14-3-3 Mediates Transcriptional Regulation by Modulating Nucleocytoplasmic Shuttling of Tobacco DNA-binding Protein Phosphatase-1*

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Tobacco DBP1 is the founding member of a novel class of plant transcription factors featuring sequence-specific DNA binding and protein phosphatase activity. To understand the mechanisms underlying the function of this family of transcriptional regulators, we have identified the tobacco 14-3-3 isoform G as the first protein interacting with a DBP factor. 14-3-3 recognition involves the N-terminal region of DBP1, which also supports the DNA binding activity attributed to DBP1. The relevance of this interaction is reinforced by its conservation in Arabidopsis plants, where the closest relative of DBP1 in this species also interacts with a homologous 14-3-3 protein through its N-terminal region. Furthermore, we show that in planta 14-3-3 G is directly involved in regulating DBP1 function by promoting nuclear export and subsequent cytoplasmic retention of DBP1 under conditions that in turn alleviate DBP1-mediated repression of target gene expression.

To cope with adverse environmental growth conditions, plants have evolved efficient defense mechanisms that imply changes in the expression of numerous genes that are mostly controlled at the transcriptional level (1, 2). Transcription factors are key components of these signaling processes as they are responsible for ultimately executing the transcriptional outputs necessary to mount the pursued adaptation. Upon signal perception, the earliest changes in transcription must be the result of the activity of pre-existing transcription factors that are functionally modulated in response to the corresponding signal. Post-translational modifications, particularly protein phosphorylation-dephosphorylation and protein-protein interactions largely account for the regulation of transcription factor function and affect their subcellular localization, stability, DNA binding activity, and/or interactions with additional protein partners.

DBP1 (DNA-binding protein phosphatase 1) was recently identified in tobacco (Nicotiana tabacum) as the first representative of a novel class of transcription factors endowed with protein phosphatase activity (3). DBP1 was shown to be involved in the transcriptional modulation of a plant defense gene, the CEVII gene, whose transcription is activated in response to detachment (in excised leaf sections) as well as during the course of a compatible plant-virus interaction (4). DBP1-related proteins have been found only in plants, in both monocot and dicot species, and within each species different family members can be identified (5). In all cases, the protein phosphatase 2C catalytic domain resides in the C-terminal part of the protein, whereas the N-terminal region supports the sequence-specific DNA binding activity of DBP factors (5). Within this latter region a highly conserved DNC3 motif (for DBP N-terminal Core) was identified. This motif recognizes the previously identified 3a4 cis-acting element present in the promoter region of the CEVI-1 gene and might represent a structural element critical for the function of DBP proteins as transcriptional repressors (3, 5). Toward the elucidation of the mechanistic basis underlying the regulatory function of DBP factors we report on the identification of the specific interaction of tobacco DBP1 with 14-3-3 isoform G and demonstrate that this interaction mediates a nucleocytoplasmic shuttling of DBP1.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**—The coding sequence of tobacco DBP1 was cloned into the yeast shuttle vector pAS2–1 (Clontech) in-frame with the GAL4 DNA-binding domain (GAL4BD). The resulting construct was used to transform the yeast strain PJ69–4A (6). A tobacco cDNA library constructed in the vector pAD–GAL4–2.1 (Stratagene) was screened following the polystyrene glycol/LiAc/single-stranded DNA protocol developed by Gietz et al. (7). Positive interacting clones were selected on histidine-lacking medium supplemented with 4 mM 3-amino-triazol (a competitive inhibitor of the HIS3 reporter gene product) and analyzed for activation of the second reporter gene, the lacZ gene, by β-galactosidase filter lift assays. Veracity and specificity of the interaction were confirmed by retransformation of the library plasmids into the original bait strain used in the screening and additional control strains. β-galactosidase activity was then measured by a quantitative liquid culture assay.

