A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription

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The transcription factor VP1 regulates maturation and dormancy in plant seeds by activating genes responsive to the stress hormone abscisic acid (ABA). Although activation involves ABA-responsive elements (ABREs), VP1 itself does not specifically bind ABREs. Instead, we have identified and cloned a basic region leucine zipper (bZIP) factor, TRAB1, that interacts with both VP1 and ABREs. Transcription from a chimeric promoter with GAL4-binding sites was ABA-inducible if cells expressed a GAL4 DNA-binding domain::TRAB1 fusion protein. Results indicate that TRAB1 is a true trans-acting factor involved in ABA-regulated transcription and reveal a molecular mechanism for the VP1-dependent, ABA-inducible transcription that controls maturation and dormancy in plant embryos.

Two major roles of the phytohormone abscisic acid (ABA) are known. First, ABA mediates responses of plants to environmental stress, such as high salinity, drought, and low temperature (1). Second, ABA is required for key events during seed maturation, which include accumulation of storage proteins and other reserve materials, acquisition of desiccation tolerance, arrest of embryonic development, and achievement of dormancy (2, 3). Many ABA-regulated genes associated with these processes have been identified. Detailed studies of cis-acting promoter elements required for ABA-induced transcriptional activation have been conducted with various ABA-inducible genes, including the rice *Osem* (4–8). These studies have revealed ABA-responsive elements (ABREs) with a consensus sequence (T/G/C)ACGT(G/T)GC. Because multimerized ABREs can confer ABA responsiveness to a heterologous, minimal promoter (7, 9), this sequence intrinsically possesses the capacity to mediate ABA signals. In a natural promoter context, an ABRE functions with a second sequence element called a coupling element. The two elements together constitute an ABA-responsive cis-element complex (ABRC), which can synergistically activate transcription in response to ABA (6, 8). Two distinct coupling elements, namely, CE1 (6) and CE3 (8), have been identified.

Several basic region leucine zipper (bZIP) factors that bind ABREs have been cloned as candidates for ABA-responsive transcription factors. These proteins include the wheat EmBP1 (10), the tobacco TAF-1 (11), and the rice OSBZ8 (12) and osZIP-1a (13). OSBZ8 mRNA is induced rapidly by ABA and precedes induction of other ABA-responsive genes (12). OSBZ8 and osZIP-1a also can slightly transactivate the ABA-inducible promoter (ref. 13; T.H. and T.H., unpublished results). However, none of these proteins have been demonstrated conclusively to function as actual transcription factors in ABA-responsive gene expression.

On the other hand, molecular genetic studies have clearly established the involvement of another class of transcription factor, VP1/ABI3, for ABA-regulated gene expression in seed tissues (1, 3). The maize *VP1* and *Arabidopsis ABI3* are seed-specific, ABA-insensitive loci, and are considered orthologous with one another (14, 15). Other orthologues of VP1 and ABI3 also have been cloned from various plant species (16–19) and include the rice OSVP1 used in the present study (19). Because VP1 and OSVP1 have been shown to act through ABREs in

ABA-regulated genes and to do so without specifically binding to them, it has been proposed that VP1 functions by interacting with an unidentified factor that directly binds to ABREs (5, 9, 20, 21). Therefore, the cloning of such a factor is essential to understand the mechanism for VP1-dependent ABA-regulated transcription. Here, we describe the cloning and characterization of a bZIP protein that interacts with both VP1 and ABREs and mediates ABA signals.

Materials and Methods

Yeast Two-Hybrid Screening. Yeast two-hybrid screening was conducted by using a Matchmaker Two-Hybrid system kit (CLONTECH). Poly(A)$^+$ RNA prepared from developing rice embryo (10–11 days after flowering) was used to synthesize cDNA. HybriZAP Two-Hybrid vector (Stratagene) was used to construct a GAL4 activation domain (GAD) fusion cDNA library, which was converted to a yeast plasmid (pAD-GAL4) library by *in vivo* excision. The entire coding region of OSVP1ΔAD (9) was amplified via PCR by using the universal M13 and 5′-CAGAATTCATGGACGCCTCCGCCG-3′ primers to create an *Eco*RI site at the N terminus. The PCR products were digested with *Eco*RI and Eco47III (internal to OSVP1) and subcloned into *Eco*RI/*Sma*I-digested pBluescript to produce pBlue-VP1-N. The pBlue-VP1-N insert was excised with *Eco*RI and *Bam*HI and ligated into *Eco*RI/*Bam*HI-digested pGBT9 (22) to produce the bait plasmid (pGBD::VP1-N). The yeast strain Y190 (23) was used for screening and LacZ assays (24). The yeast strain YRG-2 (Stratagene) was used for HIS3 assays, described in Fig. 1. Primary yeast screening was conducted on plates with medium containing 25 mM 3-aminotriazole (3-AT).

