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TINY, a Dehydration-responsive Element (DRE)-binding Protein-like Transcription Factor Connecting the DRE- and Ethylene-responsive Element-mediated Signaling Pathways in *Arabidopsis*[*]

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Dehydration-responsive element-binding proteins (DREBs) and ethylene-responsive element (ERE) binding factors are two major subfamilies of the AP2/ethylene-responsive element-binding protein family and play crucial roles in the regulation of abiotic- and biotic-stress responses, respectively. In the present work, we have reported a previously identified DREB-like factor, TINY, that was involved in both abiotic- and biotic-stress signaling pathways. TINY was capable of binding to both DRE and ERE with similar affinity and could activate the expression of reporter genes driven by either of these two elements in tobacco cells. The 15th amino acid in the APETALA2 (AP2)/ethylene-responsive element-binding factor domain was demonstrated to be essential for its specific binding to ERE, whereas the 14th and 19th amino acids were responsible for the binding to DRE. The expression of *TINY* was greatly activated by drought, cold, ethylene, and slightly by methyl jasmonate. Additionally, overexpression of *TINY* in *Arabidopsis* resulted in elevated expressions of both the DRE- and the ERE-containing genes. Moreover, the expression of DRE-regulated genes, such as *COR6.6* and *ERD10*, was up-regulated upon ethylene treatment, and the expression of ERE-regulated genes, such as *HLS1*, was also increased by cold stress, when the expression of *TINY* was being induced. These results strongly suggested that TINY might play a role in the cross-talk between abiotic- and biotic-stress-responsive gene expressions by connecting the DRE- and ERE-mediated signaling pathways. The results herein might promote the understanding of the mechanisms of specific DNA recognition and gene expression regulation by DREBs.

With the completion of the *Arabidopsis thaliana* genome sequence, it is possible to identify and analyze the entire complement of transcription factors in plant. *Arabidopsis* dedicates ~5.9% of its estimated total number of genes to code for transcription factors, which is 1.3 times that of *Drosophila* and 1.7 times that of *Caenorhabditis elegans* and yeast (1). APETALA2 (AP2)[2]/ethylene-responsive element-binding protein is among the three largest families of transcription factors in *Arabidopsis*, and this family of transcription factors is plant-specific and contains the highly conserved AP2/ethylene-responsive element binding factor (ERF) DNA-binding domain (1, 2). Based on the similarities of the amino acid sequences in the AP2/ERF domain, the members of this family are classified into five subfamilies: AP2, dehydration-responsive element-binding protein (DREB, A1–A6), ERF (B1–B6), related to ABI3/VP1 (RAV), and others (3). The DREB and ERF subfamilies have received considerable attention and have been extensively researched over the years due to their participation in plant responses to abiotic and biotic stresses. The DREB subfamily was demonstrated to play a major role in cold-stress and osmotic-stress signal transduction pathways by recognizing the dehydration-responsive element (DRE)/C-repeat with a core sequence of A/GCCGAC (4–9), whereas the ERF subfamily was mainly involved in plant responses to biotic stresses, such as pathogen-stress, wounding-stress and ethylene signal, by recognizing the ethylene-responsive element (ERE), also known as the GCC-box, with a core sequence of AGCCGCC (10–14). However, recent works have revealed some new characteristics of these two subfamily members. For example, ABA-insensitive 4, the only member of the A-3 group, was recently reported to regulate the nuclear gene expression in the plant response to plastid-to-nucleus retrograde signals through binding a conserved motif of CCAC in the promoter of a retrograde-regulated gene (15). In addition, Sasaki *et al.* isolated two B-4 group genes from tobacco, named wound-responsive AP2/ERF-like factors 1 and 2, and indicated that they were positive regulators for wound-induced expression of a tobacco peroxidase gene through specifically interacting with the vascular system-specific and wound-responsive *cis*-element (16). These findings suggest that the DREB and ERF proteins play multiple roles during the

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[2] The abbreviations used are: AP2, APETALA2; ERF, ethylene-responsive element binding factor; ERE, ethylene-responsive element; DRE, dehydration-responsive element; DREB, DRE-binding protein; CBF, C-repeat binding factor; wDRE, wide-type DRE element with the sequence 5′-GATATACTACCGACATGAGTTC-3′; mDRE, mutated DRE element with the sequence 5′-GATATACTATTTTCATGAGTTC-3′; wERE, wide-type ERE element with the sequence 5′-CGCAGACATAGCCGCCATTT-3′; mERE, mutated ERE element with the sequence 5′-CGCAGACATATCC-TCCATTT-3′; SD, synthetic dropout; CaMV 35S, cauliflower mosaic virus 35S; MeJA, methyl jasmonate; AVG, l-α-(2-aminoethoxyvinyl)-glycine; GST, glutathione *S*-transferase.

course of plant growth and that the differences in their DNA-binding specificities are accompanied by the functional diversity.

The AP2/ERF domains of the DREB and ERF proteins are closely related to each other, but their target DNA-binding sites are different. The solution structure of AtERF1 in complex with ERE revealed that seven amino acids in the AP2/ERF domain made direct contact with DNA (17). Interestingly, these amino acids were completely conserved in all reported DREBs and ERFs except Trp10, which was also highly conserved (18). Thus, the determinants of the different DNA-binding specificity were those divergent residues in the AP2/ERF domain between DREBs and ERFs. Two conserved amino acids in the AP2/ERF domain differ between them: the 14th Val and the 19th Glu in the DREBs and Ala and Asp in the corresponding positions of the ERFs. Previous studies have shown that the Val-14 and Glu-19, especially Val-14, were essential for specific binding to DRE (3, 18–20). In addition, Ala-37 was reported to play a key role in binding to both DRE and ERE (21). However, there is no report about the amino acids that are crucial in the specific recognition of ERE by far.

TINY gene was first isolated through a transposon-mutagenesis experiment designed to recover dominant gain-of-function alleles in *Arabidopsis* (22) and then identified as a DREB-like transcription factor binding to DRE (23, 24) and classified into the A-4 group (3), which suggested that, like other DREB proteins, TINY might play a role in response to abiotic stresses. Besides, enhancement of the expression of *TINY* gene caused a partial constitutive triple response, such as the short, thick hypocotyl and short root of the 3-day-old dark-grown seedling, which implied that TINY might be involved in ethylene response (22). Here, detailed work was carried out to explore the function of TINY in plants. We analyzed its stress-inducible expression profile, the DNA-binding and trans-activation properties, and the effect of overexpressing *TINY* in *Arabidopsis* plants. *TINY* was induced by not only abiotic stresses, but also the defense signal molecules ethylene and methyl jasmonate (MeJA). Interestingly, TINY could bind to both DRE and ERE with similar affinity to further activate the expression of downstream genes driven by either of these two *cis*-elements, suggesting that TINY might play a role in the cross-talk between the DRE- and ERE-mediated signaling pathways. Additionally, we found that Ser-15 in the AP2/ERF domain was essential for specific binding to ERE of TINY. The results herein may improve our understanding of the roles of AP2/ethylene-responsive element-binding protein transcription factors in plant signal transduction pathways.

EXPERIMENTAL PROCEDURES

Plant Materials and Stress Treatment—Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates as described previously (25) for 3 weeks and subjected to stress treatments. For treatment with water, salts, and ethylene, plants were grown hydroponically in dH_2O, 250 mM NaCl, or 1 mM ethephon solution. For cold stress treatment, the plants were kept in a 4 °C refrigerator. For drought stress treatment, the plants were placed on filter paper on a clean bench under dim light. For treatment with MeJA, 4-week-old plants grown in soil were sprayed with 50 μM MeJA solution. MeJA was dissolved in 50% ethanol as a 10 mM stock solution. The MeJA stock solution was diluted to 50 μM with water. In each case, the plants were subjected to the stress treatment for designated periods and frozen in liquid nitrogen. For $AgNO_3$ (Sigma) and l-α-(2-aminoethoxyvinyl)glycine (AVG, Sigma) treatment, 19-day-old plants were transferred to plates containing 10 μM $AgNO_3$ or 20 μM AVG, and assays were performed after additional 2 days of growth.