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3 The abbreviations used are: DNC, DBP N-terminal core; MBP, maltose-binding protein; GFP, green fluorescent protein; MES, 4-morpholineethanesulfonic acid.
An Arabidopsis thaliana two-hybrid cDNA library in the vector pACTII (Clontech) was transformed into PJ69–4a/H9251 and screened following the interaction mating protocol developed by Sölllick and Uhrig (8), using as bait the N-terminal region of AtDBP1 expressed in PJ69–4a as a GAL4BD fusion from the pAS2–1 vector. Reporter gene expression was measured and verified as described above.

In Vitro Pulldown Assay—Tobacco DBP1 was expressed in Escherichia coli as a maltose-binding protein (MBP) fusion protein and purified by affinity chromatography on amylose resin (New England Biolabs) according to the manufacturer’s instructions. 14-3-3 G was expressed bearing a hexahistidine tag and purified by affinity chromatography on nickel-agarose (Qiagen). Purified proteins were combined in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 0.2% (v/v) Nonidet P-40, incubated for 1 h at 4 °C under gentle shaking, and sedimented with amylose resin. After extensive washing, resin-bound proteins were eluted with loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis. A tobacco crude protein extract was prepared by grinding tobacco leaves under liquid nitrogen, suspending the ground powder in the aforementioned buffer, and removing cell debris by centrifugation. Subsequent incubations were as above. The eluted fraction was subjected to Western blot detection with a polyclonal antiserum against Arabidopsis 14-3-3 kindly provided by Robert J. Ferl (University of Florida).

Yeast Nuclear Import Assay—Nuclear targeting was analyzed in vivo using the yeast system developed by Rhee et al. (9). Briefly, the protein of interest is translationally fused to a chimeric transcriptional activator, devoid of any nuclear localization signal and consisting of the DNA-binding domain of the bacterial repressor LexA and the activation domain of the yeast transcriptional activator GAL4. Thus, nuclear import will rely on the presence of a functional nuclear localization signal in the protein under study and will result in the transcriptional activation of the HIS3 and lacZ reporter genes.

Plant Transient Expression Assay by Leaf Infiltration of Agrobacterium tumefaciens—The cDNA sequences encoding the full-length DBP1 protein and the C-terminal region were cloned in-frame with the smGFP4 coding sequence into the pVR-GFP-Ct Gateway vector. The 14-3-3 isoform G coding sequence was cloned into a binary vector under the control of the CaMV 35S promoter. Tobacco plants (Nicotiana benthamiana) were grown in a phytochamber under short day conditions at 23 °C/19 °C. Mid, almost fully expanded leaves were infiltrated with a suspension of A. tumefaciens C58 bearing the relevant construct in 10 mM MES, pH 5.6, 10 mM MgCl2, 150 µM acetoxyringone at an A600 of 0.5. After 3–4 days, GFP fluorescence was analyzed in infiltrated leaves by confocal microscopy. For co-infiltration, Agrobac-
The tobacco protein of unknown function (LRP) (10). The identified GAL4BD alone, and a fusion of this domain to an unrelated different baits, the original GAL4BD-DBP1 fusion, the reporter gene activation was assayed in combination with three genes in a DBP1-dependent manner. As shown in Fig. 1, reporter gene activation was assayed in combination with three different baits, the original GAL4BD-DBP1 fusion, the GAL4BD alone, and a fusion of this domain to an unrelated tobacco protein of unknown function (LRP) (10). The identified cDNA clone supported growth on histidine-lacking medium (Fig. 1A) and deployment of β-galactosidase activity (Fig. 1B) only in the presence of DBP1. The isolated clone was sequenced and found to encode the 14-3-3 isoform G (GenBankTM accession number AF299256) (11). 14-3-3’s are ubiquitous and conserved euarkyotic proteins encoded by multigene families, and they are recognized as important mediators in the regulation of diverse biological processes, in particular signal transduction and transcription, through direct protein-protein interactions (12–15). Plant 14-3-3 proteins were first identified as components of sequence-specific DNA-protein complexes in Arabidopsis and maize (16, 17). More recently they have been found to bind components of the general transcription machinery like TATA-binding protein and TFIIB (18), as well as specific transcription factors and other regulatory proteins with a role in signaling processes related to both developmental and stress response pathways (14).

