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Isolation and characterization of a fourth *Arabidopsis thaliana* G-box-binding factor, which has similarities to Fos oncoprotein

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ABSTRACT **A fourth member of the *Arabidopsis* G-box-binding factor (GBF) family of bZIP proteins, GBF4, has been isolated and characterized. In a manner reminiscent of the Fos-related oncoproteins of mammalian systems, GBF4 cannot bind to DNA as a homodimer, although it contains a basic region capable of specifically recognizing the G-box and G-box-like elements. However, GBF4 can interact with GBF2 and GBF3 to bind DNA as heterodimers. Mutagenesis of the leucine zipper of GBF4 indicates that the mutation of a single amino acid confers upon the protein the ability to recognize the G-box as a homodimer, apparently by altering the charge distribution within the leucine zipper.**

The mechanisms of regulated eukaryotic gene expression are topics of intense examination. These regulatory pathways require the coordination of highly specific DNA–protein and protein–protein interactions, many of which are not fully understood. One goal is to understand the individual contribution of seemingly similar DNA elements to regulated gene expression when these conserved elements are present in promoters responding to diverse regulatory controls. In plant systems, one example of this complexity is illustrated by the hexameric G-box (5′-CACGTG-3′) and those DNA elements that contain small variations on this palindromic sequence, which we refer to collectively as G-box-like elements. In some cases these elements have been identified as targets for nuclear DNA-binding factors, examples of which include the light-regulated genes of ribulose-1,5-bisphosphate carboxylase/oxygenase (1), chlorophyll a/b binding proteins (2), and chalcone synthase (3), genes regulated by abscisic acid such as the *Em* gene of wheat (4) and the *rab16A* gene of rice (5), as well as the stress-induced *Adh* gene of maize and *Arabidopsis* (6, 7). The identification of these nuclear factors has prompted the isolation and characterization of 10 DNA-binding proteins specific for the G-box and G-box-like elements from four different plant species (4, 8–11).

To understand how a seemingly ubiquitous element can be involved in such diverse regulatory pathways, it is essential to understand the individual contributions made by each of the relevant DNA-binding proteins. To date, all of the plant DNA-binding proteins that recognize the G-box belong to the bZIP family of transcription factors (12, 13). These proteins are characterized by the presence of the basic region, a subdomain of ≈20 residues rich in basic amino acids that mediates DNA binding. Immediately adjacent to the basic region is the leucine zipper, a dimerization motif defined by a 4–3 repeat of typically four or five leucine residues interspersed with other hydrophobic amino acids, which align in parallel to form a coiled coil, with the leucine and additional hydrophobic residues forming a hydrophobic interface (14–18).

A well-documented example of the intricate interactions between members of a family of related bZIP proteins is illustrated with the products of the *fos* and *jun* oncogenes (12, 13, 19). There are three Jun-related proteins, each of which is capable of binding DNA as a homodimer, as well as forming heterodimers with each of the other Jun proteins via dimerization of the compatible leucine zippers. In striking contrast, four Fos-related proteins are incapable of forming homodimers, although the leucine zippers of Fos proteins are capable of interacting with the Jun leucine zippers to form heterodimers (20). Thermodynamic and mutational analyses have demonstrated that the specificity of dimerization of these leucine zippers is dictated by the distribution of the charged amino acids that lie adjacent to the hydrophobic interface (21–23).

The potential complexity arising from the specific interaction of multiple proteins with similar DNA-binding specificities, as evidenced with the Fos/Jun system in mammals, is also observed in plant systems. In *Arabidopsis*, parsley, and wheat, small families of bZIP proteins that recognize G-box and G-box-like elements have been characterized (4, 8, 10, 11). The leucine zipper of each of these proteins is capable of interacting to form homodimers. However, among families, the formation of heterodimers can be either promiscuous (11) or selective (24).