Transient Expression Assay Using Rice Protoplasts. Electroporation of rice cultured-cell protoplasts was carried out as described previously (5). Five micrograms of a ubiquitin promoter-luciferase plasmid (25) was included in each electroporation as an internal standard. The β-glucuronidase (GUS) activities reported were normalized to the luciferase activity. Each treatment was repeated four times in an experiment by using a single preparation of protoplasts.
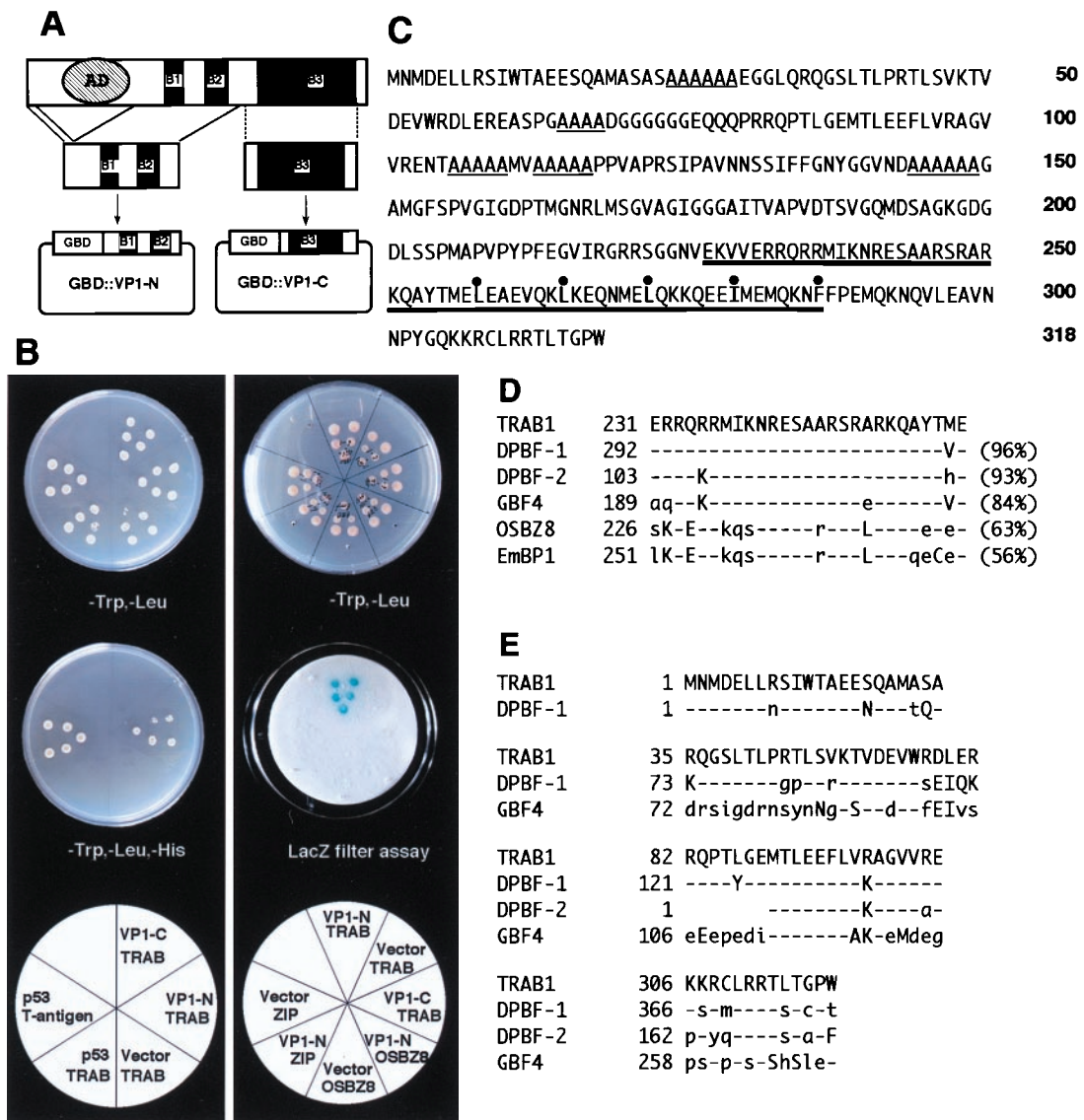
Plasmid Construction for Transient Expression Assays. The ABRC-TATA construct was prepared by ligating *Hind*III/*Xho*I-digested pIG46 (26) and a double-stranded synthetic oligonucleotide with the sequence corresponding to −179 to −125 of the *Osem* promoter (5) and 5′ overhangs compatible with *Hind*III and *Xho*I cut ends. Expression plasmids for GAD, GAL4

