Redox-mediated mechanisms regulate DNA-binding activity of the G-group of bZIP transcription factors in Arabidopsis

Jehad Shaikhali1, Louise Norén1, Juan de Dios Barajas-López1, Vaibhav Srivastava1,2, Janine König3, Uwe H. Sauer4, Gunnar Wingsle2, Karl-Josef Dietz3, Åsa Strand1

From the 1Department of Plant Physiology and Umeå Plant Science Centre, Umeå University, SE-901 87 Umeå, Sweden  
2Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, SE-901 87 Umeå, Sweden  
3Biochemistry and Physiology of Plants, Faculty of Biology, W5, Bielefeld University, 33501 Bielefeld, Germany  
4Department of Chemistry, Center of Chemical Biology and Computational Life Science Cluster Umeå University, SE-901 87 Umeå, Sweden

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To whom corresponding should be addressed: Åsa Strand, Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden, Tel: +46 (0)90 786 9314; Fax: +46 (0)90 786 66 76; Email: Asa.Strand@plantphys.umu.se. 
‡Current address: Division of Glycoscience, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Centre, SE-10691 Stockholm, Sweden

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Background: The Gbox cis-element is enriched in promoters of genes responding to light and to high light. 
Results: DTT induces DNA-binding activity of bZIP transcription factors by reducing a disulfide bond. 
Conclusion: Redox regulation is crucial for DNA-binding of the G-group of Arabidopsis bZIP transcription factors. 
Significance: Redox-dependent mechanisms modulate the activity of plant bZIPs in response to environmental signals. 

SUMMARY 

Plant genes which contain the Gbox in their promoters are responsive to a variety of environmental stimuli. Bioinformatic analysis of transcriptome data revealed that the Gbox element is significantly enriched in promoters of high light (HL)-responsive genes. From nuclear extracts of HL-treated Arabidopsis plants we identified the AtbZIP16 transcription factor as a component binding to the Gbox containing promoter fragment of LIGHT-HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN2.4 (LHCBI2.4). AtbZIP16 belongs to the G-group of Arabidopsis basic region leucine zipper (bZIP) type transcription factors. While AtbZIP16 and its close homologues AtbZIP68 and AtGBF1 bind the Gbox, they do not bind the mutated half sites of the Gbox palindrome. In addition, AtbZIP16 interacts with AtbZIP68 and AtGBF1 in the yeast two hybrid system. A conserved Cys residue was shown to be necessary for redox
regulation and enhancement of DNA-binding activity in all three proteins. Furthermore, transgenic Arabidopsis lines over-expressing the wild type version of bZIP16 and T-DNA insertion mutants for bZIP68 and GBF1 demonstrated impaired regulation of LHC2B2.4 expression. Finally, over expression lines for the mutated Cys variant of bZIP16 provided support for the biological significance of Cys330 in redox regulation of gene expression. Thus, our results suggest that environmentally induced changes in redox state regulate the activity of members of the G-group of bZIP transcription factors.

Light is not only the primary energy source for plants but it also provides them with information to modulate a wide range of developmental processes (1,2). In response to light, large reorganization of the transcriptional program is initiated to modulate plant growth and development (2). However, sometimes photon fluence exceeds the photon utilization capacity of the chloroplast. Absorption of excess light leads to redox changes in the chloroplast and production of reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$) and to inactivation of photosynthesis (3). These ROS are generated as the consequence of an overreduced electron transport system and consecutive transfer of electrons to oxygen (4). Accumulation of ROS causes damage to structural proteins, lipids, DNA and to enzymes important for the function of the chloroplasts (4,5). To protect themselves against extensive damage, plants have the ability to sense when photon fluence exceeds the photon utilization capacity of the chloroplast and communicate this information to stimulate changes in nuclear and chloroplast gene expression. The redox state of the plastoquinone pool (PQ) (6,7), the acceptor availability at photosystem I (8,9) and accumulation of specific ROS (10) are proposed as possible sources for plastid signals involved in communicating the redox changes in the chloroplast to the nucleus (11).

Redox reactions are basic to all cellular processes and the redox environment of the cell controls the activity of numerous metabolic processes by regulating protein function. The buffering capacity of the cellular redox environment is therefore very critical, and as a result different mechanisms are used to balance the redox state e.g. by the action of redox pairs such as glutathione, ferredoxin and thioredoxin, antioxidant systems such as superoxide dismutase, ascorbate peroxidase and peroxiredoxin and secondary metabolites such as flavonoids, alkaloids, and carotenoids (12). DNA binding activity of transcription factors has also been shown to be altered by redox-dependent posttranslational modifications. The plant R2R3 MYB family transcription factor is regulated in a redox-dependent manner. Using the P1 regulator of maize flavonoid biosynthesis, it was shown that two cysteines of the R2R3 domain, Cys-49 and Cys-53, are essential for DNA binding. Thus, under non-reducing conditions, both cysteines form a disulfide bond that prevents the R2R3 MYB domain from binding to DNA (13,14). DNA-binding activity of another MYB family transcription factor, AtMYB2 which lacks the first Cys residue, is controlled by an alternative mechanism that involves cysteine S-nitrosylation (15). Thus, both oxidation and S-nitrosylation negatively influence DNA-binding activity of the MYB family transcription factors described (14,15). Another example of redox regulation of transcription factors is a
group of plant homeodomain proteins, HAHR1 and HAHB10, that contain a set of conserved Cys residues. In the oxidized state, the homeodomain transcription factors form intermolecular disulfide bonds resulting in inefficient DNA binding activity. However, under reducing conditions the DNA binding activity of these proteins is strongly enhanced (16). Also the localization of transcription factors can be influenced by redox modifications as was shown for NPR1. The NPR1 monomers are translocated at a low rate to the nucleus preventing activation of the target genes in the absence of inducing stimulus (17). Whereas following a pathogen attack, salicylic acid-mediated redox changes lead to translocation of NPR1 to the nucleus where it interacts with several transcription factors (18) such as TGA1 and activates its DNA binding (19). Rap2.4a was the first plant transcription factor shown to be directly regulated by the redox status of the nucleus (20). Rap2.4a is an AP2-domain-containing transcription factor involved in the expression of the chloroplast 2-Cys peroxiredoxin A and the activity of the Rap2.4a transcription factor was demonstrated to be controlled both by reducing and oxidizing conditions (20). The homodimeric structure of Rap2.4a stabilized by an intermolecular disulfide bond is the active form necessary for DNA-binding. Oxidation of the dimer by H2O2 or reduction by DTT strongly reduces its DNA-binding affinity. Rap2.4a was proposed to act as a redox sensor and transducer (20).

Our aim was to isolate nuclear components responding to redox changes in the chloroplast following exposure to high light. Using a biochemical approach we identified the AtbZIP16 protein, a member of the G-group of bZIP transcription factors from Arabidopsis thaliana as a protein binding to the Gbox-containing LHCb2.4 promoter fragment. DTT induces DNA-binding activity of AtbZIP16 and two other members of the G-group bZIP transcription factors, bZIP68 and GFB1 by reducing a disulfide bond formed through a conserved cysteine. Transgenic Arabidopsis lines over-expressing the wild type version of bZIP16 and T-DNA insertion mutants for bZIP68 and GFB1 demonstrated impaired regulation of LHCb2.4 expression in response to light. Transgenic lines over-expressing the mutated Cys variant of bZIP16 supported a biological significance for the conserved Cys residue in redox regulation of gene expression. Thus, we propose that a redox-dependent mechanism is necessary to modulate the activity of these transcription factors in response to environmental signals.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions—Seeds of Arabidopsis thaliana Col-0 wild type were grown on soil at 23°C (16 h light 100 µmol quanta m⁻² s⁻¹) and 18 °C (8 h dark) at 60% relative humidity. For high light treatment for nuclear preparations, 4-week-old plants were subjected to 3 h 1000 µmol quanta m⁻² s⁻¹ (metal halide HQI-T 400 W day light bulbs, Orsam). Seedlings for hypocotyl measurements and LHCB2.4 gene expression were grown on MS-plates without sucrose. The plates were vernalized for 24h in 4°C and darkness, placed in 150 µmol m⁻², s⁻¹ constant white light for 12h to induce germination and then dark-adapted for 24h prior the 5 days growth in 10 µmol m⁻², s⁻¹ constant white light. Seeds were obtained from TAIR for bzip68 (Salk_147015) and gfb1 (Salk_144534) T-DNA insertion lines (21). Homozygous plants were checked for transcript levels using primers for actin: Act2.1: 5’-GGAAGGATCTGTACGGTAC-3’ and Act2.2: 5’-TGTGAACGATTCCCTGGACCT-3’,
bZIP68  F:  5'-CACCATTGGTAGCAGTGAGATG-3' and bZIP68  R:  5'-CTACGCAACATCCTGACGTGTA-3',
GBF1  F:  5'-CACCATGGGAACGAGCGAAGACAAG-3' and G BF1  R:  5'-TTAAATTTGTCTCCTCACCAC-3'.

