Optimizing the binding activity of the AP2/ERF transcription factor with the GCC box element from *Brassica napus* by directed evolution

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In this study, we cloned the ERF-B3 subfamily transcription factor gene *BnaERF-B3-hy15* from *Brassica napus* L. Huyou15. This 600 bp gene encodes a 199 amino acid classic ethylene responsive factor (ERF), which shown no binding or very weak binding GCC box-binding activity by the yeast one-hybrid assay. We used gene shuffling and the yeast one-hybrid system to obtain three mutated sequences that can bind to the GCC box. Sequence analysis indicated that two residues, Gly156 in the AP2 domain and Phe62 at the N-terminal domain were mutated to arginine and serine, respectively. Changes of Gly156 to arginine and Phe62 to serine increased the GCC-binding activity of BnaERF-B3-hy15 and the alter of Gly156 to arginine changed the AP2-domain structure of BnaERF-B3-hy15. [BMB reports 2010; 43(8): 567-572]

INTRODUCTION

Transcription factors (TFs) are important regulators of many biological processes, such as the response to stress caused by exposure to drought, cold or high salt. TFs bind specifically to their DNA-binding sites near target genes and then activate or repress gene transcription. The AP2/ERF superfamily is defined by the presence of the AP2 domain, which consists of 60-70 amino acids and is involved in DNA binding (1), and several AP2/ERF proteins have been suggested play roles in plant growth and development. According to Sakuma's classification, AP2/ERF proteins are classified into five subfamilies: AP2, RAV, DREB, ERF and others (1). The AP2 domain in the ERF subfamily was first identified as a conserved motif in four DNA-binding proteins from tobacco; namely, ethylene-responsive element-binding proteins 1-4 (EREBP1-4, currently renamed ERF1-4), was shown to bind specifically to the GCC box AGCCGCC, which involved in the ethylene-responsive transcription of genes (2).

Directed evolution has emerged as a powerful tool for protein engineering and for fundamental studies of protein structure-function relations (3). A number of different mutagenesis strategies exist, such as oligonucleotide cassette mutagenesis, semi-rational mutagenesis, point mutagenesis by error-prone PCR and the use of mutator strains, as well as DNA shuffling (4). Directed evolution in the laboratory (*in vitro*) mimics natural evolution but operates at the molecular level (i.e. no new organism is created) and focuses on specific molecular properties (5).

TFs contain the activated domain and the binding domain, and directed evolution can be used to change the activation and binding activity. For example, site-directed mutagenesis was used to identify the key amino acid for *trans*-activation of BnDREBII-I (6). A single amino acid substitution at Val14 by alanine or Glu19 by aspartic acid in the AP2 domain of DREB2A reduced the DNA binding activity and changed the DNA binding specificity of the protein (1). Liu *et al*. reported that the GCC and DRE-binding ability of DREB1A and AtERF1 were reduced when Ala37 and Ala38 in the AP2 domain were mutated to Val (7).

In this study, BnaERF-B3-hy15 (an ERF subfamily TF belonging to the B3 group from *Brassica napus*) was cloned and its binding to the GCC box was measured. Unlike the homologous genes of the ERF-B3 subgroup in *Arabidopsis*, such as AtERF1 and AtERF5, BnaERF-B3-hy15 binds very weakly to the GCC box. Sequence alignment between BnaERF-B3-hy15 and the ERF-B3 subgroup TFs in *Arabidopsis* indicated that they are highly similar in the AP2 domain but we did not know which amino acid was crucial for the GCC box-binding activity. Gene shuffling was used to make random mutations of *BnaERF-B3-hy15*. Yeast one-hybrid and colony-lift filter assays were performed and three blue clones were isolated for further analysis. Alignment between BnaERF-B3-hy15 and the mutated

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sequences indicated that there were two sites, one of which is in the AP2 domain, which might be crucial for GCC box-binding ability.