Site-directed Mutagenesis—The *TINY* gene (22) was kindly provided by Dr. George Coupland (Max Planck Institute for Plant Breeding, Cologne, Germany). The 14th and 19th residues in TINY were singly or doubly replaced by alanine and aspartic acid named T_V14A, T_E19D, and T_V14AE19D, respectively. The point mutation in 15th amino acid residue of TINY was obtained by singly replaced serine by cysteine and named T_S15C. All mutants were generated by PCR-mediated overlapping, and the mutants were sequenced and cloned into pGADT7 for yeast one-hybrid assay or pGEX-4T-1 for GST fusion protein preparation.

In Vivo DNA-binding Experiment Using the Yeast One-hybrid System—Construction of DRE reporter plasmids and selection of the yeast reporter system were performed as described previously (4). Construction of ERE reporter plasmids used the same method. A 70-bp region containing two ERE sequences from the *Arabidopsis HLS1* gene (26) (5′-TAATAATGAGTTAACGCAGACATAGCCGCCATTTTTAATAATGAGTTAACGCAGACATAGCCGCCATTTT-3′, the core sequence of ERE element is underlined) was synthesized and ligated into two tandemly repeated copies, and then inserted into the SmaI site of the multiple cloning site upstream of the *HIS3* minimal promoter in the pHISi-1 expression vector (Clontech, Palo Alto, CA) and the *LacZ* minimal promoter in the pLacZi expression vector (Clontech), respectively. The wERE reporter system was constructed by simultaneously transforming these two plasmids into yeast strain YM4271. The reporter yeast containing the mERE with the substitution of AGCCGCC with ATCCTCC was constructed by using the same method.

The full-length coding regions of *TINY* and its mutants were cloned into the EcoRI and SalI sites of the pGADT7 vector (Clontech). The constructs were transformed into the reporter yeast cells as described previously (4). The growth status of the transformed yeast cells was compared on synthetic dextrose (SD) medium without His, Ura, and Leu (SD/-His-/Ura/-Leu) with 0 mM, 30 mM, or 50 mM 3-aminotriazole (a competitive inhibitor of the *HIS3* gene product). The colony-lift filter assay used to measure β-galactosidase activity was performed to test the expression of the *LacZ* reporter gene as described in the Yeast Protocols Handbook (Clontech).

GST Fusion Protein Preparation and Gel Mobility Shift Assays—The 363-bp (1–363) fragments of *TINY* and its mutants containing the DNA-binding domain were prepared by the primer pairs: 5′-AAAAGAATTCATGATAGCTTCAGAGAGTAC-3′ (forward), 5′-AAAAGTCGACTTAGGTCTCCATGTGTGCGGCTTTG-3′ (reverse). Each of the fragments was cloned into the EcoRI-SalI sites of the pGEX-4T-1 vector (Amersham Biosciences), and the constructs were transformed into *Escherichia coli* strain BL21(DE3) to produce the GST

fusion protein. The GST fusion protein was separated using a glutathione-Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions. Gel mobility shift assays were performed as described previously (4). The 71-bp DNA fragment containing wild-type or mutated DRE sequence and 29-bp DNA fragment containing wild-type or mutated ERE sequence (Fig. 2*A*) were labeled by filling in 5′ overhangs with [^{32}P]dCTP and the Klenow fragment, respectively. The DNA-binding reaction was constructed as described previously (4). For competition experiments, unlabeled competitors were incubated with the protein at 25 °C for 5 min before the addition of labeled probes, which were further incubated for 30 min (9).

Single Molecule Force Measurement Using AFM—The DNA sequences used in all force measurements were custom synthesized from SBS Genetech Co. Ltd. (Beijing, China). These include the DRE element sequence, 5′-NH_2-GATATACTACCGACATGAGTTC-3′, and its complementary single strand DNA, 3′-CTATATGATGGCTGTACTCAAG-5′; the ERE element sequence, 5′-NH_2-CGCAGACATAGCCGCCATTT-3′, and its complementary single strand DNA, 3′-GCGTCTGTATCGGCGGTAAA-5′ (the element sequences are underlined). Chemical modification of the AFM tips and substrates and force measurements were performed according to previously reported procedures (23).

Trans-activation Activity of the DRE- and ERE-LUC Reporter Genes in Plant Cells—The trans-activation activity of TINY was measured in a constructed dual reporter system (Fig. 4*A*) as described previously (21). The DRE dual reporter system was established by our previous work (27). The ERE reporter construct containing a 4×HLS GCC-box (12) was the generous gift from Dr. Ohme-Takagi (Gene Function Research Center, National Institute of Advanced Industrial Science and Technology, Japan). For effector plasmids, the β-glucuronidase (*GUS*) gene in pBI221 (Clontech) was replaced by the coding region of *TINY*. The tobacco mosaic virus Ω sequence (28) was inserted downstream of the *CaMV 35S* promoter in pBI221 to enhance the efficiency of translation. The effector plasmid consisting of *TINY* and either DRE reporter plasmid or ERE reporter plasmid were delivered into the protoplasts. The plasmid carrying the *CaMV 35S* promoter-*Renilla* luciferase gene (R reporter plasmid) was co-transfected with both reporter and effector plasmids as an internal control. The isolation of tobacco protoplasts from the BY2 suspension cultures and the polyethylene glycol-mediated DNA transformation of tobacco protoplasts were performed as described previously (21, 29). The *Renilla* and firefly luciferase activities were measured according to the manufacturer's instructions (Promega, Dual-Luciferase Reporter Assay System).

Trans-activation Activity Analysis in Yeast Cells—A yeast one-hybrid system derived from the GAL4 two-hybrid system (Fig. 4*C*) was developed to test whether the C-terminal of TINY protein functions as a transcriptional activation domain. The effector plasmids were constructed by inserting the 333-bp (322–654) fragment of *TINY* or the full-length coding region of *DREB1A* (a positive control) downstream of the yeast GAL4 DNA-binding domain of pGBKT7 (Clontech). The fusion plasmids and the vector as a negative control were transformed into yeast strain AH109 (Clontech). The transformants were analyzed on the synthetic dextrose medium without Trp, His, and Ade (SD/-Trp/-His/-Ade) to test the expression of the *HIS3* and *ADE2* reporter genes. The colony-lift filter assay used to measure β-galactosidase activity was performed subsequently to test the expression of the *LacZ* reporter gene.

Quantitative Real-time PCR Analysis—Total RNA was prepared by TRIzol Reagent (Invitrogen) as instructed. cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Promega) with the oligo(dT)$_{15}$ primer according to the manufacturer's instructions. Quantitative real-time PCR using SYBR Green I Dye (Bio-V) was performed on Mx3000PTM (Stratagene, La Jolla, CA). Three replicate PCR amplifications were performed for each sample. The *Actin2* gene was also amplified as an internal control (30). The amount of the transcripts of each gene, normalized to the internal reference *Actin2*, is analyzed using $2^{-\Delta\Delta Ct}$ method (31). The amount of the transcripts of each target gene under normal condition, or in control transgenic plants (vec), was designated as 1.0. The primers used for the real-time PCR were: *TINY*, 5′-CACAGTCTTCTTCTTCGCTAGAGTC-3′ (forward), 5′-GTGATAACGAGGCAGGAATCAT-3′ (reverse); *Actin2*, 5′-GACCTTTAACTCTCCCGCTATGTA-3′ (forward), 5′-GTGGTGAACATGTAACCTCTCTCTG-3′ (reverse); *COR6.6*, 5′-AGTATATCGGATGCGGCAGT-3′ (forward), 5′-CAAACGTAGTACATCTAAAGGGAGA-3′ (reverse); *COR15A*, 5′-AAAACTCAGTTCGTCGTCGTTT-3′ (forward), 5′-GCTTCTTTACCCAATGTATCTGC-3′ (reverse); *COR78*, 5′-CAAAACAGAGCACTTACACAGAGAA-3′ (forward), 5′-CATAATCTCTACCCGACACACTTTT-3′ (reverse); *ERD10*, 5′-AAGGGATTTATGGACAAGATCAAA-3′ (forward), 5′-CACAAACTTGGAGAACAGCTAGAA-3′ (reverse); *PDF1.2* (32), 5′-TTGCTGCTTTCGACGCA-3′ (forward), 5′-TGTCCCACTTGGCTTCTCG-3′ (reverse); and *HLS1*, 5′-TCGAATATCCACCCGAGTCATG-3′ (forward), 5′-CTTCTCCTCCGATTCCATACATAA-3′ (reverse).