Nuclear protein extraction and DNA-affinity trapping of DNA-binding proteins– Using CelLytic™ PN-Plant nuclei isolation/extraction kit (Sigma), control and 3 h high light-exposed leaves of 4-week-old Arabidopsis plants were used for nuclear protein extraction. The tissue was ground in liquid nitrogen and resuspended in nuclei extraction buffer. The pellets were collected by centrifugation after the tissue mix was filtered through nylon net. To solubilize lipid membrane, Triton X-100 was added at 0.3% final concentration and protein extraction buffer was used to extract nuclear proteins from crude nuclei. The promoter region –1530 to –1674, containing G-box, of LHCB2.4 was used as a probe to trap DNA-binding proteins. To amplify a biotinylated DNA promoter fragment by PCR, Gbox-F (biotin-5' - CTTATTGTCGAGGATGGTCT-3') and Gbox-R (5'-AGATTCACGTGCCTGAGATA-3') primers were used. DNA-affinity trapping of DNA-binding proteins was performed as described previously (22). 2 mg beads (Dynabeads® Streptavidin; Invitrogen) were used for DNA immobilization in 2× binding buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA, 2 M NaCl). After the beads were resuspended in protein binding buffer (20 mM Tris HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 1 M NaCl, 0.05% Triton X-100, 1 mM DTT) and mixed with Arabidopsis nuclear protein extracts, DNA-protein binding was allowed by 15 minutes incubation at 25°C. At least three washes with protein binding buffer were performed on the Dynabeads to eliminate unspecific protein binding. DNA-binding proteins were collected in elution buffer (20 mM Tris HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 1 M NaCl, 0.05% Triton X-100, 1 mM DTT).

Mass Spectrometry– Proteins were incubated, in presence of 0.1 M NH₄HCO₃ and 10 mM DTT, at 95°C for 15 min, then cooled to room temperature before they were mixed with 8 M urea and incubated for 1 h. After addition of 55 mM of iodoacetamide, alkylation reaction was performed at 37°C for 30 min in the dark. Urea concentration was reduced to 0.8 M with 50mM NH₄HCO₃ and tryptic digestion (enzyme-to-substrate ratio, 1:50) was performed overnight at 37°C. The resulting peptides were lyophilized, re-suspended in 1% TFA and desalted by using a Poros 50 reverse-phase R2 microcolumn (PerSeptive Biosystems). Reversed-phase ultra-performance liquid chromatography using a nanoACQUITY UPLC™ system (Waters, Milford, MA, USA) was used to separate the desalted tryptic peptides prior to MS analysis. A C18 trap column (Symmetry 180 μm x 20 mm 5 μm; Waters, Milford, MA, USA) was used to concentrate each sample (peptides) and then washed with 2% acetonitrile, 0.1% formic acid at 15 μl/min for 2 min. After elution, samples were separated on a C18 analytical column (Symmetry 180 μm x 100 mm 1.7 μm; Waters, Milford, MA, USA) at 350 nl/min using 0.1% formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B, in a gradient. The gradients used were: linear from 0 to 40 % B in 25 min, linear from 40 to 80 % B in 1 min, isocratic at 80 % B in 1 min, linear from 80 to 5% B in 1 min and isocratic at 5% B for 7 min. The eluting peptides were sprayed into the mass spectrometer (Q-Tof Ultima™; Waters, Milford, MA, USA) with the capillary voltage set to 2.6 kV and cone voltage to 40 V. The
instrument was operated in data-dependent mode as described (23) without any further changes. For database searching, raw data was converted into peak lists using Protein Lynx Global Server software (V2.2.5). For identification of proteins, a local version of Mascot search program was used (V2.1.04, Matrix Science Limited, http://www.matrixscience.com), using Arabidopsis protein database from The Arabidopsis Information Resource (TAIR version 9.0; July 19, 2009; 33,410 sequences). Settings used for the database search were trypsin-specific digestion with two missed cleavage allowed, carbamidomethylated cysteine set as fixed modification, oxidized methionine in variable mode, peptide tolerance of 80 ppm and fragment tolerance of 0.1 Da. Peptides with Mascot ion scores exceeding the threshold for statistical significance of p<0.05 were selected and also reprocessed manually to validate their significance.

Site-directed mutagenesis of Cys residues of AtbZIP16, AtbZIP68 and GBF1 proteins– Site-directed mutagenesis was performed through three PCR steps as described previously in Montemartini et al. (24). To generate the different mutants two PCR products that overlap in the sequence containing the same mutation were synthesized in two separate PCRs. In the first PCR a mutation in the reverse primer was used and in the second PCR the same mutation was included in the forward primer. The two PCR products were separated on agarose gel and the DNA was excised and purified with the gel extraction kit (EZNA). The purified DNA (PCR1 and PCR2) served as template for a third PCR performed with the forward and reverse primers used to amplify wild type proteins (Table 1). The presence of the desired mutations was confirmed by sequencing the entire genes.

Cloning, expression and purification of recombinant proteins– The PCR products of AtbZIP16-wt, AtbZIP16-C1, AtbZIP16-C2, AtbZIP16-C1C2, GBF1-wt, GBF1-C1 and GBF1-C2 were cloned into the pET100D TOPO vector according to the manufacturer’s instructions (Invitrogen). PCR-amplified cDNAs of AtbZIP68-wt, AtbZIP68-C1 and AtbZIP68-C2 were cloned into BamHI/XhoI sites of pET32a (+) vector (Novagen). The resulting plasmids were transformed into E. coli BL21 (DE3) star. After 5-16 h induction with 2 mM IPTG expressed proteins were affinity purified on Ni²⁺-NTA agarose resin (Qiagen).

Electrophoretic mobility shift assays– The promoter region –1530 to –1674 upstream of ATG start codon of LHCb2.4 gene was PCR amplified to generate 144 bp fragment containing G-box using the primers (Gbox-F: 5‘CTATTGTCGAGGATGGTCT-3’; Gbox-R: 5‘AGATTCACGTGCCCTGAGATA-3’). To generate Gbox cis-element and its mutant variants, forward primers namely Gboxcis-F (5‘TCAACTGACACGTGGCATAGATAAC-3’), GboxcisM1-F (5‘TCAACTGACACGTGGCATAGATAAC-3’) and Gboxcis-M2-F (5‘TCAACTGACACACACAGGATAAC-3’) were annealed with their complementary oligonucleotides at room temperature after they were incubated at 70°C for 5 min. DNA probes were labeled with biotin-14-dCTP at their 3’end in 50 µL labeling reaction contained 1x TdT reaction buffer, 100 nM unlabeled DNA, 0.5 µM biotin-14-dCTP (Invitrogen) and 0.2 U/µL TdT (Terminal Deoxynucleotidyl Transferase, Invitrogen). After 30 min incubation at 37°C, reactions were stopped by addition of 5 mM EDTA and TdT was extracted with an equal volume of
chloroform/isoamyl alcohol. DNA-protein interactions and biotin detection were performed using Light shift chemiluminescent EMSA kit (Pierce) and Chemiluminescent Nucleic acid Detection Module (Pierce), respectively, according to the supplier’s instructions.

Subcellular localization of AtbZIP16wt— Isolation and transfection of A. thaliana mesophyll protoplasts was performed as described in (25). AtbZIP16-wt full length coding sequence, but lacking the stop codon, was amplified by PCR using the primers BamHI-AtbZIP16-F (5’-AAAAGGATCCATGGCTAGCAATGAGATGG-3’) and KpnI-AtbZIP16-R (5’-AAAAGGTACCCGTTGTAGCTTTGTATGAATTGTAAT-3’). The amplified product was fused to the N-terminus of CFP in the 35S-CFP vector to generate the construct AtbZIP16-CFP. AtbZIP16-CFP and the nuclear marker ABI5-YFP (20) were co-transfected in the protoplasts and incubated for 16 h in the dark. Expression in protoplasts was analyzed by confocal microscopy using SP2 confocal laser scanning microscope (Leica). All micrographs were taken with X63 water immersion objective with a numerical aperture of 0.75. Images were taken at 433 nm and 514 nm specific for CFP and YFP, respectively. Chlorophyll was excited with a 543 nm laser and detected using a 560–700 nm filter.

