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Expression, purification and characterization of a novel bZIP protein from sugarcane

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Abstract

The basic leucine zipper (bZIP) proteins form a large family of transcriptional factors in plants and other eukaryotes. Plant bZIP transcriptional factors are divided into subfamilies and are involved in regulating a large range of physiological processes, from plant development to responses to biotic and abiotic stimuli. In this work, we cloned a novel bZIP of sugarcane into the pET3c vector and expressed the recombinant SCbZIP1 (66-170) protein in *Escherichia coli* BL21 (DE3) plysS. The recombinant protein was purified by heat-treatment and reversed phase chromatography. Northern blot analysis showed that *SCbZIP1* was expressed early in development on day 4, but was not induced by abscisic acid (ABA) or exposure to cold. The identity of the recombinant protein was confirmed by mass spectrometry and CD spectroscopy showed an alpha-helical content of 33%. Electrophoretic mobility assays showed that SCbZIP1 (66-170) bound strongly to G-box, Hex and C-box DNA motifs. SCbZIP1 (66-170) was phosphorylated in vitro by a series of protein kinases and its DNA-binding affinity was strongly decreased after phosphorylation by CKII. SCbZIP1 (66-170) also underwent homo- and heterodimerization with truncated forms of the bZIP transcription factor Opaque 2 from *Coix*.

Keywords: Plant bZIPs; Binding affinity; G-box DNA motifs; Phosphorylation; Dimerization

1. Introduction

Transcription factors regulate the expression of genes and are involved in the control of many intracellular processes. These factors can be defined as sequence-specific, DNA-binding proteins that recognize regulatory sequences in the promoter of a gene and are capable of modulating transcription via interaction with basal components of the transcriptional machinery [1]. Structural and functional studies of transcription factors have shown that they have a modular protein structure consisting of DNA-binding and transcription activation domains, and usually contain nuclear localization signals (NLS) and dimerization or multimerization domains [2–5]. Most transcription factors can be grouped into a handful of different gene families based on their type of DNA-binding domain. The members of each family interact with related or identical DNA motifs found in a variety of different promoters which are regulated by external stimuli, in an endogenous, tissue-specific manner

✠ This paper is dedicated to the memory of Dr. Adilson Leite (08.04.1960–28.02.2003), who passed away after a long fight with cancer before this project was finished. This work would not have been possible without the insight, encouragement and collaboration provided by Dr. Adilson Leite, who will always be remembered as an enthusiastic researcher and an amiable colleague.

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or by in response to phytohormones such as abscisic acid (ABA) [6,7].

Members of the basic leucine zipper (bZIP) family of transcriptional regulators have been described in eukaryotes and are characterized by a conserved region rich in basic amino acid residues that binds to the target DNA and contains the NLS [3]. Close to the basic region there is a leucine zipper region which consists of several heptad repeats of hydrophobic residues. The leucine zipper region is alpha-helical and prone to dimer formation via a coiled-coil arrangement [3]. The crystal structure of the bZIP domain of the yeast GCN4 protein complexed with DNA demonstrated that the bZIP motif resembled a helical forceps "gripping" the major groove of the DNA [8].

Mutational analyses and domain swapping studies have shown that the DNA-binding specificity of bZIP proteins is determined primarily by the amino acid sequence of the basic region [9–11]. However, the hinge and the leucine zipper regions, as well as residues outside the basic and leucine zipper domains, may also be involved in determining the binding specificity by juxtaposition of the basic region or by other mechanisms [6]. Thus, the sequence of the basic region alone is insufficient for predicting the DNA-binding specificity of this class of proteins [11,12]. Genetic and biochemical studies of bZIPs in plants have shown that they are important regulators of several processes, including the responses to hormones, light, and developmental programs [6,7].

A phylogenetic classification of 50 higher plant bZIP factors has been reported [13]. The *Arabidopsis* genome contains 75 members of the bZIP family classified into 13 sub-families [14]. Another classification has been obtained based on the sequence similarity of the basic region and shared domains [6], and the results are in agreement with the phylogenetic classification. Comparison of the amino acid sequence of the basic regions of plant bZIPs has shown that they are more similar to each other than to bZIPs from other organisms, thus indicating that they are an evolutionary-related subfamily [13].

