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AtbZIP16 and AtbZIP68, two new members of GBFs, can interact with other G group bZIPs in *Arabidopsis thaliana*

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AtbZIP16 and AtbZIP68 are two putative G group bZIP transcription factors in *Arabidopsis thaliana*, the other three members of G group bZIPs are GBF1-3 which can bind G-box. Members of G group have conservative protein structure: highly homological basic region and a proline-rich domain in the N-terminal region. Here, we report that AtbZIP16 and AtbZIP68 could bind cis elements with ACGT core, such as G-box, Hex, C-box and As-1, but with different binding affinities which from high to low were G-box > Hex > C-box > As-1; AtbZIP16 and AtbZIP68 could form homodimer and form heterodimer with other members of G group; N-terminal proline rich domain of AtbZIP16 had transactivation activity in yeast cells while that of AtbZIP68 did not; AtbZIP16 and AtbZIP68 GFP fusion protein localized in the nucleus of onion epidermal cells. These results indicated that AtbZIP16 and AtbZIP68 were two new members of GBFs. In *Arabidopsis*, AtbZIP16 and AtbZIP68 may also participate in light-responsive process in which GBF1-3 are involved. [BMB reports 2008; 41(2): 132-138]

INTRODUCTION

Transcription factors are capable of activating or repressing gene transcription by binding specific cis elements in the promoter (1). In plants, Basic region/leucine zipper (bZIP) transcription factors may involve in multiple biological processes such as biotic and abiotic stress, light signaling, phytohormone response, seed maturation, *et al*. (2-7). The bZIP transcription factors have a basic region that binds DNA, close to the basic region there is a leucine zipper region which consists of several heptad repeats of leucines. The leucine zipper region is alpha-helical and can form dimer via a coiled-coil structure (8). Many plant bZIP proteins can bind or preferentially bind cis elements which have an ACGT core, such as G-box (CACGTG), C-box (GACGTC), and A-box (TACGTA) *et al*. (9, 10).

In the *Arabidopsis thaliana* genome approximately 5.9% of the total number of predicted genes is transcriptional regulators (1). The family of bZIP transcription factors in *Arabidopsis thaliana* has 75 members, which are divided into ten group (A, B, C, D, E, F, G, H, I and S group) according to their protein structure (10). G group has five members: GBF1-3, AtbZIP16 and AtbZIP68. Members in G group all have conservative protein structure: a highly homological DNA binding domain and a proline-rich domain in the N-terminal. GBF is the name of G-box binding factor. So, GBF1-3 could bind G-box (11, 12) Although there are three other GBFs (GBF4-6) in *Arabidopsis thaliana* which also could bind G-box, the structure of them is different from the former three.

bZIPs can form homo and/or heterodimers. In *Arabidopsis thaliana*, GBF1-3 could form homodimer and form heterodimers with each other (11); GBF4 could not form homodimer but can form heterodimers with GBF2,3 (13).

The other two members of G group are AtbZIP16 and AtbZIP68, they contain a parallel protein structure with GBF1-3, but whether they can bind G-box or whether they can form heterodimer with GBF1-3 remains unknown. In this study, we analyzed the characters of dimerization and DNA binding specificities of these two bZIP transcription factors.

RESULTS

Bioinformation analysis

Members of G group have a conserved basic region, the homology of basic region of GBF2, GBF3, AtbZIP16 and AtbZIP68 to GBF1 is 83.9%, 83.9%, 87.1% and 87.1%, respectively; The N-terminal region of members of G group all have a proline-rich domain, but the positions of the proline residue are only slightly conserved (Fig. 1A). The overall amino acid homology among members of G group is not very high, AtbZIP16 and AtbZIP68 have the highest homology which reaches 78.6%, but homology of others is lower than 50%. GBF4 (At1g03970) shares little overall homology with members of G group, GBF4 and members of G group are clustered into different clades in phylogenic tree (Fig. 1B).