Yeast two hybrid assays— cDNA of AtbZIP16 gene was cloned into pLEXA-N vector to generate a fusion protein with LexA DNA binding domain (Bait). AtbZIP68 and AtGBF1 full-length cDNAs were cloned into pGAD-HA vector to generate fusions of the prey protein with the GAL4 activation domain (prey). Bait and prey vectors were co-transformed into the NMY51 and the transformants were selected on selective media lacking tryptophan, leucine, histidine and adenine (SD/-Trp-Leu-HIS-Ade). The competitive inhibitor 3-aminotriazole (3AT) was used to suppress the leaky expression of the HIS gene when needed. The yeast two hybrid assay was performed with the DUALhybrid system (Biotech) according to the manufacturer’s instructions. β-Galactosidase overlay assay to detect LacZ activation was performed with an overlay buffer containing 0.5 M potassium phosphate, pH 7.0, 6% DMF, 0.1% SDS, 50 µL/100mL β-mercaptoethanol, 5 mg/mL low melting agarose and 0.05% X-gal (Fermentas). Overlay buffer was spread on top of the cells and the yeast plates were incubated at 30ºC until blue color was developed. Primer sequences for AtbZIP16 used in the bait constructs are: EcoRI-bZIP16-F 5’-AAAAGGATCCATGGCTAGCAATGAGATGG-3’, KpnI-bZIP16-R 5’-AAAAGGTACCCGTTGTAGCTTTGTATGAATTGTAAT-3’. The primers used to generate cDNAs for prey cloning are the following: BamHI-bZIP68-F: 5’-AAAAGGATCCATGGGTAGCAGTGAAG-3’ and XhoI-bZIP68-R: 5’-AAAACTCGAGCTACGCAACATCTGA-3’ for AtbZIP68 and BamHI-GBF1-F: 5’-AAAAGGATCCATGGGAACGAGCGAAGAC-3’ and XhoI-GBF1-R: 5’-AAAACTCGAGTTAATTTGTCCTTCACC-3’ for AtGBF1.

Midpoint redox potential— The recombinant AtbZIP16wt and its mutant variants, AtbZIP16C1 and AtbZIP16C2, were used for redox midpoint potential measurements as described in Hirasawa et al., (26). Samples contained 100 mM HEPES buffer pH 7.0, 100 µg recombinant protein and 2 mM total DTT (different ratios of reduced:oxidized DTT) were incubated at ambient temperature. After 3 h incubation, monobromobimane (mBBR; Sigma) was added at a final concentration of 10 mM. Proteins were precipitated with 20%
trichloroacetic acid after 30 min dark incubation. Protein pellets were collected by centrifugation at 13000 rpm and then were washed with 1% trichloroacetic acid before they were resuspended in 250 µL buffer containing 100 mM Tris-HCl, pH 8.0 and 1% SDS. Fluorescence was measured using Spectra Max Gemini plate reader (Molecular Devices) with excitation at 380 nm and emission at 450 nm. All  are calculated based on a value of -330 mV for the of DTT at pH 7.0. The data were fitted to the Nernst equation with n = 1 or with n not fixed. Best-fit values for  were determined by fitting titration data to the Nernst equation using Grafit 5.0 software.

Homology modeling of bZIP domain– Modeling was performed using the Swiss-Model (http://swissmodel.expasy.org/) and the LOMETS (http://zhanglab.ccmb.med.umich.edu/LOMETS/) servers (27,28). Two peptide sequence variants of the AtbZIP16 bZIP domain were used in order to identify structures with sufficient similarity to perform homology modeling. A Blast search with the sequence variant R304–N365 (62 amino acids) was performed against the protein data bank, PDB (www.pdb.org) (29), in order to identify structural templates. The highest ranking match with a sequence identity of 41% over 49 aligned amino acids was obtained to the structure of mouse CREB341 bound to DNA (PDB ID: 1DH3). The AtbZIP16 fragment R304–N365 was submitted to the Swiss-Model server together with the coordinates of a mouse CREB341 monomer (PDB ID: 1DH3_A) as the structural template. After generating the homology model, its quality was scrutinized. The final model contains AtbZIP16 amino acids K307 to E359 (53 amino acids). The likely AtbZIP16 “dimer” was constructed by duplicating the monomer model coordinates and superimposing each of the duplicates onto the structure of the mouse CREB341 dimer (PDB ID: 1DH3). The second, shorter sequence variant Q327–N365 (39 amino acids), which lacks the basic region (BR) did not yield any matches in a Blast search against the PDB. However, the LOMETS server identified the synthetic leucine zipper structure with pdb-id: 3HE4_A as the best structural template. For the shorter model of the AtbZIP16, bZIP16 was superimposed onto the structure of the mouse CREB341 dimer. In order to confirm the correctness of the AtbZIP16 homology models, the longer model was in addition superimposed onto the DNA bound GCN4 basic Leu-zipper structure (1YSA) and the shorter AtbZIP16 model was overlaid with the GCN4 Leu-zipper structure (2ZTA) which lacks the basic region. In all cases, Swiss-PDBViewer 4.0.1 (http://www.expasy.org/spdbv/) was used to superimpose the coordinates of the 3D structures and the homology models using the “Magic Fit” routine followed by the “Iterative Magic Fit” option, which minimizes the root mean square distance (RMSD) between corresponding atom positions. The ribbon diagrams of figure 8A and 8B were generated using the ICM-Browser (http://www.molsoft.com/icm_browser.html) which was also used to determine the distances between the Cys330 of the bZIP16 monomers.

Over-expression of bZIP16 wild type and mutant variants in Arabidopsis transgenic lines– To generate 35S:bZIP16wt (WT OX), 35S:bZIP16C1 (C1 OX) and 35S:bZIP16C1C2 (C1C2 OX) over-expressor lines, the PCR products of full length coding sequence of bZIP16 wild type or mutant variants were cloned at first into the GATEWAY entry vector pDONR207 (Invitrogen) and recombined with pH2GW7,0 destination binary vector. Recombinations were performed using the GATEWAY LR
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Clonase enzyme mix according to the manufacturer’s instructions (Invitrogen). Wild type Arabidopsis plants were transformed with Agrobacterium strain GV3101, harboring the respective constructs, using the floral-dip method (31). Transgenic plants (T1) were screened on MS plates containing 25 μg hygromycin. Segregation analysis of hygromycin resistant versus sensitive ratios (3:1) was performed to select individual lines with single T-DNA insertion and homozygous transgenic plants were selected for further analysis.

RNA isolation, cDNA synthesis and Real-Time PCR– To isolate total RNA, plant RNA mini kit (EZNA) was used according to the supplier’s instructions. iScript cDNA synthesis kit (Biorad) was used for cDNA synthesis according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed in 20 μL reactions containing 2 μL cDNA (1:10 dilution) using iQSYBR Green Supermix (Biorad) and the amplification was performed using CFX96 Real-Time system (C1000 Thermal Cycler, Biorad). All reactions were performed in triplicates and the relative transcript abundance of each tested gene was normalized to the expression level of ubiquitin as described in (32). The primers used in the qPCR were the following: Ubiquitin like protein F: 5’-CTGTTCACGGAACCCAATTCC-3’ and Ubiquitin like protein R: 5’-GGAAAAAGGTCTGACCGACA-3’, LHCB2.4 F: 5’-GGAAAAAGGTCTGACCGACA-3’ and LHCB2.4 R: 5’-GGAAAAAGGTCTGACCGACA-3’. The data were analyzed using LinRegPCR software.

RESULTS

AtbZIP16 transcription factor binds Gbox containing LHCB2.4 promoter– The Gbox (CACGTG) cis-element was shown to be enriched in the promoter sequences of genes responding to HL or redox changes in the chloroplast (33). This element was significantly enriched both in the promoters of genes induced and in the promoters of genes repressed by HL suggesting interaction with both activators and repressors of gene expression (33). In order to isolate transcription factors responding to changes in light intensity/redox changes and interacting with the Gbox element, a 144 bp Gbox containing LHCB2.4 promoter fragment was used. The LHCB2.4 gene (At3g22840) belongs to the LHC super gene family and is representative for genes responding to light and for genes repressed in response to redox changes (33). We used a biochemical DNA affinity trapping approach to isolate transcription factors. This approach was previously proven successful to isolate transcription factors from simple bacterial nuclear protein mixtures (34) and from complex mixtures from plants (35). Nuclear proteins prepared from wild type plants exposed to control and high light conditions were incubated together with the biotin-labelled DNA fragment after it was immobilized on magnetic beads coated with streptavidin. Using this affinity approach, a protein from HL-treated plants was identified to bind the DNA fragment containing the Gbox element (Fig. 1A). Q-TOF mass spectroscopic analysis identified the protein as AtbZIP16 (At2g35530) (Fig. S1). The Arabidopsis bZIP family is subdivided into 10 groups according to sequence similarities of their basic region, conserved motifs and additional features such as the size of the leucine zipper. The high similarity between the proteins in each group suggests that
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members of a given group probably bind similar cis-elements (36). AthZIP16, named after Jakoby et al. (36) was clustered in group G, together with the putative AthZIP68, GBF1, GBF2 and GBF3 proteins (36). The three GBF proteins have been demonstrated to specifically recognize and bind to the Gbox cis-element (37).