A

C

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MNMDELLRSIWTAEEESQAMASASAAAAAAAEGGLQRQGSLTLPRTLSVKTV      50
DEVWRDLEREASPGAAAADGGGGGGEQQQPRRQPTLGEMTLEEFLVRAGV       100
VRENTAAAAAMVAAAAAAPPVAPRSIPAVNNSSIFFGNYGGVNDAAAAAAG     150
AMGFSPVGIGDPTMGNRLMSGVAGIGGGAITVAPVDTSVGQMDSAGKGDG     200
DLSSPMAPVPYPFEGVIRGRRSGGNVEKVVERRQRRMIKNRESAARSRAR     250
KQAYTMELEAEVQKLKEQNMELQKKQEEIMEMQKNFFPEMQKNQVLEAVN     300
NPYGQKKRCLRRTLTGPW                                     318
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B

D

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TRAB1   231 ERRQRRMIKNRESAARSRARKQAYTME
DPBF-1  292 ------------------------V-  (96%)
DPBF-2  103 ----K-------------------h-  (93%)
GBF4    189 aq--K------------e------V-  (84%)
OSBZ8   226 sK-E--kqs-----r---L----e-e- (63%)
EmBP1   251 lK-E--kqs-----r---L---qeCe- (56%)
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E

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TRAB1     1 MNMDELLRSIWTAEEESQAMASA
DPBF-1    1 -------n-------N---tQ-

TRAB1    35 RQGSLTLPRTLSVKTVDEVWRDLER
DPBF-1   73 K-------gp--r--------sEIQK
GBF4     72 drsigdrnsynNg-S--d--fEIvs

TRAB1    82 RQPTLGEMTLEEFLVRAGVVRE
DPBF-1  121 ----Y----------K------
DPBF-2    1         --------K----a-
GBF4    106 eEepedi-------AK-eMdeg

TRAB1   306 KKRCLRRTLTGPW
DPBF-1  366 -s-m----s-c-t
DPBF-2  162 p-yq----s-a-F
GBF4    258 ps-p-s-ShSle-
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Fig. 1. Isolation and characterization of TRAB1 cDNA. (*A*) Schematic illustration of the bait construct (pGBD::VP1-N) used for yeast two-hybrid screening. pGBD::VP1-C was used as a control bait. The conserved basic regions (B1, B2, and B3) and the acidic transcriptional activation domain of OSVP1 are indicated by solid boxes and a hatched oval, respectively. (*B*) Yeast two-hybrid assays showing that TRAB1 specifically interacts with the VP1-N fragment. (*Left*) HIS3 assays. Five independent yeast transformants harboring various combinations of a bait (GBD fusion) and prey (GAD fusion) plasmids were grown on an SD medium plate containing 30 mM 3-aminotriazole, lacking Trp and Leu (*Top*) or lacking Trp, Leu, and His (*Middle*). At the bottom, the combination of bait (top line) and prey (bottom line) fragments fused to GBD and GAD, respectively, is indicated in each sector. The combination of the T antigen prey and p53 bait was used as a control for positive interaction. ''Vector'' indicates a negative control bait of GBD alone (pGBT9). Note that only transformants with the combination of pGBD::VP1-N and pGAD::TRAB1 or the positive controls grew on the minus His medium. (*Right*) LacZ assays. Yeast transformants harboring the indicated combinations of plasmids were grown on an SD medium plate lacking Trp and Leu (*Top*), and replicate filters were used for LacZ filter assays with X-Gal (*Middle*). OSBZ8 (12) and ZIP are negative control preys. The cDNA for ZIP was obtained by a yeast one-hybrid screen of the same cDNA library by using the 55-bp ABRC of *Osem* promoter as a target site and found to encode a protein with a bZIP structure very similar to that of TRAB1 in-frame with GAD. Note that positive signals were obtained only with the combination of GBD::VP1-N and GAD::TRAB1. (*C*) Amino acid sequence of TRAB1 deduced from the nucleotide sequence of the cDNA. The bZIP region and short stretches of Ala residues are indicated by thick and thin underlines, respectively. Hydrophobic amino acid residues that constitute a leucine zipper structure are marked with dots. (*D*) Comparison between the sequence of the basic region of TRAB1 and those of DPBF-1, DPBF-2, GBF4, OSBZ8, and EmBP1. Percentages of identical amino acids to those of TRAB1 are shown in parentheses. Amino acids identical, similar, and dissimilar to those of TRAB1 are indicated by dashes and uppercase and lowercase letters, respectively. (*E*) Amino acid sequence blocks conserved between TRAB1, DPBF-1, DPBF-2, and GBF4. Amino acids identical, similar, and dissimilar to those of TRAB1 are indicated by dashes and uppercase and lowercase letters, respectively.

