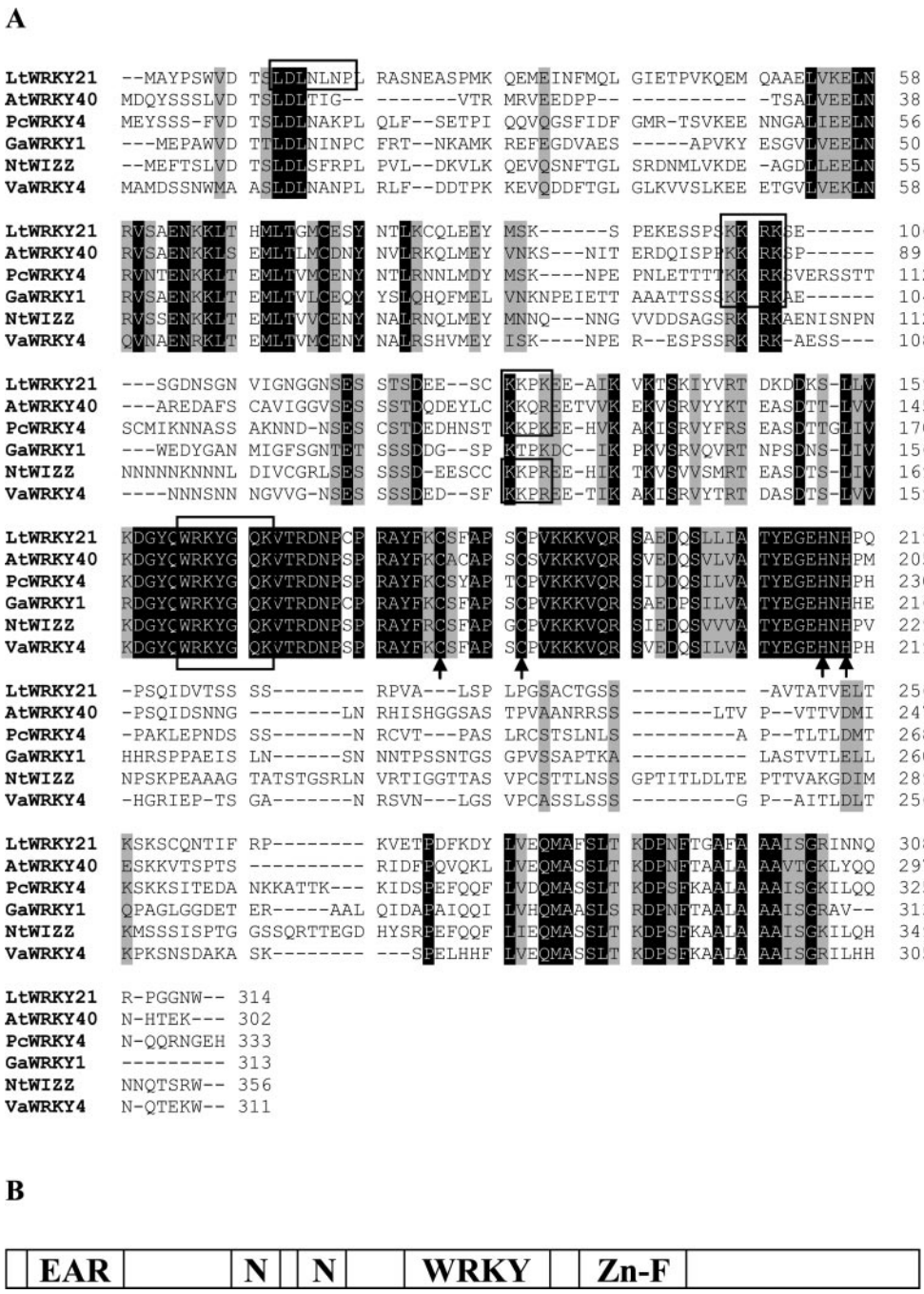

FIG. 2. **Protein sequence alignment of LtWRKY21 with its homologues.** *A*, the alignment of LtWRKY21 with its homologues. The deduced amino acid sequences were aligned by using ClustalW. Identical residues are *shaded* in *black*, and residues chemically similar are in *gray*. The putative EAR motif (LDLNLNP), nuclear localization signal (K(RK)*X*(RK)) and WRKY amino acid residues are labeled with *rectangles*, and amino acids residues potentially interacting with zinc ligands are pointed to with *arrows*. *B*, the schematic diagram of the LtWRKY21 protein showing the EAR motif, nuclear localization signals (*N*), WRKY motif, and zinc finger motif (*Zn-F*). It is not drawn to scale.

WRKY1 (accession number AY507929.2) (79), tobacco WIZZ (accession number AB028022.1) (69), and grapevine WRKY4 (accession number AY484579.1) were aligned with the ClustalW program at the default settings. LtWRKY21 shares 44–48% identity and 57–62% chemical similarity at the amino acid level with these WRKY proteins (Fig. 2). The WRKY, zinc finger motif, nucleus targeting signal sequence, and putative leucine zipper domain are highly conserved. Interestingly, among this group of homologues, only LtWRKY21 contains an EAR motif, with a consensus sequence of (L/F)DLN(L/F)*X*P. In LtWRKY21, this motif is LDLNLNP (Fig. 2). EAR resides in the 59-amino acid ERF domain of ERFs (80). In *Arabidopsis*, there are five ERFs, all of which bind to the GCC box (GCCGCC). AtERF1, AtERF2, and AtERF5 function as activators of GCC box-dependent transcription in *Arabidopsis* leaves, whereas AtERF3 and AtERF4 act as repressors (81). Both AtERF3 and AtERF4 contain the EAR motif, which is also present other unrelated proteins such as SUPERMAN that regulates flowering (82). A related motif, L*X*L*X*L*X*, is necessary for the activity of some Aux/IAA repressors mediating auxin signaling (83).