Here we report the isolation and characterization of GBF4, another member of the G-box-binding factor (GBF) family of *Arabidopsis thaliana*.† GBF4 is distinct from GBF1, -2, and -3 in a number of ways. GBF4 is unable to recognize the palindromic G-box as a homodimer, although "domain-swap" experiments demonstrated that the basic region of GBF4 has the potential to specifically recognize G-box elements. A mutational analysis of the GBF4 leucine zipper revealed that homodimer formation is apparently inhibited by the charge distribution within the leucine zipper in a manner similar to the Fos and Fos-related proteins.

MATERIALS AND METHODS

Isolation and Characterization of GBF4. A cDNA library constructed from 3-day-old *Arabidopsis* seedling hypocotyls was screened by using as a radiolabeled probe a DNA sequence derived from the basic region of GBF1, the details of which are described by Schindler *et al.* (11). DNA sequence analysis was determined by using double-stranded DNA templates and the modified T7 DNA polymerase Sequenase (United States Biochemical).

DNA Templates. DNA templates encoding proteins of the lengths indicated in each figure were generated using PCR [50 pmol each primer, 50 μM (each) deoxyribonucleotide, 20 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl] with cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles. In each case, a T7 promoter sequence (5′-CGAAA-

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Abbreviations: GBF, G-box-binding factor; RRL, rabbit reticulocyte lysate.

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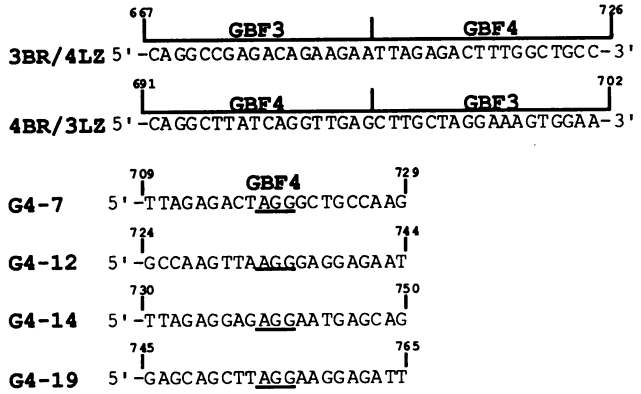
†The sequence reported in this paper has been deposited in the GenBank data base (accession no. UO1823).

TTAATACGACTCACTATAGGGACACC-3') was included at the 5′ end of each "upstream" primer. Proteins for "domain swap" and mutagenesis experiments were generated by recombinant PCR and contained the following alterations, which are numbered with respect to either the GBF4 (Fig. 1) or GBF3 (11) sequences:



Transcription/Translation of Proteins *in Vitro*. RNA templates were generated from DNA templates *in vitro* using T7 RNA polymerase as described (26). The resulting RNA templates were then translated *in vitro* by using a rabbit reticulocyte lysate (RRL) system as described by the manufacturer (Promega). Synthesis of protein was monitored by translating equal amounts of the respective RNA templates in a reaction containing [^{35}S]methionine, and the resulting radiolabeled proteins were assayed by SDS/PAGE (data not shown).

Gel-Mobility-Shift Assays. The volume of each reaction was 20 μl, which included 1 μg of poly(dI·dC), 0.1 pmol of random single-stranded DNA, 10 mM Tris·HCl (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 4% (vol/vol) glycerol. Homodimer formation was assayed by mixing either 2 μl of the longer protein (GBF1M, GBF2M, or GBF3M) or 1 μl of the short proteins in the reaction mixture containing 1 × 10^4 cpm of radiolabeled probe (2–10 fmol), which were incubated for 30 min at room temperature. Heterodimer formation was assayed by mixing 2 μl of the longer protein (GBF1M, GBF2M, GBF3M) with 1 μl of the indicated shorter protein, which were incubated for 30 min at room temperature before the addition of radiolabeled probe. All reactions were then subjected to 6% nondenaturing PAGE.