Electrophoretic Mobility Shift Assay (EMSA) confirms binding of AthZIP16 to the Gbox—EMSA was performed to confirm the interaction between AthZIP16 and the 144 bp promoter fragment used in the affinity trapping assay. Full-length coding sequence of AthZIP16 was expressed in E. coli as HIS-tagged recombinant protein (Fig. S2). Binding of AthZIP16 to the 144 bp Gbox containing DNA fragment (Fig. 1B) was confirmed with EMSA (Fig. 1B). The observed multiple shifts, resembling the DNA-protein complex, are most likely caused by either binding of bZIP16 protein to both of the binding sites within the 144 bp target DNA (Fig. 1B) or by the DNA binding to multiple forms of the bZIP16 protein such as monomer, dimer, etc (Fig. 1B).

To assess the specificity of the interaction between AthZIP16 and its putative Gbox target sequence EMSA was performed again using 21 bp oligonucleotides containing the Gbox element of the Arabidopsis LHCb2.4 promoter as a probe (Gboxcis) (Fig. 1C). Recombinant AthZIP16 fusion protein demonstrated clear binding to the 21 bp DNA-fragment (Fig. 1C). Moreover, 75x excess unlabeled Gbox fragment was able to compete, although not completely, for the binding activity of AthZIP16 (Fig. 1C) and complete competition was revealed when 100x excess unlabeled Gbox fragment was used (Fig. S3). The G-box cis-element is a palindromic DNA motif that is composed of two identical half sites (C^-3A^-2C^-1G+1T+2G+3). Here we have numbered the base pairs as -3 to +3 (starting from 5’ to 3’) of the Gbox. Binding activity of recombinant AthZIP16 protein was abolished when either the first (M1, -3 to -1) or the second half site (M2, +1 to +3) were mutated (Fig. 1D). Taken together, these results indicate high specificity of the interaction between AthZIP16 and the Gbox core sequence.

Subcellular localization of AthZIP16—Sequence analysis of AthZIP16 protein revealed the presence of KRQRRKQSNRESARRSR amino acid sequence indicating a putative bipartite nuclear localization sequences (NLS). NLS overlaps with the basic region of the bZIP DNA-binding domain. We analyzed the intracellular distribution of AthZIP16-CFP fusion protein in transiently transformed Arabidopsis mesophyll protoplasts. The CFP fusion plasmid was transfected into protoplasts and CFP localization examined by confocal microscopy following overnight expression in the dark. The AthZIP16-CFP fusion protein was exclusively localized to the nucleus (Fig. 2). As a nuclear marker we used ABI5-YFP fusion protein (20) which exclusively co-localized with AthZIP16 in the nucleus (Fig. 2).

GBF1 and AthZIP68 proteins bind Gbox and interact with AthZIP16—The cluster of group G contains, in addition to AthZIP16, the putative AthZIP68, GBF1, GBF2 and GBF3 proteins (36). The bZIP domain shares high similarity (more than 85%) among all members of the G group (Fig 3A). Moreover, AthZIP16 shares 78% overall similarity with AthZIP68, 48% with GBF1 and less than 40% with GBF2 and GBF3 (Fig. 3B). The high sequence similarity between AthZIP16, AthZIP68 and GBF1 suggests that these proteins might bind the same cis-element. To test this assumption, recombinant AthZIP68 and AtGBF1 proteins were expressed in
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E. coli (Fig. S2) and incubated with the 21 bp oligonucleotides containing the Gbox element of the Arabidopsis LHCb2.4 promoter (Gboxcis). The two proteins interacted with Gboxcis oligonucleotides (Fig. 4A & B). GBF1 has been isolated from Arabidopsis cDNA expression library based on its DNA-binding activity to a synthetic oligonucleotide from the tomato RBCS-3A G-box-like element, G-3A (37). GBF1 has also been shown to interact strongly with the Gbox containing promoter of the Arabidopsis RBCS-1A (38). Like AtbZIP16, binding activity of AtbZIP68 and AtGBF1 recombinant proteins was abolished when incubated with M1 and M2 mutagenized half sites of oligonucleotides containing the Gbox element of the Arabidopsis LHCb2.4 promoter (Fig. 4A & B).

The bZIP transcription factors are dimeric proteins. We therefore tested interaction between AbZIP16, AtbZIP68 and AtGBF1 using the yeast two hybrid assay. We expressed AtbZIP16 as a fusion to the GAL4-DNA binding domain in pLexAN vector (BD-bZIP16) and AtbZIP68 and AtGBF1 as fusions to the GAL4 activation domain in pGADHA vector (AD-bZIP68 and AD-GBF1), respectively. We introduced them into NMY51 yeast strain containing HIS and/or LacZ genes under the control of GAL4 binding sites (Fig. 4C). Transformants were grown on selective media lacking the nutritional selective markers Trp, Leu and His. Leaky expression of the HIS gene, due to self-activating capacity of bZIP16, was eliminated with 3-AT. Like BD-p53 interacted with AD-LargeT (positive control), BD-bZIP16 bait was able to interact with the preys AD-bZIP68 and AD-GBF1 but not with the empty AD vector (Fig. 4C). The interactions were confirmed in β-galactosidase overlay assays demonstrating LacZ activation. Similarly to the positive control (P53 interaction with LargeT), strong interaction between AtbZIP16 and its homologues AtbZIP68 and GBF1 was demonstrated by the LacZ reporter activity (Fig. 4C). AtbZIP16 was also previously shown to form heterodimers with AtbZIP68, GBF1; and the other members of the G-group, in the presence of DNA (39).

Redox regulation of AtbZIP16– AtbZIP16 protein contains two cysteine residues at positions 330 and 358 within its bZIP domain (Fig. 3A) suggesting the possibility to form disulfide bonds. To investigate this possibility, we performed denaturing polyacrylamide gels (SDS-PAGE) with AtbZIP16 protein subjected to different reducing or oxidizing conditions. Under non-reducing conditions several bands were detected in the regions corresponding to monomeric, dimeric and oligomeric forms of AtbZIP16 (Fig. 5A). Increasing concentrations of DTT reduced the oligomers and the dimers (Fig. 5A) and led to complete conversion to monomers (Fig. 5A). In contrast, addition of increasing concentrations of H2O2 resulted in a gradual loss of the different detected forms suggesting formation of very high molecular mass oligomers which were beyond the migration capacity of the gel and/or the transfer limit of the membrane (Fig 5A). Restoring reducing conditions by addition of a reducing agent should reverse the effect of H2O2 and break up the high molecular mass oligomers. AtbZIP16 was incubated in the presence of 50 mM H2O2 for 10 min before the addition of the reductant DTT which resulted in high amounts of AtbZIP16 monomer demonstrating that H2O2 formed reversible high molecular weight complexes (Fig 5A).

Redox regulation of DNA-binding activity of AtbZIP16– DNA-binding activity of plant transcription factors has been shown to be directly
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(14,16,20) or indirectly (19) regulated by the redox environment. To determine whether DNA-binding activity of AtbZIP16 to the Gbox is regulated by changes in the redox state, we treated binding reactions with fixed amounts of DTT and H₂O₂ before determining DNA-binding activity by EMSA. As shown in Fig. 5B, the binding activity of AtbZIP16 was greatly enhanced following DTT treatment compared with that obtained under control conditions or H₂O₂ treatment (Fig. 5B) suggesting that cysteines in the reduced state are required for efficient DNA binding.