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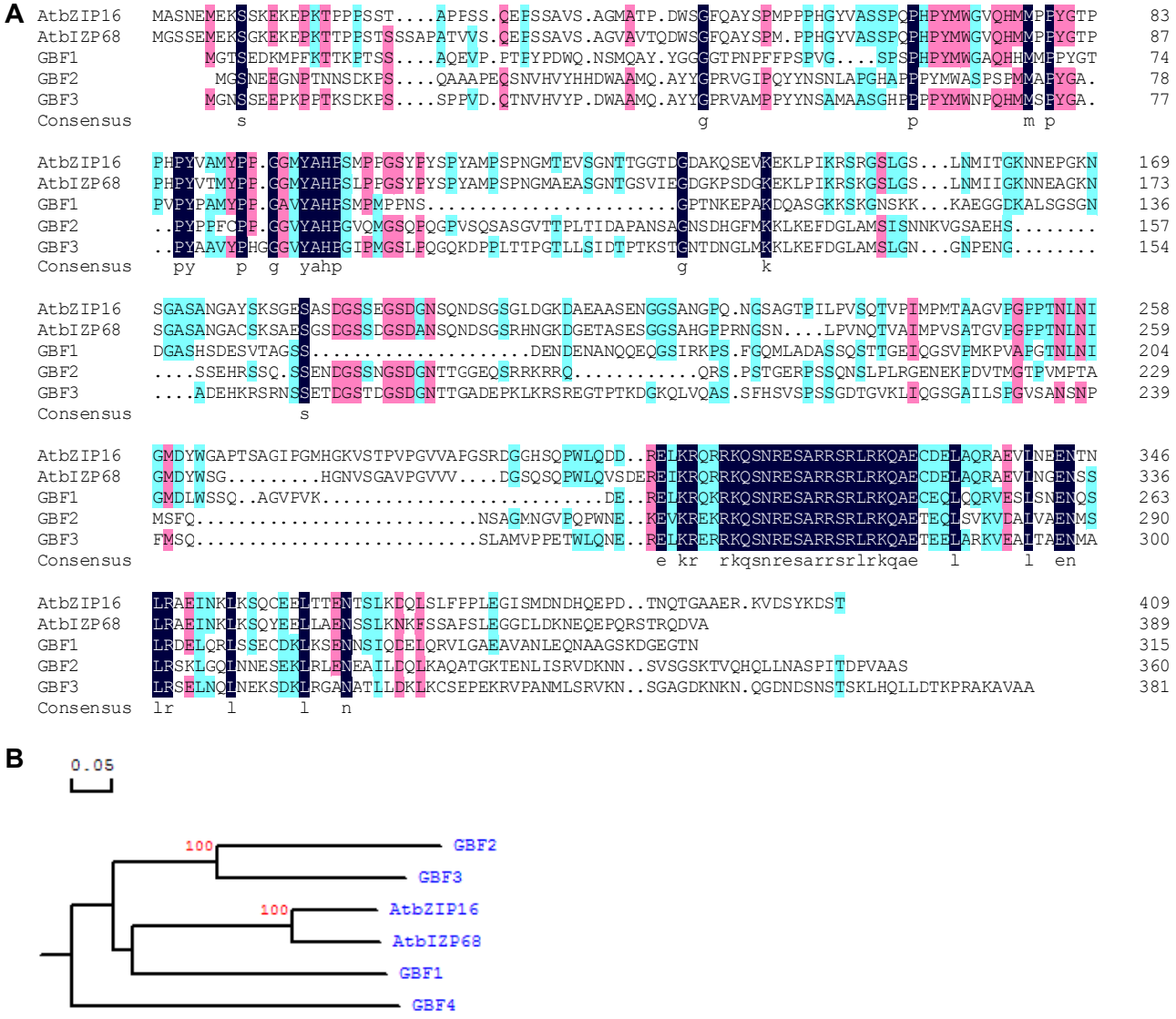

Fig. 1. (A) Protein sequence alignment of members of G group. The alignment was made using the program DNAMAN 5.2.2. The letters in blue background represent amino acids conserved between three or four family members. The letters in red background are amino acids conserved between four or five family members. The letters in deep blue background are amino acids that are common among all five members. (B) The phylogenic tree of members of G group and GBF4. Tree was created using the program DNAMAN5.2.2.

