Plant Molecular Biology **38:** 539–549, 1998.

Overexpression of ΔEmBP, a truncated dominant negative version of the wheat G-box binding protein EmBP-1, alters vegetative development in transgenic tobacco

Nancy A. Eckardt[1], Lauren McHenry[2] and Mark J. Guiltinan[1,*]
[1]*Department of Horticulture and The Life Sciences Consortium, Penn State University, University Park , PA 16802, USA (*author for correspondence)* [2]*Loyola College, Biology Department, 4501 N. Charles St., Baltimore, MD 21210, USA*

Received 18 December 1997; accepted in revised form 13 April 1998

Key words: bZIP proteins, EmBP-1, G-box, transgenic tobacco

Abstract

As a first step toward elucidating the *in vivo* function of plant bZIP proteins and their related G-box *cis* elements, we have introduced a dominant negative inhibitor of G-box-dependent transcriptional activation into tobacco plants by transforming them with a truncated EmBP-1 gene (ΔEmBP) containing the DNA binding and dimerization domains under the control of the CaMV 35S promoter. Five independent lines of transgenic plants expressing ΔEmBP were identified, as demonstrated by immunodetection of the transgenic protein in leaf extracts, and the ability of the protein to bind a target G-box DNA sequence. The transgenic plants exhibited an abnormal phenotype characterized by interveinal chlorosis, growth inhibition and weakening of stems and petioles, the severity of which positively correlated with ΔEmBP expression and G-box DNA binding capability. Furthermore, development of chlorosis and growth inhibition was dependent on growth irradiance. Low light promoted the development of interveinal chlorosis and growth inhibition in the transgenic plants, whereas high light conditions led to near-complete amelioration of the abnormal phenotype. Transgenic plants under both light regimes showed signs of impaired stem and petiole function which was not observed in wild-type tobacco. *RbcS* gene expression was not significantly altered by ΔEmBP expression, suggesting that down-regulation of this gene was not responsible for the altered phenotype. The results suggest that G-box elements specific for the EmBP-1 class of bZIP proteins have an important developmental function in vegetative plant tissues, and that the *trans*-dominant negative mutant approach is a useful tool for continued *in vivo* functional analysis of bZIP transcription factors and their corresponding *cis* elements in plants.

Introduction

The G-box is a *cis*-acting DNA sequence (CACGTG) present in a number of plant promoters that are regulated by diverse developmental and environmental signals including light, abscisic acid (ABA), hypoxia, and a variety of other stresses [11]. Mutational analyses coupled with transient and stable gene expression assays have demonstrated a G-box requirement for optimal inducibility in many promoters which contain these elements [30]. G-box and related elements are specifically recognized by G-box binding factors (GBFs), which in most cases belong to the basic leucine zipper (bZIP) family of DNA binding proteins [20]. The bZIP proteins are characterized by a bipartite DNA binding domain consisting of a basic region, involved in sequence-specific binding to DNA, and a leucine repeat or zipper region, required for dimerization [5]. Transcriptional activation domains, often containing proline-rich or acidic regions, have been localized to regions both N- and C-terminal to the bZIP domain [21]. DNA binding specificity is complex, involving formation of heterodimers between related GBFs, the identity of nucleotides both within and im-

mediately flanking the ACGT core, and the presence of other nearby *cis* elements [5]. While *in vitro* studies have elucidated many of the details of bZIP structure and binding specificity, little is definitively known about the *in vivo* function and activity of most GBFs and their related G-box elements. A notable exception is the opaque2 (O2) protein, which is involved in regulation of zein gene expression [40].

The plant bZIP protein EmBP-1 was isolated from a wheat embryo cDNA expression library utilizing a double-stranded DNA probe, Em1a, present in the ABA responsive element (ABRE) of the wheat *Em* gene [15]. Methylation interference footprinting demonstrated that EmBP-1 interacts with an 8 bp sequence (5′-ACACGTGG-3′) which contains a G-box core at the center of the ABRE. A 2 bp mutation in this sequence prevented binding of EmBP-1 and reduced the ability of the ABRE to confer ABA inducibility on the CaMV 35S promoter [15]. Molecular-genetic mapping and cDNA cloning indicated that there are at least seven closely related genes in the wheat EmBP-1 family [6, 31], and two in maize [3]. In addition to Em1a, EmBP-1 binds with high affinity to a number of sites with G-box or G/C-box cores [31]. EmBP-1 binding to Em1a can also be competitively inhibited by various related oligonucleotides [6]. These include the Hex recognition site GGTGACGTGGC for the wheat transcription factor HBP-1 [39], and a related element in the promoter of an Arabidopsis gene encoding the small subunit of Rubisco (TCCACGTGGC) [22]. The ABA-regulated *Em* gene is expressed in response to rising ABA levels during late embryo maturation, and encodes a small hydrophilic protein believed to function in protecting the embryo from desiccation during dormancy [34]. However, EmBP-1 mRNA is present in wheat seeds throughout development, as well as in leaves and roots [34], suggesting that EmBP-1 might play a role in regulation of genes in vegetative as well as reproductive tissues.