DNA-binding domain (GBD), and their fusions were constructed in a plant expression vector p35S-shΔ-stop, which, from 5′ to 3′, consists of the CaMV 35S promoter, internally deleted first intron of *Sh1*, multiple cloning sites, stop codons in three frames, and a nopaline synthase terminator (M. Suzuki and D. R. McCarty, personal communication). A GAD fragment prepared from pGAD424 (22) by digestion with *Hin*dIII and *Sal*I was subcloned into similarly digested pBluescript, excised with *Eco*RV and *Sal*I, then ligated into *Sma*I/*Sal*I-digested p35S-ShΔ-stop to produce p35S-ShΔ-GAD, an expression plasmid for GAD alone. An expression plasmid, p35S-ShΔ-GBD, for GBD alone was constructed as for p35S-ShΔ-GAD, using a GBD fragment from pGBT9. p35S-ShΔ-GBD::TRAB1 and p35S-ShΔ-GAD::TRAB1 for expression of GBD::TRAB1 and
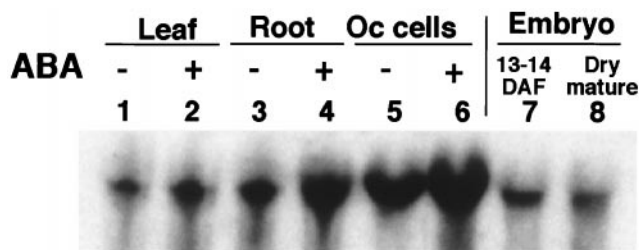
Fig. 2. Northern hybridization analysis of TRAB1. Thirty micrograms of RNA was subjected to Northern hybridization analysis by using a TRAB1 cDNA probe. RNA was from leaves (lanes 1 and 2) and roots (lanes 3 and 4) of young rice plants (14 days old) treated with 10^{-4} M ABA for 6 h (lanes 2 and 4) or receiving no ABA (lanes 1 and 3), suspension-cultured cells (Oc cells) treated with 5×10^{-5} M ABA for 6 h (lane 6) or receiving no ABA (lane 5), and rice embryos at 13–14 days after flowering (lane 7) or at dry mature stage (lane 8). The entire TRAB1 cDNA was used for the probe.

GAD::TRAB1 fusion proteins were constructed by ligating the *Sal*I-*Eco*RI fragment of TRAB1 cDNA into *Sal*I/*Eco*RI-digested p35S-ShΔ-GBD and p35S-ShΔ-GAD, respectively. p35S-ShΔ-GBD::VP1-N for expression of GBD::VP1-N fusion protein was constructed by ligating the *Sal*I-*Eco*RI insert of pBlue-VP1-N into the *Sal*I/*Eco*RI-digested p35S-ShΔ-GBD.

**Electrophoretic Mobility-Shift Assays Using *in Vitro*-Translated TRAB1.** An *Eco*RI-*Sal*I fragment of TRAB1 cDNA (the entire fragment) was cloned into *Eco*RI/*Sal*I-digested pCITE-4a (Novagen), linearized with *Spe*I (3′ to the *Sal*I site), and transcribed with T7 RNA polymerase. The transcribed RNA was translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. A 3-μl aliquot of the translation reaction was included in a 20-μl DNA-binding reaction and resolved on a 5% polyacrylamide gel as described previously (12). Control binding reactions were performed by using translation reaction medium without template.

Other Methods. Treatments of plants and cells, RNA preparation, Northern hybridization analysis, and DNA sequence determination were conducted as described previously (12).

Results

Cloning of TRAB1. To clone DNA-binding factors that specifically interact with VP1, we carried out a yeast two-hybrid screen. For the bait construct (GBD::VP1-N), amino acid residues 1–17 and 188–517 of the rice VP1 (OSVP1) (19) were fused to the GBD (Fig. 1*A*). This was used to screen a GAD fusion cDNA library prepared from developing rice embryo. The GBD::VP1-N bait fragment contained two conserved basic regions of VP1, B1, and B2, but not the N-terminal transcriptional activation domain or the highly conserved C-terminal B3 region (shown to have *Sph* element-binding activity; ref. 20). Both *HIS3* and *lacZ* reporters were used to identify positive interactions. Approximately 4×10^6 yeast transformants were screened, and one reproducibly positive clone was identified from both HIS3 and LacZ assays. A specific interaction between the OSVP1 fragment and the cDNA-encoded protein was implicated by the presence of $HIS^+/LacZ^+$ phenotypes only when the positive cDNA clone plasmid and the GBD::VP1-N construct were cotransformed (Fig. 1*B*).