Consistent with this high level of conservation, all plant bZIPs can bind sequences with a conserved ACGT core, even though they may do so with different affinities [15–18]. To facilitate comparisons among different eukaryotes, the nomenclature used for the DNA-binding sequence of the yeast transcription factor GCN4 has been adopted, in which the two central nucleotides, C and G, in the ACGT element are designated −0 and +0, respectively [19]. The nucleotide at +2 defines the box class, with many plant bZIPs binding preferentially to either G- (ACGTG), A- (ACGTA) or C-boxes (ACGTC), all of which are present in the promoters of a wide variety of plant genes [17,18]. There are two classes of G-boxes and of the corresponding G-box-binding factors (GBFs) in cauliflower nuclear extract [15]. Type A activity interacts with the class I G-box, whereas type B activity binds to the class II G-box. The positions ±3 define the class of the G-box, with the palindromic class I having either C or A at −3, while the class II element has G or T at −3. The different affinity observed for each class is dictated primarily by the nucleotides at positions ±4 [17]. The ACGT element is necessary for maximal transcriptional activation, and plant bZIPs have been classified according to their binding affinities for ACGT elements and their flanking sequences [17]. Thus, characterization of the specificity of a newly isolated DNA-binding protein is a key prerequisite for understanding its physiological function.

All bZIPs can form homo and/or heterodimers, but the mechanisms that determine whether homo or heterodimerization occurs are still poorly understood. Nevertheless, the amphipathic nature of the leucine zipper domain favors the hypothesis that charged or polar residues at positions *a*, *e* and *g* of the heptad of one monomer interact with the corresponding residues at the same position on the opposite monomer, probably via hydrogen bonds [20,21].

After data-mining the Sugarcane EST Genome Project (SUCEST; http://sucest.lad.ic.unicamp.br) [22] for possible sugarcane bZIPs, we cloned and expressed the bZIP region of a novel bZIP family protein, which we named SCbZIP1. We confirmed the correct molecular weight of this novel protein by mass spectroscopy of tryptic peptides and assessed the secondary structure content of the recombinant protein by far UV CD spectroscopy. We also studied the phosphorylation of SCbZIP1 in vitro, its binding to different DNA probes via gel-shift assays and its hetero-dimerization with different truncated forms of Opaque 2 from *Coix lacryma-job*. SCbZIP1 binds strongly to G-box DNA elements, has a basic region and a leucine zipper motif with at least eight heptad repeats, and lacks some motifs that are well conserved in other G-box-binding factors. Northern blot analysis showed that this new sugarcane bZIP was expressed in the early stages of plant development and was not induced by abscisic acid or exposure to cold.

2. Materials and methods

2.1. Bioinformatic

Forty-three plant G-box-binding factors were aligned using 70 amino acid residues corresponding to the basic and leucine zipper domains containing at least six heptad repeats. The protein sequences were aligned with the CLUSTAL X program [23]. The full length protein was used to search for motifs with the MEME program (http://meme.sdsc.edu/meme/website). The theoretical protease digestion profile, the determination of the peptide masses (http://ca.expasy.org/tools/peptide-mass.html) and the theoretical p*I* calculations were done using the ExPASy Proteomic Tools (http://ca.expasy.org/tools/pi_tool.html). The putative phosphorylation sites were predicted using the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/).

2.2. Plant treatment and RNA extraction

Sugarcane plantlets (*Saccharum* sp. cv SP80-3280) employed in this works were grown like described previously [24]. The cold exposure was done according described formerly [24]. The ABA treatment was done adding 100 μM of ABA to the medium and the leaves of the plantlets were harvested 0, 3, 6 and 12 h afterwards. Total RNA was isolated from different plant organs of the sugarcane plantlets using Trizol reagent (GibcoBRL, USA) according to the manufacturer's instructions to analyze expression of SCbZIP1 transcripts.

2.3. RNA blot analysis

Ten micrograms of total RNA were run on a 1% (w/v) agarose gel containing formaldehyde and transferred to a Hybond-N+ filter (Amersham Pharmacia Biotech., USA) as described elsewhere [25]. The filters were hybridized with an α-^{32}P dCTP radiolabeled *SCbZIP1* cDNA at 42 °C. Blots were washed at high stringency and exposed to imaging plates. Signals of the digitalized images were quantified using the Image Gauge software (Fuji Film, Japan).

2.4. Cloning and expression vector construction

A cDNA encoding a truncated polypeptide of 12.5 kDa, representing the bZIP motif of SCbZIP1 [=SCbZIP (66-170)] was amplified by a standard polymerase chain reaction (PCR) using DNA of the EST clone isolated from the SUCEST cDNA-library (SUCEST; http://sucest.lad.ic.unicamp.br). Primers containing the cloning restriction sites (in bold) for NDEI and *Bam*HI in the sense and anti-sense primers were used: 5′-CAAATACCATATGGAGGAGTC-3′ and 5′-CTGGATCCTTCAAGAGTCAG-3′. The amplified cDNA insert was cloned into the pET3c expression vector (Novagen, USA).