Transcription activation in the yeast cell

In order to test whether AtbZIP16 and AtbZIP68 have transcriptional activation activity, the full-length coding region of AtbZIP16 and AtbZIP68 was fused in frame to yeast GAL4 DNA-binding domain (BD). Yeast cells which transformed with BDbZIP16 could grow in SD/-Trp-His-Ade medium; it indicated that AtbZIP16 was able to activate transcription in yeast. The proline rich region of AtGBF1 serves as a transcriptional activator in plant protoplasts and mammalian cells (11). Yeast cells with BDbZIP16ΔN which N-terminal proline rich domain was deleted could not grow in SD/-Trp-His-Ade medium. So, the proline rich domain of AtbZIP16 also serves as a transcriptional activator (Fig. 2A, 2B).

Though AtbZIP68 has highest homology with AtbZIP16 among members of G group, yeast cells which transformed with BDbZIP68 could not grow in SD/-Trp-His-Ade medium. Transcriptional activation strength was quantified with β-gal assay making use of the lacZ reporter gene. It is interesting that β-gal activity in yeast cells with BDbZIP68ΔN which was also deleted N-terminal proline rich domain was 1.7 fold higher

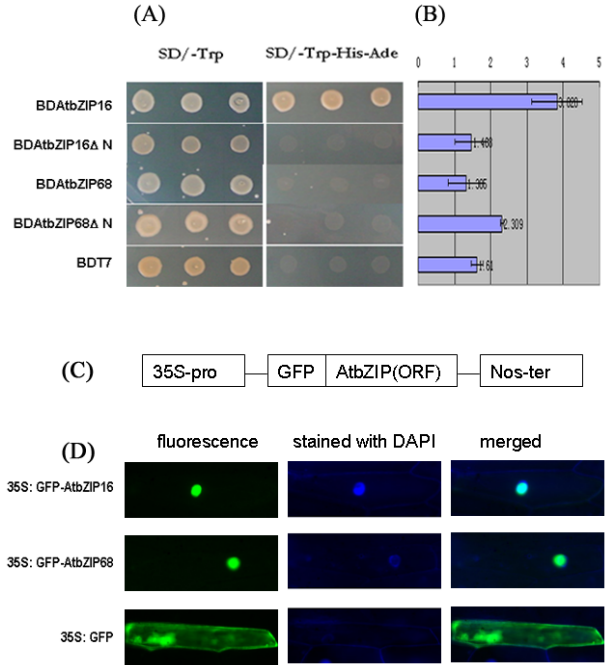

Fig. 2. Transcriptional activation analysis and subcelluar localization of AtbZIP16 and AtbZIP68. (A) Transformants growing on SD/-Trp and SD/-Trp-His -Ade plates; (B) β-gal activity test of different transformants. The β-gal units were shown as a mean value of three independent measurements. Error bar represented standard deviation; (C) Construction of 35S:GFP-AtbZIP16 (or AtbZIP68) fusion protein. The AtbZIP16 or AtbZIP68 coding region was fused in frame to the GFP coding region in the plant expression vector; (D) Onion epidermal cells were transfected by bombardments with plasmids expressing 35S: GFP-AtbZIP16 (or AtbZIP68) fusion protein. The same cell layer was briefly stained with DAPI (100 μg/ml) to visualize nuclei.

than that with BDbZIP68. It might be that N-terminal proline rich domain of AtbZIP68 serves as a transcriptional repressor.

Subcellular localization in the onion epidermal cells

In the basic region, AtbZIP16 and AtbZIP68 have nuclear localization signal (NLS). As putative transcription factors, they are likely to be localized in the nucleus. To verify the intracellular localization of AtbZIP16 and AtbZIP68 protein, we used whole length CDS in frame fusion with sequence encoding the green fluorescent protein (GFP). The plasmid containing 35S: GFP-bZIP16 (or bZIP68) was bombarded into onion epidermal cells. The recombinant GFP-bZIP16 and GFP-bZIP68 accumulated exclusively in the nucleus of onion epidermal cells, while GFP accumulated in both cytoplasm and nucleus of onion epidermal cells in GFP control (Fig. 2D). The result suggested that AtbZIP16 and AtbZIP68 were nuclear-localized proteins.