Plant Transformation—The plasmid used for the transformation of *Arabidopsis* was constructed with the coding region of the *TINY* cDNA. The coding region fragment was cloned into a multicloning site of the pBI121 vector (Clontech) (4). The plasmid and the vector as the transgenic control were then introduced into *Agrobacterium tumefaciens* strain EHA105. Plants were transformed using a vacuum infiltration method as described previously (4).

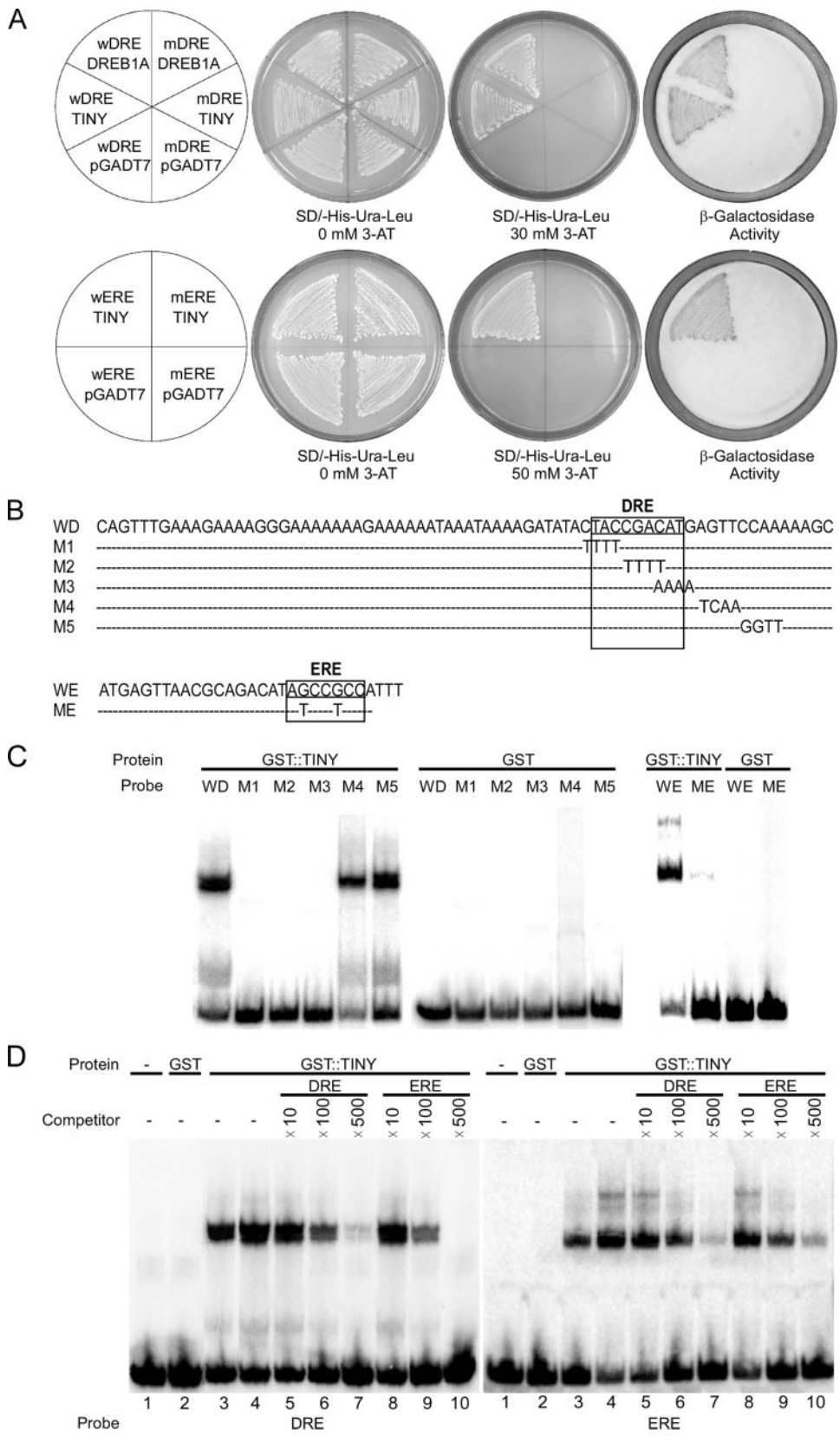
RESULTS

TINY Can Bind to Both DRE and ERE Elements with Similar Affinity—To analyze the DNA-binding property of TINY, the entire coding region of *TINY* was fused in-frame with the GAL4 activation domain in a pGADT7 vector and subjected to yeast one-hybrid assay using four reporter yeast strains, which carried the dual reporter gene *HIS3* and *LacZ* under the control of wild-type DRE, mutated DRE, wild-type ERE, and mutated ERE sequences, respectively. The growth status of these transformed yeast cells was analyzed. As shown in Fig. 1*A* (*upper panel*), in wild-type DRE reporter yeasts, the transformants carrying the recombinant plasmid TINY/pGADT7 and DREB1A(CBF3)/pGADT7 (a positive control) could grow well on SD/-His/-Ura/-Leu containing 30 mM 3-aminotriazole, and

show the expression of LacZ activity, whereas the transformants with the vector plasmid pGADT7, as a negative control, could neither grow on SD/-His/-Ura/-Leu nor show the expression of *lacZ*. To make a further confirmation, TINY/pGADT7 was transformed into mutated DRE reporter yeasts, and the transformants could not grow on SD/-His/-Ura/-Leu, which indicated that TINY could specifically bind to wild-type DRE in *vivo*. Similar results were obtained from the ERE reporter system (Fig. 1*A*, *lower panel*), showing that TINY could also specifically bind to wild-type ERE. Then the results here indicated that TINY could specifically bind to both DRE and ERE elements.

To further confirm the results above, a gel mobility shift assay was carried out to test the binding affinity and specificity of TINY to these two *cis*-elements *in vitro*. The 121 amino acids of the DNA-binding domain of TINY were expressed in *E. coli* as a fusion protein with GST and was used for gel mobility shift assay with ^{32}P-labeled probes, WD and M1–M5 (Fig. 1*B*). As shown in Fig. 1*C*, the recombinant protein bound the wild-type 71-bp fragment containing DRE element, but not the base-substituted 71-bp fragments M1, M2, and M3, in which the DRE element was mutated. By contrast, the protein still bound fragments M4 and M5, which still contained the DRE element. Therefore, TINY could specifically bind to the DRE element. Likewise, the TINY fusion protein bound the wild-type 29-bp fragment-containing ERE element, but not the base-substituted fragment containing mutated ERE element (Fig. 1*C*). As the negative control, no retardation band was detected when the pure GST protein was tested using any of these probes (Fig. 1*C*). These results indicate that TINY could specifically interact with both DRE and ERE elements.