RESULTS

RT-PCR cloning of BnaERFB3-hy15

The ERF-B3 subfamily conserved domain amino acid sequence of AtERF1 was used as a probe to search against the UniGene database of *B. napus*, and one corresponding UniGene group called Bna.14208 was obtained. One full-length cDNA was produced by using the CAP3 program to splice and analyse the sequences in this database. Using primers designed on the basis of this sequence, we amplified a fragment of about 600 bp from the cDNA of *B. napus* L. Huyou15 seedlings by RT-PCR (Supplementary Fig. 1A), and the gene was named *BnaERF-B3-hy15*. The sequencing results indicated that *BnaERF-B3-hy15* contains a 600 bp open reading frame (ORF), and encodes a 199 amino acid protein (Supplementary Fig. 1B).

BnaERF-B3-hy15 belongs to the ERF-B3 subfamily

We cloned the *BnaERF-B3-hy15* gene based on the AP2 conserved amino acid sequence of the ERF-B3 subfamily in *Arabidopsis*, however its classification was unclear. To classify

it, we collected forty of the AP2/ERF proteins to align with BnaERF-B3-hy15 and created the phylogenetic tree that shown in Fig. 1. Based on the tree analysis, BnaERF-B3-hy15 belongs to the ERF-B3 subfamily. Furthermore, these 18 ERF-B3 proteins were aligned with the 60 amino acid sequences around the AP2 domain (Supplementary Fig. 2). The degree of similarity of BnaERF-B3-hy15 to AtERF1 (At4g17500), AtERF5 (At5g47230), AtERF6 (At4g17490) and AtERF15 (At2g31230) was 24.18%, 23.10%, 27.66% and 21.35%, respectively. We found that BnaERF-B3-hy15 was highly conserved in the three β sheets (strands 1, 2 and 3) compared to the other 17 *Arabidopsis* ERF-B3 subfamily sequences. However, in α helix-encoding region, two amino acids that did not agree with the conserved amino acids that include glutamic acid E151 and glycine 156. The corresponding amino acids in these two positions were alanine and arginine, respectively. BnaERF-B3-hy15 also has a high degree of variation among the ERF conserved sequences (data not shown).

GCC box-binding activity of BnaERF-B3-hy15 and the mutants

Members of the B-3 ERF group were shown to bind to the GCC box target sequence. We used the yeast one-hybrid system to investigate the binding activity of BnaERF-B3-hy15. The full-length cDNA of BnaERF-B3-hy15 was introduced into the pPC87 expressing vector, which had the *Bam* HI and *Sac* I restriction enzyme sites that facilitated the introduction. This construction was transformed to yeast cells that had another vector containing the GCC box sequence. After the colony-lift filter assay, clones grown on an X-gal-containing plate did not turn blue visibly (Fig. 2A), which indicated that BnaERF-B3-hy15 has no binding or very weak binding ability to the GCC box and to promote the expression of the *LacZ* reporter gene. The yeast one-hybrid results suggested that there is possible crucial residue(s) controlling the binding activity of BnaERF-B3-hy15, thus gene shuffling was applied to obtain random mutant sequences and a mutant DNA library was constructed as described above. The yeast one-hybrid system was used to screen the mutant DNA library to identify the GCC box-binding proteins. We obtained about 20,000 transformants, and about a quarter of them were blue colonies (data not shown).

Three blue clones, Mu-1 (lighter blue than the wild type), Mu-2 (darker blue than the wild type) and Mu-3 (the darkest blue among the three colonies), were selected for further analysis. When the constructions containing Mu-1, Mu-2 or Mu-3 were again subjected to the yeast one-hybrid assay, the cells turned blue (Fig. 2A). β-Galactosidase activity was measured by setting the relative activity of the negative control GCC to 1, the activity of GCC + BnaERF-B3-hy15 was only 1.02, indicating that there is no, or very weak, binding between BnaERF-B3-hy15 and the GCC box. The activity of mutants GCC + Mu1, GCC + Mu2 and GCC + Mu3 were measured as 3.98, 11.76 and 18.83, respectively (Fig. 2B).