The GFP-LtWRKY21 Fusion Protein Was Localized in Nuclei—To examine the subcellular localization of the LtWRKY21 protein, we used GFP as a reporter and a red fluorescent nucleic acid stain, SYTO17, for nuclear localization. *GFP* was fused in frame to the 5′ end of the *LtWRKY21* coding sequence.

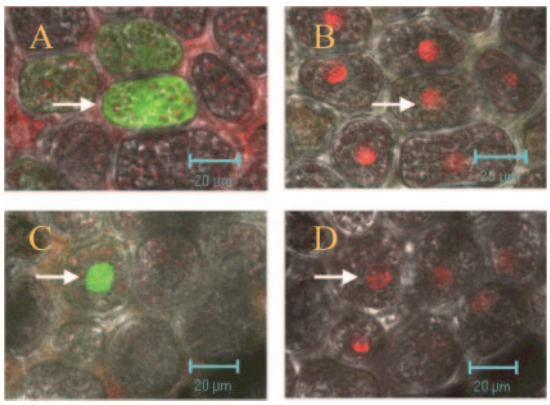

FIG. 3. **The GFP-LtWRKY21 fusion protein was localized in the nuclei.** *A*, the GFP fluorescence from cells bombarded with the *UBI-GFP* construct. *C*, the GFP fluorescence from *UBI-GFP-LtWRKY21*. *B* and *D*, nuclei in the same cells as in *A* and *C* that were stained with SYTO17, respectively. The *bars* represent 20 μm.

UBI-GFP or *UBI-GFP-LtWRKY21* plasmids were introduced into the barley aleurone cells by particle bombardment, and the GFP fluorescence was visualized using confocal microscopy. In control, GFP fluorescence was observed throughout the cells (Fig. 3*A*). In contrast, GFP-LtWRKY21 fusion proteins were localized exclusively in the nuclei (Fig. 3*C*), as confirmed by SYTO17 staining (Fig. 3, *B* and *D*).

LtWRKY21 Transactivates the HVA22 Promoter—To test the function of LtWRKY21 on ABA signal transduction pathways, we used a reporter construct that contains the *GUS* reporter gene driven by the promoter of *HVA22*, an ABA-responsive gene in barley (32). The effector construct, *UBI-LtWRKY21*, was co-introduced to evaluate its effect on ABA signaling. As shown in Fig. 4, a very low level of GUS activity was observed in the absence of ABA. The exogenous ABA (20 μM) treatment resulted in a 30-fold enhancement of GUS activity over that found with the ABA-untreated control. Expression of *UBI-LtWRKY21* resulted in a 7-fold induction in the absence of ABA. Interestingly, *LtWRKY21* synergistically interacted with ABA to transactivate the expression of the *HVA22* promoter, leading to a 47-fold induction.

The Activating Effect of LtWRKY21 on the Expression of the HVA22 Promoter Is Dosage-dependent—The activating effect of LtWRKY21 was further confirmed by a dosage experiment, in which the amount of reporter plasmid was always constant, whereas that of the effector varied from 0 to 100% (Fig. 5). As expected, when the *HVA22-GUS* construct was transformed alone, the treatment with 20 μM ABA led to a 31-fold induction of *HVA22-GUS*. The expression of the *HVA22-GUS* in response to ABA treatment increased gradually with the increment of the *UBI-LtWRKY21* effector construct. When the relative amount of effector to reporter was 25 and 50%, the *GUS* expression, in reference to the control (no ABA, no effector), was induced by a factor of 57 and 62, respectively. The GUS activities increased to 74-fold and reached a plateau with the higher amounts of the effector construct (75 or 100%). These data indicated that under these conditions, LtWRKY21 is a transcriptional activator of ABA signaling. To our knowledge, this is the first report of such activity by a WRKY protein.

The EAR Motif at the N Terminus and the C-terminal Region Containing the WRKY Domain of LtWRKY21 Are Essential for Its Transactivating Activity—To further demonstrate the specificity of LtWRKY21 on activating ABA induction, mutagenesis experiments were carried out to try to change its activity. A stop codon was introduced at amino acid 165 (mutant 1), which is upstream from the WRKY domain. The purpose was to produce a truncated protein missing the WRKY domain and