This positive clone contained a 1,167-bp cDNA sequence encoding a protein of 318 aa (Fig. 1*C*), joined in-frame with GAD in the cloning vector. The cDNA-encoded protein was designated TRAB1 (for transcription factor responsible for ABA regulation), based on the results described below. Database analysis indicated that TRAB1 contains a bZIP structure in
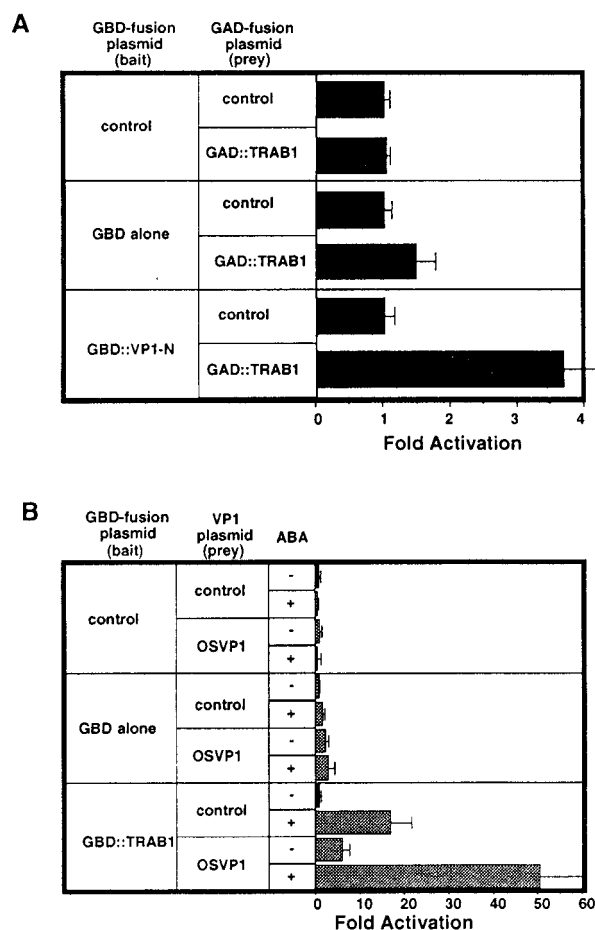
Fig. 3. Interaction between TRAB1 and VP1 in rice protoplast cells. (*A*) Two-hybrid interactions in the plant cell. Ten micrograms each of bait plasmid [p35S-ShΔ-stop (control), p35S-ShΔ-GBD (GBD alone), or p35S-ShΔ-GBD-VP1-N (GBD::VP1-N)], prey plasmid [p35S-ShΔ-stop (control) or p35S-ShΔ-GAD::TRAB1 (GAD::TRAB1)], and the UAS-TATA-GUS reporter plasmid were cotransfected into protoplasts of rice suspension-cultured cells by electroporation. The electroporated cells were cultured for 40 h. The control plasmid (p35S-ShΔ-stop) consisted of CaMV 35S promoter, *Sh1* first intron with an internal deletion, and NOS terminator, but no coding region. Each transfection also included 5 μg of a ubiquitin promoter::luciferase plasmid as an internal standard. GUS activities were normalized by luciferase activity. The effects of GAD::TRAB1 (prey) fusion on the expression of the reporter gene are expressed by fold activation of normalized GUS activities relative to that obtained with a combination of indicated bait and control plasmids. Each value is the mean of four replicate electroporations with standard error. (*B*) Interaction between VP1 and GBD::TRAB1 and the capacity of GBD::TRAB1 fusion to confer ABA responsiveness to a promoter carrying GAL4-binding sites. Ten micrograms each of bait (GBD fusion) plasmid [p35S-ShΔ-stop (control), p35S-ShΔ-GBD (GBD alone), or p35S-ShΔ-GBD-TRAB1 (GBD::TRAB1)], prey plasmid [p35-S-ShΔ-stop (control) or p35-S-Sh-OSVP1 (OSVP1)], and the UAS-TATA-GUS reporter plasmid were cotransfected into protoplasts of rice suspension-cultured cells by electroporation, and the cells were cultured for 40 h in the absence (−) or presence (+) of 5×10^{-5} M ABA. The effects of OSVP1 and ABA on the expression of the reporter gene are expressed by fold activation of normalized GUS activities relative to that obtained with a combination of indicated GBD (bait) fusion and control plasmid in the absence of ABA. Other details are as described for *A*.