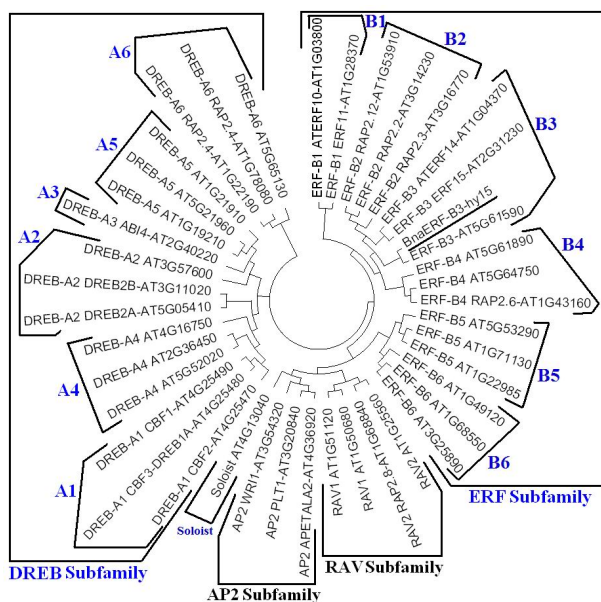The difference in β-galactosidase activity between BnaERF-

Fig. 1. The phylogenetic tree of BnaERF-B3-hy15. Phylogenetic tree was constructed with the aligned BnaERF-B3-hy15 and forty *Arabidopsis* AP2/ERF protein sequences with MEGA version 4.0 using the neighbor-joining (NJ) method and the following parameters: Poisson correction, pairwise deletion and bootstrap (1,000 replicates). The *Arabidopsis* AP2/ERF family sequences were downloaded from Database of *Arabidopsis* Transcription Factors.
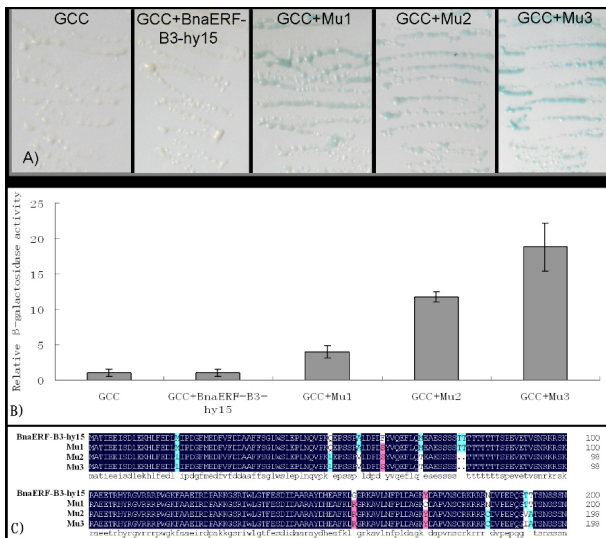
Fig. 2. The yeast one-hybrid and β-galactosidase activity assay. (A) GCC box-binding ability of BnaERF-B3-hy15 and the mutants. The pictures were captured after incubation for 10 h at 30°C with 80 μg/ml X-gal. (B) The relative activity of β-galactosidase. Data are the mean ± SD of three replicates. (C) Sequence alignment between BnaERF-B3-hy15 and the three mutants. The numbers marked represent the potion of the corresponding residues. The black background represents conserved amino acid residues. The red and blue backgrounds represent the mutant amino acid residues.

B3-hy15 and the mutants suggested that changes in residues play a key role in GCC box-binding activity. Full alignment of the encoding regions of BnaERF-B3-hy15 and the mutated sequences was perforemd. Fig. 2C shows a total of 12 differences, two of which (Phe62 changed to serine and Gly156 changed to arginine) occurred in all three mutants.

Mutation in the AP2 domain changes the structure

Among the two mutated sites F62 and G156, only G156 is in the AP2 domain where it changes the structural model significantly. Fig. 3 shows that the 3D structures of BnaERF-B3-hy15 and its mutants have no difference in the β sheet regions, but there is obvious distinction in the α helix region at glycine 156 of BnaERF-B3-hy15, and the α helix region is relatively loose (Fig. 3A). When position 156 was occupied by arginine, the α helix region was more similar to the wild type, and the helices appeared more compact and slender (Fig. 3B).

