FEBS Letters 580 (2006) 1303–1308

The conserved Ala37 in the ERF/AP2 domain is essential for binding with the DRE element and the GCC box

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Received 2 November 2005; revised 16 January 2006; accepted 17 January 2006

Available online 26 January 2006

Edited by Michael R. Sussman

Abstract **Four AP2/EREBP genes encoding putative ethylene-responsive element binding factor (ERF)/AP2 domains were cloned from *Brassica napus*, and these genes could be induced by low temperature, ethylene, drought, high salinity, abscisic acid and jasmonate treatments. These four genes, named *BnDREBIII-1* to *BnDREBIII-4*, were highly homologous and the 37th amino acid was the only difference among their ERF/AP2 domains. BnDREBIII-1 was demonstrated to be able to bind to both dehydration-responsive element and the GCC box and transactivate the expression of downstream genes, while BnDREBIII-4 could bind neither. Further results suggested that Ala37 might play a crucial role in the DNA binding or the stability of the ERF/AP2 domain.**

Keywords: BnDREBIII; Dehydration-responsive element; GCC box; ERF/AP2 domain; *Brassica napus*

1. Introduction

AP2/EREBP is a family of plant-specific transcription factors containing the highly conserved ethylene-responsive element binding factor (ERF)/AP2 DNA binding domain [1,2]. The AP2/EREBP genes constitute a large multigene family, and they are involved in a variety of regulation mechanisms throughout the plant life cycle [3–6]. Based on the number of ERF/AP2 domains and the function of the genes, the AP2/EREBP family could be divided into four subfamilies named the APETALA2 (AP2), dehydration-responsive element binding proteins (DREB), ERF and RAV subfamilies [7]. The DREB and ERF subfamilies are of particular interest due to their involvement in plant response to stresses. The genes in the DREB subfamily play a crucial role in the resistance of plants to abiotic stresses by recognizing the dehydration-responsive element (DRE) with a core motif of A/GCCGAC [8–11]. The ERF subfamily includes a large number of ERFs [5,12], which are mainly involved in the plant response to biotic stresses like pathogenesis by recognizing the *cis*-acting element AGCCGCC, known as the GCC box [5,13].

The solution structure of the ERF/AP2 domain of AtERF1 reveals that this highly conserved domain consists of a three-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet, which is similar to the topology of the well-known zinc-fingers [14]. However, unlike zinc-fingers, the ERF/AP2 domain recognizes its target DNA via the conserved Arg and Trp residues in the β-sheet [14]. Previous studies have shown that the Val14 and Glu19 conserved in the β-sheet of the ERF/AP2 domain of DREBs were indispensable for binding with DRE element, and the valanine residue was the more important one [7,15]. However, besides these residues, it is unclear whether there are still other residues essential for the binding activity of the ERF/AP2 domain. In the present study, four AP2/EREBPs of *Brassica napus* (named *BnDREBIII*s) were cloned, and their binding and activation properties were analyzed. Interestingly, it was found that three of these genes, BnDREBIII-1 to BnDREBIII-3, were able to bind to both DRE and the GCC box, while BnDREBIII-4 could bind to neither. Sequence alignment of the four BnDREBIIIs indicates that they are highly homologous and the ERF/AP2 domains are almost identical. Particularly, only one residue, Ala37 located on the α-helix, is different between the ERF/AP2 domains of BnDREBIII-4 and other BnDREBIIIs. These results suggested that the Ala37 in the α-helix might play a crucial role in the DNA binding or the stability of the ERF/AP2 domain.

2. Materials and methods

2.1. Plant materials and stress treatments

Seeds of *B. napus* were sterilized and were germinated in GM media (Murashige and Skoog medium) solidified with 0.8% (w/v) agar. The plants were developed at 25 °C under a long-day photoperiod. Various treatments were performed on 2-week-old plants as described previously [9,10,16].