RESULTS

Isolation of *GBF4*. The 137-bp DNA fragment encoding the basic region of GBF1 was radiolabeled and used to probe an *Arabidopsis* cDNA library at low stringency (11). As a result of this screen, three additional members of the GBF family were isolated. GBF2 and GBF3 have been described (11). A fourth cDNA clone, GBF4, was also isolated. The complete nucleotide sequence of clone GBF4 was determined (Fig. 1). The total length of the cDNA clone was 900 bp; an open reading frame encoded a putative protein of 270 amino acids. This protein contains a basic region and leucine zipper, domains that define the bZIP family of proteins.

Comparison of the amino acid sequence of the bZIP region of each of the four *Arabidopsis* GBF proteins is shown in Fig. 2. The basic region of GBF4 contains the two amino acids that are absolutely conserved within all bZIP proteins, Asn-15 and Arg-23. Three additional residues at positions 18, 19, and 23 are common to most bZIP proteins (27) and are absolutely conserved among the four GBF proteins. However, the leucine zipper region of GBF4 is quite distinct from GBF1, -2, and -3 (Fig. 2). The established nomenclature of a leucine zipper refers to each position within the heptad repeat as positions a–g (noted above the amino acid sequence in Fig. 2).

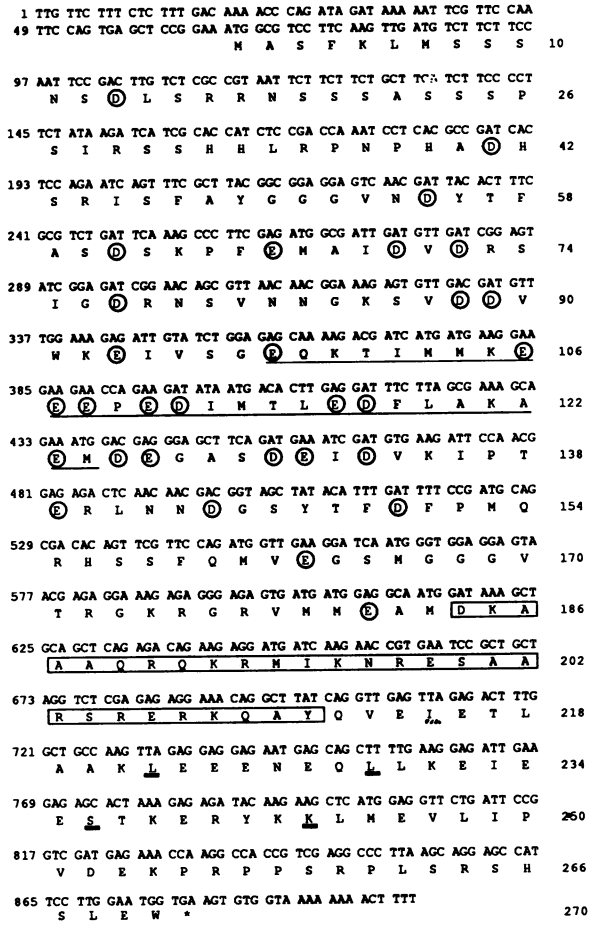


FIG. 1. DNA sequence of *GBF4* and the deduced amino acid sequence of the protein. The bZIP domain is represented by the basic region (boxed) and the leucine zipper (thick underlinings). A region rich in acidic amino acids (circled) contains a small subregion (underlined) that forms a putative α-helix as predicted by Chou–Fasman analysis.

In general, the leucine residues that define the zipper are present at the d positions, with the alternating hydrophobic residues at the a positions, and GBF1, -2, and -3 meet these criteria. In contrast, GBF4 contains a serine residue at the fourth d position and a lysine at the fifth position. The a positions each contain a hydrophobic residue except for the fifth a position, which is occupied by the basic residue arginine. Mutational analysis of other leucine zippers has demonstrated that substitution at one of these positions by a nonpolar amino acid is acceptable for function (28–30). In addition, because functional leucine zippers exist that contain only four heptad repeats and the polar residues of lysine and arginine are within the fifth heptad repeat, we considered that these residues might not disrupt the structural integrity of the α-helix (16, 28, 31–34).