We performed cysteine mutagenesis to probe the importance of these residues for disulfide formation in AtbZIP16. As shown in Fig. 5C, mutant proteins with C330L and C330LC358L but not C358L alone were converted into a monomer under non reducing conditions (in the absence of β-mercaptoethanol). Thus, the redox-regulated intermolecular disulfide formation is specifically targeted to the cysteine residue at position 330. Furthermore, DNA-binding activity was greatly enhanced in both the C330L and C330LC358L mutant variants compared to the wild type protein (Fig. 5D). The C358L mutant protein resulted in a slight increase in DNA-binding activity (Fig. 5D). Taken together, these results greatly support the importance of the Cys330 disulfide bridge for redox regulation of AtbZIP16 DNA-binding activity. The different behavior of the single mutants, C330L and C358L may reflect differences in redox potential of the respective cysteines.

Monomer and dimer pathways of DNA-binding have been proposed for bZIP and other transcription factors where the monomeric forms can recognize and bind the DNA. These monomers then dimerize while bound to the DNA (40-42). To test the possibility that bZIP16 could bind its DNA target sequence as a monomer, EMSA was performed using the Gbox element and recombinant bZIP16 protein containing the basic region but lacking the leucine zipper; bZIP16BR (Fig. 5E). The truncated form of bZIP16 was able to bind the DNA as a monomer (Fig. 5E). Specificity might be increased by the monomer DNA-binding pathway which prevent trapping of transcription factors at non specific DNA sites (40-42).

Midpoint redox potential of AtbZIP16—A shift of the redox potential to oxidative conditions may induce the formation of intermolecular disulfide bonds between vicinal cysteine residues of protein subunits. Cys330 is responsible for the intermolecular disulfide bond formation in AtbZIP16 and likely stimulates its DNA-binding activity (Fig. 5C & D). The redox state of AtbZIP16wt and its mutant variants, AtbZIP16C1 (lacking Cys330) and AtbZIP16C2 (lacking Cys358), was titrated in the presence of varying ratios of DTT_oxidized/DTT_reduced to adjust defined thiol redox potentials. After labeling with monobromobimane, the redox state was determined in a fluorometric assay. Titration of the redox state of AtbZIP16wt revealed two midpoint redox potentials of -281 mV and -327 mV, respectively (Fig. 6A) indicating a redox regulatory role for both cysteines. To examine the effect of the cysteine mutations on the $E_m$ value of AtbZIP16, additional titrations were performed (Fig. 6B & C). Redox titration experiments with the two Cys mutants (C1 and C2) yielded $E_m$ values similar to those obtained with the wild type protein, C1 ($E_m = -287 \pm 2$ mV) and C2 ($E_m = -324 \pm 1$ mV). The C330L (C1) mutant titration characterizes the properties of the Cys358 whereas the C358L (C2) mutant titration characterizes the properties of Cys330 (C1). The Cys330 residue, which is responsible for disulfide formation has a midpoint redox potential of -324 mV.
that is within the range of values (-290 mV to -330 mV) reported for other redox-active proteins (26). In addition, the second cysteine of AtbZIP16, Cys358 has a midpoint redox potential of -287 mV indicating that it could also play a redox regulatory role possibly through glutathionylation. Taken together, AtbZIP16 conformation and activity respond to changes in the intracellular redox environment similarly to other plant proteins with established roles in redox-regulated systems (26).

**Redox-dependent DNA-binding activity of AtbZIP68 and AtGBF1** – Analysis of the conserved cysteines in the other members of the G group of bZIP transcription factors revealed that C1 and C2 of AtbZIP16 are also conserved in AtGBF1. C2 of AtbZIP68 resembles C1 in AtbZIP16 and AtGBF1. In AtbZIP68 C1 is located at position 182 in the N-terminal and upstream of the bZIP domain (Fig. 3A). The other members, AtGBF2 and AtGBF3 contain threonine and serine instead of cysteines at the respective positions (Fig. 3A). To determine whether DNA-binding activity of AtbZIP68 and AtGBF1 also is subjected to redox-dependent modulation, we expressed these proteins in *E. coli* and performed similar studies by EMSA as those described above for AtbZIP16. Similar to AtbZIP16, DTT greatly enhanced DNA binding of both AtbZIP68 and AtGBF1 (Fig. 7A & C) suggesting involvement of the cysteines in redox-dependent regulation. The role of these cysteines was studied by the mutant proteins AtbZIP68C1 (C182L), AtbZIP68C2 (C320L), AtGBF1C1 (C247L) and AtGBF1C2 (C275L) where the cysteines were exchanged for leucines. The AtbZIP68C2 and AtGBF1C1 mutant forms revealed significantly increased DNA-binding activity (Fig. 7B & D) consistent with the result of DTT-induced DNA binding activity. AtbZIP68C1 and AtGBF1C2 mutant proteins resulted in a slight increase in DNA-binding activity (Fig. 7B & D). These results indicate that the behavior of AtbZIP68 and AtGBF1 resembles that observed for AtbZIP16 (Fig. 5).

**Modeling of the bZIP domain of AtbZIP16** – Comparative modeling produced 3D models of the free and DNA-bound bZIP domain of AtbZIP16 (Fig. 8). The longer variant comprising the basic, DNA binding region (residues R304-N365) was modeled onto the DNA bound cAMP response element-binding (CREB341) bZIP protein from mouse (pdb:1DH3_A). The shorter variant, lacking the basic region (residues Q327–N365) was modeled onto the structure of a synthetic Leu-zipper (pdb: 3HE4_A). The predicted AtbZIP16 structures show that Cys330 (C1) is positioned at the transition between the zipper and the basic region, just outside the direct DNA contact sites of the basic region (BR). In the DNA-free bZIP form the Cys330 (C1) of the respective monomers are located in close proximity of each other. The distance between the two corresponding Cys330 Ca-atoms is estimated to 5.5 Å. The sulphur atoms of the cysteine residues would then be sufficiently close to allow disulfide bond formation. The Sγ to Sγ atom separation (S-S distance) in this model is estimated to 1.5 Å which is slightly closer than the most commonly observed Sγ-Sγ distance of 2.02 Å in disulfide bond forming proteins (Fig. 8A) (43). However, upon DNA-binding the separation between the Cys330 on the two monomers is predicted to increase to about 6.3 Å which will most likely not allow formation of the disulfide bond (Fig. 8B).
Gbox element and bZIP16, and the Cys330 mediated redox regulation of bZIP activity are biologically significant.

We generated over-expression lines of wild type bZIP16 (bZIP16WTOX) and bZIP16 Cys mutants (bZIP16C1OX and bZIP16C1C2OX). Homozygote lines carrying only single T-DNA insertions were identified and used for the experiments. The bZIP16 transcript levels were significantly higher compared to wild type in all produced lines (Fig. 9A). GBF1 was shown previously to play a role during the early light response in Arabidopsis (44) and we therefore monitored the growth of 5-day-old seedlings of the over-expression lines in constant white light. Examination of the hypocotyl length revealed that the bZIP16WTOX line displayed a significant reduction of inhibition of hypocotyl elongation in response to white light (Fig. 9B). In contrast, the over-expression lines with the cysteine mutations bZIP16C1OX and bZIP16C1C2OX displayed hypocotyl length similar to the wild type. These results indicate a biological significance and a functional involvement of the redox status of Cys330 in planta (Fig. 9B). Furthermore, we investigated whether bZIP16 plays a role in light-regulated gene expression of LHCb2.4 in planta and whether Cys330 is important for such a regulation. Expression of LHCb2.4 gene was investigated in 5-day-old seedlings of the bZIP16WTOX, bZIP16C1OX and bZIP16C1C2OX lines grown in constant white light and compared to wild type (Fig. 9C). The expression level of LHCb2.4 was significantly lower in the bZIP16WTOX line compared to wild type suggesting that bZIP16 acts as a repressor of LHCb2.4. Similarly to the hypocotyl elongation, the bZIP16C1OX and bZIP16C1C2OX lines displayed wild type levels of the LHCb2.4 transcript (Fig. 9C). Taken together, the results provide biological relevance for the interaction between bZIP16 and the Gbox and support the proposed regulatory role of C330.

In addition to the study of the bZIP16 over-expresser lines, we also investigated LHCb2.4 expression in T-DNA insertion mutants of the other two G-group members, bZIP68 and GBF1. Eliminating the bZIP68 and GBF1 proteins resulted in a decrease of LHCb2.4 transcript compared to wild type suggesting repression of the light-regulated gene expression (Fig. 9D and 9E). Surprisingly, this regulation was similar to the over-expression of the bZIP16 protein in bZIP16WTOX (Fig. 9C) which implies that in contrast to bZIP16, bZIP68 and GBF1 function as activators of LHCb2.4 expression. Furthermore, this de-regulation of gene expression suggests that despite the high similarity among the three G-group members bZIP16, bZIP68 and GBF1, their function is not redundant.