Although EmBP-1 binds with high specificity to the ABRE *in vitro*, the multiplicity of related transcription factors and promoter elements make it difficult to ascertain the *in vivo* function of such factors. As a first step toward elucidating the *in vivo* function of G-box binding proteins and their related *cis* elements, we have introduced a dominant negative inhibitor of G-box-dependent transcriptional activation into tobacco plants by transforming them with a truncated EmBP-1 gene (ΔEmBP) containing the DNA binding and dimerization domains under the control of the CaMV 35S promoter. We produced several independent lines of transgenic plants that express ΔEmBP, and demonstrated the G-box-binding capability of the transgenic protein isolated from tobacco leaf tissue.

Materials and methods

Production of ΔEmBP transformants

Standard protocols were used for DNA isolation and manipulation [36]. The *Hin*dIII/*Sp*eI fragment of pMG99.23 [14], containing sequences specifying amino acids 410–419 of the human c-*Myc* gene [8], fused to EmBP-1 sequences from the *Eco*RI insert of λGC19 [15], was rendered blunt with T4 polymerase and subcloned into the similarly blunt-ended *Eco*RI site of the plant expression vector pMON881 (Monsanto, St. Louis, MO). The construct incorporates an ATG start codon directly preceding the c-*Myc* epitope tag. The fusion gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3′-untranslated region. For details on vector construction and availability, see [14].

Leaf disk transformation of *Nicotiana tabacum* cv. Petite Havana SR1 [25] was performed as previously described [18], and regenerated shoots were selected for their ability to root on MSO media (MS salts plus Gamborg's vitamins (Sigma Co., St. Louis, MO), 1% sucrose, 0.3% phytagel) containing 100 μg/ml kanamycin monosulfate (Sigma). Stable transformation was confirmed by segregation analysis of the *nptII* selectable marker in seed lots from self-fertilized primary transformants plated on germination medium (MS salts plus Gamborg's vitamins, 0.7% glucose, 0.3% sucrose, 0.3% phytagel) containing 100 μg/ml kanamycin monosulfate.

Protein extraction and analysis

Total soluble protein was extracted from tobacco leaf tissue in 100 mM Bicine pH 8.0, 1 mM EDTA, 1 mM PMSF, 10 μM leupeptin sulfate, separated by SDS-PAGE (BioRad, Hercules, CA), and transferred to Immobilon-P membrane (Amersham, Arlington Heights, IL) using a semi-dry electroblotter (Fisher Scientific, Pittsburgh, PA). Total SDS-soluble protein was extracted from tobacco seed, electrophoresed and blotted onto Immobilon-P as previously described [14]. Protein expression for co-segregation analysis was determined by tissue printing [4]. Leaves or roots of T_2 seedlings propagated on germination media were

rinsed with sterile water and blotted directly onto Immobilon-P.

Immunodetection was performed as previously described [14], utilizing as primary antibody the undiluted supernatant from cultures of a mouse cell line producing monoclonal antibody raised against the human c-*Myc* protein, Myc1-9E10 [8], available from the American Type Culture Collection (CRL 1729; Lineberger Cancer Research Facility, University of North Carolina, Chapel Hill, NC). Immunoblots of tissue prints and SDS-PAGE-separated seed proteins were incubated with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI) and developed with BCIP/NPT. Immunoblots of SDS-PAGE-separated foliar proteins were incubated with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody, developed with ECL reagents (Amersham), and exposed to Kodak-XAR film for 1–5 min.

Sequence-specific DNA binding capability of foliar proteins was determined following SDS-PAGE and transfer onto Immobilon-P (as described above) by hybridization with the ^{32}P-labeled DNA fragment GCCACGTGTC, which corresponds to the ABRE from the wheat Em promoter (probe 2 in [31]). Radionucleotide labeling, incubation and washing were carried out as described [15] and the blots exposed to Kodak XAR film for 24 h at −80 °C.

RNA gel blot analysis

Total RNA was isolated from leaves of SR1 and exp2 plants as described [28], fractionated on a 2.2 M formaldehyde, 1.2% (w/v) agarose gel, capillary-transferred onto Hybond-N membrane (Amersham), and fixed by exposure to 120 mJ UV light in a GS Gene Linker UV Chamber (BioRad). Membranes were prehybridized and hybridized at 65 °C in 10 ml 6× SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 100 μg/ml salmon sperm DNA, and 5× Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) PVP, 0.1% (w/v) BSA). Nucleic acid probes were labeled with [^{32}P]dCTP using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN), and purified with Sephadex G-50 spin columns [36]. Blots were stripped of the labeled *rbcS* probe by repeated washes in 0.1× SSC, 0.1% SDS at 72 °C, and hybridized with a ^{32}P-labeled tomato cDNA probe hybridizing to the 16S rRNA to account for minor RNA loading differences between samples. Relative quantity of *rbcS* and 26S rRNA was assessed with Phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). The probe for *rbcS* was the 2.5 kb *Hin*dIII insert of genomic clone pNTSS23 from tobacco [27]. The rRNA probe was the 400 bp *Eco*RI insert of ASCD-7D from petunia [19].