the C-terminal region (Fig. 1*C*). This basic region of TRAB1 is highly homologous (amino acid identity: 84–96%) to that of a *Helianthus Dc3* promoter-binding factor, DPBF-1 (27), as well as those of related DPBF-2 (27) and *Arabidopsis* GBF4 (28), compared with those of OSBZ8 (12) and EmBP1 (10) (amino acid identity: 63 and 56%, respectively), which previously have
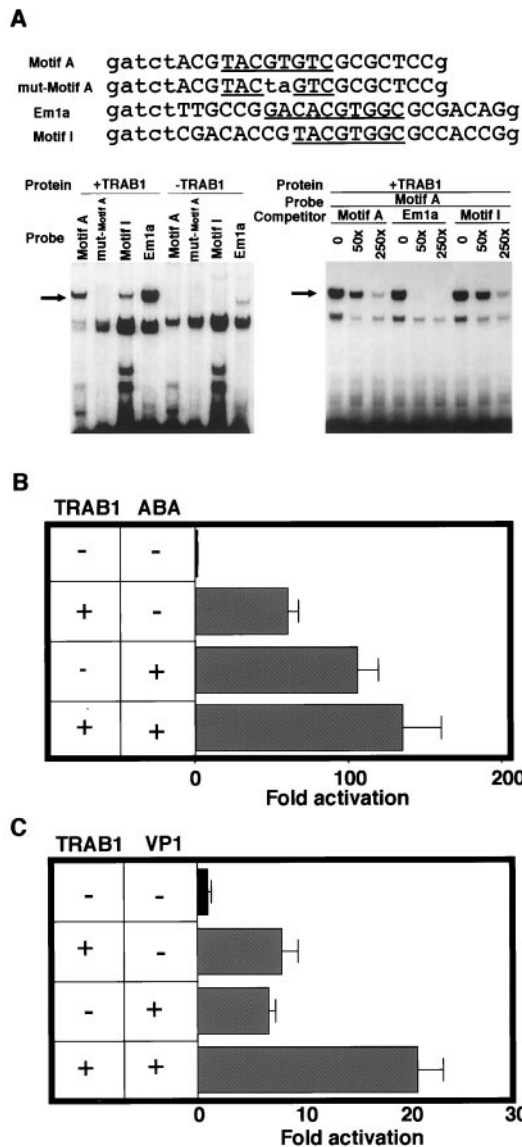
Fig. 4. Interaction of TRAB1 with ABREs and activation of the ABRC of *Osem* promoter consisting of ABRE and CE3. (*A*) Electrophoretic mobility-shift assays of TRAB1 synthesized *in vitro*. Nucleotide sequences of ABREs (underlined) and its mutant used for probes of electrophoretic mobility-shift assays are listed on the top. The synthetic double-strand DNA fragments carried 4-bp 5′ overhangs at each end for fill-in labeling. Each different probe without specific competitors (*Left*) or motif A probe with an unlabeled competitor as indicated (*Right*) was incubated with TRAB1 protein (+TRAB1) synthesized in a rabbit reticulocyte cell-free system. "−TRAB1" indicates the control binding reactions with the reticulocyte lysate after protein synthesis reaction without added RNA templates. "×" indicates fold-molar excess. DNA–protein complexes were separated from free probes by PAGE. Arrows indicate the position of the bands of DNA–protein complexes specific to TRAB1 protein. Other bands derive from the protein present in the reticulocyte lysate. (*B*) Activation of the 55-bp ABRC of *Osem* by TRAB1. Ten micrograms of the ABRC-TATA-GUS reporter plasmid was cotransfected with the same amount of TRAB1 expression (p35S-ShΔ-TRAB1) or control (p35S-ShΔ-stop) plasmids, as indicated, into rice cell protoplasts by electroporation, and protoplasts were cultured in the absence (−) or presence (+) of 5×10^{-5} M ABA. The effects of TRAB1 and ABA are indicated by fold activation of normalized GUS activities relative to that obtained with the control plasmid in the absence of ABA. Other details are as described for Fig. 3. (*C*) The effect of VP1 overexpression on the activation of the 55-bp ABRC by TRAB1. Cotransfection experiments were conducted as in *B* except for the following: the amount of p35S-ShΔ-TRAB1 or its control plasmid was reduced to 5 μg; 30 μg of VP1 expression plasmid (p35S-Sh-OSVP1) or its corresponding control plasmid was included; and protoplasts were cultured only in the absence of ABA.

been implicated in ABA regulation (Fig. 1*D*). In addition to the bZIP region, TRAB1 contains three short-sequence blocks that are conserved among these bZIP factors (Fig. 1*E*). Another feature of the TRAB1 sequence is that short stretches of alanine residues (four to six) are dispersed in the N-terminal half of the protein. Such alanine stretches are not present in DPBF-1 but are found in other bZIP proteins (29–31) such as HBP-1b, OHP2, and HALF1, all of which have been isolated from monocot plant species.