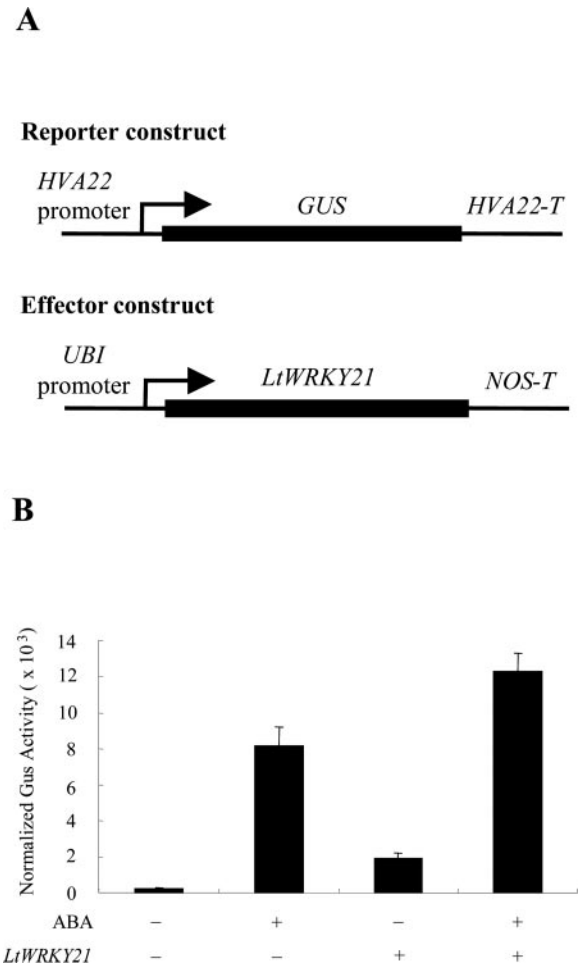

FIG. 4. **LtWRKY21 synergistically interacts with ABA to transactivate the expression of the *HVA22* promoter.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. *HVA22* is an ABA-responsive gene. *UBI* promoter is from the maize ubiquitin gene. *HVA22-T* represents the terminator sequence of *HVA22*. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector construct (*UBI-LtWRKY21*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seed with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

the rest of the C-terminal region. As shown in Fig. 6, the expression of the *HVA22* promoter increased 35-fold after ABA treatment. The wild type *LtWRKY21* gene alone activated the expression of the *HVA22* promoter by 5-fold. ABA treatment along with *LtWRKY21* expression resulted in a 60-fold induction. However, when the *LtWRKY21* mutant 1 was co-expressed, the induction of GUS was 28-fold, which is comparable with that of the ABA treatment alone (35-fold; Fig. 6).

Interestingly, the EAR motif (Fig. 2) is reported to be present in transcriptional repressors only. The presence of such a motif in LtWRKY21 (a clear activator under the experimental conditions) is intriguing. Thus, the DLN residues at the 12^{th}–14^{th} positions were mutated to ARV (mutant 2; Fig. 6). In the presence of ABA, wild type LtWRKY21 activated *HVA22-GUS* expression by 60-fold. However, mutation of the EAR motif decreased the induction level to 21-fold (Fig. 6). In summary, the results presented in Fig. 6 suggested that the EAR domain and C-terminal region of LtWRKY21 are necessary for its transactivating activity in ABA signaling.

LtWRKY21 Interacts Synergistically with ABA and VP1 to

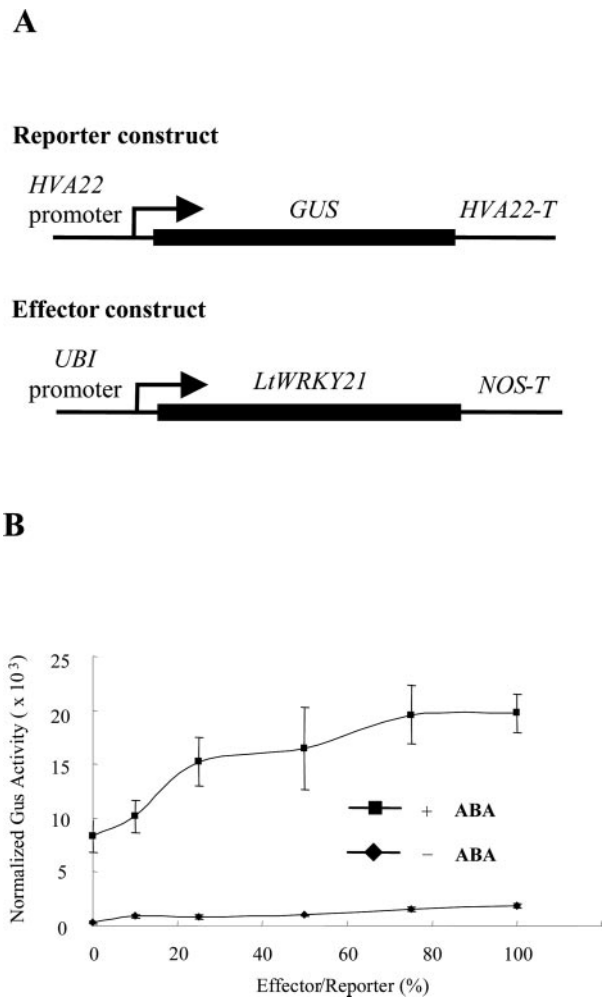

FIG. 5. **The synergistic effect of LtWRKY21 is dosage-dependent.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. The annotations are the same as in Fig. 4. *B*, the effector construct, *UBI-LtWRKY21*, was co-bombarded into barley half-seeds along with the reporter construct, *HVA22-GUS*, and the internal control construct, *UBI-luciferase*. The amount of reporter and internal control plasmid DNA was always constant (1.43 μg/shot), whereas that of the effector varied with respect to the reporter as shown in the *x* axis. 100% means the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