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Abbreviations: DREB, dehydration-responsive element binding proteins; ERF, ethylene-responsive element binding factors; ABA, abscisic acid; JA, jasmonate; PDI, protein disulfide isomerase; EMSA, electrophoresis mobility shift assay; AD, activation domain

0014-5793/$32.00
doi:10.1016/j.febslet.2006.01.048

2.2. Isolation and identification of BnDREBIII cDNAs

The DNA sequence of ERF/AP2 domain of DREB1A/CBF3 was used as a query sequence for BLAST search of *B. napus* sequences available on GenBank. The search returned a *B. napus* EST (BN20.049L02F011220), which encoded a putative ERF/AP2 domain. Then 5′-RACE and 3′-RACE were conducted to obtain the 5′- and 3′-end of the EST according to the manufacturer's instructions (Takara, Japan). The coding regions of BnDREBIIIs were amplified by consensus primers (5′-GCAGGAATTCATGAAAAAACTCAAACTCATGG-3′ and 5′-CGACCTCGAGCTAGTAAGTTCCCGAGAAGAAAG-3′), and cloned into the *Eco*RI–*Xho*I sites of the pBluescript II SK+ vector.

2.3. RT-PCR analysis of the expression level

Two-week-old seedlings were subjected to various stress treatments for 5 h and total RNAs were extracted. In RNA analysis, the actin gene was used as an internal control. The consensus primers used in amplification of BnDREBIIIs were 5′-CTCATGGATCACTCATCCTGCCG-3′ and 5′-CTAGTAAGTTCCCGAGAAGAAAG-3′. The primers used in amplification of the actin gene were 5′-CATGTTCGAAACTTTCAATGTCC-3′ and 5′-CCTTGATCTTCATGCTGCTTG-3′.

2.4. DNA binding analysis by fluorescence quenching experiment and EMSA

The production and purification of recombinant BnDREBIIIs-protein disulfide isomerase (PDI) fusion proteins were performed as described previously [17]. The following double-stranded oligonucleotides were synthesized as wild-type (W) or mutated (m) DNA elements in electrophoresis mobility shift assay (EMSA) and the fluorescence quenching experiment: WDRE, AGCTACCGACATAAGGC; mDRE, AGCTATTTTCATAAGGC; WGCC, CATAAGAGCCGCCACT; and mGCC, CATAAGATCCTCCACT. For EMSA, 0.5 μg DNA elements and 15 μg BnDREBIIIs-PDI fusion proteins were mixed in 30 mM Tris–HCl buffer (pH 8.0) at room temperature for 20 min, and then were loaded onto an 8% non-denaturing polyacrylamide gel. After electrophoresis in 0.5 × TBE buffer, the gel was stained with ethidium bromide for visualization of DNA bands. The DNA binding affinity of BnDREBIIIs was monitored by the quenching of fluorescence spectra as described previously [17]. The apparent binding constants were calculated according to the algorithm described previously [18].

2.5. Transient expression assay

The transactivation activity of BnDREBIIIs was measured in a constructed dual reporter system (Fig. 5A). For the DRE reporter construct, the 3-fold multimerized DRE and the upstream of the minimal TATA box from the cauliflower mosaic virus (CaMV) 35S promoter, were transcriptionally fused to the firefly *luciferase* gene (Promega). For the R reporter construct, the *β-glucuronidase* (*GUS*) gene in pBI221 (Clontech) was replaced with the *Renilla luciferase* gene (Promega). The tobacco mosaic virus Ω sequence [19] was inserted downstream of the CaMV 35S promoter in pBI221 to enhance the efficiency of transcription. For effector plasmids, the *R. luciferase* gene in the R reporter was replaced by the coding region of various AP2/EREBPs and constructs. The plasmid AtERF1/SK containing the AtERF1 gene and the GCC reporter construct containing a 4 × *HLS* GCC box [12] were generous gifts from Professor Ohme-Takagi (Gene Function Research Center, AIST, Japan). For AD-III-4 effector plasmid, the GAL4 activation domain (AD) in the pGADT7 vector was fused upstream of BnDREBIII-4 in BnDREBIII-4 effector plasmid. The tobacco protoplasts were isolated from the BY2 suspension cultures. The PEG-mediated DNA transformation of tobacco protoplasts was performed according to a previous study [20]. The *Renilla* and firefly *luciferase* activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

3. Results

3.1. Isolation and characterization of BnDREBIII cDNAs

To clone new AP2/EREBP genes from *B. napus*, the DNA sequence encoding the ERF/AP2 domain of *DREB1A/CBF3* was used as a probe to blast the EST database on NCBI, and a *B. napus* EST (BN20.049L02F011220) was found to be a putative AP2/EREBP gene. Then 5′-RACE and 3′-RACE were carried out, and four genes were isolated, named *BnDREBIII-1* to *BnDREBIII-4*, respectively. The coding regions of the four genes were all 615 bp, encoding a 22.6 kDa protein with a conserved ERF/AP2 domain. Sequence alignment of the four BnDREBIIIs indicated that they were highly homologous, about 98.3% identity (Fig. 1). The most noticeable difference among these sequences is that the 37th amino acid in the ERF/AP2 domain is Val in BnDREBIII-4, while it is Ala in the other three proteins.