Interaction of DBP1 with 14-3-3 isoform G was confirmed in vitro in a pulldown assay using purified recombinant proteins. DBP1 was expressed in E. coli as a translational fusion to the maltose-binding protein, and 14-3-3 G was expressed bearing a hexa-histidine tag (14-3-3 G-His6). As shown in Fig. 1C, purified MBP-DBP1 was able to pull along 14-3-3 G-His6 when bound to amylose resin (lane 4), whereas MBP alone was not (lane 5). Furthermore, using a crude protein extract from tobacco leaves, a polyclonal antiserum raised against Arabidopsis 14-3-3 recognized a Western blot a band of the correct size in the fraction sedimented with amylose resin after incubation with MBP-DBP1 (Fig. 1D, lane 1), but not with MBP alone (lane 2).

Identification of 14-3-3 G-interacting Domains within DBP1—14-3-3 target recognition largely involves conserved phosphoserine-containing binding sites, and phosphopeptide motifs providing optimal 14-3-3 binding have been identified from peptide libraries (19). Although no sequence motif was found in the DBP1 amino acid sequence conforming to these consensus sites, some 14-3-3-binding proteins interact through phosphorylated motifs that diverge from these consensus sites, and even non-phosphorylated motifs may also provide high affinity binding by 14-3-3 proteins (13). To locate those regions in DBP1 involved in the interaction with 14-3-3 G, reporter gene activation was assayed in the two-hybrid system using as baits different deletions of DBP1, as depicted in Fig. 2A. When in combination with 14-3-3 G, growth on histidine-lacking medium was observed for full-length DBP1 and the truncated proteins del1 and del2 (Fig. 2B). del1 comprises the first 87 amino acid residues that roughly encompass the N-terminal region of DBP1 previously shown to directly mediate sequence-specific DNA binding (5). In contrast, del3 and del4, which both lack this region, failed to sustain the interaction. Therefore, the N-terminal region of DBP1 is necessary and sufficient for recognition of 14-3-3 G. To further delineate the motifs involved in the interaction, one additional deletion construct (del5) was assayed in the two-hybrid system for its capacity to bind 14-3-3 G. As shown in Fig. 2C, deletion of the N-terminal region up to position 64 in the context of the full-length DBP1 protein did not prevent 14-3-3 G recognition, narrowing the interaction site to the region encompassing amino acid residues from 64 to 87. We had previously shown that the highly conserved DNC motif at the end of the N-terminal region is engaged in DNA binding (5). It would be possible to envision a mechanism by which DNA binding could contribute to retaining DBP1 in the nucleus. Then, 14-3-3 G interaction might displace DBP1 from its target DNA sequence by binding to DBP1 at an overlapping site, thereby promoting DBP1 nuclear export. del1 construct lacks the C-terminal half of the DNC motif and still binds

**RESULTS AND DISCUSSION**

Identification of DBP1-interacting Proteins—To identify proteins involved in the regulatory mechanism mediated by DBP1, we performed a yeast two-hybrid screen of a tobacco GAL4AD-cDNA library using full-length tobacco DBP1 as bait. From 1.5 × 10⁶ transformants analyzed, one clone was identified that activated transcription of the HIS3 and lacZ reporter genes in a DBP1-dependent manner. As shown in Fig. 1, reporter gene activation was assayed in combination with three different baits, the original GAL4BD-DBP1 fusion, the GAL4BD alone, and a fusion of this domain to an unrelated tobacco protein of unknown function (LRP) (10). The identified cDNA clone supported growth on histidine-lacking medium (Fig. 1A) and deployment of β-galactosidase activity (Fig. 1B) only in the presence of DBP1. The isolated clone was sequenced and found to encode the 14-3-3 isoform G (GenBankTM accession number AF299256) (11). 14-3-3’s are ubiquitous and conserved euarkyotic proteins encoded by multigene families, and they are recognized as important mediators in the regulation of diverse biological processes, in particular signal transduction and transcription, through direct protein-protein interactions (12–15). Plant 14-3-3 proteins were first identified as components of sequence-specific DNA-protein complexes in Arabidopsis and maize (16, 17). More recently they have been found to bind components of the general transcription machinery like TATA-binding protein and TFIIB (18), as well as specific transcription factors and other regulatory proteins with a role in signaling processes related to both developmental and stress response pathways (14).