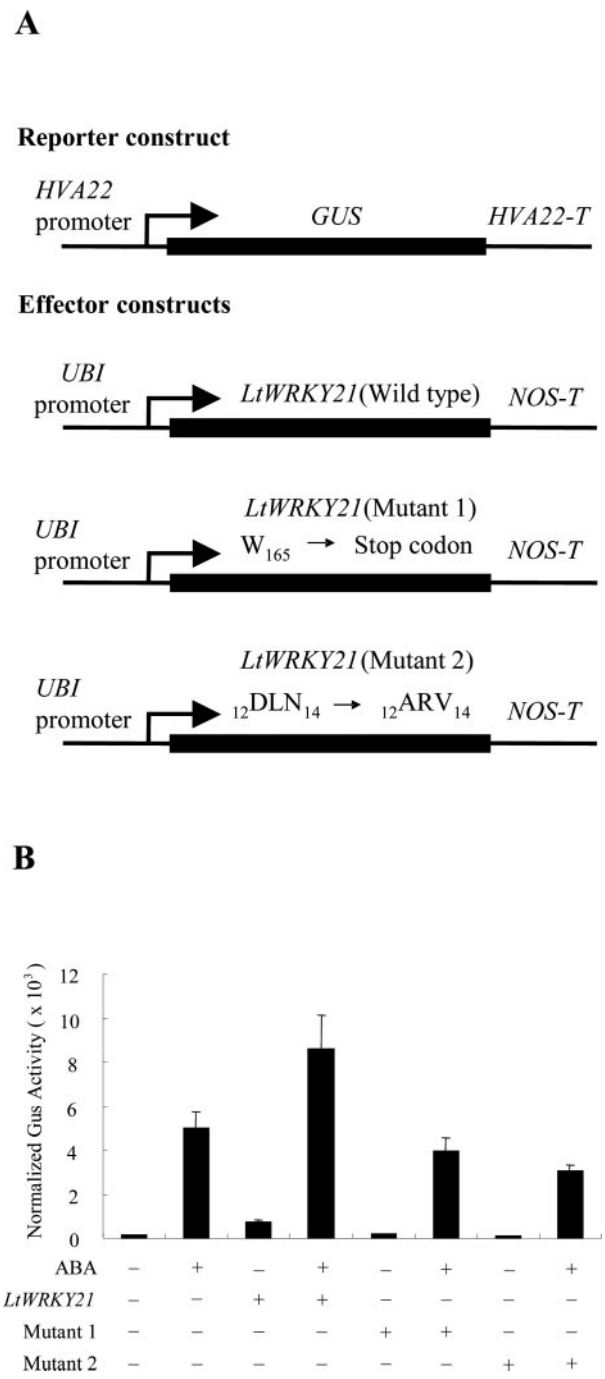

FIG. 6. **The EAR motif and C-terminal region containing the WRKY domain of LtWRKY21 are essential for its transactivating activity.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. The mutant 1 was made by introducing a stop codon at amino acid 165, which is a tryptophan in the wild type protein. The mutant 2 was made by changing residues 12–14 from aspartate-leucine-asparagine to alanine-arginine-valine. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector constructs (*UBI-LtWRKY21* or *UBI-LtWRKY21* mutants) by using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

Transactivate the Expression of the HVA22 Promoter—VP1 encodes a transcription activator that up-regulates ABA responsive genes (19). Fig. 7 shows the results of a functional interaction of LtWRKY21 with VP1 on regulating ABA responses. Expression of *VP1* promoted a small induction (2-fold) of *HVA22-GUS*, in the absence of ABA. ABA treatment along with *VP1* expression resulted in a 16-fold induction. Interestingly, expression of *LtWRKY21* also led to a 2-fold induction. ABA treatment along with *LtWRKY21* expression led to a 53-fold induction. Co-expression of *LtWRKY21* and *VP1* resulted in a 21-fold induction, which is even higher than ABA treatment along with *VP1* expression (16-fold). ABA treatment along with *VP1* and *LtWRKY21* co-expression gave a 62-fold induction (Fig. 7).

LtWRKY21 Interacts Synergistically with ABA and ABI5 to Transactivate the Expression of the HVA22 Promoter—ABI5 encodes a transcription activator on the ABA pathway (24). As reported before (43), expression of *ABI5* promoted a small induction of *HVA22-GUS* in the absence of ABA (Fig. 8). A similar level of induction (5-fold) was achieved with the expression of *LtWRKY21*. Like *ABI5*, *LtWRKY21* synergistically in-

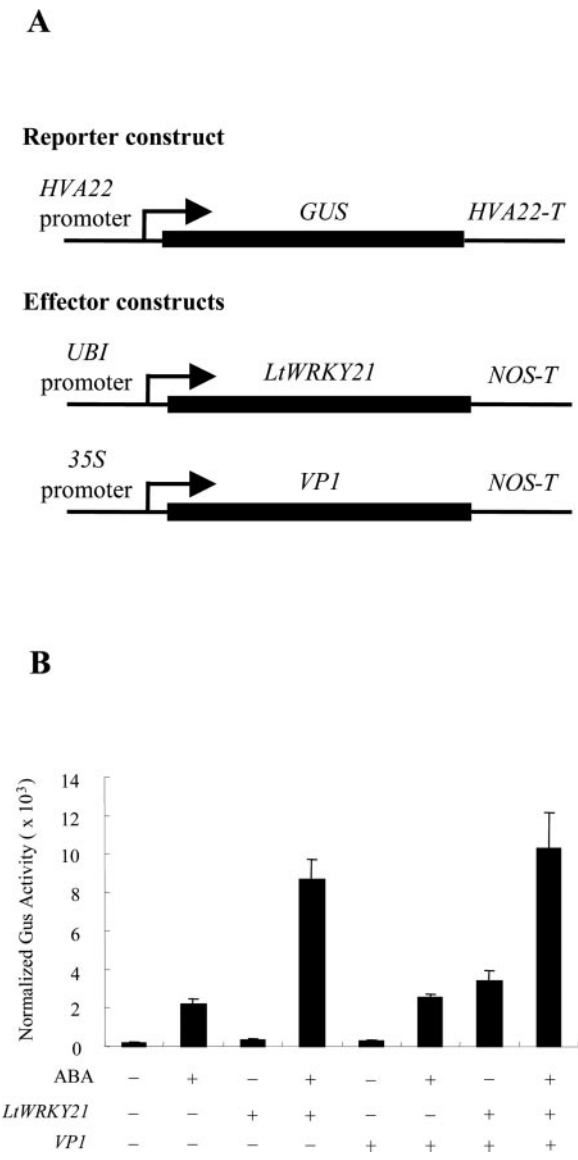

FIG. 7. **LtWRKY21 interacts synergistically with ABA and VP1 to transactivate the expression of the *HVA22* promoter.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. *VP1* is the *Viviparous 1* gene from maize. The 35S promoter is from the cauliflower mosaic virus. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector constructs (*UBI-LtWRKY21* or *35S-VP1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