A further alignment in the ERF/AP2 domains of the four BnDREBIIIs with other published AP2/EREBPs was made (Fig. 2A), and a phylogenetic tree was generated based on the alignment (Fig. 2B). Interestingly, the BnDREBIIIs here not only contain the conserved Val14 and Glu19 in the ERF/AP2 domains of the DREB subfamily [7,15], but they also contain all the key residues reported to bind to the GCC box [14]. It is also noteworthy that the 37th amino acid is uniquely Ala in all the AP2/EREBPs except BnDREBIII-4 (Fig. 2A). Phylogenetic analysis of the ERF/AP2 domains revealed that these four genes belonged to the DREB subfamily, but they were different from DREB1/CBF and DREB2 (Fig. 2B). They were evolutionarily close to TINY in *Arabidopsis thaliana* [21] and DBF2 in *Zea mays* [22].

3.2. BnDREBIIIs could be induced by various treatments

To investigate the expression pattern of *BnDREBIIIs* in response to different abiotic stress, total RNAs were extracted from *B. napus* subjected to various treatments. A time course study indicated that BnDREBIIIs were rapidly induced within 30 min and lasted for at least 24 h (data not shown). Thus an induction time of 5 h was used for further analysis. The consensus primers were designed to detect the expression of *BnDREBIIIs*, and the gene of actin, which was constitutively expressed, was amplified as an internal control. Interestingly, *BnDREBIIIs* could be induced by low temperature, ethylene, drought, high salinity, abscisic acid (ABA) and jasmonate (JA) (Fig. 3), different from the commonly reported AP2/EREBPs.

3.3. DNA binding ability of BnDREBIIIs

Since the BnDREBIIIs contained the key amino acids to interact with both DRE element and the GCC box

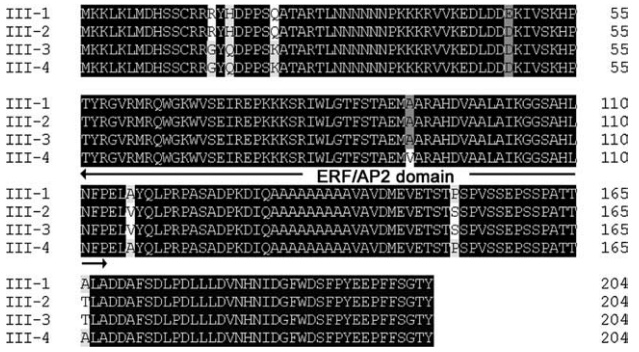

Fig. 1. Alignment of the amino acid sequences of the BnDREBIIIs in *B. napus*. The putative ERF/AP2 domains are noted. *BnDREBIII-1*, *BnDREBIII-2*, *BnDREBIII-3*, and *BnDREBIII-4* are accessible in GenBank databases under Accession Nos. AY842306, DQ199615, DQ199616, and DQ199617, respectively.