"Domain-Swap" Analysis Indicates That GBF4 Contains a Basic Region Capable of Binding to a G-Box, but the Leucine Zipper Is Incapable of Forming Functional Homodimers. We sought to examine the DNA-binding properties of GBF4. Full-length *GBF4* was transcribed/translated *in vitro*, and the resulting protein was used in gel-mobility-shift assays with a radiolabeled probe containing the G-box (5′-CACGTG-3′) to determine the DNA-binding specificity of GBF4. No DNA–protein complex was seen in those experiments (data not shown). There were two obvious possibilities that could account for this result. (*i*) The basic region might be incapable of recognizing a G-box and require an alternative binding site to form a DNA–protein complex. (*ii*) The leucine zipper of

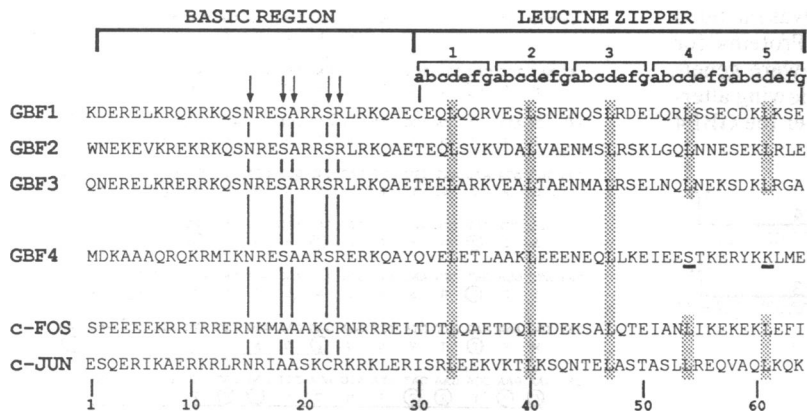


FIG. 2. Alignment of the bZIP regions of the *Arabidopsis* GBF proteins with those of c-Fos and c-Jun. The two subdomains of the basic region and leucine zipper are indicated. Within the basic region, those amino acids that are conserved in most bZIP proteins are indicated by arrows. The leucine zipper region is divided into five heptad repeats, defined as positions a–g, with the leucine residues at position d (highlighted). The two d positions within GBF4 lacking the leucine residue are underlined. The numbering indicated below is arbitrary and does not reflect the absolute position of the bZIP domain within the respective protein.

GBF4 might be incapable of forming homodimers, in a manner reminiscent of the Fos oncoprotein.

To distinguish between these two possibilities, we carried out domain-swap experiments (35–39), using recombinant PCR to generate the appropriate templates for the production of *in vitro* transcribed/translated proteins (Fig. 3). In these experiments, truncated versions of GBF4 and GBF3 containing the basic region and leucine zipper domain were divided into two cassettes each (Fig. 3*A*). The basic region of each protein included 32 amino acids (aa 1–32, Fig. 2), sufficient to mediate specific DNA binding. The second cassette of each protein contained the leucine zipper (aa 33–64, Fig. 2), as well as 14 amino acids of the carboxyl-terminal region of GBF3 or the 24 carboxyl-terminal amino acids of GBF4. Primers specific to the basic region incorporated a T7 promoter sequence to direct synthesis of the proteins *in vitro*. The truncated GBF3 protein could form a DNA–protein complex with a radiolabeled G-box, whereas the truncated GBF4 could not form a complex (Fig. 3*B*, lanes 3 and 4). When the leucine zipper of GBF4 was fused to the basic region of GBF3, no apparent DNA–protein complex was formed (Fig. 3, lane 5). In contrast, when the basic region of GBF4 was fused to the leucine zipper of GBF3, the appropriate complex formed (Fig. 3, lane 6). These results indicated that although the basic region of GBF4 could recognize the G-box, the leucine zipper was apparently incapable of interacting to form homodimers. Competitive gel-mobility-shift analysis has shown that the DNA-binding specificities of the basic region of GBF4 are essentially indistinguishable from those of GBF1 (11), GBF2, and GBF3 (data not shown).