Growth analysis

T_2 seed was germinated in 32-cell flats in Metro-Mix 350 (Grace Sierra, Milpitas, CA). Seedlings were thinned to one per cell after 10–12 days, and transplanted to 1-liter pots after 3 weeks. Plants were watered as needed and fertilized twice weekly with 150 mg/l Peters 20-10-20 Peat Lite Special (W.R. Grace and Co., Fogelsville, PA). Plant height was determined weekly between 6 and 15 weeks after planting on six plants per line for transgenic lines exp2, exp3, exp4, and exp5 and six SR1 untransformed controls grown in a greenhouse without supplemental lighting during the months of October to January. The mean midday irradiance during this period was ca. 350 μmol photons m^{-2} s^{-1}, with an average day length of about 10 h.

Controlled-environment growth studies were carried out in a ECG growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) set at 24 °C 16 h day/19 °C night, 70% RH). High Light (HL, 600 μmol PAR) was provided with a combination of 160 W cool white fluorescent and 60 W incandescent bulbs, and shade cloth was used to provide half the chamber with Low Light (LL, 100 μmol PAR). T_2 seedlings of SR1 wild-type and transgenic line exp2 were germinated in 32-cell flats as described above, and allowed to grow under HL for 2 weeks, when plants were thinned and two plants per line were transferred to LL or maintained under HL. The seedlings were transplanted to 1-liter pots at 3 weeks after planting. Plants were kept well-watered and fertilized as described above. In some experiments, plants were transferred to LL after 5 weeks of growth at HL (see Results).

Photosynthesis and chlorophyll fluorescence measurements

Gas exchange measurements were conducted with a Licor 6200 closed-loop photosynthesis system (Licor, Lincoln, NE). In this system, mass balance equations for water and CO_2 are used to calculate net photosynthesis and stomatal conductance (Li-6200 Technical Reference, 1986). Measurements were conducted in the greenhouse under full sun (ca. 1200 μmol m^{-2} s^{-1}

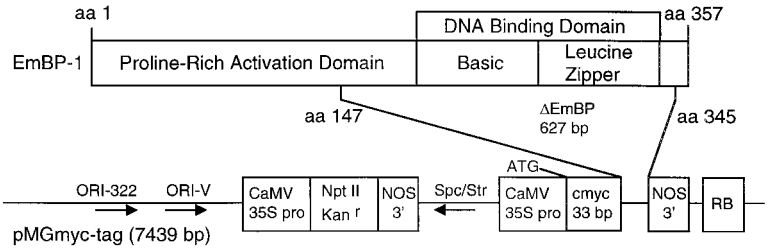


Figure 1. Construction of c-Myc tagged ΔEmBP plant expression vector pMGmyc-tag. Sequences encoding amino acids 410–419 of the human c-*Myc* gene preceded by an ATG start codon were fused to the 627 bp insert from lambda clone GC19 [14] containing sequences encoding the basic and leucine repeat regions of EmBP-1 (ΔEmBP). The ΔEmBP gene fusion was inserted into the Eco RI site of the plant expression vector pMON881 [13], which includes the nptII coding region for kanamycin resistance, placing it under the control of the CaMV 35S promoter and the nopaline synthase 3′-untranslated region.

PPFD inside the greenhouse), ambient RH 40–45%, and leaf temperature of $27.0 \pm 0.5\,^{\circ}C$.

Chlorophyll fluorescence was measured with a Walz PAM-101 chlorophyll fluorescence system (Walz, Effeltrich, Germany). Dark- and light-adapted fluorescence parameters were measured according to Genty *et al.* [12].

Results

Transgenic tobacco plants expressing ΔEmBP protein show an abnormal vegetative phenotype that cosegregates with ΔEmBP expression

A dominant negative mutant version of the wheat bZIP protein EmBP-1 was expressed in tobacco plants in an effort to evaluate its function *in vivo*. Figure 1 shows the construction of the fusion gene, made by placing a DNA fragment encoding the DNA binding and leucine zipper domains of EmBP-1 (ΔEmBP), under the transcriptional control of the CaMV 35S promoter. To allow for detection of the expressed protein in transgenic plant tissues, an epitope tag consisting of sequences specifying 10 amino acids of the human c-*Myc* gene [8], for which an antibody is available, was translationally fused to the N-terminus of ΔEmBP [14]. This construct was introduced into tobacco via *Agrobacterium*-mediated transformation, and resulted in the stable integration of the ΔEmBP gene into nine lines of plants derived from independent transformation events as demonstrated by genomic Southern analysis (data not shown) and by segregation of the kanamycin resistance marker gene in offspring produced by the self fertilization of the primary transformants.

Table 1 shows the correspondence of severity of the phenotype with ΔEmBP expression, and the segregation ratios for kanamycin resistance in the offspring from self-pollinated primary transformants. The phenotype of seedlings grown on agar was characterized by a stunted growth habit and chlorosis of primary leaves, and was distinct from kanamycin sensitivity. The approximate 3:1 (Kan^R:Kan^S) segregation ratio obtained for seven of the lines is consistent with a single genomic insertion of the t-DNA in those lines. Segregation of kanamycin resistance in lines exp3 and non4 indicates insertion of the t-DNA at two separate loci. The near 15:1 ration in line non4 indicates two unlinked insertions, while the 10:1 ratio in exp3 suggests that the loci may be linked on a single chromosome or that the presence of three or more copies of the ΔEmBP gene may be lethal in that line.