DISCUSSION

AP2/ERF TF is a superfamily with a wide range of functions. The members of the DREB subfamily can regulate cold or osmotic stress-induced genes and can participate in these pathways. ERF subfamily members are involved in ethylene-mediated defence signalling and ERFs in plant are related to biotic stress (8, 9). *B. napus* growth and productivity are great-
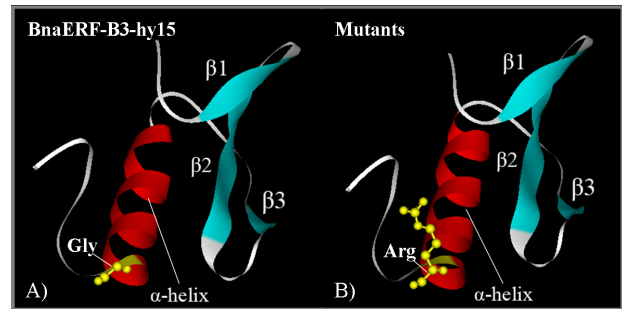

Fig. 3. The structural models of BnaERF-B3-hy15 and the mutant. (A) The structural model of BnaERF-B3-hy15. The red region represents α helix, the blue region represents β sheet, and the yellow region represents the residue 156. (B) The structural model of the mutant.

ly affected by environmental stresses. *B. napus* defend themselves from different sources of biotic (such as insects and pathogen attack) and abiotic stresses (such as drought, high salinity and extreme temperature) by activating various defence mechanisms. The expression of *BnaERF-B3-hy15* was analyzed by using real-time PCR to examine the role of *BnaERF-B3-hy15* under stress conditions in *B. napus* L. cv HuYou15. The *BnaERF-B3-hy15* gene was induced by cold, drought, high salt and ABA. The expression of *BnaERF-B3-hy15* was detected in root and pod at the stage of flowering and seed developing (data not show). These observations suggested that *BnaERF-B3-hy15* was strongly induced by cold and salt stresses, and also induced by drought.

Arabidopsis (the model dicotyledonous plant) is closely related to *Brassica* species (10). BLAST with all AP2/ERF proteins in *Arabidopsis* suggested that BnaERF-B3-hy15 is related closely with AT5G61590, which is an ERF-B3 subfamily factor (Fig. 2). The amino acid length of AT5G61590 is 201. There were high similarity between AT5G61590 and BnaERF-B3-hy15 full amino acid sequence. The EST sequence of BnaERF-B3 is also confirmed that the 200 amino acid sequence is the full length of BnaERF-B3-hy15 (data not shown). Full cDNA sequence alignments revealed that the sequence similarity among the members of the AP2/ERF family TFs is limited to the DNA-binding domain. It was the conclusion of earlier studies that the Tsi1 protein from tobacco contains a highly conserved AP2-type DNA-binding domain, but this is the only part of the protein that exhibits significant sequence homology with many known proteins (11). So, it is responsible for the low degree of similarity between BnaERF-B3-hy15 and AtERF1 (24.18%), AtERF5 (23.10%), AtERF6 (27.66%) and AtERF15 (21.35%), although BnaERF-B3-hy15 has a high level of similarity to these proteins in the AP2 domain (Supplementary Fig. 2).

GCC box-binding activity is one property of ERF proteins, and it has been suggested to be an advantageous function in multiple stress tolerance in plants (12). In this study, the yeast one-hybrid system was used to demonstrate the GCC box-

binding ability of BnaERF-B3-hy15. It binds with the GCC box only very weakly (Fig. 2A), which was contrary to our original expectation for it and for the other members of this subgroup. AtERF1, AtERF2, and AtERF5 functioned as activators of GCC box-dependent transcription in *Arabidopsis* leaves (13). Allen *et al.* determined the 3D solution structure of the AP2 domain of AtERF1 and found it to consist of a three-stranded, anti-parallel β sheet and an α helix packed approximately parallel with the β sheet (14). They found that arginine and tryptophan residues in the β sheet contact eight of the nine consecutive base pairs in the major groove. This indicates that the β sheet in the AP2 domain has important roles in the formation of the domain-GCC box complex. Liu suggested that the two conserved amino acids are Val14 and Glu19 in the AP2 domain of the DREB proteins, whereas alanine and aspartic acid occupy the corresponding positions of the ERF proteins are Ala14 and Asp19, which have important roles in recognition of the DNA-binding sequence. Further analysis indicated that Glu19 might be more important than Val14 for the recognition of the DNA-binding sequence in the DREB1 proteins, and some other amino acids in the DREB2A DNA-binding domain are important for recognition of the binding sequence (1). These results pointed to the binding between the ERF-GCC are necessary but complicated. BnaERF-B3-hy15 in this study was highly conserved in the ERF domain compared to other group members. There was some difference in several residues (Supplementary Fig. 2). However, the key residue to maybe affect the binding ability is not known with certainty.