AtbZIP16 and AtbZIP68 bind G-box and G-box-like elements in EMSA

It was known that GBF1, GBF2 and GBF3 are able to bind G-box and G-box-like elements (11). The high sequence similarities among all five members of G group within their DNA binding domain suggest that AtbZIP16 and AtbZIP68 may also bind G-box and G-box-like elements. To this end, EMSA was performed. The full length proteins of AtbZIP16 and AtbZIP68 were expressed in vitro using T_NT coupled transcription/translation reticulocyte lysate system (Promga). The translated products were incubated with radio-labeled oligonucleotide containing G-box. As shown in Fig. 3A, AtbZIP16 and AtbZIP68 could bind G box specifically and form protein-DNA complex.

Plant bZIP proteins bind to DNA exhibiting dyad symmetry with an ACGT core sequence, but flanking sequences strongly influence the affinity of particular bZIP proteins. The bZIP region of AtbZIP68 (from 292aa to 365aa) was translated in vitro, this truncated version of AtbZIP68, named AtbZIP68bz, was incubated with different radio-labeled cis elements: G-box, Hex, C-box and As-1, all these cis elements have an ACGT core. As shown in Fig. 3B, these elements could be specifically bound by AtbZIP68bz. To test binding affinity of AtbZIP68bz with these elements, competent assay was performed. AtbZIP68bz first was incubated with radio-labeled G-box, then adding different amount of non-radio-labeled G-box, Hex, C-box and As-1 as competent, respectively. As shown in Fig. 3C, a 50 fold amount of non-radio-labeled G-box could efficiently compete with radio-labeled G-box, 100-fold was needed for Hex, and much more than 100-fold was needed for C-box and As-1 box. These results indicated that although the *cis*-elements, such as G-box, Hex, C-box and As-1, all have an ACGT core sequence, AtbZIP68bz has highest binding affinity to G-box, much lower affinity to Hex and C-box, and little affinity to As-1 element.

AtbZIP16 and AtbZIP68 could form heterodimers with other members of G group

It has been shown GBF1, GBF2 and GBF3 form heterodimer promiscuously in the presence of DNA (11). To determine whether AtbZIP16 or AtbZIP68 can form heterodimer with other members of G group including GBF1, GBF2 and GBF3, EMSA was performed. Both full length of AtbZIP16, AtbZIP68 and truncated version of all five members of G group were translated in vitro and EMSA was performed. As shown in Fig. 4A, when full length protein of AtbZIP16 and the truncated version proteins of all five members of G group were individually test for of G-box binding assay, only one major band which represents protein-DNA complex was observed in each case (Fig. 4A, lanes 02, 04, 05, 06, 08, 10, 11, 13, 14 and 16). When full length protein of AtbZIP16 was mixed with the truncated version proteins of all five members of G group in the presence of G-box, respectively, a new band of protein-DNA complexes with intermediate mobility was observed