2.5. Protein expression and purification

Escherichia coli BL21 (DE3) plysS cells were transformed with the pET3c vector containing the SCbZIP1 (66-170) cDNA. The bacteria were cultured in 50 mL of 2× YT broth [25] with 50 μg of ampicillin/mL and grown overnight at 37 °C and 300 rpm. The pre-culture was transferred to 2 L of 2× YT broth with ampicillin and grown to an OD_{600} of 0.6 at 37 °C and 300 rpm. The culture was then induced for protein expression by the addition of 50 mM lactose (Merck, Germany) for 4 h. The cells were harvested, resuspended and lysed in: 50 mM Tris–HCl pH 7.5, containing 5 mM EDTA (Gibco-BRL, USA), 5 mM Benzamidine (Sigma, USA) and 5 mM DTT (Gibco-BRL, USA). After five cycles of sonication the bacterial extract was centrifuged at 13,000 × *g* for 30 min at 4 °C. The supernatant was heated to 80 °C for 3 min and centrifuged at 13,000 × *g* for 30 min at 4 °C. Ammonium sulfate was added to the supernatant to a final concentration of 80%, which was then stirred at 4 °C overnight. After a further centrifugation at 13,000 × *g* for 30 min at 4 °C, the pellet was dissolved in 20 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 2 mM DTT, 1 M NaCl and 5% of polyethyleneimine (PEI; Sigma, USA) and then stirred at 4 °C for 15 min and centrifuged again at 13,000 × *g* for 30 min at 4 °C. The ammonium sulfate precipitation step was repeated three times to ensure total removal of the PEI. The final pellet was dissolved in 20 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 2 mM DTT and 80% ammonium sulfate. The supernatant was centrifuged, filtered and applied to an AP-1 column (10 mm × 100 mm, Waters, USA) filled with 6 mL of Poros 50R2 resin (PerSeptive Biosystems, USA) for further purification by reversed phase chromatography. The column was washed and equilibrated with 50 mL of buffer A [2% acetonitrile and 0.065% trifluoroacetic acid (both from Merck, Germany)]. The protein was eluted with a gradient of acetonitrile (2–100%). The fractions obtained in all pre-purification steps and the chromatographic fractions were analyzed by SDS–PAGE. The Opaque 2 proteins from *Coix* [O2_BL (180–319) and O2_L (239–319)] were generated and expressed as described in a previous report [14]. The expression and purification steps were the same as described here for SCbZIP1. Lyophilized SCbZIP1 protein was dissolved in 500 μl of water and the protein concentration was determined by the Bradford assay [26].

2.6. MALDI-TOF

The recombinant protein (1.5 μg) was analyzed by mass spectrometry (MS). MS was done using matrix-assisted laser desorption ionization (MALDI) and time-of-flight (TOF) mass measurements in a MALDI LR spectrometer (Micromass, UK) operated in the reflector mode (10,000 *m*/*z* resolution with 50 ppm mass accuracy). The recombinant protein was desalted with a C18 ZipTip pipette tip (Millipore, USA), eluted with acetonitrile-TFA and then co-crystallized on a MALDI target plate with α-cyano-4-hydroxycinnamic acid (CHCA). The mass spectrum was acquired in the positive ion mode. Recombinant protein (1.5 μg) was also digested with 1 μg of trypsin (Sigma, USA) in 10 mM ammonium bicarbonate at 37 °C for 18 h and then analyzed by mass spectrometry (MS) under the same conditions as for the intact recombinant protein.

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) experiments were done using a JASCO J-715 (Jasco, USA) spectropolarimeter with a 2 nm bandwidth and an optical path length of 0.2 cm. For thermal denaturation experiments, the temperature ramp (1 °C/min) was produced with a Peltier unit.

2.8. Phosphorylation

One microgram of SCbZIP1 was incubated with 20 mM Tris–HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM DTT, 2 μCi [γ-^{32}P]-ATP, 50 nM of unlabeled ATP, and 1 μl of protein kinase in a final volume of 10 μl. For the kinase P-34^{cdc2} (Calbiochem, USA) the buffer was supplemented with 1 mM EGTA and 0.01% Brij. The reactions were done at 30 °C for 30 min and were then heated to 100 °C for 3 min with Laemmli buffer [27]. All samples were electrophoresed by SDS–PAGE using 15% gels that were subsequently stained with Coomassie blue, dried and autoradiographed.