Figure 2 illustrates the correspondence of the transgenic phenotype with expression and DNA-binding activity of ΔEmBP, from experiments conducted with homozygous Kan^R T_2 progeny of lines exp2, exp3, and exp5. The phenotype of plants grown under ambient greenhouse conditions in January (maximum day length of 10 h and mean midday PPFD of ca. 350 μmol m^{-2} s^{-1}) corresponded to the level of ΔEmBP expression (Figure 2A–C). Immunoblot analysis showed that transformants from five of the lines (exp1-exp5) produced detectable amounts of a c-*Myc* reactive protein of the size expected of ΔEmBP (Figure 2C shows SR1 wild-type and three exp lines which were used in subsequent analyses; Table 1 shows protein expression characteristics of all transgenic lines). The c-*Myc* tag was not detected in untransformed SR1 plants nor in four lines of low or non-expressing ΔEmBP transgenics (lines non1-4, data not shown). The ΔEmBP protein was not detected in seed tissue of any of the transgenic lines, nor was there any evidence of a seed phenotype, such as precocious germination or ability to germinate in the presence of exogenously applied ABA (data not

Table 1. Summary of characteristics of ΔEmBP transformants of the T_1 generation [offspring resulting from self-pollination of primary (R_0) transformants].

1° transformant (R_0)				Marker gene segregation[2]	
line number	name	phenotype[1]	ΔEmBP expression[a]	Kan^R:Kan^S	progeny scored
30	exp1	++++	++++	2.8	325
4	exp2	+++	+++	2.7	188
13	exp3	++	++	10.0	265
35	exp4	++	++	3.4	449
20	exp5	+	+	2.8	451
31	non1	–	–	3.0	475
39	non2	–	–	2.5	494
44	non3	–	–	3.6	487
49	non4	–	–	13.8	488

[1] + indicates relative severity of phenotype/level of ΔEmBP expression. See text for description of phenotype. Expression of the ΔEmBP protein is demonstrated in Figure 2B.
[2] Kan^R:Kan^S indicates ratio of kanamycin-resistant to kanamycin-sensitive individuals.

shown). Figure 2D shows the DNA-binding activity of the ΔEmBP protein, demonstrated by transfer of SDS-PAGE-separated soluble leaf proteins to PVDF membrane and incubation of the membrane with a radio-labeled double-stranded DNA fragment containing the ABRE *cis* element originally used to isolate EmBP-1 from a wheat embryo cDNA library [15]. Line exp2 showed a high level of ΔEmBP protein expression and ΔEmBP DNA binding activity, which corresponded with development of severe chlorosis and dramatic reduction in shoot growth. Line exp5 showed the least amount ΔEmBP protein and DNA binding activity of the transgenic lines tested, and no signs of chlorosis or reduction in shoot growth relative to SR1 were evident in this line. Line exp3 showed intermediate characteristics of ΔEmBP protein expression, DNA binding activity, and expression of the ΔEmBP phenotype. Due to the severity of the abnormal phenotype, homozygous T_2 seeds were not obtained from transgenic exp1 plants. In control transformations with the parent Ti plasmid pMON881, a full-length EmBP-1 construct, or with a mutated version of ΔEmBP with alterations in the basic (DNA binding) domain, no transgenic plants were recovered with a ΔEmBP phenotype (data not shown).

Co-segregation of kanamycin resistance, ΔEmBP expression and expression of the phenotype was formally demonstrated for line exp1 to confirm that the observed phenotype was dependent on expression of the ΔEmBP protein. Line exp1 was used for this analysis because it produced a viable phenotype (leaf chlorosis) which could be scored only four weeks after germination on agar plates and it exhibited a single insertional locus for ΔEmBP (Table 1). After 4 weeks of growth on agar, seedlings were scored for the ΔEmBP phenotype and kanamycin resistance. Single leaves were assayed for the presence of ΔEmBP protein by tissue printing using the c-*Myc* antibody [14]. Other leaves were placed on kanamycin-containing media to test for kanamycin resistance. Of 100 exp1 seedlings from a self-cross of primary transformant 30, 27 were phenotypically normal, did not express the ΔEmBP protein, and were kanamycin-sensitive indicating that the ΔEmBP gene had been segregated out of approximately one quarter of the progeny. Another phenotypic class consisted of 49 individuals which were kanamycin-resistant, expressed ΔEmBP protein, and exhibited moderate chlorosis of cotyledons and primary leaves of reduced size relative to the first phenotypic class. The remaining 24 segregants exhibited severely chlorotic leaves and completely impaired shoot growth by 4 weeks. All seedlings in this class tested positive for the presence of ΔEmBP protein. The small amount of live tissue produced by the last group precluded testing them additionally for kanamycin resistance. However, the presence of ΔEmBP protein and phenotype distinct from kanamycin sensitivity suggested that this class was likely resistant to kanamycin. Furthermore, when seedlings from the same population were grown on kanamycin containing media, they segregated into three distinct phenotypic classes identical to those described above: ca. 25% clearly kanamycin-sensitive (white cotyledons, and no primary leaf pro-