DNA shuffling was used to address this problem because it can make mutations, and a mutant DNA library was constructed. The yeast one-hybrid and the colony-lift filter assays were used to select clones that contained the mutated GCC box binding protein. The measurement of β-galactosidase activity confirmed the GCC box-binding activity of these three mutations (Fig. 2A, B). Sequence alignment suggested that two residues, Phe62 and Gly156, might influence the GCC box-binding ability of BnaERF-B3-hy15 (Fig. 2C). Phe62 is in the N segment of the full cDNA sequence, which is outside the AP2 domain, and Gly156 is located in the end of α helix formation region. The α helix has been implicated in protein-protein interactions to facilitate DNA binding (15). The crucial amino acid for DRE and GCC binding activity in Liu's study was Ala37, which is also located in the α helix of BnDREBIII-4 (7). Deletion experiments showed that the conserved N-terminal half of the 59 amino acid AP2 domain is likely to be responsible for formation of a stable complex with the GCC box and the divergent C-terminal half for modulating the specificity (16). In this study, Gly156 was mutated to arginine (Fig. 2C), which resulted in structural alteration of the α helix region (Fig. 3). Comparing Fig. 3A with Fig. 3B, it can be seen that the difference of glycine and arginine makes the helix region more compact, which might occur because the molecular characteristic of arginine can influence the spatial arrangement of the other residues. The results of the yeast one-hybrid assays con-

firm that the mutated structure can bind to the GCC box more easily. Although we did not investigate the function of Phe62 in this study, it is certain that the change of Gly156 to arginine is important for the GCC box-binding ability of BnaERF-B3-hy15, which maybe a new crucial site for AP2-GCC combination.

Although the two mutations of Phe62Ser and Gly156Arg are potential good candidates for optimizing the original activity of the wild type BnaERF-B3-hy15 factor, there are still ten other mutations that are simultaneously found in the mutated proteins. Those amino acid mutations may influence GCC-box binding activity as well. The conventional view is that changes of amino acids near the active sites are more likely to alter the function more easily. However, many experiments have demonstrated that specific and random amino acid changes distant from the active site can also affect protein function (17, 18). Are the two mutant residues of Phe62 and Gly156 optimizing the GCC-box binding activity? Do the other ten mutant residues affect the GGC-box binding activity? Moreover, does the modified factor improve the TF's function in plant are remain to be seen.

Those questions should be answered by producing a series of modified BnaERF-B3-hy15 proteins by site-directed mutagenesis harbouring these 12 mutant amino acid residues, and comparing their GCC-binding activity levels in relation to the wild type and the BnaERF-B3-hy15-mu3 proteins. Another useful control would be to produce "reverse" mutants of the mutant proteins, by modifying these 12 specific residues, in order to see if these "reverse" mutant proteins have lost the ability to bind to the GCC-box. Furthermore, direct gel shift assay experiments would be demonstrated if these residues are really involved in direct DNA binding. Over-expression of those mutant genes and the original *BnaERF-B3-hy15* gene in transgenic *Arabidopsis* and *B. napus* plants shall also be used to study the function of those factors in future.

MATERIALS AND METHODS

Growth of *B. napus* L. Huyou15 and cDNA preparation
The *B. napus* L. Huyou15 seeds were sterilized in 1% (v/v) NaOCl and then planted in a mixture of vermiculite, peat moss, and perlite (18 : 6 : 1 by volume) and kept at room temperature for 20d under a 16 h light/8 h dark cycle. The RNA from vigorous seedlings was extracted as described (19). The first strand of cDNA was synthesized using 2 μg of total RNA as template with the AMV reverse transcription system (TOYOBO, Japan) in a 20 μl reaction volume and was used as the template for PCR amplification.