The DNA-binding specificity of TINY was further confirmed by the competition experiments. The specific interaction between TINY and DRE element was effectively competed by either unlabeled DRE or ERE, and competitive abilities of DRE and ERE were almost the same. Similarly, unlabeled DRE and ERE showed almost the same abilities to compete with the labeled ERE to bind to TINY (Fig. 1*D*). These observations clearly show that TINY interacted



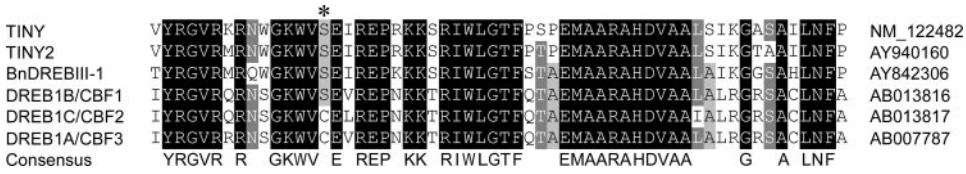


FIGURE 2. **Alignment of the amino acid sequences of AP2/ERF domains of TINY, TINY2, BnDREBIII-1, DREB1B/CBF1, DREB1C/CBF2, and DREB1A/CBF3.** The accession numbers of these DREBs are listed on the *right-hand side*. *, the 15th amino acid.

specifically with both the DRE and ERE sequences with similar binding affinity.

To make a quantitative analysis of the binding affinity of TINY with DRE and ERE, we used AFM to quantify the binding strength, according to our previously established system (23). By fitting a Gaussian distribution to the single peak in the force distribution histogram, the most probable force, 91.3 ± 6.1 piconewtons, for the specific single molecular interaction force of TINY-GST/ERE was calculated (Fig. 3*B*, *left panel*). This value was similar to that of the single molecule force between TINY and DRE obtained under the same experiment conditions before, which was 83.5 ± 3.4 piconewtons (23). The results here further confirmed that TINY could bind to DRE and ERE with similar affinity.

Ser-15 Was the Crucial Amino Acid in Binding with ERE—Different from the most other DREB proteins, TINY could bind to the DRE element as well as the ERE element with similar binding affinity. Then it is interesting to figure out which of the one or more amino acids bestows TINY the ability to bind to ERE. Therefore, we made a sequence alignment among the AP2/ERF domains of TINY and some other DREBs such as TINY2 (33), BnDREBIII-1 (21), and CBF1/DREB1B (18), which were also demonstrated to bind to both of the two elements. CBF2/DREB1C and CBF3/DREB1A were also analyzed, because they can only bind to DRE. As shown in Fig. 2, the AP2/ERF domains of these proteins are highly homologous with ~86.5% identity, and the most noticeable difference among these sequences was that the 15th amino acid was Ser in TINY, TINY2, BnDREBIII-1, and CBF1/DREB1B, whereas it was Cys in CBF2/DREB1C and CBF3/DREB1A. Thus it is reasonable to deduce that Ser-15 is crucial for the specific binding of ERE element.

Then a site-directed mutant, T_S15C, was constructed to explore the role of the 15th amino acid in determining the target DNA sequence using the AFM method *in vitro*. The force measurement was carried out with the protein immobilized AFM tips and the DNA element modified substrates. The representative force-distance curves are shown in Fig. 3*A*. Distinguished from the random appearances of the first peaks caused by the nonspecific interaction between the AFM tip and substrate (34), the second peaks, which appeared ~20–40 nm away from separation of the tip and substrate, represented the specific force between T_S15C-GST and DRE element (Fig. 3*A*, *left panel*). If a solution of free T_S15C-GST protein or DRE element was injected for the blocking experiment, the second peaks were hardly detected, but the random appearance of the first peaks did not change much (Fig. 3*A*, *right panel*), and the binding probability, which is defined as the ratio between the number of force curves showing adhesion events to the total number of curves, decreased greatly to about 5% from 22% (Fig. 3*C*), which confirmed the specific interaction force measured by AFM. By fitting a Gaussian distribution to the single peak in the force distribution histogram, the reliable mean value, 82.8 ± 3.0 piconewtons, for the specific single molecular interaction force of T_S15C-GST/DRE was calculated (Fig. 3*B*, *right panel*). This value was almost the same as that of the wild-type TINY. But, when we used ERE element as substrates, the peak represented the specific interaction of protein and DNA rarely appeared, and most of the force-distance curves were like that obtained in the blocking experiment shown in Fig. 3*A* (*right panel*). Moreover, not only did the binding probability of T_S15C-GST and ERE element decreased greatly to ~4%, when compared with 23% of TINY-GST with ERE (Fig. 3*C*), but also the force distribution of the rarely detected forces due to the nonspecific interaction did not fit to Gaussian distribution. Therefore, the results here strongly suggested that Ser-15 in the AP2/ERF domain of TINY is crucial in specific binding with ERE.

The *in vivo* yeast one-hybrid assay was carried out to further confirm the results above. As shown in Fig. 3*D*, in wild-type DRE reporter yeasts, the transformants carrying the plasmid T_S15C/pGADT7 could grow well on SD/-His/-Ura/-Leu in the presence of 50 mM 3-aminotriazole, whereas in wild-type ERE reporter yeasts, the transformants harboring the same plasmid could not grow on the same selective medium. As a negative control, the mutated DRE or ERE reporter yeast cells transformed with T_S15C/pGADT7 could not grow on these media. The results indicated that T_S15C could bind to DRE but not the ERE element, which further confirmed that Ser-15 was crucial in specific binding with ERE, instead of DRE.

Previously, we have shown that the 14th and 19th amino acids in the AP2/ERF domain were essential for binding with

FIGURE 1. **Analysis of the DNA-binding property of TINY *in vivo* and *in vitro*.** *A*, detecting the DNA-binding property of TINY by yeast one-hybrid assay. *Upper panel*: the wild-type DRE (*wDRE*) or mutated DRE (*mDRE*) reporter yeast cells transformed by DREB1A/pGADT7 (a positive control), TINY/pGADT7, or pGADT7 vector (a negative control), as indicated in the *first panel*, were examined for growth on SD/-His-/Ura/-Leu with or without 30 mM 3-aminotriazole (*3-AT*) and tested for β-galactosidase activity. *Lower panel*: the wild-type ERE (*wERE*) or mutated ERE (*mERE*) reporter yeast cells transformed by plasmids containing TINY/pGADT7 or pGADT7 vector were examined for growth on SD/-His-/Ura/-Leu with or without 50 mM 3-aminotriazole and tested for β-galactosidase activity. *B*, sequences of the 71-bp fragment of the *rd29A* promoter (*WD*) and its mutated fragments (*M1* to *M5*), and the 29-bp fragment of the *HLS* promoter (*WE*) and its mutated fragment (*ME*) were used as probes in gel mobility shift assay (4, 12). *C*, gel mobility shift assay of sequence specificity of the recombinant TINY protein. GST was used as a negative control. *D*, competitive binding assay. TINY recombinant protein was preincubated with or without competitors at 25 °C for 5 min. Then the ^{32}P-labeled probe was added and the mixture incubated at 25 °C for 30 min. As competitors, 10-fold (×10), 100-fold (×100), or 500-fold (×500) molar excess amounts of the unlabeled DRE or ERE fragments were used. *Lane 1* contained only the free probes, and *lane 2* contained GST only. 0.4 μg of proteins was loaded to *lane 3*, and 0.8 μg of proteins was loaded to *lane 2* and *lanes 4–10*.