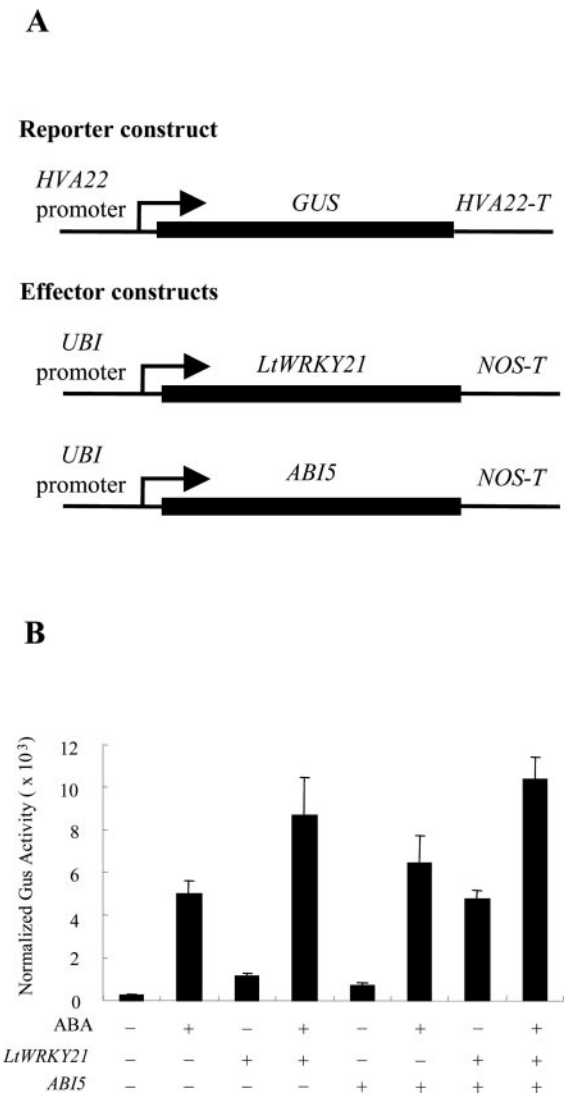

FIG. 8. **LtWRKY21 interacts synergistically with ABA and ABI5 to transactivate the expression of the *HVA22* promoter.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. *ABI5* is the *ABA Insensitive 5* gene from barley. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector constructs (*UBI-LtWRKY21* or *UBI-ABI5*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

teracted with ABA to induce the expression of *HVA22-GUS*. Co-expression of *LtWRKY21* and *ABI5* resulted in a 21-fold induction, which is similar to the ABA treatment (22-fold). The highest level of induction was achieved with the co-expression of *LtWRKY21* and *ABI5* in the presence of ABA (Fig. 8).

1-Butanol Blocks the Synergistic Effect of ABA and LtWRKY21—Phospholipase D (PLD) is a phosphodiesterase that hydrolyzes phospholipids to produce phosphatidic acid. PLD has been demonstrated to be up-regulated by ABA (84). As reported (85), 1-butanol, a specific inhibitor of PLD, inhibits ABA-inducible gene expression. Indeed, ABA induction of *HVA22-GUS* dropped from 32- to 2-fold (Fig. 9). In this experiment, *LtWRKY21* expression led to 4-fold induction in the absence of ABA. 1-Butanol treatment prevented the induction of the reporter by LtWRKY21. This chemical also blocks the synergistic interaction of ABA and LtWRKY21, decreasing the induction level from 61-fold to only 10-fold.

ABI1 Blocks the Synergistic Effect of ABA and LtWRKY21—*ABI1* encodes a protein phosphatase 2C, a negative regulator of ABA signaling (12, 14). A mutation of this gene, *abi1-1*, causes a reduction of phosphatase activity (86), and this mutation is dominant negative in blocking ABA responses in *Arabidopsis* (14) and barley (87). Indeed, abi1-1 prevented the ABA induction of *HVA22-GUS* from 14-fold to the background level in this experiment (Fig. 10). In the absence of ABA, abi1-1 did not appear to affect the activity of LtWRKY21. However, the synergistic effect of ABA and LtWRKY21 was essentially abolished by abi1-1, with the induction level dropping from 53- to 3-fold.