AP2/ERF-B3 TF cloning from *B. napus* L. Huyou15
We used the conserved domain amino acid sequence: YRGVR RRPWGKFAAEIRDSTRNGIRVWLGTFESAEEAALAYDQAAFS MRGSSAILNFSAER of *A. thaliana* ERF-B3 subfamily AtERF1 (AT3G23240.1) to search against the UniGene database of *B.*

napus from NCBI to find the ERF-B3-related EST fragments. The CAP3 (http://pbil.univ1yonl.fr/cap3.php) program was used to splice and analyse the sequences. According to the sequence of the *BnaERF-B3* gene established by *in silico* cloning, we designed a pair of primers with *Bam* HI and *Sac* I enzyme sites at the end of each primer. BnaERF-B3 z1: 5'-GGATCCAT GGCAACTATTGAGGAAATC-3'; BnaERF-B3 f1:5'-GAGCTCT CAGAATTCGTTTGATGATGAATTGC-3'. The RT-PCR protocol was: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s, and a final extension at 72°C for 10 min in a 50 μl reaction volume. The PCR product was retrieved and cloned into the pMD-18-Simple T vector for enzymatic and sequencing identification.

The deduced amino acid sequence of the AP2/ERF gene was obtained from the NCBI DNA database. Multi-sequence alignments were obtained with the program ClustalW (http://bioinformatics.ubc.ca/resources/tools/clustalx). Phylogenetic tree was constructed using the Neighbor-Joining (NJ) method and diagrams of the trees were drawn with the help of MEGA software (20). The sequence analysis and the construction of a phylogenetic tree were done essentially as described (21). The *Arabidopsis* AP2/ERF family sequences were downloaded from DATF, which is maintained by Beijing University (http://datf.cbi.pku.edu.cn/).

Gene mutation and mutant DNA library construction

The gene shuffling used to generate the *BnaERFB3-hy15* mutant was done essentially as described (4, 22) but with modifications as follows. Fragments were purified by 10% PAGE and dialysis. After the primerless PCR and primer PCR using the oligonucleotides BnaERF-B3 f1 and BnaERF-B3 z1 as primers, a collection of full-length *BnaERF-B3-hy15* mutants was obtained and digested with *Bam* HI and *Sac* I enzymes and the isolated fragments were cloned into the pPC87 expression vector to construct the mutant DNA library. Because of the poor efficiency of ligation and because it would degrade the subsequent transformation efficiency, the mutant DNA library was delivered into *Escherichia coli* strain DH5α by electroporation to be amplified. The transformants were placed onto LB plates containing 100 mg/ml ampicillin. After 16 h of growth at 37°C, colonies were scraped down and the plasmid DNA was extracted.

Yeast one-hybrid and β-galactosidase activity assay

The entire *BnaERF-B3-hy15* coding region or each of the mutations was fused into the *Bam* HI-*Sac* I site of the pPC87 expression vector in order to compare the GCC box-binding activity of the wild type and mutated proteins. The yeast pPC87 expression plasmid was reconstructed from pPC86, which contains a galactose-inducible, protein-activating domain (GAL4) under the control of the yeast alcohol dehydrogenase (ADH1) promoter (23-25). Only pPC87 changed the enzyme sites to facilitate the *Bam* HI-*Sac* I ligation, and there was no other alteration. The constructions were transformed into the yeast strain

EGY48, which already carried the vector that could synthesize Ura and had the reporter gene *LacZ* under the control of the GCC element: 5'accctcgagcggataacaatttcacacaggggcggctctt agg cggctcttataagagccg ccggatccgggccc3' (GenBank No. AF394909). The transformants were cultured on SD/-Ura/-Trp plates at 30°C for 3d. A colony-lift filter assay was used to make a qualitative assay of the *trans*-activation activity of the constructs. For the quantitative analysis of β-galactosidase activity, three clones of each transformants were cultured in liquid SD/-Ura/-Trp medium and grown to 0.2 < OD_{600} < 1.0, and the assay used o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate (Clontech Laboratories, Inc.).

Molecular modelling

The Swiss model was used for molecular modelling of the wild type BnaERF-B3-hy15 and its mutants (http://swissmodel.expasy.org/). The structural models of wild type BnaERF-B3-hy15 and the mutants were aligned with the template structure of AtERF1, whose structure was known from earlier multidimensional NMR experiments (14).

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