Expression of TRAB1. Northern analysis (Fig. 2) revealed that TRAB1 mRNA was expressed not only in developing embryos but also in dry, mature embryos, leaves and roots of young plants, and in the cultured cells used for transient assays, described in Figs. 3 and 4. Among these cells and tissues, relative abundance of TRAB1 mRNA was highest in the cultured cells. Expression of the *TRAB1* gene was slightly up-regulated by exogenously applied ABA (Fig. 2).

TRAB1 Interacts with VP1 in Plant Cells. To assess the interaction between VP1 and TRAB1 independently, the same bait protein (GBD::VP1-N fusion) was expressed in cultured rice cells together with the prey protein (GAD::TRAB1 fusion). Two-hybrid interaction in these cells was monitored by activation of a UAS-TATA-GUS (a GUS reporter gene placed downstream of a chimeric promoter consisting of GAL4-binding sites and the CaMV 35S minimal promoter; ref. 32). With GBD alone (p35S-ShΔ-GBD) or without a bait protein (p35S-ShΔ-stop), no significant activation of the reporter gene was observed by cotransfection of the GAD::TRAB1 expression plasmid (p35S-ΔSh-GAD::TRAB1) (Fig. 3*A*). However, with the GBD::VP1-N bait (p35S-ShΔ-GBD::VP1-N), cotransfection of the GAD::TRAB1 plasmid resulted in a significant activation of the reporter (Fig. 3*A*). TRAB1, therefore, can specifically interact with VP1 in plant cells. The specific interaction was evidenced further by an experiment in which the bait and prey were exchanged. The VP1 prey used in this experiment was not in the form of GAD fusion but, instead, in the native form of OSVP1 because VP1 itself has a strong acidic transcriptional activation domain (14). Cotransfection of VP1 (p35S-Sh-OSVP1; ref. 19) together with GBD::TRAB1 resulted in a 7-fold activation of UAS-TATA-GUS (Fig. 3*B*; in the absence of ABA). On the other hand, essentially no or only a slight activation was observed by VP1 in the combination with control or GBD expression plasmid. The result confirmed the specific interaction between VP1 and TRAB1 in plant cells. It also suggests that TRAB1 activates a target gene by recruiting VP1 that possesses an transcriptional activation domain.

Specific interaction *in vitro* between VP1 and TRAB1 was not detectable by using either *in vitro*-translated or *Escherichia coli*-produced proteins in glutathione *S*-transferase pull-down experiments or electrophoretic mobility-shift assays (supershift). If the interaction *in vivo* depends on an ABA signal under normal conditions as discussed below, some modification(s) of either VP1 or TRAB1 or both may be required for interaction *in vitro*. Because the two-hybrid interaction was observed in yeast, yeast may have some degree of such a modification activity. Other accessory factors also might be required for stable formation of a complex (33). Such factors may assist interaction between VP1 and TRAB1 or constitute additional protein members of a larger complex. Technical problems may also be involved, such as an improper folding of *E. coli*-produced protein and a high, nonspecific binding of the *in vitro*-synthesized TRAB1 to glutathione-Sepharose.

TRAB1 Can Mediate ABA Signals. In addition to the specific interaction between TRAB1 and VP1, the experiment using the GBD::TRAB1 fusion construct gave another important result

that revealed the function of TRAB1 (Fig. 3*B*). The expression of UAS-TATA-GUS was activated strongly (17-fold) by ABA when the GBD::TRAB1 expression plasmid was cotransfected even without the VP1 expression plasmid (Fig. 3*B*). In contrast, essentially no ABA induction was detected either with the expression of GBD alone or an empty vector (Fig. 3*B*). These results show that TRAB1 is a transcription factor that truly mediates ABA signals. The ABA induction of the reporter gene in the cells transfected with the GBD::TRAB1 alone (Fig. 3*B*) is considered to be supported by the endogenous VP1 protein in our rice protoplasts (the cultured rice cells used for the protoplast preparation express OSVP1 at a level equal to or higher than that in developing embryo; ref. 34). In the presence of overdriven VP1 and GBD::TRAB1, the ABA induction was enhanced further (Fig. 3*B*).

TRAB1 Interacts with ABREs Both *in Vitro* and *in Vivo*. To test whether TRAB1 interacts directly with ABREs, we conducted electrophoretic mobility-shift assays (35) by using *in vitro*-translated TRAB1 protein (Fig. 4*A*). TRAB1 was found to bind to the ABREs such as motif A and motif I of the rice *Osem* (5) and *Rab16A* (7), respectively, and Em1a of the wheat *Em* (10) (Fig. 4*A Left*). When a mutation was introduced in motif A, the binding was abolished. The binding to motif A was inhibited by excess amounts of unlabeled motif A as well as other ABREs (Fig. 4*A Right*). These results show that TRAB1 specifically binds to ABREs. The competition assays also indicated that TRAB1 has a higher relative affinity to Em1a, which contains a palindromic G-box, compared with motif A and motif I, which contain an A-box/G-box hybrid.