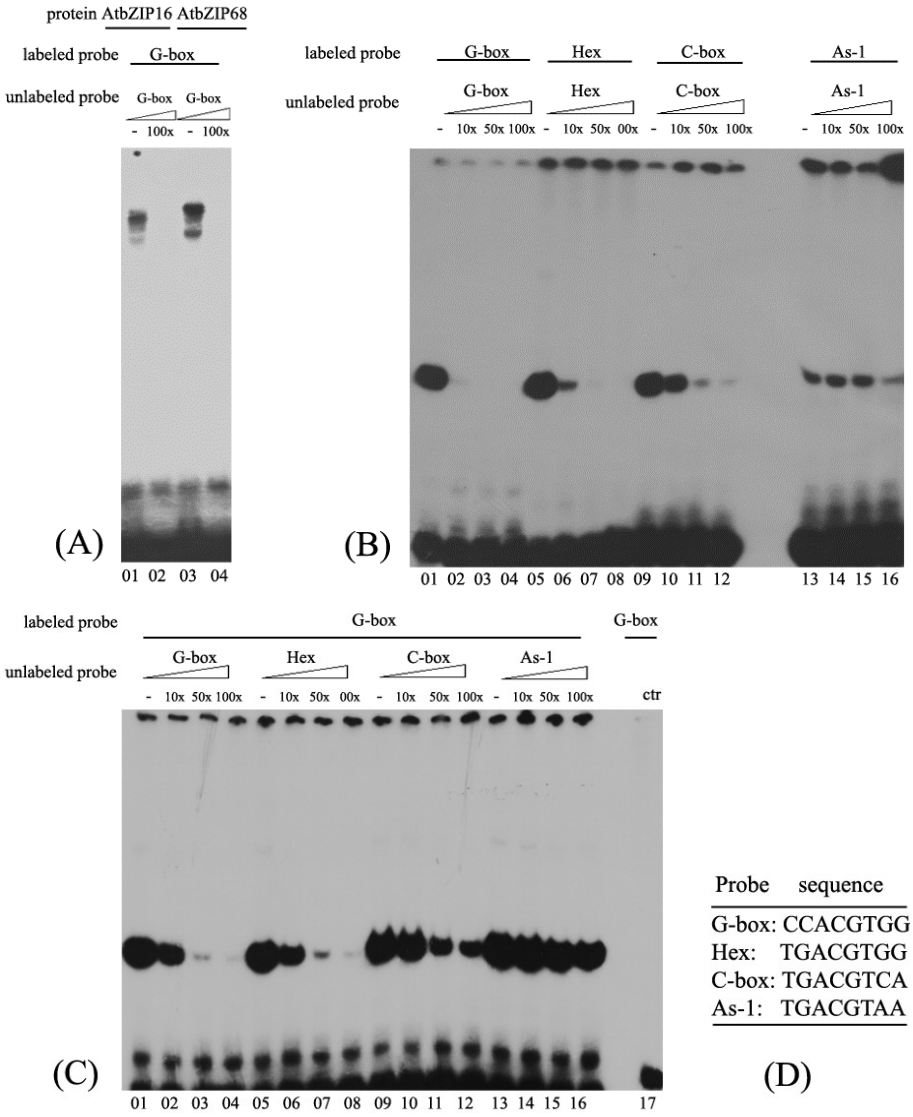

Fig. 3. AtbZIP16 and AtbZIP68 binding G-box and G-box-like elements. (A) AtbZIP16 (lanes 01, 02) and AtbZIP68 (lanes 03, 04) interacted with G-box; (B) AtbZIP68bz could specifically bind G-box (lanes01-04), Hex (lanes05-08), C-box (lanes 09-12) and As-1 (lanes 13-16); (C) Different affinity for AtbZIP68bz binding G-box (lanes01-04), Hex (lanes05-08), C-box (lanes 09-12) and As-1 (lanes 13-16). lanes 17: control (the binding reactions containing reticulocyte lysate only). (D) DNA sequences of the oligonucleotides employed in DNA binding studies.

in each case (Fig. 4A, lanes 03, 07, 09, 12 and 15). These intermediate protein-DNA complexes indicated homodimer formed by AtbZIP16 and AtbZIP16bz (Fig. 4A, lanes 03) or heterodimers formed by AtbZIP16 and other members of G group, respectively (Fig. 4A, lanes 07, 09, 12 and 15). Similar results were obtained for AtbZIP68 (Fig. 4B).

We also tested dimerization of AtbZIP16 and AtbZIP68 by yeast two-hybrid assay. AtbZIP16ΔN which deleted N-terminal proline-rich domain and full length AtbZIP68 fused to the GAL4 DNA-binding domain as bait (BDbZIP16ΔN, BDbZIP68). Full length of members of G group fused behind a GAL4 transcriptional activation domain as prey. Bait and prey were co-introduced into the yeast strain AH109. The strength of interaction was quantified with β-Gal assay. As shown in Fig. 4D, the interactions between AtbZIP16 and all five members of G group resulted in at least 7.0 fold higher β-gal activity than that of negative control; the corresponding result of AtbZIP68 was 6.5 fold higher. The result indicated that AtbZIP16 and AtbZIP68 could interact with all five members of G group. In other words, AtbZIP16 and AtbZIP68 could form homodimer and form heterodimers with other members of G group. This was consistent with the result of EMSA.