GBF4 Can Interact with GBF2 and GBF3 to Form Heterodimers. We were interested in determining whether the GBF4 leucine zipper was, in fact, able to interact with the leucine zippers of each of the other GBF proteins to form heterodimers. Long versions of GBF1, -2, or -3 were incubated with the truncated GBF4 containing the bZIP domain for 30 min before addition of a radiolabeled G-box (5′-CACGTG-3′) fragment, and the reactions were then subjected to gel-mobility-shift analysis (Fig. 4). In this assay the formation of DNA–protein complex with an intermediate mobility indicates heterodimer formation (40). The results of the experiments with GBF2 and GBF3 demonstrated that a new protein–DNA complex of altered mobility was formed when these long proteins were incubated with the short GBF4 (Fig. 4, compare lanes 8 and 10; lanes 13 and 15). The absence of formation of the homodimeric GBF2 and GBF3 complexes in the presence of GBF4 may reflect different protein–protein affinities. Similar observations were made in our studies with GBF1, -2, and -3 homo- and heterodimers (11), and in the case of Fos and Jun, heterodimers are also preferentially formed (23). In contrast to GBF2 and GBF3, there was little, if any, formation of heterodimers between GBF4 and GBF1 (Fig. 4, compare lanes 3 and 5). The observations with GBF2 and GBF3 showed that although the leucine zipper of GBF4 could not form homodimers, it was capable of interacting with other proteins to form heterodimers. A deletion analysis of the carboxyl-terminal region has shown that the ability of GBF4 to heterodimerize with GBF2 and GBF3 requires only the first four

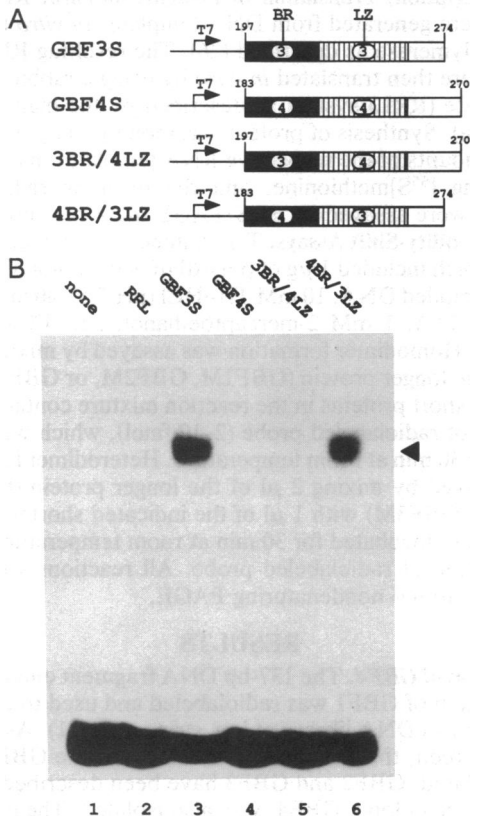


FIG. 3. "Domain-swap" experiments of GBF4 with GBF3. (*A*) DNA templates encoding the bZIP region of GBF4 or GBF3 or the indicated chimeric proteins were generated by recombinant PCR. These templates, each of which included the T7 promoter, were transcribed/translated *in vitro* by using a RRL system. BR, basic region; LZ, leucine zipper. (*B*) The resulting proteins were then subjected to gel-mobility-shift analysis by using a radiolabeled probe derived from the *Arabidopsis rbcS1A* promoter that contains the G-box (5′-gatcttatcttcCACGTGgcattattcg-3′). A DNA–protein complex is formed by using the truncated GBF3 (lane 3) but is absent when the truncated GBF4 protein is used (lane 4). In contrast, a fusion of the GBF3 basic region to the GBF4 leucine zipper failed to form a complex (3BR/4LZ; lane 5), where a fusion of the GBF4 basic region with the GBF3 leucine zipper formed a complex (4BR/3LZ; lane 6). Control lanes contained DNA probe only (lane 1) or DNA incubated with RRL alone (lane 2). DNA–protein complexes are marked by an arrowhead.