A second line of evidence for TRAB1 interaction with ABREs was obtained by cotransfection assays by using a GUS reporter under the control of a fusion promoter consisting of the 55-bp ABRC of *Osem* directed from a chimeric promoter (ABRC-TATA-GUS). The 55-bp ABRC is a minimal-length fragment of the *Osem* promoter (-179 to -124) (5) that can confer both ABA and VP1 responsiveness to a heterologous minimal promoter and that contains an ABRE (motif A) and a CE3 (8). As shown in Fig. 4*B*, overexpression of TRAB1 activated the expression of ABRC-TATA-GUS by more than 50-fold. Reporter gene expression also was increased somewhat above its ABA-induced level when TRAB1 was expressed in the presence of ABA. Simultaneous overexpression of VP1 and TRAB1 (at unsaturating levels; see legend to Fig. 4*C*) led to a further activation of the target promoter (Fig. 4*C*). This again confirmed the specific interaction between VP1 and TRAB1. These results, together with those of electrophoretic mobility-shift experiments, indicate that TRAB1 can function as a trans-acting factor for ABRE. The interaction between ABRE and TRAB1 provides a physical basis for the link between ABRE and VP1, which has been revealed by the observation that VP1 activation is achieved via ABRE (9).

Discussion

In the present study, we have cloned and characterized an ABRE-binding bZIP factor that interacts with VP1. The results provide compelling evidence that VP1 functions through interaction with a factor that directly binds to ABRE, thereby regulating ABA-induced transcription. In addition, together with previous work (20, 21), this research shows that VP1 functions as a transcriptional activator by two distinct mechanisms depending on target cis-elements: VP1 interacts with the *Sph* element of the *C1* promoter by directly binding through its B3 domain, and it acts on ABREs via TRAB1.

We observed a significant ABA enhancement of the interaction between GBD::TRAB1 and VP1 in cotransfection assays (Fig. 3*B*). This may suggest that the interaction between TRAB1 and VP1 is regulated by an ABA signal. This hypothesis is highly attractive because ABA-dependent interaction between VP1 and TRAB1 would serve as an excellent molecular switch for ABA-regulated transcription. Although we were not able to observe an ABA enhancement of the two-hybrid interaction between GBD::VP1-N and GAD::TRAB1 (data not shown), this does not necessarily rule out the hypothesis, considering that only a part of VP1 is contained in GBD::VP1-N and that the topology of the two-hybrid complex relative to DNA is opposite of that of a natural TRAB1-VP1 complex. Alternatively, the activation function of VP1 is regulated by ABA. However, we consider this possibility less likely because of our preliminary observation that the activation of the reporter by VP1::GBD (a fusion protein between the entire VP1 and GBD) was not enhanced by ABA.

Although several bZIP-type transcription factors have been cloned as candidates for an ABRE-binding trans-acting factor (10–13), none of them have been demonstrated conclusively to be involved in ABA-regulated transcription. G-box or G-box-like elements very similar or identical to ABREs also have been identified as cis-elements for other regulations (6). In addition, there exist many bZIP-type transcription factors with similar binding specificities. These facts have made it difficult to identify an ABRE-binding factor that actually functions in ABA-regulated transcription. However, our result that GBD::TRAB1 can confer ABA responsiveness to a promoter with GAL4-binding sites (UASs) (Fig. 3) provides solid evidence that TRAB1 can truly mediate ABA-regulated transcription. The specific interaction with both ABREs and VP1 further supports this conclusion. In addition, the demonstration that the ABA activation of the reporter gene through a heterologous DNA-binding domain and its target sequence indicates that the major regulatory step for ABA-induced transcription through TRAB1 does not lie at the level of DNA binding. This is consistent with the observation that *in vivo* footprints on ABREs are not altered by ABA stimulation or the presence/absence of VP1 (36).

The expression of the *TRAB1* gene in the leaf and root tissues (Fig. 2) suggests that TRAB1 mediates ABA-regulated transcription not only in seeds but also in vegetative tissues. Because VP1 is known to be expressed only in seed tissues (15, 18), TRAB1 may function in vegetative tissues by interacting with an as yet unidentified protein similar to VP1.

By establishing the physical link between the VP1 activator and G-box-type ABREs, the identification of TRAB1 fills a long-standing gap in our understanding of ABA signaling. Moreover, TRAB1 identifies a firm endpoint from which we will be able to trace upward along the ABA signaling pathway of higher plants.

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