DISCUSSION

As two putative transcription factors, AtbZIP16 and AtbZIP68 have the similar protein structure with GBF1, GBF2 and GBF3, all of them constitute G group bZIPs in *Arabidopsis thaliana*.

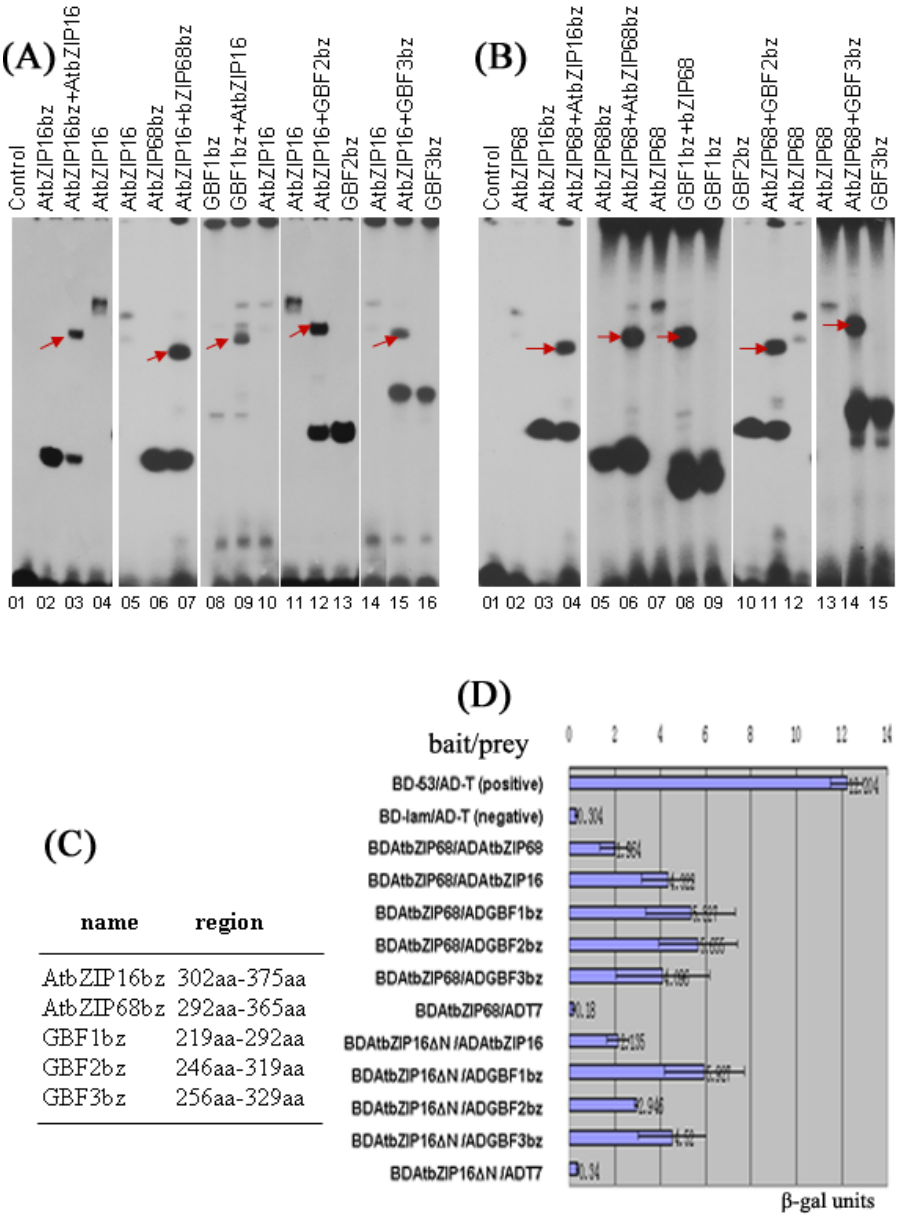

Fig. 4. Heterodimerization between AtbZIP16 and other members of G group. (A) Heterodimer formation between AtbZIP16 and members of G group. Lanes 02, 04, 05, 06, 08, 10, 11, 13, 14 and 16 contained the individual protein-DNA complexes obtained with the full-length and truncated version proteins (bZIP region of members of G group) as indicated above each lane. For lanes 03, 07, 09, 12 and 15, the indicated proteins were mixed and incubated for 30 min at 25°C prior to the addition of the binding site DNA. The heterodimeric protein-DNA complex of intermediate mobility was marked by an arrowhead. For lane 1, only labeled DNA was added as control. (B) Heterodimer formation between AtbZIP68 and members of G group. Lanes 02, 03, 05, 07, 09, 10, 12, 13 and 15 contained the individual protein-DNA complexes obtained with the full-length or truncated version proteins as indicated above each lane. For lanes 04, 06, 08, 11 and 14, the indicated proteins were mixed and incubated for 30 min at 25°C prior to the addition of the binding site DNA. The heterodimeric protein-DNA complex of intermediate mobility was marked by an arrowhead. For lane 1, only labeled DNA was added as control. (C) The bZIP regions of members of G group employed in EMSA. (D) AtbZIP16 and AtbZIP68 interacted with members of G group in yeast cells. The β-gal units were shown as a mean value of three independent measurements. Error bar represented standard deviation.

By several assays, we found AtbZIP16 and AtbZIP68 GFP fusion proteins were localized in the nucleus of onion epidermal cells; proline-rich domain in AtbZIP16 was a transcriptional activator, which in AtbZIP68 might be a transcriptional repressor though we were not very sure; both AtbZIP16 and AtbZIP68 could bind G-box. In addition, we tested AtbZIP68 could also bind G-box-like elements, such as Hex, C-box and As-1, but had different affinity with these cis elements; AtbZIP16 and AtbZIP68 could form homodimer and form heterodimer with other members of G group. From these results, we concluded that AtbZIP16 and AtbZIP68 were two new members of GBFs.