**DISCUSSION**

Exposure to redox changes induced by exposure to excess light results in dramatic changes in gene expression (6,33). Analysis of the 500 bp promoter sequences of genes responding to exposure to high light or redox changes in the chloroplast revealed that the Gbox was enriched in the promoters of genes repressed by excess light such as LHCb2.4 (At3g27690) (33). Using a biochemical approach we identified AtbZIP16 as a transcriptional regulator involved in the light and/or redox triggered regulation of LHCb2.4 expression in Arabidopsis.

The *A. thaliana* genome encodes at least 75 predicted bZIP transcription factors (36,45,46) which are clustered into 10 subgroups (A-I and S) (36). Plant bZIP transcription factors have been shown to bind the Gbox, Cbox and Abox elements all containing the functional
ACGT core (47). AtbZIP16 belongs to the G-group and clusters with the AtbZIP68, AtGBF1, AtGBF2 and AtGBF3 proteins. AtGBF1, AtGBF2 and AtGBF3 have been shown to bind the Gbox element (37). We further demonstrated that AtbZIP16, AtbZIP68 and AtGBF1 recognize and specifically bind to the oligonucleotide sequences containing the CAGTG Gbox derived from LHC2.4 promoter. Site directed mutations of the ACGT core abolished binding of AtbZIP16, AtbZIP68 and AtGBF1 to DNA.

Regulatory proteins and transcription factors are subject to post-translational modifications necessary to modulate their activities. Dithiol/disulfide exchange is a key component responding fast to changes in the redox environment and modifying the activity of the protein (48). The EMSA for AtbZIP16, AtbZIP68 and AtGBF1 clearly demonstrated that binding to the DNA target is significantly affected by redox conditions and that DNA binding is enhanced under reducing conditions (Fig. 5 and 7). Analysis of Cys330, Cys247 and Cys320 residues in the basic region of AtbZIP16, AtGBF1 and AtbZIP68, respectively, demonstrated that these residues account for the observed changes in DNA-binding activities of the respective proteins. Furthermore, our data suggests that Cys330 in bZIP16 is essential for both regulation of DNA-binding activity (Fig. 5D) and for the disulfide bond formation (Fig. 5C). The midpoint redox potential for Cys330 was determined to -324 mV which is biologically relevant and falls within the range of values (-290 mV to -330 mV) reported for other redox-active proteins, including thioredoxins, ferredoxin-thioredoxin reductase, and thioredoxin regulated proteins (26). In addition, the midpoint redox potential for the second cysteine of bZIP16, Cys358 (C2), was determined to -287 mV although it does not form intra- or inter-molecular disulfide bonds (Fig. 5). This redox potential is more negative than that reported for glutathione -240 mV (49), suggesting that this cysteine is prone to redox regulation possibly through glutathionylation or mixed disulfide bonds. The midpoint redox potential of the redox-regulated AP2-domain containing transcription factor, Rap2.4a was determined to -269 mV. Moderate oxidation of the glutathione pool was suggested to activate Rap2.4a-dependent gene expression, whereas stronger deviations from the normal cellular redox states inactivate Rap2.4a by aggregate formation (20).

Structural analysis of bZIP transcription factors revealed that these proteins bind DNA as dimers formed by interaction of two α-helical stretches which consist of 7 amino acid repeats per DNA turn (50). Under reducing and non-reducing conditions the DNA-protein complexes formed between AtbZIP16 and its target DNA migrated similarly in the EMSA suggesting that the homodimer of the protein is still formed and bound to the DNA. DTT induced monomerization of the AtbZIP16 proteins in vitro and DTT was shown to stimulate DNA-binding activity (Fig. 5). The conventional model for DNA binding of bZIP transcription factors proposes that dimerization of the leucine zipper protein is a prerequisite to specific recognition and DNA binding (51). However, more recent reports suggest that there are two possible pathways for DNA binding of bZIP transcription factors: in pathway I (dimer pathway), bZIP dimer formation precedes DNA-binding while pathway II (monomer pathway) involves the initial formation of the bZIP monomer-DNA complex which then binds a second bZIP monomer in a subsequent step (40-42,52). DTT stimulates monomerization which might improve DNA-binding activity of
AtbZIP16 through pathway II where the monomer, formed by the disruption of disulfide bond, binds DNA, the monomer-DNA complex binds a second AtbZIP16 monomer and subsequent dimerization takes place through the leucine zippers of the proteins. We show that the truncated form of AtbZIP16, containing only the basic BR of the DNA binding domain, has DNA binding activity (Fig. 5E). The DNA binding of this truncated variant of AtbZIP16, unable to form a dimer via the leucine zipper, suggests that AtbZIP16 indeed can bind DNA as a monomer. Thus, it is likely that DNA-binding of AtbZIP16 occurs via pathway II. Furthermore, it has been proposed that the monomer pathway not only allows for rapid identification of a specific DNA site in response to cellular stimuli and faster assembly of the bZIP dimer DNA-complex, but it could also provide an efficient mean for discriminating between specific and non specific DNA target sites (42). We hypothesize that the monomeric form of AtbZIP16 binds to the DNA before the formation of the functional dimer to enhance the response to changes in the environment such as changing light conditions.

A model of the bZIP domain from AtbZIP16 was generated using the Swiss Model and LOMETS softwares. From this model the theoretical structural position of the regulatory Cys330 was assigned to a critical location within the basic region but just outside the direct contact site between BR and the DNA (Fig. 8). In the free bZIP form the position of C330 in each monomer and the distance between them allows for formation of a disulfide bond (Fig. 8A). Thus, the redox status of the Cys330 could potentially be critical for DNA-binding. If a disulfide bridge is formed between the Cys330-residues of the two bZIP16 monomers, the configuration of the zipper may not be open or flexible enough to allow DNA-binding. Furthermore, upon DNA-binding the distance between the two C330-residues increases significantly and may not allow formation of the disulfide bond (Fig. 8B). Thus, the theoretical model supports the experimental evidence for a stimulation of DNA binding activity under reducing conditions (Fig. 5 & 7).

When the early light response was investigated in the bZIP16WT-OX lines impaired induction of LHC2.4 expression compared to wild type was observed. In the bZIP16WT-OX line the levels of LHC2.4 transcript was significantly reduced compared to wild type suggesting that bZIP16 act as a repressor of LHC2.4. Expression of LHC2.4 is strongly repressed in response to redox changes in the chloroplast (7) and possibly bZIP16 plays a role in mediating this repression. Interestingly, when the mutated forms of bZIP16, OXC1 and OXC1C2 were overexpressed no effect on LHC2.4 expression was observed. This suggests that redox regulation of Cys330 is important for the activity of bZIP16. In support of the gene expression data and of the proposed role for bZIP16 as a repressor of photosynthetic gene expression, the bZIP16WT-OX line demonstrated elongated hypocotyls compared to wild type in response to light. This phenotype was observed neither in the OXC1 nor the OXC1C2 lines (Fig. 9). The T-DNA insertion lines for bZIP68 and GFB1 also demonstrated reduced LHC2.4 expression levels in response to light compared to wild type suggesting a role as activators for bZIP68 and GFB1. GFB1 has been shown to function as transcriptional repressor of RBCS and CAT2, but as an activator of LHCs (21,44). In addition, gfb1 mutants have also been shown to exhibit elongated hypocotyls in response to white and blue light (44). It is clear that AtbZIP16, AtbZIP68 and AtGBF1 form...
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homo- and heterodimers (Fig. 4) (39). Heterodimer formation increases the diversity of functional Gbox binding combinations and possibly the monomeric form of AtbZIP16 binds to the DNA and depending on the recruited partner a different output is generated. DNA-binding affinity and specificity, transactivation potential and overall cell physiological function has been suggested to be altered by heterodimerization (53). Thus, our results suggest that combinatorial interactions between bZIP16, bZIP68 and GBF1 play a role in generating light signaling outputs and that these are possibly regulated by redox modifications to the proteins via the conserved Cys residues. The formation of bZIP homo- or heterodimers offers flexibility to a regulatory system enabling a redox-controlled response to changes in the environment.

In conclusion, changes in intracellular redox potential triggers changes of the activity of many proteins (12). Thus, redox regulation of Gbox binding transcription factors offers a fundamental and mechanistic link between photosynthesis-dependent changes in the redox environment and regulation of nuclear encoded photosynthesis genes.