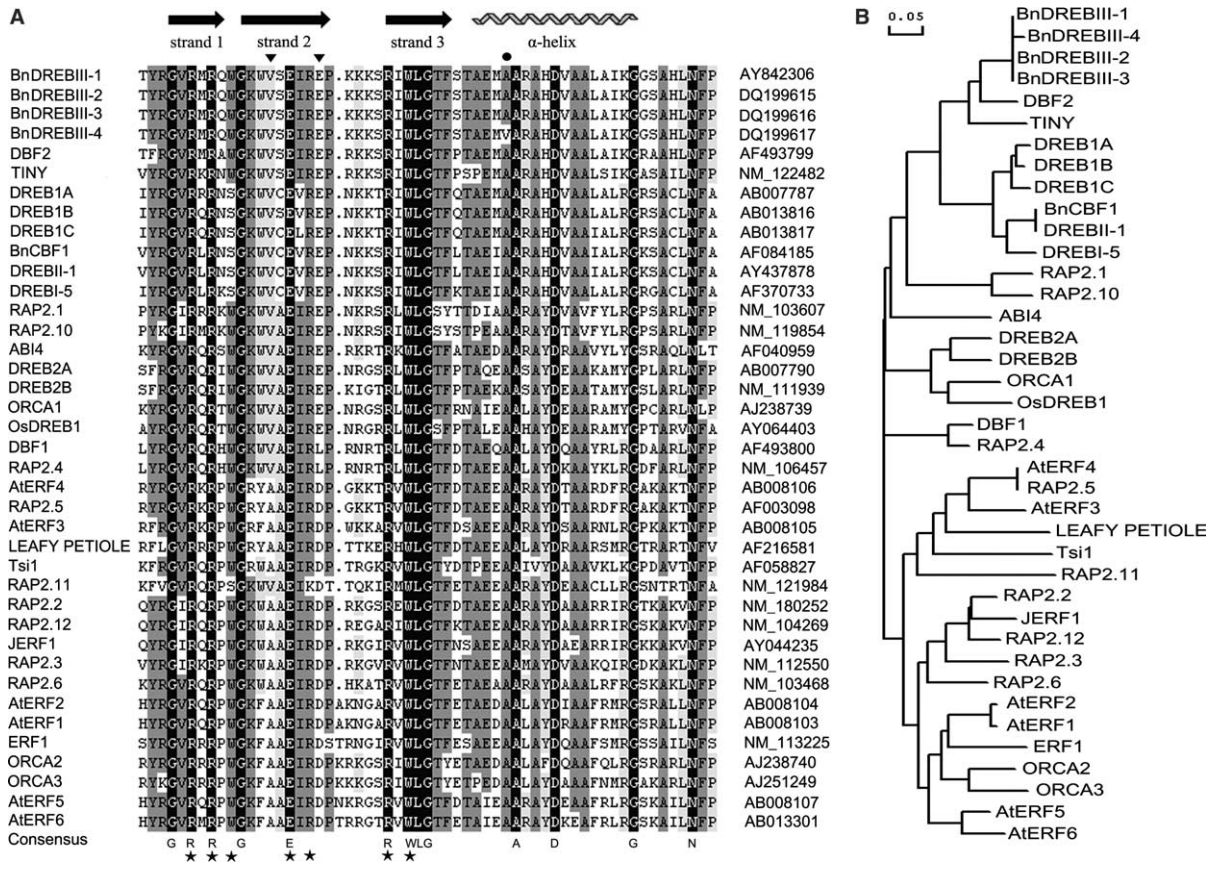

Fig. 2. Alignment and phylogenic tree of ERF/AP2 domain of AP2/EREBP family. (A) Comparison of the deduced ERF/AP2 domains of BnDREBIIIs and other AP2/EREBP proteins from *Arabidopsis thaliana*, *Brassica napus*, *Zea mays*, *Catharanthus roseus*, *Nicotiana tabacum*, *Lycopersicon esculentum* and *Oryza sativa*. The amino acids with asterisks represent the residues that interact with the GCC box [14]. Triangles indicate crucial amino acids for determining the DRE-binding specificity [7,15]. The circle shows the difference between BnDREBIII-1 and BnDREBIII-4. (B) Phylogenic tree of the ERF/AP2 domains of AP2/EREBP family developed by DNAMAN.

(Fig. 2A), it is meaningful to explore whether they could indeed bind to these two *cis*-elements. Because the ERF/AP2 domains of BnDREBIII-1 to BnDREBIII-3 are identical, and are different from that of BnDREBIII-4 (Fig. 1), BnDREBIII-1 and BnDREBIII-4 were selected for the binding ability analysis. As was shown in EMSA (Fig. 4A), the BnDREBIII-1-PDI was able to specifically bind to both the wild-type DRE element and GCC box, and it had no interaction with either the mutated DRE or GCC box. Surprisingly, BnDREBIII-4-PDI could bind to neither the DRE element nor the GCC box. Fluorescence quenching assay was carried out to confirm the results above and to quantitatively analyze the binding affinity of BnDREBIII-1 on DRE and GCC box (Fig. 4B). The apparent binding constant was $(4.8 \pm 1.1) \times 10^7\ M^{-1}$ for DRE and $(1.2 \pm 0.2) \times 10^7\ M^{-1}$ for the GCC box at 25 °C, indicating the binding of DRE with BnDREBIII-1 might be stronger than that of the GCC box. Consistent with the results in Fig. 4A, neither DRE nor GCC box could quench the fluorescence intensity of BnDREBIII-4 (data not shown). Since the 37th amino acid is the only difference in the ERF/AP2 DNA binding domains among these four BnDREBIIIs (Val in BnDREBIII-4, and Ala in the others), it is reasonable to deduce that Ala37 is crucial for the binding of both DRE and the GCC box.