Interaction among LtWRKY21, VP1, ABI5, and ABI1—Because abi1-1 functions upstream of ABI5 and VP1 in modulat-

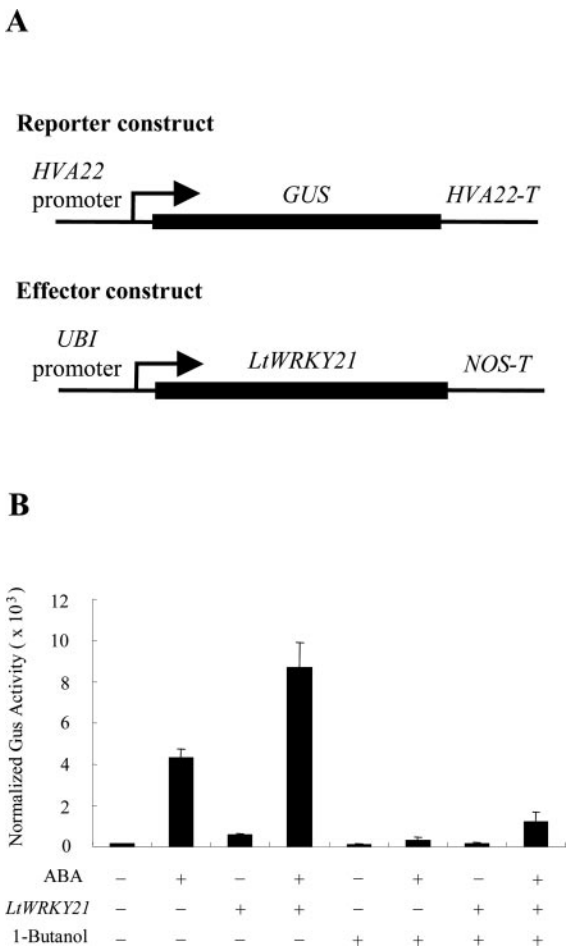

FIG. 9. **1-Butanol blocks the synergistic effect of ABA and LtWRKY21.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. The annotations are the same as in Fig. 4. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector construct (*UBI-LtWRKY21*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seed with (+) or without (−) 1% 1-butanol and 20 μM ABA. The data are the means ± S.E. of four replicates.

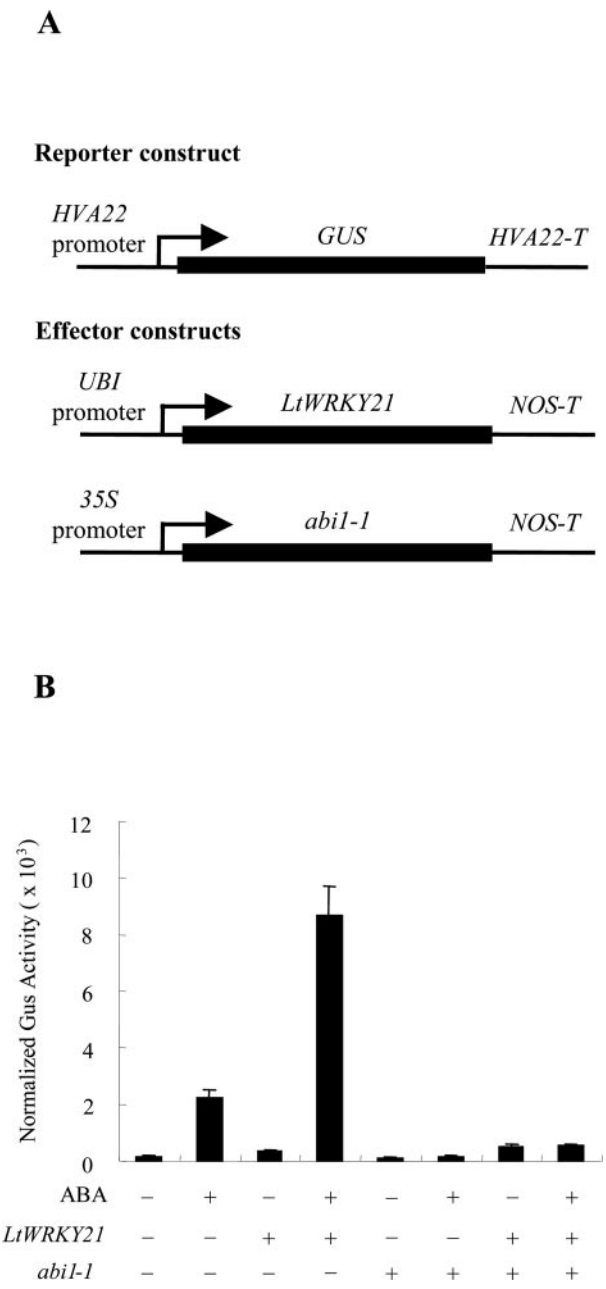

FIG. 10. **ABI1 blocks the synergistic effect of ABA and LtWRKY21.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. The 35S promoter is from the cauliflower mosaic virus. *abi1-1* is the dominant mutant gene of *ABI1* from *Arabidopsis*. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector constructs (*UBI-LtWRKY21* or *UBI-abi1-1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seed with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

ing the ABA signaling (43, 87), we studied the effect of co-expressing *abi1-1*, *LtWRKY21*, *VP1*, and *ABI5* on regulating the *HVA22* promoter. As shown in Fig. 11, co-expression of *LtWRKY21*, *VP1*, and *ABI5* led to a 122-fold induction of *HVA22-GUS,* which is much higher than that of ABA treatment alone (33-fold in this experiment). ABA treatment did not further enhance the induction of the *HVA22* promoter by co-expression of *LtWRKY21*, *VP1*, and *ABI5*. Interestingly, abi1-1 did not block the synergistic effect of LtWRKY21, VP1, and ABI5 on inducing the *HVA22* promoter, either in the absence or in the presence of ABA (Fig. 11).