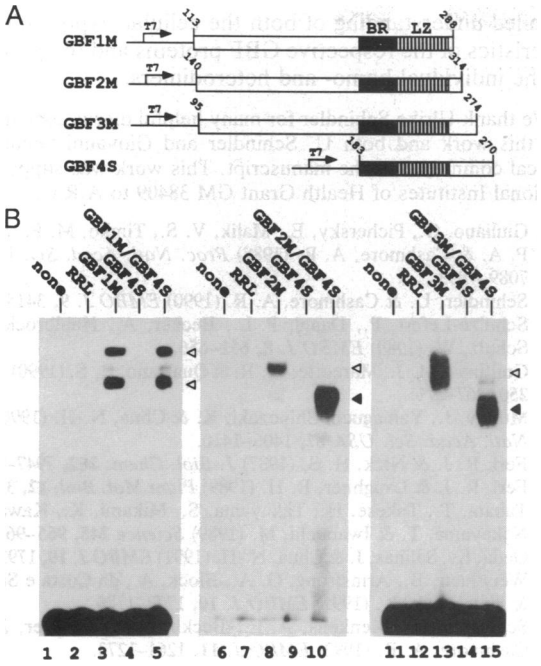


FIG. 4. GBF4 can interact with GBF2 and GBF3 to form heterodimers that recognize the G-box. (*A*) DNA templates were generated by PCR that encoded GBF1, GBF2, GBF3, and GBF4 proteins of the indicated lengths. These templates were then used for the *in vitro* transcription/translation of protein in a RRL system. BR, basic region; LZ, leucine zipper. (*B*) Homodimer formation was assayed by incubating GBF1, GBF2, and GBF3 proteins (lanes 3, 8, and 13, respectively) or the truncated GBF4 protein (lanes 4, 9, and 14) with radiolabeled G-box probe (5′-gatcttatcttcCACGTGgcattattcg-3′). In this experiment, two DNA–protein complexes were formed, by using the intact GBF1 template (lanes 3 and 5). This result is experimentally variable, with one or two GBF complexes being formed, and apparently reflects some variable of the RRL batch used. To assay for heterodimer formation, the longer protein was incubated with the shorter protein for 30 min before the addition of radiolabeled probe. Heterodimer formation, as detected by a DNA–protein complex of altered mobility, is apparent with GBF2 (lane 10) and GBF3 (lane 15) but is not apparent with GBF1 (lane 5). Control lanes contain radiolabeled DNA probe alone (lanes 1, 6, and 11) or 2 μl of rabbit reticulocyte lysate alone (lanes 2, 7, and 12). Homodimer protein–DNA complexes are indicated by open arrowheads (◁), where heterodimer protein DNA complexes are indicated by filled arrowheads (◀).

heptad repeats of the leucine zipper (Fig. 2, aa 30–57) immediately adjacent to the basic region (data not shown).

Mutation of a Single Amino Acid Within the Leucine Zipper Renders GBF4 Capable of Binding to DNA as a Homodimer. The Fos oncoprotein is a well-characterized case of a bZIP protein that is incapable of forming homodimers, yet in conjunction with the Jun protein forms heterodimers to produce the functional AP-1 protein. We examined closely the similarity of the leucine zipper of GBF4 with the leucine zipper of Fos and contrasted it with the leucine zippers of the other GBF proteins. It was apparent that the distribution of charged amino acids within the leucine zipper of GBF4 is distinct from that of GBF1, -2, and -3. GBF4 has only negatively charged glutamate residues at positions e and g, demonstrated as being involved in electrostatic interactions within the leucine zipper of other bZIP proteins, whereas the other GBF proteins have a distribution of positively and negatively charged amino acids at these positions (Fig. 5*A*; see Fig. 2 for comparison with GBF1, -2, and -3). Significantly, Fos is similar to GBF4 in that all charged amino acids at the e and g positions are glutamate residues (23).