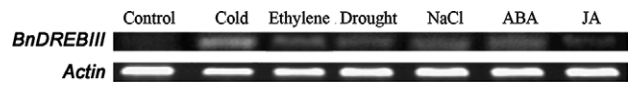

Fig. 3. RT-PCR analysis of *BnDREBIII*s expression pattern. Total RNA was extracted from 2-week-old *B. napus* treated with low temperature, ethylene, drought, NaCl, ABA and JA for 5 h. Untreated plants were used as a control. Actin gene was selected as an internal control.

3.4. Transient expression assay

The binding and transactivation activities of the BnDREBIIIs were explored in the transient expression assay. Two dual reporter systems were constructed (Fig. 5A), among which the effector plasmid and R Reporter (intrinsic control) were common. The DRE and GCC Reporter were used to test the activities of the genes in DREB- and ERF-mediated signaling pathways, respectively. In both the DRE and GCC reporter systems (Fig. 5B and C), BnDREBIII-1 was able to transactivate the expression of downstream genes, though the transactivation activity of BnDREBIII-1 was somewhat weak, about 20% (DRE) of that of DREB1A, a well-known DREB gene in *Arabidopsis* [9], and 40% (GCC) of that of AtERF1, a typical ERF gene in *Arabidopsis* [12]. In contrast, BnDREBIII-4 showed no transactivation activity in either DRE or GCC reporter systems, even when it was fused with the AD domain of GAL4. This result suggested that the Ala37 is crucial for the efficient binding of DRE and the GCC box. This

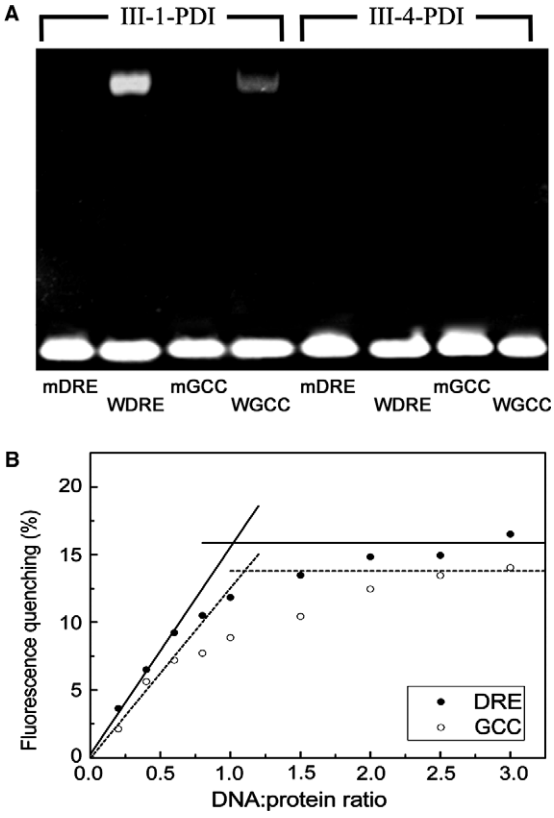

Fig. 4. Characterization of BnDREBIII-1 and BnDREBIII-4 DNA binding affinity to DRE element and GCC box. (A) EMSA of BnDREBIII-1-PDI and BnDREBIII-4-PDI fusion protein with the wild-type (W) and mutated (m) DRE element or GCC box as probes. (B) Fluorescence titration curves for the binding of DRE element (●) or GCC box (○) to BnDREBIII-1-PDI fusion protein.