REFERENCES
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FOOTNOTES

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1To whom corresponding should be addressed: Åsa Strand, Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden, Tel: +46 (0)90 786 9314; Fax: +46 (0)90 786 66 76; Email: Asa.Strand@plantphys.umu.se

2Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, S901 87 Umeå, Sweden

3Biochemistry and Physiology of Plants, Faculty of Biology, W5, Bielefeld University, 33501 Bielefeld, Germany

4Department of Chemistry and Center of Chemical Biology, Umeå University, SE-901 87 Umeå, Sweden

5The abbreviations used are: BR: basic region; HL, high light; LHC2.4, Light-harvesting chlorophyll A/B-binding protein2.4; bZIP, basic region leucine zipper; Leu, leucine; ROS, reactive oxygen species; H2O2, hydrogen peroxide; O2-, superoxide; OH, hydroxyl radical; 1O2, singlet oxygen; PQ, plastoquinone pool; EMSA, electrophoretic mobility shift assay; mBBR, monobromobimane; Quantitative PCR: qPCR.
FIGURE 1. DNA-affinity trapping of AtbZIP16 using LHC24 promoter. A, Biotinylated ligands were immobilized on streptavidin-coated beads and mixed with samples to allow binding. Magnetic separation was used and the target proteins collected for subsequent analysis. B, Electrophoretic mobility shift assay (EMSA) verified binding of recombinant AtbZIP16 protein to the 144 bp fragment of the LHC24 promoter containing the G-box (positions -1530 to -1674). C, EMSA with complementary synthetic oligonucleotides representing Gbox element and recombinant AtbZIP16 protein. Competition assay was performed by adding unlabeled Gbox probe at 75 times in excess. D, Sequence of complementary synthetic oligonucleotides representing Gbox and its mutagenized half sites used with AtbZIP16 in EMSA. The biotin-labeled DNA probes were incubated in the presence (+) or the absence (-) of 4 µg recombinant AtbZIP16 protein and the DNA-protein complexes were separated from free DNA by non-denaturing polyacrylamide gel electrophoresis. The signals were detected with chemiluminescent nucleic acid detection method. Positions of free DNA and protein-DNA complexes are indicated by an arrow.

FIGURE 2. Subcellular localization of bZIP16 fusion protein. AtbZIP16-CFP and ABI5-YFP were co-transfected into A. thaliana mesophyll protoplasts. Confocal images are shown as follows: A, bright field transmission B, AtbZIP16-CFP fluorescence C, ABI5-YFP fluorescence D, AtbZIP16-CFP/ ABI5-YFP overlay E, chlorophyll autofluorescence and F, the merged image of A-E. Bar length is 10 µm.

FIGURE 3. Sequence comparison of the G group of the bZIP family in A. thaliana. A, Alignment of the predicted amino acid sequence of bZIP16 (At2g35530) with bZIP68 (At1g32150), GBF1 (At4g36730), GBF2 (At4g01120) and GBF3 (At2g46270) which comprise the G group of bZIP family. The asterisks (*) and the gray shading indicate fully conserved amino acid residues, (:) indicates general similarities, similarity among 2 to 4 is represented by (.) and dashes (-) indicate gaps introduced to maximize alignment. Cys residues are indicated by boxes and light gray shading and the NLS of bZIP16 is underlined. The black rectangle indicates the location of the bZIP domain. B, The phylogenetic tree was produced using Mega5.0 software.

FIGURE 4. DNA-binding of AtbZIP68 and AtGBF1 and interaction between bZIP16 and AtbZIP68 and AtGBF1. Complementary synthetic oligonucleotides representing Gbox and its mutagenized half sites were labeled with biotin and incubated in the presence (+) or the absence (-) of A, AtGBF1 or B, AtbZIP68. Binding reactions were separated by 6% native PAGE. The signals were detected with chemiluminescent nucleic acid detection module and positions of free DNA and protein-DNA complexes are indicated by arrows. C, Interaction between AtbZIP16, AtbZIP68 and AtGBF1 in yeast two hybrid system. Full length coding sequence of AtbZIP16 was fused to Gal4 binding domain in pLexA-N vector to generate the bait BD-bZIP16. AtbZIP68 or AtGBF1 were fused to the Gal4 activation domain in pGADHA vector to produce the prey clones, respectively. NMY51 yeast strain was cotransformed with bZIP16 bait and pGADHA empty prey vector (negative control), bZIP16 bait and bZIP68 prey, bZIP16 bait and GBF1 prey and p53 bait and Large T prey (positive control). Interaction is indicated by the activation of HIS3 reporter gene.
and by the *LacZ* activation. Growth due to the activation of *HIS3* reporter gene was examined in the presence of 3-AT.

**FIGURE 5. Redox regulation of AtbZIP16 and its DNA binding activity.** A, quaternary structure of AtbZIP16 protein was analyzed under reducing and oxidizing conditions by incubating AtbZIP16 either in the presence of 0-10 mM DTT or 0-50 mM H$_2$O$_2$. Proteins were detected using anti-HIS antibody and positions of monomers, dimers and oligomers are indicated by arrows. B, effect of DTT and H$_2$O$_2$ on DNA-binding activity of AtbZIP16 was analyzed by EMSA. Binding reactions in the absence or the presence of AtbZIP16, 10 mM DTT and 10 mM H$_2$O$_2$. Positions of free DNA and protein-DNA complexes are indicated by arrows. C, intermolecular disulfide bond formation in AtbZIP16. AtbZIP16 protein or its mutant variants was either reduced with β-mercaptoethanol or left in its unreduced form before loading on non-reducing SDS-PAGE. Location of the monomers, dimers and oligomers are indicated by arrows for AtbZIP16-wt protein, AtbZIP16 containing a Cys to Leu mutation at position 330, AtbZIP16 containing a Cys to Leu mutation at position 358 and AtbZIP16 containing a Cys to Leu mutations at position 330 and 358. D, Binding of AtbZIP16-wt protein and its mutant variants to the Gbox element. EMSA was performed using biotin-labeled Gbox element without protein, AtbZIP16-wt, AtbZIP16 containing a Cys to Leu mutation at position 358, AtbZIP16 containing a Cys to Leu mutation at position 330 and AtbZIP16 containing a Cys to Leu mutations at positions 330 and 358. Positions of free DNA and protein-DNA complexes are indicated by arrows. E, Coomassie staining of purified HIS-tagged bZIP16 protein containing only the basic region and lacking the leucine zipper (bZIP16BR) The band corresponding to the respective protein is indicated by an arrow. bZIP16BR was analyzed for the formation of DNA-protein complex with Gbox by EMSA. Binding reactions contained biotin-labeled Gbox element in the absence or the presence of AtbZIP16BR. Positions of free DNA and protein-DNA complexes are indicated by arrows.

**FIGURE 6. Mid-point redox potential of AtbZIP16 and its mutant variants.** The ambient redox potential, adjusted by defined ratios of DTT$_{oxidized}$/DTT$_{reduced}$, ranged from -260 to -360 mV. After labeling the reduced protein thiols with mBBr, the fluorescence was quantified in each sample. The redox potential of A, AtbZIP16wt B, AtbZIP16C1 (C330L) and C, AtbZIP16C2 (C358L) was assessed using the Nernst equation (see details under experimental procedures).

**FIGURE 7. Redox modulation of AtbZIP68 and AtGBF1 binding affinities to the Gbox.** A, DTT and H$_2$O$_2$ effect on DNA-binding activity of GBF1 was tested by EMSA. EMSA was performed using biotin-labeled complementary synthetic oligonucleotides representing Gbox element without protein, GBF1-wt, 10 mM DTT and 10 mM H$_2$O$_2$. B, Binding of GBF1-wt protein and its mutant variants to the Gbox element. Binding reactions in the absence of GBF1 protein, GBF1-wt, GBF1 containing a Cys to Leu mutation at position 257, GBF1 containing a Cys to Leu mutation at position 247. C, Effect of DTT and H$_2$O$_2$ on DNA-binding activity of bZIP68 was analyzed by EMSA. Binding reactions in the absence of bZIP68 protein, the presence of bZIP68, 10 mM DTT and 10 mM H$_2$O$_2$. D, Binding of bZIP68-wt protein and its mutant variants to the Gbox element. EMSA was performed using biotin-labeled Gbox element without protein, bZIP68-wt, bZIP86 containing a Cys to Leu mutation at position 320, bZIP68 containing a Cys to Leu mutation at position...
182. Biotin-labeled probes were detected with chemiluminescent nucleic acid detection module and positions of free DNA and protein-DNA complexes are indicated by arrows

**FIGURE 8. Ribbon diagrams of the theoretical 3D structures (comparative models) of the bZIP domain of AtbZIP16.** The figures show the proximity of the cysteine residues at position 330 of the AtbZIP16 sequence without the basic DNA-binding region (A) and when bound to DNA (B). A, A model based on amino acids Gln327 to Asn365 built onto a DNA-free template (3HE4_A). The position of Cys330 is indicated and the distance between the two corresponding Cys330 Cα-atoms is determined to roughly 5.5 Å. At this Ca-Ca distance the sulphur atoms (Sγ) of the cysteine residues would be sufficiently close to form a disulphide bond. The S-S separation in the model is about 1.5 Å. B, A homology model of residues Lys307 to Glu359 built onto the DNA bound structure CREB from mouse (1DH3_A). In this case the distance between the Cys330 Ca-atoms is about 6.3 Å, which would be too far for allowing a disulphide bond to form. Color coding: dark blue depicts the basic region and cyan marks the leucine zipper part of AtbZIP16. Amino acids are depicted in gray with the sulphur atoms (Sγ) in yellow and carbonyl oxygen atoms in red.