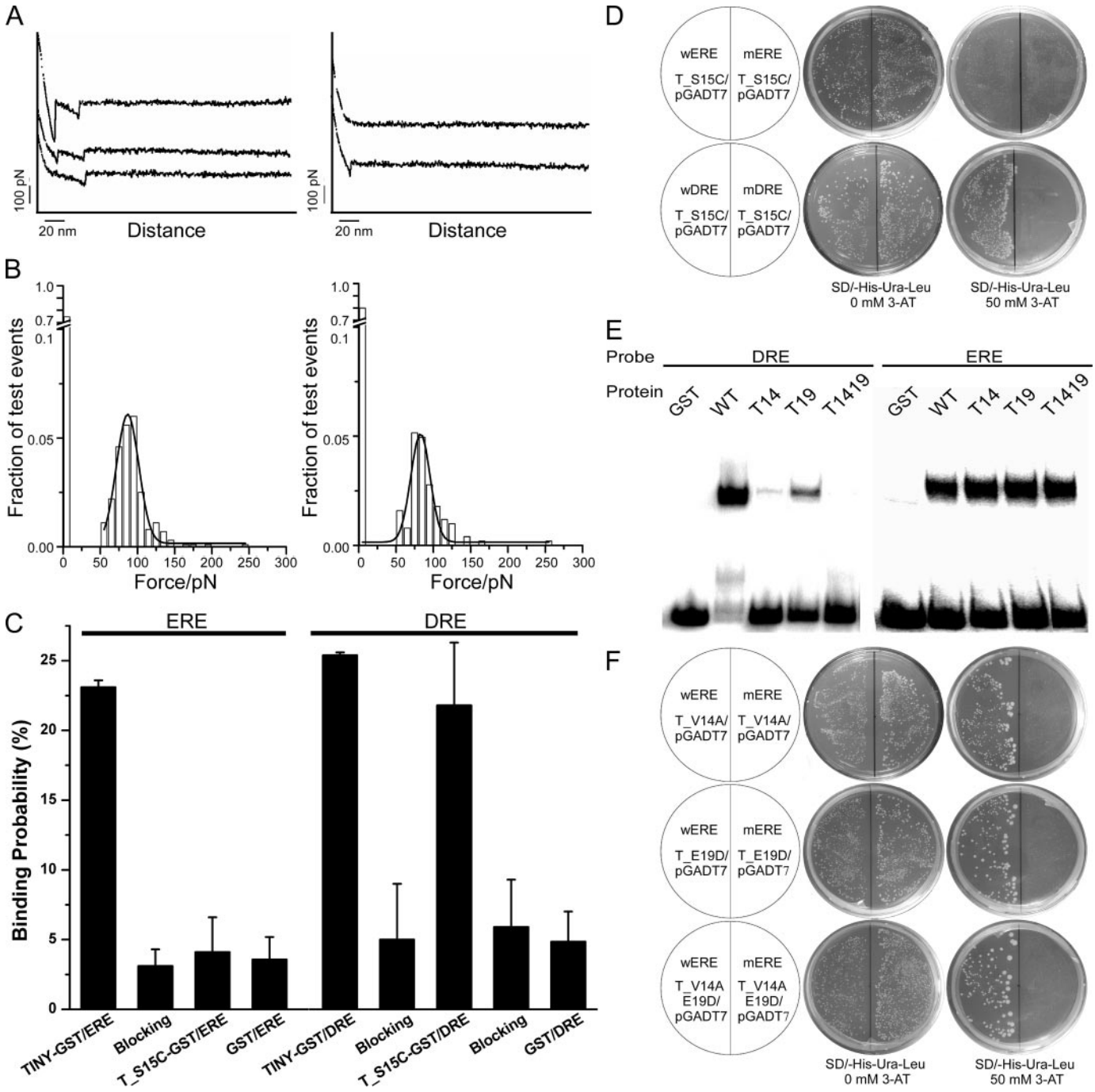


FIGURE 3. **Ser-15 was crucial in binding with ERE, whereas Val-14 and Glu-19 were essential for binding with DRE.** *A*, representative force-distance curves between T_S15C-GST-modified AFM tip and DRE element-modified substrate in the phosphate-buffered saline buffer (*left panel*) and after blocking the specific binding of T_S15C-GST to DRE with the solution of free T_S15C-GST (*right panel*). *B*, histograms of the binding forces of TINY-GST/ERE (*left panel*) and T_S15C-GST/DRE (*right panel*). The *open bar* indicates the experimental data, and the *solid line* indicates the theoretical Gaussian distribution curve. *C*, Ser-15 was specific to bind with ERE, as indicated by the binding probabilities measured using the AFM method. The *left panel* shows the binding with ERE, and the *right panel* shows the binding with DRE. The solution of free protein (2 μg/ml) was injected into the reaction liquid cell for the blocking experiment. The data for TINY-GST/DRE was from our previous work (23). GST/ERE and GST/DRE were used as negative controls. *D*, yeast one-hybrid analysis of DNA-binding ability of T_S15C. The reporter yeast cells carrying wERE, mERE, wDRE, or mDRE were transformed by the plasmid of T_S15C/pGADT7. *E*, gel mobility shift assay of wild-type TINY and its mutants of T_V14A (*T14*), T_E19D (*T19*), and T_V14AE19D (*T1419*) proteins with the DRE or ERE element as probes. *F*, yeast one-hybrid analysis of ERE element-binding ability of T_V14A, T_E19D, and T_V14AE19D. The reporter yeast cells carrying wERE or mERE were transformed by plasmids containing these mutants, respectively. *3-AT*, 3-aminotriazole.

DRE (23). Then it is necessary to explore the role of these two amino acids in binding with ERE. As can be expected, gel mobility shift assay indicated that the mutations at the 14th and 19th amino acids decreased the binding capability of TINY to DRE element (Fig. 3*E*, *left panel*). However, these mutations did not alter the binding ability of TINY with ERE (Fig. 3*E*, *right panel*), which was further confirmed by yeast one-hybrid assay in ERE reporter yeast cells (Fig. 3*F*). These results indicated that Val-14 and Glu-19 were significant for the binding capability of TINY to the DRE element, but not the ERE element.