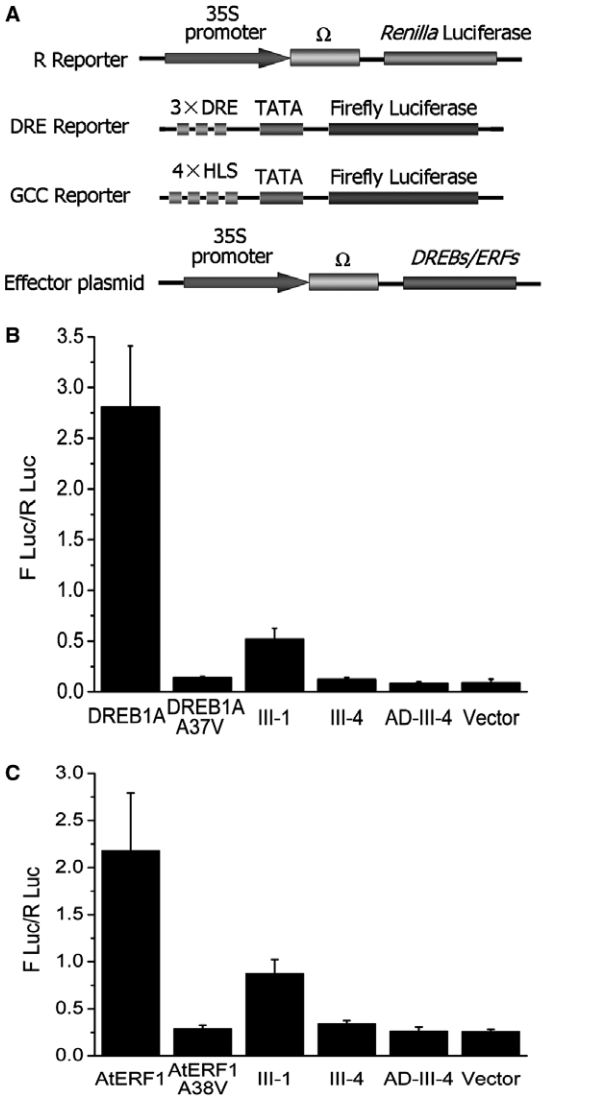

Fig. 5. Transient expression assay in tobacco BY2 suspension cells. (A) The dual reporter system constructed for transient expression assay. (B) Transactivation of the 3 × DRE-LUC reporter gene. (C) Transactivation of the 4 × *HLS* GCC box-LUC reporter gene. DREB1A, AtERF1, III-1, and III-4 indicate the effector plasmids containing DREB1A, AtERF1, BnDREBIII-1, and BnDREBIII-4, respectively. The GAL4 AD was fused to the N-terminal of BnDREBIII-4 to produce AD-III-4 effector plasmid. The Ala37 and Ala38 in the ERF/AP2 domain of DREB1A and AtERF1 were mutated to Val to produce DREB1A/A37V and AtERF1/A38V effector plasmid, respectively. The DRE and R reporter genes, the GCC and R reporter genes were co-transformed with each effector plasmid in DRE and GCC reporter system, respectively. F Luc, firefly *luciferase* activity; R Luc, *Renilla luciferase luciferase* activity.

conclusion was further confirmed by the fact that in the DRE reporter system, protoplasts transformed with DREB1A showed high expression of the downstream firefly *luciferase*, while those transformed with DREB1A/A37V, a site-directed mutagenesis in the 37th Ala in the ERF/AP2 domain of DREB1A to Val, did not show any expression (Fig. 5B). Similar result was also observed in the GCC reporter system when the 38th Ala in the ERF/AP2 domain of AtERF1 (the counterpart of Ala37 in BnDREBIII-1) was mutated to Val (Fig. 5C). The transactivation abilities of *BnDREBIII-1* to *BnDREBIII-4* were also characterized by yeast one-hybrid assay and similar results were obtained (data not shown).

4. Discussion

DREB and ERF are two major subfamilies in the AP2/EREBP family, and they play significant roles in plant resistance to environmental and abiotic stresses. The DREB subfamily has been demonstrated to play a crucial role in the resistance of plants to abiotic stresses such as cold, high salinity and drought [6,10,11]. Also some *DREBs* are responsive to ABA, such as *DBF1*, *DBF2* [22] and *CBF4* [23]. In contrast, genes of the ERF subfamily are usually induced by ethylene and JA, and they are crucial in plant defense against pathogenesis [5,12,24]. Interestingly, the expression of *BnDREBIIIs* was responsive to not only the conditions sufficient for the induction of DREBs (low temperature, drought, high salinity and ABA), but to those for induction of ERFs (ethylene and JA) (Fig. 3). Further experiments indicated that BnDREBIII-1 was able to bind to both DRE and the GCC box (Fig. 4) and transactivate the expression of downstream genes (Fig. 5). Thus, these genes might be involved in both the DREB- and ERF-mediated signaling pathways. As the transactivation activity of BnDREBIII-1, compared with DREB1A and AtERF-1, was relatively weak (Fig. 5), these genes might not play a major role in either DREB- or ERF-mediated plant