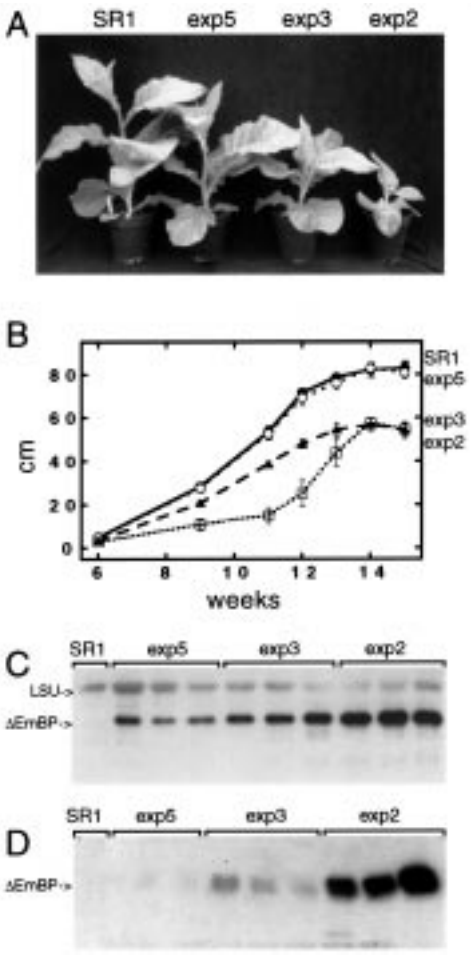


Figure 2. Severity of phenotype corresponds to expression and DNA-binding activity of ΔEmBP. A. SR1 Wt and homozygous T_2 transgenic ΔEmBP tobacco grown under natural greenhouse light in the winter, taken at ca. 9 weeks after planting. B. Height of plants from 6–14 weeks after planting, grown as in A. Values represent measurements taken on 6 plants per line per day ± SE. C. Immunoblot of SDS-PAGE-separated total soluble leaf proteins from 12-week old plants described in B, incubated with antibody to the c-*Myc* epitope tag, followed by horse-radish peroxidase-conjugated secondary antibody and development with ECL reagents (Amersham) and 1 min exposure to Kodak XAR film. Lanes contain 20 μg total soluble protein. Cross-reaction of antibody with the Rubisco large subunit (LSU) shows approximate equal loading of protein across lanes. D. Southwestern blot of SDS-PAGE-separated total soluble leaf proteins from 12-week old plants described in B, incubated with ^{32}P-labeled cDNA corresponding to the wheat *Em* promoter ABRE, showing G-box-specific binding activity of ΔEmBP.

duction) and 75% kanamycin-resistant, about 1/3 of which were severely stunted and chlorotic after four weeks while 2/3 showed moderate chlorosis. The absolute co-segregation of kanamycin resistance with the ΔEmBP protein and phenotype in 100 seedlings supports the conclusion that the observed phenotype is the result of ΔEmBP expression.

Figure 3 illustrates the phenotypic characteristics of ΔEmBP-expressing tobacco. The five lines of primary transformants (R_0) expressing ΔEmBP and their kanamycin resistant offspring (T_1) exhibited a phenotype characterized (to various degrees) by reduced shoot growth, pervasive chlorosis, and weakened stems and petioles. When the R_0 plants were grown in ambient greenhouse conditions of relatively low light in November–December (mean midday irradiance of about 400 μmol m^{-2} s^{-1} with maximum 10 h day length) lines exp1, exp2, and exp3 produced very little stem elongation, the leaves exhibited severe interveinal chlorosis and premature senescence, and floral abscission was near 100%. When shifted to a high humidity and high irradiance (600 μmol m^{-2} s^{-1}, 12 h/day) growth chamber, the younger leaves did not develop severe chlorosis, and the plants flowered and set seed. T_1 and T_2 progeny grown in the greenhouse under high natural irradiance (April to September or October) showed slightly reduced to near-normal stem elongation and infrequent signs of chlorosis; however, the leaves were frequently abnormally wrinkled and pitted, the stems were prone to cracking and splitting along the vertical axis, and petioles of older leaves usually cracked and broke prior to leaf senescence (Figure 3C-F). Inhibition of stem elongation (Figure 2A and B) and chlorosis (Figure 3A and B) occurred in greenhouse-grown plants during the short-day, low light winter months (November to March). Chlorosis (identical to that in Figure 3A and B) often appeared on leaves of spring or summer greenhouse-grown plants following several consecutive days of overcast skies.