**FIGURE 9. Characterization of bZIP16 over-expression lines and bzip68 and gbf1 T-DNA insertion mutants.** A, Real time analysis of bZIP16 expression in the over-expression lines of wild type bZIP16 (bZIP16WTOX) and bZIP16 Cys mutants (bZIP16C1OX and bZIP16C1C2OX). B, 5-day-old seedlings of wild type and the over-expression lines were used to measure the hypocotyl lengths. The seedlings were grown in 10 µmol quanta m⁻² s⁻¹ constant white light and 90-100 seedlings were measured for each genotype. C and D, Real time RT-PCR analysis of *LHCB2.4* (At3g27690) transcripts levels in response to white light in 5-day-old seedlings of WT, C, bZIP16WTOX, bZIP16C1OX and bZIP16C1C2OX D, the T-DNA insertion mutants, bzip68 and gbf1. The gene expression was normalized to the expression level of At4g36800 encoding an ubiquitin-protein ligase-like protein. The mean (±SE) of at least three biological replicates is shown. The expression was significantly different from wild type in the transgenic lines as demonstrated by student T-test: **P<0.01 and ***P<0.001.E, quantitative PCR analysis of transcript levels in the bzip68 and gbf1 T-DNA insertion lines using gene specific primers. Actin (At5g09810) was used as a reference gene.
### Table 1. Primers used to generate AtbZIP16, GBF1 and AtbZIP68 wild type and mutant proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>AtbZIP16-wt</td>
<td>bZIP-F1</td>
<td>5’-CACCATGGCTAGCAATGAGATGGA-3’</td>
</tr>
<tr>
<td></td>
<td>bZIP-R</td>
<td>5’-TCACGTTGAGTCTTTGTATGAATC-3’</td>
</tr>
<tr>
<td>GBF1-wt</td>
<td>GBF1-F</td>
<td>5’-CACCATGGGAAGCGAGCAGCAAG-3’</td>
</tr>
<tr>
<td></td>
<td>GBF1-R</td>
<td>5’-TTAATTTGGTTTCCTGCCACCAC-3’</td>
</tr>
<tr>
<td>AtbZIP68-wt</td>
<td>BamHI-bZIP68-F</td>
<td>5’-AAAAGGATCCATGCTAGCAGCTGA-3’</td>
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<tr>
<td></td>
<td>XhoI-bZIP68-R</td>
<td>5’-AAAAGGATCCATGCTAGCAGCTGA-3’</td>
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<tr>
<td>AtbZIP16-C1</td>
<td>bZIP-C1-F</td>
<td>5’-CACAGCTCATCAGTTTGCTTGAAC-3’</td>
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<tr>
<td></td>
<td>bZIP-C1-R</td>
<td>5’-ACAGGGCCGAGTTTGCTTGAAC-3’</td>
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<td>bZIP-C2-F</td>
<td>5’-TCACGTTGAGTCTTTGTATGAATC-3’</td>
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<td></td>
<td>bZIP-C2-R</td>
<td>5’-TTAATTTGGTTTCCTGCCACCAC-3’</td>
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<td>GBF1-C1</td>
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<td>GBF1-C2-F</td>
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<td>bZIP16basic-R</td>
<td>5’-TCACTCATCAGTTTGCTTGAAG-3’</td>
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</table>
Redox regulation of bZIP transcription factors

**Figure 1**

A) Sample (e.g., Nuclear, cytoplasmic, chloroplastic and whole protein extract) 

B) GboxF: (144 bp) Gbox element 

-1674  -1530 

bZIP16 - + + 

GboxF + + + 

DNA-protein complex 

Free probe 

C) Gboxcis: 5′-TCAACTGACAGTGCATCAC-3′ 

Competitor - - + 75x 
bZIP16 - + + 
Gboxcis + + + 

DNA-protein complex 

Free probe 

D) Gboxcis: 5′-TCAACTGACAGTGCATCAC-3′ 

M1: 5′-ACAGTGCATCAC-3′ 

M2: 5′-CACACACATCAC-3′ 

bZIP16 

<table>
<thead>
<tr>
<th></th>
<th>DNA-protein complex</th>
<th>Free probe</th>
</tr>
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<tr>
<td>1</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td>2</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>3</td>
<td>- +</td>
<td>- +</td>
</tr>
</tbody>
</table>

M1: 5′-ACAGTGCATCAC-3′ 

M2: 5′-CACACACATCAC-3′ 

bZIP16 

<table>
<thead>
<tr>
<th></th>
<th>DNA-protein complex</th>
<th>Free probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td>5</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>6</td>
<td>- +</td>
<td>- +</td>
</tr>
</tbody>
</table>
Figure 2

Redox regulation of bZIP transcription factors

TEM

bZIP16-CFP

ABI5-YFP

bZIP16-CFP/ABI5-YFP

Chlorophyll

Merge
Figure 3

Redox regulation of bZIP transcription factors

A

GBF2

GBF3

AtbZIP16

AtbZIP68

AtbZIP68

GBF1

Figure 4

Redox regulation of bZIP transcription factors

A

GBF1

\[\begin{array}{ccc}
Gboxcis & M1 & M2 \\
- & + & - \\
+ & + & + \\
\end{array}\]

DNA-protein complex

Free probe

1 2 3 4 5 6

B

bZIP68

\[\begin{array}{ccc}
Gboxcis & M1 & M2 \\
- & + & - \\
+ & + & + \\
\end{array}\]

DNA-protein complex

Free probe

1 2 3 4 5 6

C

<table>
<thead>
<tr>
<th>BD-p53/AD-largeT</th>
<th>BD-bZIP16/pGADHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-bZIP16/AD-bZIP68</td>
<td>BD-bZIP16/AD-GBF1</td>
</tr>
</tbody>
</table>

SD-TrpLeuHIS+3AT  
LacZ
Figure 5

Redox regulation of bZIP transcription factors

A

mM DTT

0 1 5 10

mM H2O2 + 50 mM DTT

oligomers

dimer

monomer

1 2 3 4 5

B

H2O2

- - + + +

DTT

- - + + +

bZIP16

- + + + +

Gbox

+ + + + +

DNA protein complex

Free probe

C

β-ME

- +

oligomers

dimer

monomer

vit

C358L

β-ME

- +

monomer

C330L

C330LC358L

D

No protein

βZIP16

C330L

C358L

βZIP16:C330L:C358L

DNA protein complex

Free probe

E

KDa

43 34 26 17 10

Coomassie staining

bZIP16BR

DNA-protein complex

Free probe

1 2
Figure 6

Redox regulation of bZIP transcription factors

A

B

C

Em = -287 ± 2
n = 1.2 ± 0.1

Em = -324 ± 1
n = 1.3 ± 0.1
Redox regulation of bZIP transcription factors

**Figure 7**

A and C: Gbox-cis

- H$_2$O$_2$ - - - +
- DTT - - + -
- protein - + + +
- Biotin-DNA + + + +

B and D: DNA-protein complex

- No protein
- G681-6F
- G681-C76L
- G681-C297L

Free probe

GF1

bZIP68

1 2 3 4
Redox regulation of bZIP transcription factors

Figure 9

A

Relative ZIP6 expression

B

Hypocotyl length (mm)

C

Relative Lhcb2-4 expression

D

Relative Lhcb2-4 expression

E

Western blot analysis:
- bZIP68
- GBF1
  - WT bZIP68
  - WT gbfl
Redox-mediated mechanisms regulate DNA-binding activity of the G-group of bZIP transcription factors in Arabidopsis

Jehad Shaikhali, Louise Noren, Juan de Dios Barajas-Lopez, Vaibhav Srivastava, Janine Konig, Uwe H. Sauer, Gunnar Wingsle, Karl-Josef Dietz and Asa Strand

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