All GBFs can bind G-box and G-box-like elements which mainly present in light-responsive promoter such as: RBCS, CHS and CAB *et al*. (14). The expression of GBF3 is regulated by light (11), CPRF1, CPRF4a in parsley are two homologues of GBFs, their expression is also regulated by light (15, 16); GBF2 and CPRF4a translocate into nucleus upon light treatment (17, 18). However, there are hardly genetic data showing that these genes function in light-regulated signal transduction. HY5, belongs to H group of bZIP family, also binds G-box and mainly involves in morphogenesis of *Arabidopsis thaliana* (19)

It is reasonable to speculated that the overexpression of GBFs might compete with HY5 to bind G-box, which will cause HY5 completely or partially lose its function. In fact, it has been shown that overexpression transgenic lines of GBF1 have an elongational hypocotyl under blue or white light treatment which is similar to *hy5* mutant (20). In soybean, STF1, which contains a domain with high homology to HY5 in the C-terminal region, can form heterodimers with GBF in the presence of Hex elements. We also examined the possible interaction between HY5 and AtbZIP16 or AtbZIP68, but did not find any positive results (data not shown). However, AtbZIP16 and AtbZIP68 can bind G-box and form heterodimers with GBF1-3, it can be speculated that AtbZIP16 and AtbZIP68 may also be involved in light-responsive processes in *Arabidopsis*.

By RT-PCR, it was found that *AtbZIP16* and *AtbZIP68* were constitutively expressed in all tissues tested including root, stem, leaf and flower, their basal expression levels were relatively high which was quite similar to that of *Actin* gene (data not shown). From microarray date of transcription factors of Arabidopsis, the expression of *AtbZIP16* and *AtbZIP68* were not found to have distinct variation under light or dark (21). The knockout mutants of both *AtbZIP16* and *AtbZIP68* did not shown any obvious phenotypes. G group bZIPs have five members and can interact with each other and bind the same *cis*-elements, suggests the functional redundancy exists. The further analysis of double mutant of *AtbZIP16*/*AtbZIP68* or triple mutant of *AtbZIP16*/*AtbZIP68*/*GBFs* perhaps will help us to elucidate biological functions of AtbZIP16 and AtbZIP68.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) plants were cultured under controlled environmental conditions in 16 h light/8 h dark cycles, plants were maintained at 22°C.