The phenotype of ΔEmBP-expressing tobacco is affected by growth irradiance

Greenhouse growth studies suggested that irradiance during growth was a significant factor in the development and severity of the ΔEmBP phenotype. This hypothesis was tested by growing SR1 wild-type and transformed line exp2 in a controlled environment chamber under HL (600 μmol m^{-2} s^{-1}PPFD) or LL (100 μmol m^{-2} s^{-1} PPFD). In HL, exp2 plants were

Figure 3. Phenotypic details of ΔEmBP-expressing transgenic tobacco. Photographs are of homozygous T_2 transgenic line exp2 plants. A and B. Interveinal chlorosis. C. Extensive wrinkling of leaves. D. Petiole breakage on older leaves. E and F. Stem-splitting. G. Plants grown under HL (600 μmol m^{-2} s^{-1} PPFD) for 7 weeks. H. Plants grown under LL (100 μmol m^{-2} s^{-1} PPFD) for 7 weeks. I. Plants grown for 5 weeks under HL followed by 12 days under LL. In G. to I, SR1 wild-type plant is on the left and exp2 plant on the right.

nearly identical to the SR1 throughout 9–10 weeks development (Figure 3G). Leaves of Exp2 plants were slightly more wrinkled than SR1 (this can be noted by careful observation of Figure 3G), and the older leaves exhibited a marked tendency for petiole breakage (as in Figure 2D). Petiole breakage was not routinely observed in any of the SR1 plants. Under LL conditions, exp2 plants produced severely chlorotic leaves, and stem elongation was dramatically inhibited (Figure 3H). This experiment was conducted three times using three plants of each line, with identical results. Immunoblot analysis confirmed that expression of the ΔEmBP protein was unaffected by irradiance (data not shown). Two experiments also included hemizygous T_2 seedlings from line exp1 with two ΔEmBP-expressing siblings and two non-expressing normal (Kan^S)siblings. The normal siblings were virtually identical to the SR1 wild type under both HL and LL (data not shown). Transgenic exp1 plants that expressed ΔEmBP gave results identical to line exp2: exp1 plants grown in HL were almost indistinguishable from their normal siblings and SR1 plants, whereas transgenic plants grown in LL developed severe chlorosis and growth inhibition (not shown).

Based on the light-dependent and leaf-specific phenotype of the ΔEmBP transgenic plants, we hypothesized that the plants were deficient in some aspect of photosynthesis. Net photosynthesis and chlorophyll fluorescence were measured during an experiment in which SR1 and exp2 plants were transferred to LL after 5 weeks of growth under HL. Measurements of plant height, net photosynthesis and chlorophyll fluorescence were made 1, 6, and 12 days after the transfer to LL. Chlorotic lesions began to appear by 4–5 days following the transfer, and by 12 days the exp2 plants were showing severe chlorosis and inhibition of stem elongation (Figure 3I). As shown in Figure 4, there were no significant differences in plant height, net photosynthesis, or chlorophyll fluorescence between line exp2 and SR1 plants in HL.

After the shift to LL, chlorotic lesions were evident after 4 days. However, 6 days after the shift, only net photosynthesis was significantly lower in line exp2 compared to SR1. Plant height, net photosynthesis, and chlorophyll fluorescence parameters Fv/Fm and ø (quantum yield) were all significantly reduced in line exp2 by 12 days after the shift. Light-adapted qQ and qN (reflecting photochemical and nonphotochemical fluorescence quenching, respectively) were not significantly different between exp2 and SR1, even up to 12 days following the shift to LL (data not shown).

Rubisco small subunit genes (*rbcS*) contain G-boxes in their promoter regions which are likely involved in the light induction characteristic of this gene [7]. We measured the steady-state levels of *rbcS* mRNA to investigate the possibility that the decline in photosynthesis and development of chlorosis in the ΔEmBP transgenic plants result from down regulation of *rbcS*. Repeated northern analysis of total leaf mRNA showed no significant difference in steady-state *rbcS* mRNA content between SR1 and transgenic line exp2 when grown in HL or 4 days after the shift from HL to LL. Figure 5 shows an autoradiograph representative of these results.

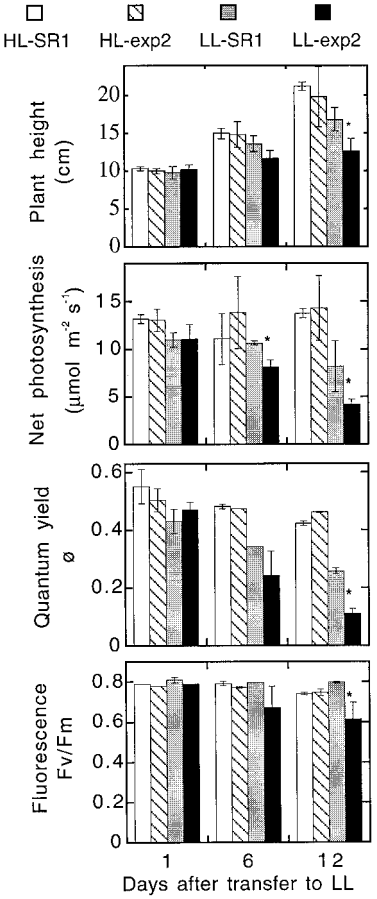
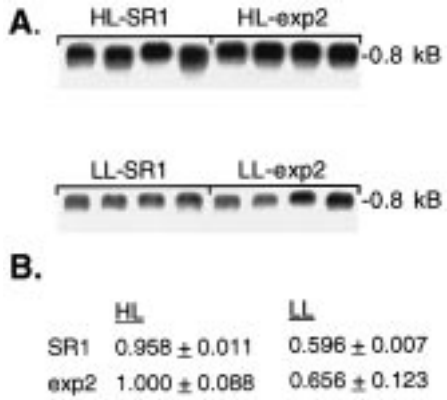