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14-3-3 G. To definitely exclude the implication of the DNC motif in the interaction with 14-3-3 G, we tested the effect of point mutations in conserved positions of the remaining part of this motif, again in the context of the full-length DBP1 protein. Simultaneously introducing alanine substitutions P83A, R86A, and S87A was previously shown to interfere with DNA recognition in vitro (5). However, these point mutations did not affect 14-3-3 G binding in the two-hybrid system (Fig. 2C, DBP1-mut1). Altogether, these results delimitate the interaction site to a 19-amino acid stretch comprised between positions 64 and 82.

14-3-3 Modulates DBP1 Nucleocytoplasmic Shuttling in Vivo—DBP1 was shown to be targeted to the nucleus in yeast using an in vivo nuclear import assay (3). The C-terminal half of the protein was also efficiently imported into the nucleus, consistent with the location in this region of a putative bipartite nuclear localization signal. Nuclear targeting was studied in planta by analyzing the subcellular localization of translational fusions to GFP in transient expression assays as driven by the 35S constitutive promoter upon infiltration of tobacco leaves with A. tumefaciens carrying the relevant gene construct (agroinfiltration) and subsequent detection of green fluorescence by confocal laser scanning microscopy. Both the full-length protein (GFP-DBP1) and the C-terminal half of DBP1 (GFP-DBP1Ct) were found distributed both in the cytosol and in the nucleus (Fig. 3A, left panels). This dual localization was also observed for GFP when expressed alone and is in agreement with previous observations (20). However, whereas GFP nucleocytoplasmic distribution may be explained by diffusion of such a small protein as GFP through the nuclear membrane, the presence of the GFP-DBP1 and GFP-DBP1Ct fusions in the nucleus of transformed plant cells suggests an active nuclear import mechanism, because the size of the fusion protein is beyond the size exclusion limit of the nuclear pore complex (21). This would be consistent with a possible dual function of DBP1 in different subcellular compartments.

One of the reported functions of 14-3-3 proteins is the modulation of subcellular localization of their target proteins (22). As shown in Fig. 3A (right panels), co-expression of 14-3-3 G clearly excluded full-length DBP1 (GFP-DBP1) from the nucleus, whereas the subcellular localization of GFP alone remained unaltered under the same conditions. As expected, a truncated DBP1 protein lacking the N-terminal region (GFP-DBP1Ct), and therefore unable to interact with 14-3-3 G, retains its nucleocytoplasmic distribution even when co-expressed with 14-3-3 G (Fig. 3A, right bottom panel). These results provide strong evidence for modulation of DBP1 nucleocytoplasmic partitioning via a direct interaction with 14-3-3 G through the N-terminal region. As discussed above, because this region is also involved in DNA recognition, 14-3-3 G might promote DBP1 nuclear export by interfering with DNA binding. Although we have excluded the direct implication of the DNC DBP1 DNA binding motif in the interaction with 14-3-3 G, the site for this interaction lies adjacent to the DNC motif, making it still possible to compromise binding to DNA. However, DNA binding seems not to directly contribute to DBP1 nuclear localization because the aforementioned mutations in DBP-mut1, which abolished DNA binding in vitro (5), did not affect DBP1 nuclear accumulation in transient expression assays (data not shown).