We used recombinant PCR to mutagenize single amino acids at four different positions within the leucine zipper of

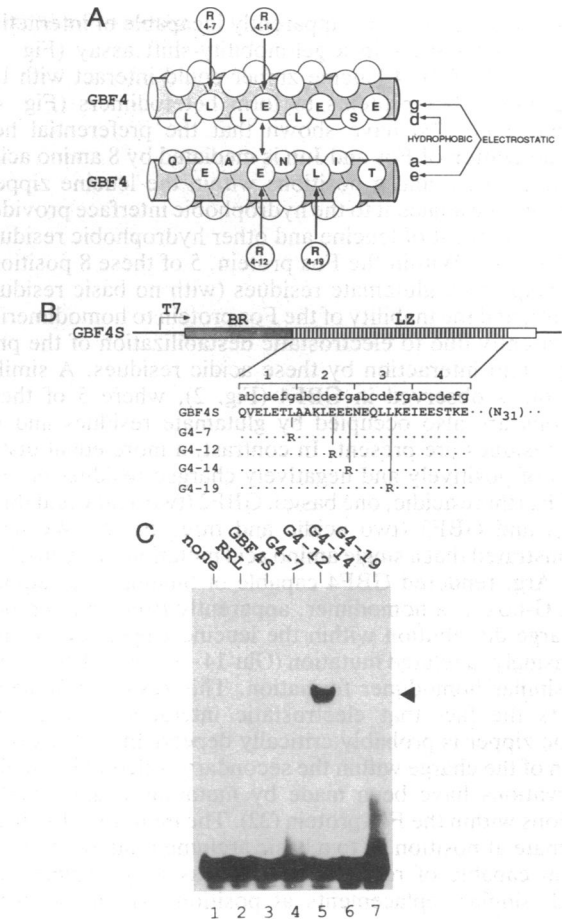


FIG. 5. Mutagenesis of the leucine zipper of GBF4. (*A*) DNA templates were generated by recombinant PCR and included the DNA sequence changes resulting in mutation of the indicated amino acids. Each template included the T7 promoter that was used to transcribe/translate the respective proteins *in vitro*. (*B*) In each reaction, the protein resulting from *in vitro* transcription/translation of the DNA template was added to radiolabeled G-box probe and subjected to gel-mobility-shift analysis. BR, basic region; LZ, leucine zipper. (*C*) The intact GBF4S (lane 3) failed to form a DNA–protein complex, as did G4-7, G4-14, and G4-19 (lanes 4, 6, and 7, respectively). A DNA–protein complex was formed using only the G4-12 protein (lane 5). Control lanes include radiolabeled probe alone (lane 1) or probe incubated with RRL alone (lane 2). DNA–protein complex formation is indicated by an arrowhead.

GBF4 to determine whether the charge distribution was responsible for destabilizing homodimer formation. The Glu-12 → Arg changed the negatively charged glutamate to the positively charged arginine allowed for the formation of a homodimer (Fig. 5*C*, lane 5). However, mutation of the glutamate residues at positions 7, 14, and 19 to arginine residues demonstrated no detectable homodimer formation (Fig. 5*C*, lanes 4, 6, and 7).

DISCUSSION

We have isolated and characterized GBF4, another bZIP protein that is a member of the *Arabidopsis thaliana* GBF family of transcription factors. Despite the fact that the amino acid sequence of this isolated GBF4 indicated the presence of a bZIP domain, the intact GBF4 protein was incapable of forming a DNA–protein complex with a radiolabeled G-box in gel-mobility-shift assays. Therefore, we sought to examine more closely the two subdomains of GBF4, the basic region and the leucine zipper.