DISCUSSION

The creosote bush survives exceptionally well in the arid desert where rainfall events only occur a few times each year. Understanding the molecular mechanism underlying its resistance to drought is biologically and agriculturally important. Because transcription factors are master switches of gene regulation, alternations in their expression levels, activities, and/or functions, as opposed to those of structural genes, are more likely to have broader impacts on the resistance of plants to environmental stresses and hence on the speciation of creosote bush. Therefore, we focused on drought stress-induced transcription factors. In *Arabidopsis*, there are 43 stress-induced transcription factor genes that have been identified, corresponding to 11% of all stress-inducible genes (77). Among these stress-inducible proteins, there are six DREBs, two ERFs, ten zinc fingers, four WRKYs, three MYBs, two bHLHs, four bZIPs, five NACs, and three homeodomain transcription factors (77). The protein sequences of these 43 transcription factors were collected and searched against the translated creosote bush expression sequence tag database. This effort led to

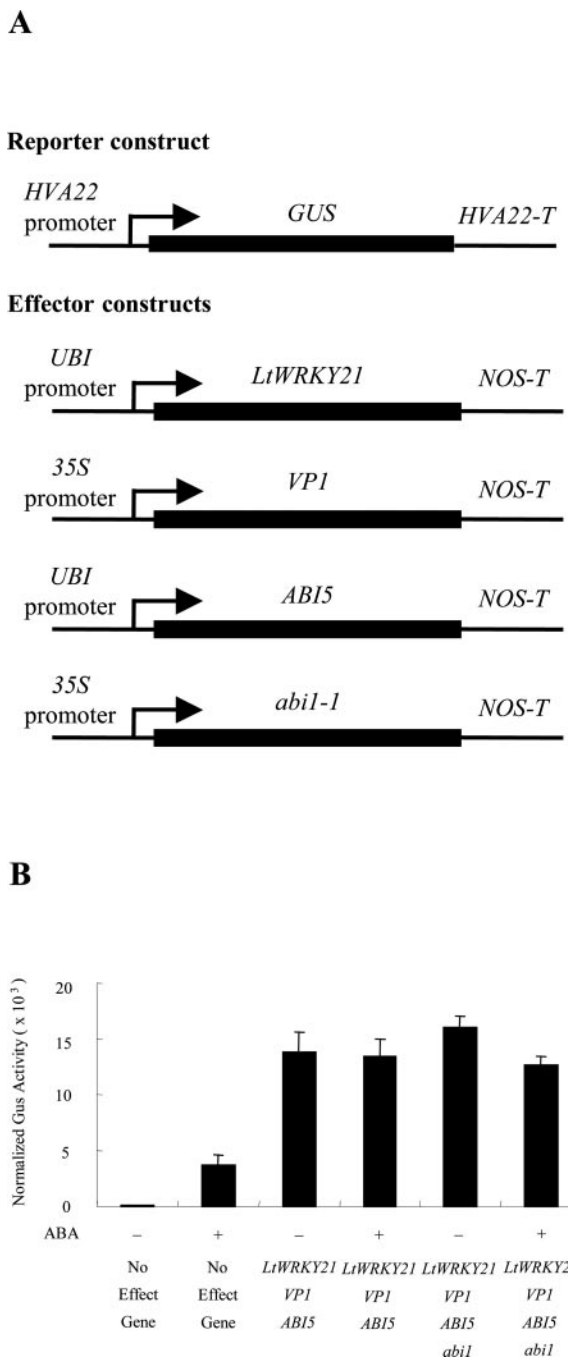

FIG. 11. **Interaction among LtWRKY21, VP1, ABI5, and ABI1.** *A*, schematic diagrams of the reporter and effector constructs used in the co-bombardment experiment. *B*, the reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (−) the effector constructs (*UBI-LtWRKY21*, *UBI-ABI5*, *35S-VP1*, or *35S-abi1-1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The *bars* indicate GUS activities ± S.E. after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. The data are the means ± S.E. of four replicates.

the identification of ten putative stress-inducible transcription factors of different families in creosote bush. One of the ten genes is *LtWRKY21*, which is highly expressed in creosote bush seeds (Fig. 1). LtWRKY21 contains a WRKY motif, a zinc finger motif, two nucleus targeting signal sequences, and a putative leucine zipper domain (Fig. 2). Consistent with its role as a transcription factor, the GFP-LtWRKY21 fusion proteins were targeted to nuclei (Fig. 3).

The homologues of LtWRKY21 are present in many other plant species such as *Arabidopsis*, parsley, cotton, tobacco, and grapevine and play a variety of roles. *AtWRKY40* is a drought and ABA response gene; *GaWRKY1*, *PsWRKY4*, and *VaWRKY4* are involved in pathogen defense, and *NtWIZZ* is a wounding-inducible gene. The goal of this study was to address the function of *LtWRKY21* in ABA responses. It has been demonstrated that the ABA signaling machinery is conserved among higher plant species and even bryophytes (8, 9). For example, the promoter of *Em*, a wheat ABA-responsive gene, responds to osmotic stress and ABA in moss, and the moss transcription factors can bind to the *Em* promoter (8). Transcription factors from maize and *Arabidopsis* function well in barley aleurone layer (87) or rice protoplasts (9) to regulate the expression of the wheat *Em*, *Arabidopsis AtEM6*, bean *β-Phaseolin*, and barley *HVA1* and *HVA22* promoters. Therefore, we studied the function of LtWRKY21 in barley aleurone cells and demonstrated that it acted as an activator of ABA signaling (Figs. 4 and 5). Unlike *AtWRKY40*, *LtWRKY21* expression did not appear to be affected by external ABA applications in the seeds (Fig. 1). Similar results were observed for other genes involved in ABA signaling such as *VP1/ABI3* and *ABI4* genes (6, 88, 89). It is speculated that LtWRKY21 activity may be regulated by post-translational modifications and/or interactions with other regulators in response to ABA.