Figure 4. Plant growth, photosynthesis and chlorophyll fluorescence in plants after a switch from HL to LL. SR1 and transgenic line exp2 plants were grown for 5 weeks under HL and then placed under LL or maintained under HL. Day 1 corresponds to the first day under LL. Values in all figures represent measurements taken on 2 plants per treatment per line ± SD ($n - 1$). A. Plant height. B. Net photosynthesis. C. Quantum yield (from chlorophyll fluorescence). D. Fluorescence parameter Fv/Fm. *indicates a value significantly lower than the SR1 value of the same treatment at $p \leq 0.05$.

Discussion

Experimental approach

The characterization of a growing number of plant bZIP proteins, combined with the ease of whole plant transformation, provides an excellent opportunity to test models of bZIP function that have been derived largely from *in vitro* analyses. Introduction of a *trans*-dominant negative inhibitor of GBF activity is one approach to *in vivo* analysis of GBF and related G-box function. We have established transgenic tobacco expressing a derivative of EmBP-1 (ΔEmBP) lacking the transcriptional activation domain to test the dominant negative mutant strategy for analyzing G-box-dependent gene regulation *in vivo.* The use of the heterologous wheat-tobacco system for the present study was supported by previous experiments showing that the wheat *Em* promoter is capable of inducing high levels of tissue-specific expression of the β-glucuronidase reporter gene (GUS) in transgenic tobacco seed [26]. Insertion of an epitope tag consisting of a 10 amino acid segment of human c-*Myc* protein allowed detection the ΔEmBP protein in transgenic plant tissue (described in detail in [14]). We reasoned that overexpression of ΔEmBP would repress genes which require a G-box for expression. Trans-dominant inhibition could result from one or more of several possible mechanisms, including formation of inactive heterodimers of ΔEmBP with endogenous factors and competitive inhibition of endogenous EmBP-class factors by ΔEmBP homodimers. Similar strategies have also been used in yeast and mammalian cell cultures to investigate the *in vivo* functional role of other bZIP transcription factors. In yeast [17] and mammalian cell culture [24] cells expressing truncated bZIP genes, encoding only the DNA binding and dimerization do-



	HL	LL
SR1	0.958 ± 0.011	0.596 ± 0.007
exp2	1.000 ± 0.088	0.656 ± 0.123

Figure 5. Quantitation of steady-state *rbcS* mRNA in transgenic tobacco grown under high light (HL) or grown under high light for 5 weeks and shifted to low light for 4 days (LL). A. Autoradiograph of RNA gel blot hybridized with ^{32}P-labeled probes for *rbcS*. Each lane contains 8 μg total RNA from SR1 wild-type or line exp2 transgenic leaf tissue. B. Relative quantities of *rbcS* mRNA from Phosphorimage analysis shown in A, corrected for minor differences in loading by calibration with a rRNA probe (see Materials and methods).

mains, failed to activate transcription of promoters containing target binding sites.

Rieping *et al.* [35] utilized the dominant negative mutant strategy to investigate the role of the TGA1a/PG13 family of bZIP proteins in transgenic tobacco expressing a mutant of potato PG13 which lacked the DNA binding domain. In this case, expression of the mutant protein resulted in titration of TGA1a/PG13 activity as wild-type and mutant proteins formed heterodimers incapable of binding to wild-type target DNA sequences [35]. Olive *et al.* [32] showed that mutant bZIP proteins which lack the DNA binding domain are more effective at reducing transcriptional activation from target promoters than mutants with truncated or altered transactivation domains and intact DNA-binding domains. It is possible that we avoided an extremely severe or lethal phenotype by employing the latter strategy, since the promoters of numerous genes, some critical for plant growth and development, contain G-boxes recognized by EmBP-1 *in vitro*. Indeed, we generated lines of tobacco with phenotypes ranging from none to homozygous lethal, and the phenotype positively correlated with ΔEmBP expression level.

The observation of a distinct vegetative phenotype in ΔEmBP transgenic tobacco suggests an important role for G-box elements specific for the EmBP-1 class of bZIP proteins in the development of vegetative tissues. We cannot rule out the possibility that ΔEmBP expressed at high levels in tobacco tissues may result in binding to promoters which do not normally bind enodgenous EmBP-1 or related GBFs, and/or the physiological effects of ΔEmBP could be an artifact of overexpressing a heterologous transcription factor. However, the lack of an observable phenotype in transgenic tobacco containing a variety of other similar constructs under the control of the CaMV 35S promoter, such as a full-length EmBP-1 construct, the parent pMON881 plasmid alone, and a mutated version of ΔEmBP with alterations in the DNA binding domain, support the hypothesis that the ΔEmBP phenotype is a specific G-box-related effect.