Gene cloning and plasmid construction

Total RNA was extracted with Trizol reagent from seedlings cultivated for 14 days, and reversely transcribed to cDNA according to the manufacturer's instruction. The ORFs of AtbZIP16 (At2g35530) and AtbZIP68 (At1g32150) were isolated by RT-PCR via specific primers designed for amplification: AtbZIP16F (5'CACCACAAAATGGCACAACTTCCTCCGAAAATC3'), AtbZIP16R (5'GACATTGAGGAGCTGTTCCTTCTC3'), AtbZIP68F (5'CACCACAAAATGGCACAACTCCCTCCTAAAATC3'), AtbZIP68R (5'TGAGACATTGAGGAGCTGCTCTGT3'). The PCR products were purified and ligated into pENTER-TOPO vectors (Invitrogen, USA) and sequencing confirmed. The gene sequences which code bZIP region of GBF1 (At4g36730), GBF2 (At4g01120) and GBF3 (At2g46270) were also isolated by RT-PCR, the specific primersdesigned for amplificaton: GBF1bzF (5'CCAGAATTCGATGAACGAGAGCTCAAGC3'), GBF1bzR (5'TGCGGATCCTACTCTCTGCAACTCATCC3'); GBF2bzF (5'CAGGAATTCAATGAAAAAGAGGTTAAAC3'), GBF2bzR (5'CCCGGATCCTTGCGCTTTCAGTTGATCC3'); GBF3bzF (5'TGGGAATTCAACGAGAGAGAACTGAAAC3'), GBF3bzR (5'TTCGGATCCCGAGCATTTCAGTTTGTCC3'). The PCR products were digested with EcoRI and BamHI and subcloned into pGBDT7 and pGADT7 which can be used in yeast two hybrid assay and the T_NT coupled transcription/translation reticulocyte lysate system.

Subcellular localization of AtbZIP16-GFP and AtbZIP68-GFP in onion epidermal cells

The AtbZIP16 and AtbZIP68 full length CDS were cloned into a pRTL2S65TGFP vector (Arnim AV and Deng XW, unpublished data). The plasmid was isolated using Tiangen kits, concentrated to about 1 μg/μl and used to coat the tungsten particles for bombardment experiments. A plasmid containing GFP alone was concentrated as above and bombarded in parallel as controls. Transformation of onion (*Allium cepa*) epidermal cells and localization of the protein were performed essentially as described in (22). After bombardment, onion cell layers were incubated for 48 hr at 22°C, the cell layers were then examined by fluorescence microscopy.

EMSA (Electrophoretic mobility shift assays)

Complementary oligonucleotides were annealed in annealing buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 70°C and cooled slowly to room temperature. Oligonucleotides (10 pmol) were end-labeled with [α-^{32}P]dATP by filling in the overhang using Klenow fragment.

Proteins were synthesized using the T_NT coupled transcription/translation reticulocyte lysate system (Promega). Binding reactions were performed mainly as described in (23). *In vitro* translated proteins (2 μl) were incubated with labeled probe (10,000-20,000 cpm; final concentration of 0.12-0.25 nM) in the standard binding reaction buffer (final volume of 10 μl) (20 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 2.5 μg of BSA, 2 ng of double-stranded poly(dI-dC) (Roche), and 200 ng of sonicated calf thymus DNA) for 30 min at 30°C. DNA-protein complexes were resolved by electrophoresis through a 5% polyacrylamide gel (acrylamide : bisacrylamide ratio of 60 : 1) in 1 × TBE for 2-2.5 h at 150 V.

Transcription activation assay and yeast two-hybrid analysis

The MATCHMAKER Two-Hybrid System 3 (Clontech) was used for transcription activation assay and the yeast two-hybrid experiments. The activation domain (AD) vector used was pGADT7 and the DNA-binding domain (BD) vector was pGBDT7. The yeast strain was YH109. The experiments were carried out following the manufacturer's specifications.

Acknowledgments

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