Our experiments show that although the basic region of GBF4 was potentially capable of recognizing the G-box, the

GBF4 leucine zipper was apparently incapable of interacting to form homodimers in a gel-mobility-shift assay (Fig. 3). However, the GBF4 leucine zipper could interact with the GBF2 and GBF3 proteins to form heterodimers (Fig. 4). O'Shea *et al.* (23) have shown that the preferential heterodimerization of Fos and Jun is mediated by 8 amino acids that lie at the e and g positions within the leucine zipper. These sites lie adjacent to the hydrophobic interface provided by the 4–3 repeat of leucine and other hydrophobic residues (see Fig. 5*A*). Within the Fos protein, 5 of these 8 positions are occupied by glutamate residues (with no basic residues present), and the inability of the Fos protein to homodimerize is apparently due to electrostatic destabilization of the protein–protein interaction by these acidic residues. A similar situation is observed in GBF4 (Fig. 2), where 5 of the 8 positions are also occupied by glutamate residues and no basic residues are present. In contrast, a more equal distribution of positively and negatively charged residues is seen in GBF1 (three acidic, one basic), GBF2 (two acidic and three basic), and GBF3 (two acidic and three basic). We have demonstrated that a single amino acid mutation, the Glu-12 → basic Arg, rendered GBF4 capable of binding to a radiolabeled G-box as a homodimer, apparently from an alteration of charge distribution within the leucine zipper. Somewhat surprisingly, a related mutation (Glu-14 → Arg) did not result in a similar homodimer formation. This result presumably reflects the fact that electrostatic interaction within the leucine zipper is probably critically dependent on the distribution of the charge within the secondary coiled-coil. Similar observations have been made by mutating single e and g positions within the Fos protein (22). The mutation of a single glutamate at position 36 to a basic arginine rendered the Fos protein capable of recognizing DNA as a homodimer, although similar replacements at positions 41 and 43 were incapable of homodimer formation.

The domain swap experiments demonstrated that although GBF4 was incapable of homodimerizing, the basic region was capable of recognizing the G-box (Fig. 3, lane 6). Preliminary competition studies using numerous G-box and G-box-like elements have demonstrated that the binding specificity of the GBF4 basic region is quite similar to that of GBF1, -2, and -3 (data not shown). However, these studies need extension to include a detailed definition of the binding properties of the respective homo- and heterodimers in a manner similar to those reported for GBF1 (41). Furthermore, the prospect that DNA bending may be affected by heterodimer formation, as described for Fos/Jun heterodimers, also needs to be considered (42).

The demonstration that GBF4 does not bind to DNA as a homodimer and yet does interact with GBF2 and GBF3 to form heterodimers has potentially interesting implications for gene regulation involving G-box regulatory elements within the *Arabidopsis* plant. GBF4 possesses an acid-rich amino-terminal domain and lacks the proline-rich domain found in GBF1, -2, and -3. This proline-rich region of GBF1 has been demonstrated to have the characteristics of a transcriptional activation domain (41). From these observations it follows that the transcriptional activation properties of GBF2/GBF4 and GBF3/GBF4 heterodimers would be expected to differ from those of the corresponding GBF2 and GBF3 homodimers. It would not be unexpected for the plant cell to have acquired mechanisms to selectively regulate the formation of homo- and heterodimers. One component of this regulation would be the nature of the expression characteristics of the respective GBF proteins. In this context, it is of interest that GBF4 is strongly expressed in root tissue (data not shown), and thus GBF2/GBF4 and GBF3/GBF4 heterodimers might be expected to be the predominant GBF protein in this tissue. Further studies in this area require a detailed understanding of both the cellular expression characteristics of the respective GBF proteins and the properties of the individual homo- and heterodimers.

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