Transgenic tobacco expressing ΔEmBP show a distinctive vegetative phenotype that is affected by irradiance during growth

The phenotype of ΔEmBP transgenic tobacco was markedly affected by irradiance during growth. Transgenic plants grown or transferred to low light developed interveinal chlorosis and showed significant reductions in photosynthesis and growth relative to wild-type, whereas plants grown in high light were almost indistinguishable from wild-type. Analyses of ΔEmBP line exp2 showed that photosynthesis and chlorophyll fluorescence were identical to wild-type in high light. When plants were transferred from high to low light, net photosynthesis in line exp2 declined significantly below SR1 within 6 days. The chlorophyll fluorescence quenching parameters qQ and qN were not significantly affected in line exp2 relative to SR1 wild-type, while Fv/Fm and quantum efficiency from chlorophyll fluorescence were significantly lower than SR1 only after 12 days. The chlorophyll fluorescence parameters reflect fluorescence quenching and efficiency of photon capture associated mainly with PSII [12]. The results suggest that genes associated with light harvesting reactions of photosynthesis were only secondarily affected and were not part of the primary lesion affecting the phenotype of the plants.

We hypothesized that ΔEmBP expression would interfere with the expression of genes which contain a G-box in their promoter regions and are induced by various environmental signals including light. G-box elements have been found in the promoters of a number of light-responsive genes, such as the Rubisco small subunit (*rbcS*) [7], the chlorophyll binding protein (*cab*) [16], and Rubisco activase (*rca*) [33]. The *rbcS* G-box element is known to interact with class I bZIP proteins (e.g. *Arabidopsis* GBF-1, -2, and -3)

[13], and exhibits *in vitro* binding activity with EmBP-1[22]. If EmBP-1 (or closely related GBF) were a transcriptional activator of genes controlling Rubisco activity, such as *rbcS* and *rca*, we might expect these genes to be down-regulated in ΔEmBP transgenic tobacco. ΔEmBP-expressing tobacco plants exhibited a distinct phenotype related to light intensity during growth, but the response of the light-inducible photosynthetic gene *rbcS* was not significantly altered relative to wild-type tobacco in HL or 4 days following transfer from HL to LL, even though the leaves sampled in LL were beginning to show signs of chlorosis. We concluded that *rbcS* expression was unaffected in the transgenic plants, and the development of chlorosis was not the result of Rubisco down-regulation.

The data may suggest that EmBP-1 is not a transcriptional regulator of *rbcS in vivo*, despite its *in vitro* binding capability. In some cases *in vitro* binding capability of transcription factors may not correlate with *in vivo* activity. For example, McKendree *et al.* [29] found evidence of *in vitro* binding activity associated with the *alcohol dehydrogenase* (*Adh*) promoter in *Arabidopsis* leaf extracts, but no comparable *in vivo* binding to the Adh B-box was observed in leaves. There is increasing evidence of cooperation between factors binding to G-box elements and factors binding to nearby unrelated elements, and it may be that ΔEmBP is impaired in its ability to form multifactor complexes. G-box elements appear to regulate the inducible expression of numerous genes in concert with other critical *cis* elements, or coupling elements, positioned nearby [7, 9, 37, 38]. The sequence of the second *cis* element is not conserved among different inducible promoters, but may be similar among promoters induced by the same signal [5]. The association of such multifactor complexes may serve to discriminate between different G-box (or ACGT-core) elements *in vivo*. For example, Kuras *et al.* [23] presented evidence of a functional relationship between the bHLH protein Cbf1 and the two bZIP factors Met4 and Met28 in yeast. Feldbrügge *et al.* [10] found that bZIP proteins may function synergistically with specific myb-like DNA binding proteins during light-mediated activation of parsley *CHS* gene expression. Cooperation between bZIP and myb proteins has also been demonstrated for the *mim*-1 promoter in animals [2]. Thus bZIP proteins may require regions outside the DNA binding and dimerization domains to form multifactor complexes for binding to subsets of gene promoters (such as light-regulated genes), and binding of endogenous EmBP-1-like proteins to these elements may not be inhibited by ΔEmBP.

It is possible that a primary lesion affecting translocation or partitioning of photosynthate would have a negative feedback effect on photosynthesis, ultimately leading to chlorosis and a reduction in growth such as that observed in the ΔEmBP transgenic plants, but this hypothesis remains to be tested. The observations of petiole weakness and stem-splitting in ΔEmBP transgenic plants are supportive of this hypothesis. It is interesting to note that these structural weaknesses were observed in HL- as well as LL-grown plants. Apparently, some aspects of the ΔEmBP phenotype are light-dependent, while others (e.g. stem splitting and petiole weakness) are not. This is consistent with the known diversity of G-box function. Although we have not yet identified the genes primarily affected by ΔEmBP expression, the distinct nature of the ΔEmBP phenotype suggests that these plants will provide a valuable tool for elucidating the *in vivo* function of the affected G-box elements using a combined physiological-molecular analysis. Further molecular and physiological analysis of ΔEmBP-expressing transgenic tobacco may provide valuable insight into the complex interactions controlling these regulatory mechanisms.

Acknowledgements

We thank Steven Crone for technical support. This work was supported in part by grants to M.J.G. from the NSF (MCB 920 6095) and from Penn State University College of Agricultural Sciences (CRIS 3278).

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