14-3-3’s have been shown to regulate the nucleocytoplasmic shuttling of human histone deacetylases (23, 24), of Xenopus protein phosphatase Cdc25, (25), and of p27, a cyclin-dependent kinase inhibitor (26). 14-3-3 proteins often promote the cytoplasmic accumulation of their partners, but also nuclear targeting in others (27, 28). In plants, direct evidence for such a role of 14-3-3 proteins is scant. For example, the tobacco transcription factor RSG is in charge of controlling the biosynthetic pathway of gibberellic acid (GA) and shows a GA-regulated nucleocytoplasmic shuttling. A mutant version of RSG not able to interact with its 14-3-3 ligand fails to shuttle and accumulates predominantly in the nucleus even in the presence of gibberellic acid (29, 30). In this regard, we offer further direct evidence, based on both loss- and gain-of-function experiments,
that a 14-3-3 protein also regulates the nucleocytoplasmic distribution of DBP1.

To complement these studies, and by means of using a yeast nuclear import system (9), we set out to test whether the tobacco 14-3-3 isoform G is intrinsically competent to enter the nucleus as a prerequisite to function in the observed shuttling of the client DBP1 protein. As shown in Fig. 3B, 14-3-3 isoform G is very efficiently imported into the yeast nucleus. VirE2, a protein from A. tumefaciens, is here used as a negative control based on its reported cytosolic localization (9). The C-terminal half of DBP1, previously shown to be targeted to the nucleus with high efficiency (3), was included in the assay as a positive control. This result is in agreement with the work by Paul et al. (31), who have very recently found 14-3-3 κ and λ, the closest Arabidopsis relatives of 14-3-3 G, to be predominantly localized in the nucleus.

14-3-3 G Expression Is Induced in Detached Leaf Sectors and Precedes CEVI1 Gene Activation—Under resting conditions DBP1 represses transcription of CEVI1 by binding to a 3a4 cis element located in the 5’-promoter region of CEVI1 (3). Egression of leaf sectors from the plant results in loss of DBP1 binding to the 3a4 element and the concomitant induction of CEVI1 gene expression in the detached leaf tissue (3). Thus, we reasoned that if 14-3-3 G is involved in relieving CEVI1 transcriptional repression mediated by DBP1, inductive stimuli should prime the interaction of these two proteins and thereby the export and exclusion of DBP1 from the nucleus. A simple model would imply a rapid induction of 14-3-3 G gene expression under inductive conditions, preceding that of the CEVI1 gene. As discussed above, de novo synthesized 14-3-3 G would be targeted to the nucleus, where it would interact with DBP1, leading to nuclear export. To test this hypothesis, we analyzed the expression of CEVI1, DBP1, and 14-3-3 G genes by Northern hybridization (Fig. 4A). Tobacco leaf disks were excised and allowed to float on Hepes buffer as already described (4). Both CEVI1 and 14-3-3 G were induced after leaf detachment but with different kinetics. Whereas 14-3-3 G mRNA showed a maximal level of accumulation as early as 6 h after detachment, CEVI1 mRNA continued to accumulate until 24 h. DBP1 expression was low and intriguingly was up-regulated at late times during detachment, after being slightly reduced at short times. A plausible explanation for this late induction might be the need for resetting the DBP1-mediated regulatory mechanism, although we cannot rule out the possibility that DBP1 plays additional roles in the detachment response. To validate the proposed model in vivo, we detached leaf tissue and monitored the effect on the subcellular partitioning of a GFP-DBP1 fusion protein in combination with different baits allowed to float on histidine-containing (left) and histidine-lacking medium supplemented with 7 mM 3AT (right). GAL4BD-AtDBP1Ct, AtDBP1 protein excluding the N-terminal region.
is bound to the CEVII promoter to repress transcription (3). Upon excision/detachment of leaf sectors, and very likely also in response to infection by a compatible viral pathogen, expression of 14-3-3 G would be primed with the corresponding accumulation of the protein in the nucleus, where it interacts with DBP1. As a result of this interaction, the protein complex would be exported from the nucleus and sequester DBP1 in the cytoplasm, thereby preventing binding to the CEVII promoter and eventually relieving CEVII repression. Whether the sole interaction of DBP1 with 14-3-3 is sufficient to trigger this mechanism or alternatively requires additional partners, and also what other defense-related genes may be regulated in the same way, remain our challenges for the future.

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