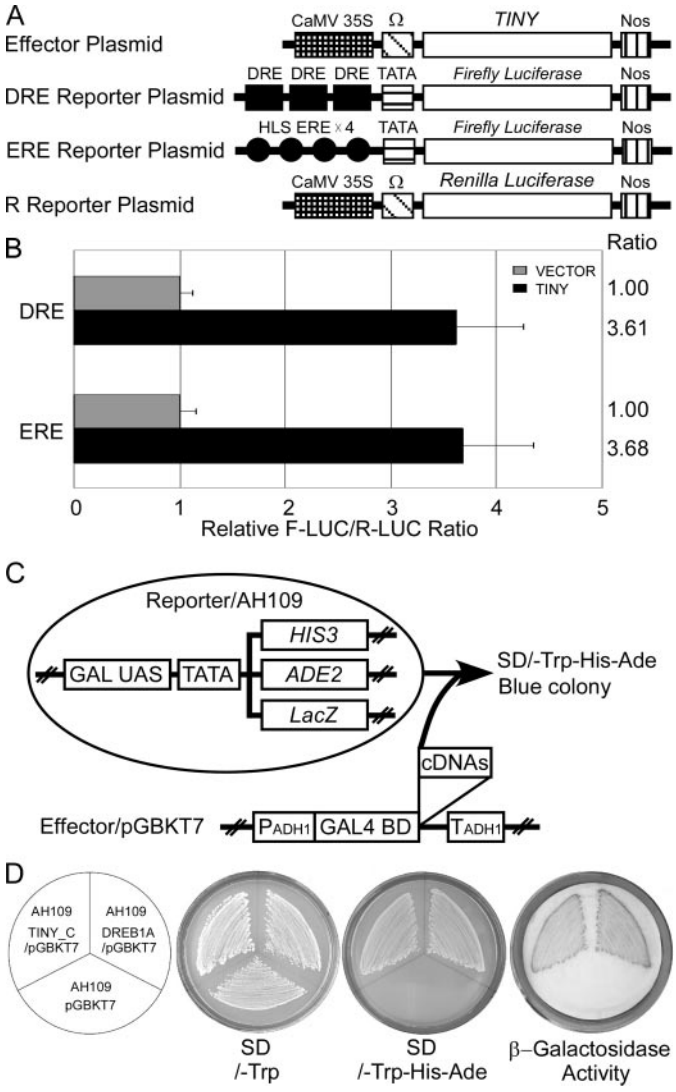


FIGURE 4. **The trans-activation activity assay of TINY.** *A*, the dual reporter system constructed for transient expression assay. *B*, trans-activation activity of the TINY protein. The vector was also transformed as a negative control. The activities of firefly luciferase and *Renilla* luciferase were designated as F-LUC and R-LUC, respectively. Trans-activation activity was determined by the value of F-LUC/R-LUC, and the relative trans-activation activity was calculated by defining the F-LUC/R-LUC value of the vector control as 1.00. *Bars* indicate the standard error of three replicates. *C*, the yeast one-hybrid system derived from the GAL4 two-hybrid system to explore whether the C-terminal of TINY protein functions as a transcriptional activation domain. pGBKT7 was effector plasmid used here. The yeast strain AH109 was used as reporter yeast. *D*, trans-activation activity analysis in yeast cells. The plasmids of TINY_C/pGBKT7, DREB1A/pGBKT7, and the vector were transformed into yeast strain AH109, respectively. The transformants were selected by growth on SD/-Trp at 30 °C for 3 days. The existence of transcriptional activation activity was confirmed by a viability test on SD/-Trp/-His/-Ade and β-galactosidase activity assay.

TINY Functions as a Transcriptional Activator—Transient expression assay in tobacco BY2 cells was carried out to determine whether TINY was capable of activating DRE-dependent and ERE-dependent transcription, according to our previously established system (Fig. 4*A*) (21). As was reflected by the ratio of the activity of Firefly luciferase to that of *Renilla* luciferase, TINY could activate the expression of both the DRE-dependent and the ERE-dependent downstream reporter genes (Fig. 4*B*), indicating that TINY might function as both the DRE-driven and the ERE-driven transcriptional activators.

To further confirm the results above, the C-terminal region, which was abundant with acidic amino acids (22) and was supposed to act as a transcriptional activation domain, was fused to the GAL4 DNA-binding domain expression vector pGBKT7 to explore its transcriptional activity in yeast (Fig. 4*C*). As was shown in Fig. 4*D*, the C-terminal of TINY effectively activated the transcription of *HIS3*, *ADE2*, and *LacZ* reporter genes, which was evidenced by the observation that yeasts containing TINY_C/pGBKT7 could grow well on the SD/-Trp/-His/-Ade and that the colonies turned blue when LacZ activity was examined with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). DREB1A (CBF3)/pGBKT7 and the vector pGBKT7 were used as positive and negative controls, respectively. The results here further confirmed that TINY was a trans-active transcription factor and that its C-terminal region might serve as a transcriptional activation domain.

TINY Was Induced by Both Abiotic and Biotic Stresses—To explore the expression patterns of *TINY* in response to various stresses, total RNAs were extracted from water-, salt-, dry-, cold-, ethylene-, and MeJA-stressed plants at different periods. Real-time PCR was performed using *TINY*-specific primers, with *Actin2* as an internal control. As was shown in Fig. 5*A*, the expression level of *TINY* was decreased by water treatment, and slightly increased by 2.7-fold after 40 min of high salinity stress. However, under dehydration stress, the *TINY* mRNA started to accumulate immediately after the treatment, reaching a maximum level at 4 h (increase of ~60-fold) and then gradually decreased. Under cold stress, the transcripts accumulated slowly and reached the maximum at 6 h (increase of ~55-fold) and then decreased rapidly. Notably, a significant induction was observed 3 h after ethylene treatment. Moreover, the expression of *TINY* was also increased by 7-fold after 10 h of MeJA treatment (Fig. 5*B*). The expression levels of the *COR78* gene under high salinity, dehydration, and cold stresses, and the *PDF1.2* gene under ethylene and MeJA treatments were also examined as positive controls for each treatment. Because ethylene and MeJA are signal molecules implicated in plant defense responses (35), the results here suggested that *TINY* gene might be not only in response to abiotic stresses but also involved in biotic stresses.

Overexpression of TINY Can Activate the Expression of Both the DRE- and the ERE-driven Genes in Arabidopsis—To further investigate whether the TINY transcription factor could also activate some downstream genes in the DRE- or ERE-mediated signaling pathway in plants, we generated transgenic *Arabidopsis* overexpressing *TINY*. The *TINY* cDNA was overexpressed by the enhanced *CaMV 35S* promoter (36) in *Arabidopsis*, and 24 *Arabidopsis* transformants were generated. T2 transgenic plants were used for further analyses. Transgenic plants with pBI121 vector (Vec) were used as the control.

As was shown in Fig. 6*A*, TINY7, which was a representative line of the *35S:TINY* plants (T2), had a dwarf phenotype under normal growth conditions. The result here was consistent with that the previously reported *tiny* mutant, in which the level of *TINY* expression was increased caused by the semidominant *tiny* mutation (22). Then, the accumulations of some downstream genes were analyzed by real-time PCR in the *TINY* overexpressing plants, TINY1 and TINY7, which expressed the