defense. However, the roles of these genes should not be neglected, and they might play a significant role in the cross-talk between the DREB- and ERF-mediated signaling pathways. In fact, some AP2/EREBP members have also been proven to be able to bind to both DRE and the GCC box, including CBF1 [25], DREB2A [7], tobacco Tsi [26] and tomato JERF1 [16], which confirmed the existence of the cross-talk between the DREB- and ERF-mediated signaling pathways. The potential physiological role of BnDREBIII-4 was unknown since it could bind with neither DRE nor GCC box, and it was unclear whether BnDREBIII-4 could function through the binding with some other *cis*-element.

Since the determination of the NMR structure of AtERF1 [14], much research has been reported investigating the role of the key amino acids in the β-sheet of the ERF/AP2 domain responsible for the binding with DNA [7,15,25]. Nevertheless, few studies were focused on the function of the α-helix in the ERF/AP2 domain. In this research, Ala37 in the α-helix was demonstrated to be essential for binding with DRE and the GCC box. A close inspection of the solution structure of the ERF/AP2 domain of AtERF1 suggested that Ala38 (the same residue to Ala37 in BnDREBIIIs) serves as an underlay to stabilize the hydrogen bonds between strand 2 and strand 3 of the β-sheet (Fig. 6). Ala38 spatially protruded into the gap between Phe13 in strand 2 and Phe32 in strand 3, and might play a role in stabilizing the relative positions of Phe13 and Phe32 to form the proper hydrogen bonds between two strands. Although Phe13 and Phe32 did not participate in the binding with DNA, the putative hydrogen bonds between them might stabilize the anti-parallel β-sheet structure. Sequence alignment indicated that these two residues are Phe, Tyr or Trp in both the DREB and ERF subfamilies (Fig. 2A), and their aromatic groups might extremely restrict the size of the 37th or 38th amino acid in the α-helix (Fig. 6). For BnDREBIII-4, Ala37 was replaced with Val, which would occupy more space than Ala. This replacement might disrupt the hydrogen bonds formed by Trp13 and Phe31, and detach strand 2 and strand 3 or affect the critical positions of the amino acids responsible for binding with the *cis*-element. Our results suggested that although the α-helix of the ERF/AP2 domain is not directly involved in DNA binding, it might also play a crucial role in the DNA binding or the stability of the ERF/AP2 domain.

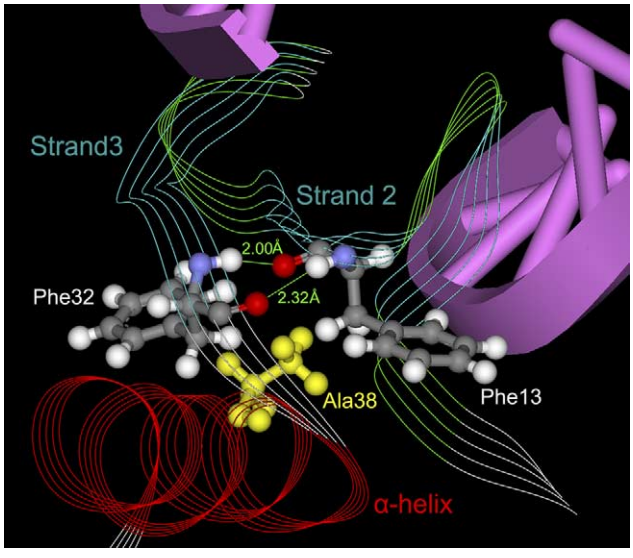

Fig. 6. Structural insights into the possible role of Ala38 in the stability of the AtERF1-GCC box (purple) (PDB entry 1GCC). Phe13 in strand 2 (bottle-green), Phe32 in strand 3 (bottle-green), and Ala38 (yellow) in α-helix (red) are highlighted in ball and stick style.

Acknowledgements: We thank Dr. Ohme-Takagi (Gene Function Research Center, AIST, Japan) for kindly providing the plasmid of AtERF1/SK and the GCC reporter construct. This work was supported by funds from the National Key Science and Technology Item (No. 2002AA224091 and 2005AA224090), and the Natural Science Foundation of China (No. 30500084 and 30221003) and Funds from Jiaxing, Zhejiang.

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