Six classes of transcription factors have been demonstrated by genetic analyses to be essential for ABA responses: VP1/ABI3, ABI4, ABI5, LEC1, LEC2, and FUS3 (reviewed in Ref. 6). We studied the interactions of LtWRKY21 with VP1 and ABI5. Like VP1 and its *Arabidopsis* orthologue ABI3, ABI5 and other bZIP transcription factors function as activators of ABA signaling (19, 20, 24, 36, 43, 87, 90, 91). Excitingly, LtWRKY21 synergistically interacted with VP1 (Fig. 7) and ABI5 (Fig. 8) in regulating ABA responses. VP1 has been shown to potentiate ABA-inducible gene expression by forming a DNA-binding complex with bZIP, 14-3-3, ring (C_3HC_3-type) zinc finger proteins, and RNA polymerase II subunit RPB5 (91–94). Our data suggest that the WRKY protein might be a new component of this complex.

WRKY proteins can bind specifically to the W box that contains a TGAC core (72, 95, 96). The putative W box has been found in the promoter regions of *HVA22* (73) and *ABF* (36). However, the 49-bp promoter fragment in the reporter construct of this study does not include this W box. Similarly, this promoter does not contain the SphI element that is bound by the C-terminal B3 domain of VP1 (48). Instead, only two elements are present in this promoter fragment: the ABRE that is bound by ABI5 or its related bZIP proteins (24, 38, 43, 91) and CE1 that is bound by a APETALA2 domain-containing transcription factor ABI4 (21, 44). It should be noted that the full-length VP1 does not bind DNA specifically *in vitro*, suggesting that it interacts with other proteins that mediate DNA binding (48). Our preliminary data suggest that LtWRKY21 does not bind to the promoter sequence of the *HVA22-GUS* reporter construct used in this study.[2] Therefore, we suggest that LtWRKY21 regulates the *HVA22* promoter as a non-DNA-binding component of the transcription complex mentioned above.

Deletion and substitution studies of LtWRKY21 should lead to the identification of domains and residues that are essential for the interaction of LtWRKY21 with the remaining components of the transcription complex. Fig. 6 shows that the C-terminal region, which contains the WRKY domain and zinc finger motif, was required for LtWRKY21 transactivating the

[2] X. Zou and J. Q. Shen, unpublished results.

expression of the *HVA22* promoter. Interestingly, LtWRKY21 also contains the EAR motif, which is necessary for the repression function of AtERF3, AtERF4, and their orthologues in ethylene signaling of *Arabidopsis* (81), wheat, and petunia plants (97). Yet the EAR motif was essential for the transactivation activity of LtWRKY21. These data suggest that the same motif might play opposite roles in different hormonal signaling pathways, and it is possible that LtWRKY21 is also involved in ethylene signaling. Transcription factors with dual activities have been found in plants. For instance, maize *VP1* promotes the ABA induction pathway yet inhibits the GA induction pathway (87, 98). *Arabidopsis WRKY6* acts as a negative regulator of its own and *WRKY42* expression; on the other hand, it positively influences the senescence- and pathogen defense-associated *PR1* promoter activity (99).

Several groups of proteins such as G proteins, phospholipases, protein kinases, and protein phosphatases are involved in the early events of ABA signaling (100–103). Phospholipases C and D produce inositol 1,4,5-trisphosphate and diacylglycerol or phosphatidic acid and the head group, respectively. These products of phospholipases act as secondary messengers in ABA signaling (101 ,104). The application of phosphatidic acid to barley aleurone inhibits α-amylase production and induces an ABA-inducible amylase inhibitor and RAB (response to ABA) protein expression (84). On the other hand, 1-butanol, a specific inhibitor of PLD (105), inhibits the accumulation of the RAB protein (84). 1-Butanol inhibits the transactivation of VP1 or ABI5 on ABA response promoters (85). Together, these data suggest that PLD is involved in ABA signaling. Fig. 9 shows that the synergistic effect of LtWRKY21 and ABA was also inhibited by 1-butanol. ABI1 and its dominant negative mutant abi1-1 act as a negative regulator of ABA signaling in *Arabidopsis* (106), barley (87), and rice (85). The ABI1-1 inhibitory effect is able to overcome the transactivation effect of VP1 or ABI5 in ABA signaling (85, 87), but it does not decrease the synergistic effect of VP1 and ABI5 on ABA induction, indicating that abi1-1 acts upstream of ABI5 in the ABA up-regulatory pathway (43). Here, we showed that abi1-1 inhibited the synergistic effect of ABA and LtWRKY21 (Fig. 10), but it had little effect when *VP1*, *ABI5* and *LtWRKY21* were co-expressed (Fig. 11). Therefore, we suggest that LtWRKY21 may form a complex with VP1, ABI4, and ABI5 to control ABA response, and this complex functions downstream of ABI1 in ABA signaling. Experiments are ongoing to further address this question.

Acknowledgments—We thank Dr. Andrew Andres and members of our lab for contributions to the improvement of this article. We also thank Dr. John Cushman (University of Nevada, Reno) for providing us with the *Larrea* cDNA clones and the sGFP construct and Drs. David Ho and Jose Casaretto (Washington University, St. Louis, MO) for sending us the ABA-responsive reporter and effector constructs.

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J. Biol. Chem. 2004, 279:55770-55779.
doi: 10.1074/jbc.M408536200 originally published online October 25, 2004