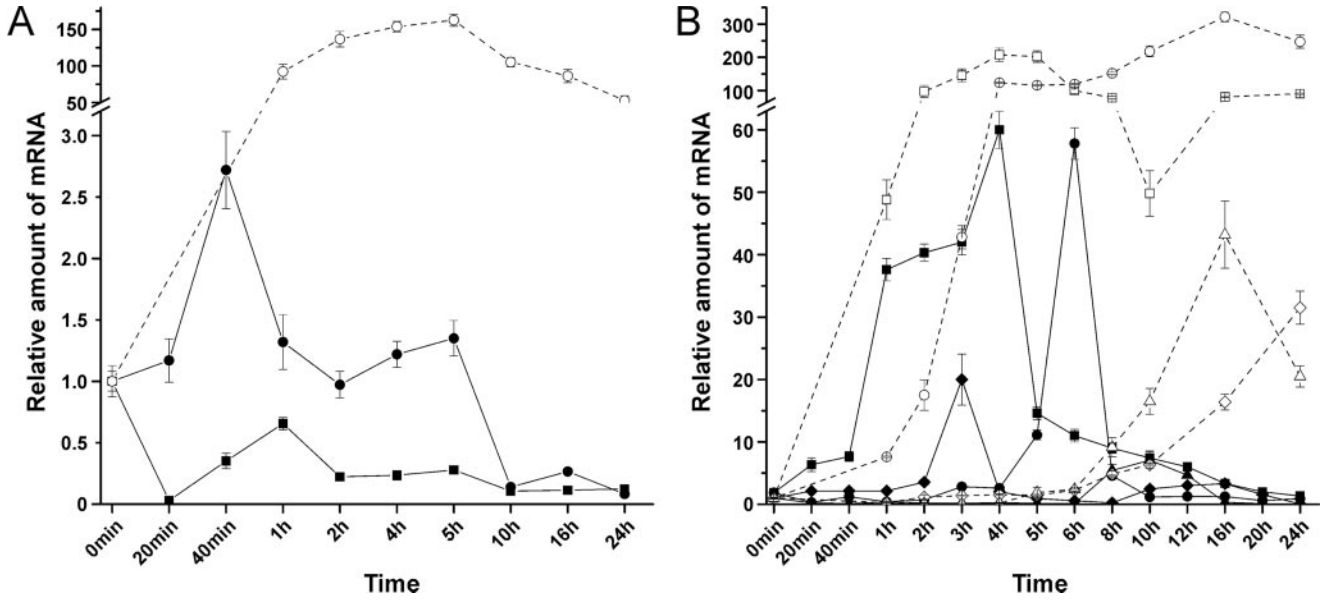


FIGURE 5. **Real-time PCR analysis of the expression of *TINY* in response to various stresses.** *A*, 21-day-old plants grown on medium plates treated by water (dH_2O) (■) and salt (250 mM NaCl) (●) for the indicated time courses were used for real-time PCR analysis. *COR78* (○) was used as a marker gene for salt treatment. *B*, 21-day-old plants grown on medium plates treated by dry (filter paper) (■), cold (4 °C) (●), and ethylene (1 mM ethephon) (◆), and 28-day-old plants grown in soil treated by MeJA (50 μM MeJA) (▲) for the indicated time courses were used for real-time PCR analysis. *COR78* was used as a marker gene for dry (□) and cold (○) treatment. *PDF1.2* was used as a marker gene for ethylene (◇) and MeJA treatment (△). *Solid* and *dashed lines* indicated the expression of *TINY* and marker genes, respectively. *Actin2* was used as an internal control. The amount of *TINY* transcripts under the normal condition was defined as 1.0. *Bars* indicate the standard error from three individual experiments.

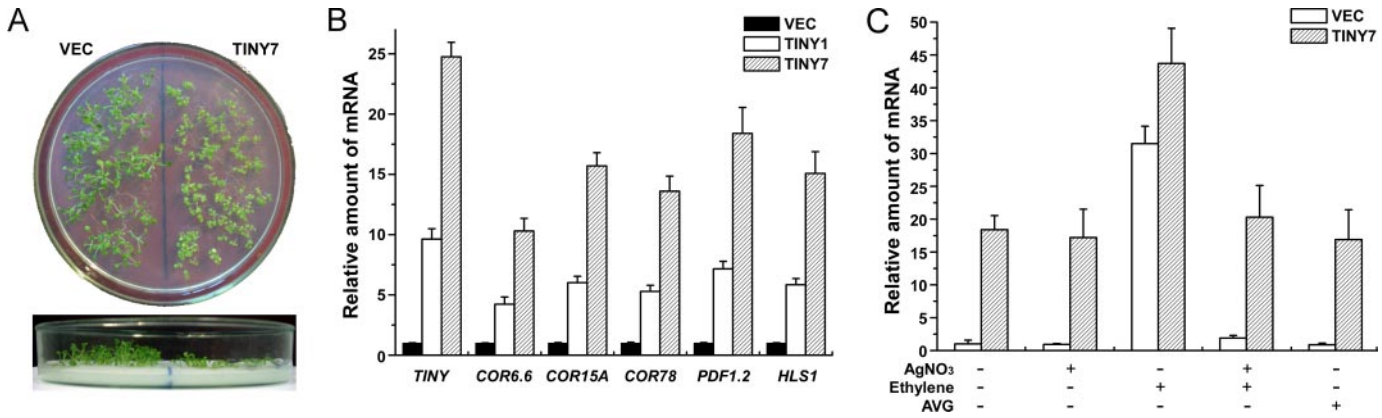


FIGURE 6. **Analysis of the transgenic plants overexpressing *TINY*.** *A*, the phenotype of 21-day-old *35S:TINY* plants of *line 7* (the *right part* of the *plate*) and control transgenic plants transformed by pBI121 empty vector (the *left part*) growing on selective germination medium. *Upper panel*: top view; *lower panel*: side view. *B*, real-time PCR analysis of the expressions of the transgene and some target genes in the control transgenic plants (*VEC*) and *35S:TINY* plants of line 1 (*TINY1*) and line 7 (*TINY7*) under normal conditions. The amount of the transcripts of each gene in the control transgenic plants was defined as 1.0. *C*, the effects of $AgNO_3$ (the ethylene perception inhibitor) and AVG (the ethylene synthesis inhibitor) on the expression of the *PDF1.2* gene in the control (*VEC*) and transgenic (*TINY7*) plants. Ethylene, plants were treated with 1 mM ethephon for 24 h. The amount of the transcripts of *PDF1.2* without treatments in control plants was defined as 1.0. *Actin2* was used as an internal control. *Bars* indicate the standard error from three individual experiments.

transgene at a medium level and a high level, respectively. As was shown in Fig. 6*B*, the expression levels of both the DRE-driven genes, *COR6.6*, *COR15A*, and *COR78* (37, 38), and the ERE-driven genes, *PDF1.2* (39) and *HLS1* (26), were elevated under non-stressed conditions in TINY1 and TINY7 when compared with those in the control plants. Moreover, the expression levels of these genes were correlated with that of *TINY*, which strongly suggested that TINY could activate both the DRE- and the ERE-driven genes *in vivo*.

It has been reported that overexpression of *TERF1*, one member of the tomato ERF subfamily, in tobacco resulted in the ethylene overproduction (40). Thus, it was important to determine whether the role of TINY in activation of ERE-driven genes was a direct effect of the interaction of TINY with the signaling pathway or an indirect effect of the activation of ethylene biosynthesis by *TINY* overexpression. If it was the latter, blocking ethylene biosynthesis or perception by using AVG or Ag^+ ($AgNO_3$) (41) would inhibit the expression of these genes. As shown in Fig. 6*C*, the expression of *PDF1.2* showed similar levels in TINY7 with or without $AgNO_3$ application, and the induced expression of this gene upon the exogenous ethylene treatment was inhibited in both control and TINY7 plants pretreated with $AgNO_3$, which confirmed the efficiency of $AgNO_3$ application. A similar result was obtained with AVG, showing that AVG application did not block the expression of *PDF1.2* in transgenic TINY7 plants (Fig. 6*C*). Therefore, these results

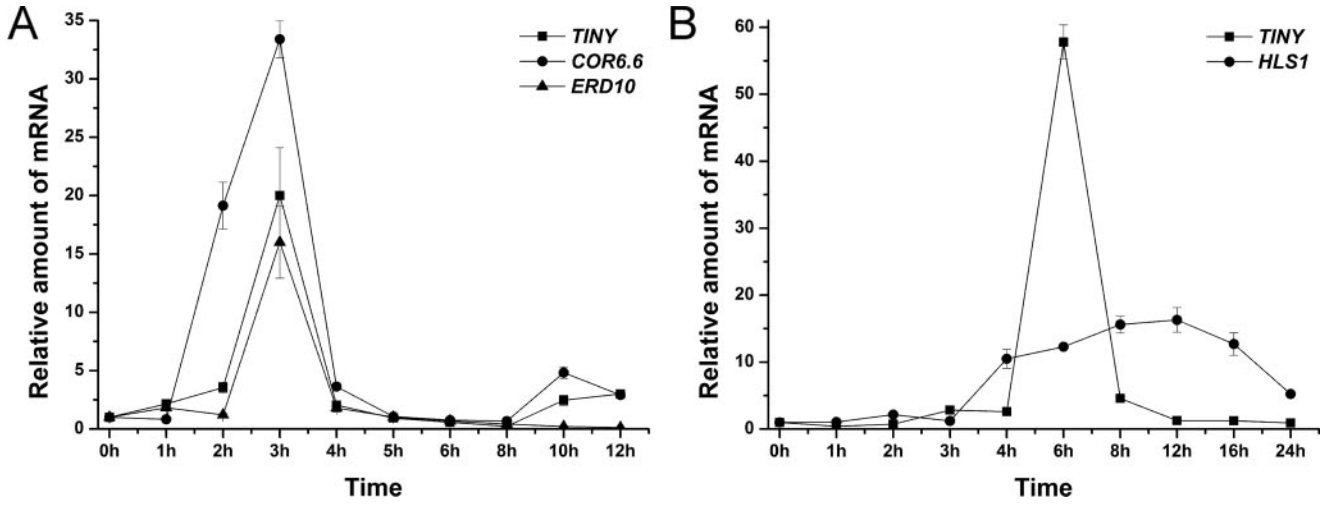


FIGURE 7. **Analysis of the expression of DRE-regulated genes upon ethylene treatment and ERE-regulated genes by cold stress.** *A*, 21-day-old plants grown on medium plates treated by ethylene (1 mM ethephon) for the indicated time courses were used for real-time PCR analysis. *B*, 21-day-old plants grown on medium plates treated by cold (4 °C) for the indicated time courses were used for real-time PCR analysis. *Actin2* was used as an internal control. The amount of the transcripts of each gene under the normal condition was defined as 1.0. *Bars* indicate the standard error from three individual experiments.

demonstrated that overexpressing TINY could directly activate the ERE-driven genes *in vivo*.

DRE-regulated Genes Are Up-regulated upon Ethylene Treatment, and ERE-regulated Genes Are Up-regulated upon Abiotic Stress Treatment—To investigate whether the abiotic stress signaling pathway involving the DRE element exhibited cross-talk with the biotic stress signaling pathway involving the ERE element, we detected the expression levels of DRE-regulated genes upon ethylene treatment and ERE-regulated genes upon abiotic stress treatment, respectively, supposing that if the cross-talk existed, the signal from one pathway would be transmitted to the other one and eventually regulate the expressions of the downstream genes in this pathway. As shown in Fig. 7, the expression of *COR6.6* and *ERD10* (37), which were both DRE-regulated genes, was induced in response to ethylene treatment, and the level of transcripts of *HLS1* (26), an ERE-regulated gene, was up-regulated by cold stress. The time course study indicated that the expression pattern of the *COR6.6* gene during ethylene treatment was similar to that of the *TINY* gene, and the expression of the *HLS1* gene during cold stress followed that of the *TINY* gene. These results strongly suggested that the cross-talk between DRE- and ERE-mediated signaling pathways existed in *Arabidopsis* and that TINY might contribute to it.

DISCUSSION

According to the similarity of the amino acid sequence in the AP2/ERF domain, the transcription factor TINY was classified into the DREB subfamily (3), and it could indeed bind to the DRE element as expected. Here for the first time, we reported that TINY was also capable of binding to ERE element (Fig. 1), and that the binding affinity was similar to that of DRE. Furthermore, TINY was demonstrated to be able to trans-activate the expression of both the DRE- and the ERE-driven genes (Figs. 4 and 6). Therefore, it is reasonable to deduce that TINY functions not only as a DREB but also an ERF. These results could also well explain the ethylene triple response of the *tiny* mutant, which showed increased *TINY* mRNA accumulation (22). According to the results here, the overexpressed TINY proteins could bind to the ERE element and activate the expression of some downstream genes involved in response to ethylene, such as *HLS1* gene (26), and finally caused the constitutive activation of the ethylene response pathway.

Usually, DREBs are induced by only abiotic stresses, such as high salinity, cold, and drought (4, 5, 7, 8), and ERFs are induced by only biotic stresses like pathogen, the defense signal molecules ethylene, and MeJA (10, 13, 42, 43). Different from them, TINY was found to be induced by both abiotic stresses and signaling molecules involved in biotic stress responses (Fig. 5), which was very similar to the characterization of *BnDREBIII-1*, one of the DREB transcription factors from *Brassica napus* (21). Because TINY was able to bind to both DRE and ERE elements and activate the downstream genes, its response to various stimuli indicated that TINY might participate in both DRE- and ERE-mediated signaling pathways. Here, the transgenic *Arabidopsis* plants overexpressing *TINY* did showed elevated expression levels of not only DRE containing genes, such as *COR6.6*, *COR15A*, and *COR78* (37, 38) in non-stressed conditions, but also some ERE containing genes, including *PDF1.2* (39) and *HLS1* (26), were directly activated in transgenic lines without ethylene treatment (Fig. 6), evidence that gives strong support to the hypothesis mentioned above.

Accumulating evidences have shown that, to adapt adverse environmental conditions, different signaling pathways could be integrated to provide a great regulatory potential for activating specific responsive genes related to multiple stresses in plants. For instance, ERF1 was simultaneously activated by both ethylene and jasmonate, and it was a key integrator of ethylene and jasmonate signaling pathways for the regulation of defense response genes (42). There are also some AP2/ethylene-responsive element-binding protein members that were reported to bind to both DRE and ERE, such as *CBF1* (18), *DREB2A* (3), *Tsi1* (44), *CaERFLP1* (45), *JERF3* (46), *CaPF1* (47) and so on, which suggested the existence of cross-talk between the DRE- and ERE-mediated signaling pathways. Actually, *CaERFLP1*

and *CaPF1*, two members of ERF subfamily, were demonstrated to be responsible to both abiotic and biotic stresses, including high salinity, pathogen infection, and ethylene, and overexpression of them resulted in enhancement of the expression of both the DRE- and the ERE-containing genes in plants (45, 47). Here, for the first time, we reported a DREB-like transcription factor, TINY, was involved not only in abiotic-, but also in biotic-induced signaling pathways. Different from CaERFLP1 and CaPF1, which showed much stronger binding affinity to ERE than DRE, TINY showed similar binding affinities to both DRE and ERE (Fig. 1). Moreover, the expression of DRE-regulated genes, such as *COR6.6* and *ERD10* (37), was activated upon ethylene treatment and the expression of ERE-regulated genes, such as *HLS1* (26), was increased by cold stress, when the expression of *TINY* was also being induced (Fig. 7). Therefore, the data presented suggested that TINY might play a role in the cross-talk between abiotic- and biotic-stress responsive gene expressions through connecting the DRE- and ERE-mediated signaling pathways. Once the plants are subjected to either abiotic or biotic stress, *TINY* will be induced, activate the expression of DREB and ERF downstream genes, and thus function as a linkage between the two signaling pathways. Global gene expression analysis of *TINY*-transgenic plants and gene expression-disrupted mutants may provide more insight into the functions of TINY in abiotic- and biotic-stress signaling in plants.

DREB and ERF regulated different gene expressions in the diverse signaling pathways due to their different DNA-binding specificity, although their DNA-binding domain shared a high degree of similarity (3, 18). Since the determination of the structure of AtERF1 and its complex with ERE (17), many studies have been conducted to reveal the key amino acids in the AP2/ERF domain responsible for the specific DNA-binding ability. The highly conserved 14th and 19th amino acids differ between DREB and ERF and have been demonstrated to be essential for the specific binding with DRE (3, 18, 19). However, neither V14A nor E19D of DREB1A showed ERE-binding activity (3). Correspondingly, both A14V and D19E of NtERF2 exhibited ERE-binding activity (18). Therefore, there must be one ore more other amino acids that contribute to the recognition of ERE. Here for the first time, we reported the Ser-15, which was crucial for the specific binding to ERE of TINY. Mutation of Ser-15 in TINY to Cys, the counterpart in DREB1A/CBF3, resulted in the total loss of its binding capability to ERE (Fig. 3). Although the AP2/ERF domain of DREB was predicted to fold into a conformation similar to that of AtERF1 on the basis of the high sequence similarity between them (17, 18), the binding mode of ERE by ERFs and that by DREBs might be different, because the second Gly in the core sequence of the ERE element was essential for stable ERF binding (11, 18) but was not necessary for binding of CBF1 to ERE (18). The evidence that the "AAEIRD" mutation of DREB2A, whose sequence is exactly the same as that in the corresponding sites of ERFs, does not allow binding of DREB2A to ERE (3) also supported the above hypothesis. Therefore, the detailed mechanism of the binding of TINY to ERE and the role of Ser-15 in such binding need to be further investigated.

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