2.9. Electrophoretic mobility shift assay (EMSA)

Single strand sense oligonucleotides were synthesized (Invitrogen, USA) and the sequences are indicated in Fig. 6, panel I. The bold region in each of these sequences corresponds to the core motif that is highly conserved in most plant bZIP DNA targets (Fig. 6, panel I). The anti-sense oligonucleotide used to produce a double-strand radiolabeled DNA oligonucleotide for the electrophoretic mobility shift assays (EMSA) was designed as follows: 3′-TGAGCTC-5′. One nmol of each of the sense anti-sense oligonucleotides were mixed with 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and heated to 94 °C and gradually cooled to room temperature. The annealed probes were extended with Klenow enzyme (Gibco-BRL, USA) in the presence of ^{32}P-labelled dCTP and were purified on Sephadex® G-25 (NAP™-5, AP-Biotech, USA) columns.

The protein–DNA-binding reaction was done by mixing different amounts of SCbZIP1 protein (15–500 ng) with 18 pmol of radiolabeled probe and 100 ng of salmon sperm DNA with gel-shift buffer (150 mM Tris–HCl, pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 5 mM DTT, 10% glycerol and 10% Ficoll) for 30 min at 30 °C. After formation of the complex 5 μL of running buffer (250 mM Tris–HCl pH 7.5, 40% glycerol, 0.2% bromophenol blue and 0.2% xylene cyanol) were added and the samples were run on a 6% PAGE with 1% glycerol in 0.25 × TBE buffer at 400 V and 4 °C for 1 h. The gel was dried and autoradiographed. The competitive assays were done by mixing 150 ng of SCbZIP1 with 18 pmol of radiolabeled probe and a 20, 30, 40 or 50-fold molar excess of non-radiolabeled probe using the same conditions as described above. The EMSA with phosphorylated SCbZIP1 was done using the G-box1 and C-box1 ^{32}P-labeled probes under the same conditions.

2.10. Dimerization reactions

For the homodimerization reactions, 1 μg of SCbZIP1 was mixed with 0.5 mM of BS3 (bis-sulfosuccimidyl suberate, Pierce, USA) in dimerization buffer (100 mM Hepes pH 7.5, 50 mM $MgCl_2$, 5 mM DTT) in a final volume of 10 μL and incubated for 30 min at 30 °C. For the heterodimerization reactions 1 μg of the O2 proteins was added. The reaction products were separated by SDS–PAGE on 15% gels that were subsequently stained with Coomassie blue and then documented with an Eagle Eye II system (Stratagene, USA).

3. Results

3.1. Purification and characterization of SCbZIP1

The strategy of data mining was used to identify the putative sugarcane bZIP proteins in the SUCEST program, as described previously [14]. As an initial target for further detailed studies, we chose to clone the cluster SCSGAM1095E12 because of its long leucine zipper domain and because of its expression in the lateral bud during the early stages of plantlet development.

The cDNA sequence of the clone SCSGAM1095E12 coded for a small protein with 170 amino acid residues (20 kDa) and a theoretical p*I* of 7. The encoded protein contained a leucine zipper with at least eight heptad repeats $(abcdefg)_8$, in which the leucine was replaced by valine in the 4th and 5th heptads at position "d" (Figs. 1 and 2A). The bZIP domain was located close to the carboxy-terminal end. Based on the DNA sequencing results and on computer searches for related proteins in GenBank, the corresponding cDNA appeared to be full length and shared sequence identity (given in %) with the clone AAK25822 from *Phaseolus vulgaris* (43%), clone AAK01953 from *Phaseolus acutifolius* (43%), AtbZIP58 (51%), AtbZIP42 (55%), and AtbZIP48 (39%) from *Arabidopsis*. According to the phylogenetic classification [14], all these plant bZIP proteins, including SCbZIP1, belong to the same family. These proteins are not yet well characterized functionally, but they are small proteins with little homology outside their basic region (data not shown). Compared to other characterized plant bZIPs (e.g. G-box-binding factors), SCbZIP1 lacks many of the conserved motifs such a proline-rich transcriptional activation domain [28]. Nevertheless, SCbZIP1 showed seven non-clustered Pro residues in its N-terminal domain (Fig. 1, residues 1–65).

Alignment of the bZIP motifs showed that all bZIPs had a well-conserved basic region with a more variable leucine zipper domain (Fig. 2A). The leucine residues were well conserved at position "d" in the heptad repeats of the zipper domain in the most of the bZIP proteins, except for the 4th and 5th heptads that had Phe, Ile, Ala or Val at this position (Fig. 2A). SCbZIP1 had two Val at these positions. Fig. 2B shows the putative nuclear localization signal (NLS), the amino acid residues that contact DNA, the sites for the regulation of DNA binding by phosphorylation, the amino acid residues indispensable for DNA binding, and the putative phosphorylation target sites for CKII and PKC.

The bZIP region (amino acids 66-170) of the cDNA clone SCSGAM1095E12 was sub-cloned in pET3c. SCbZIP1 (66-170) was expressed and purified by a procedure

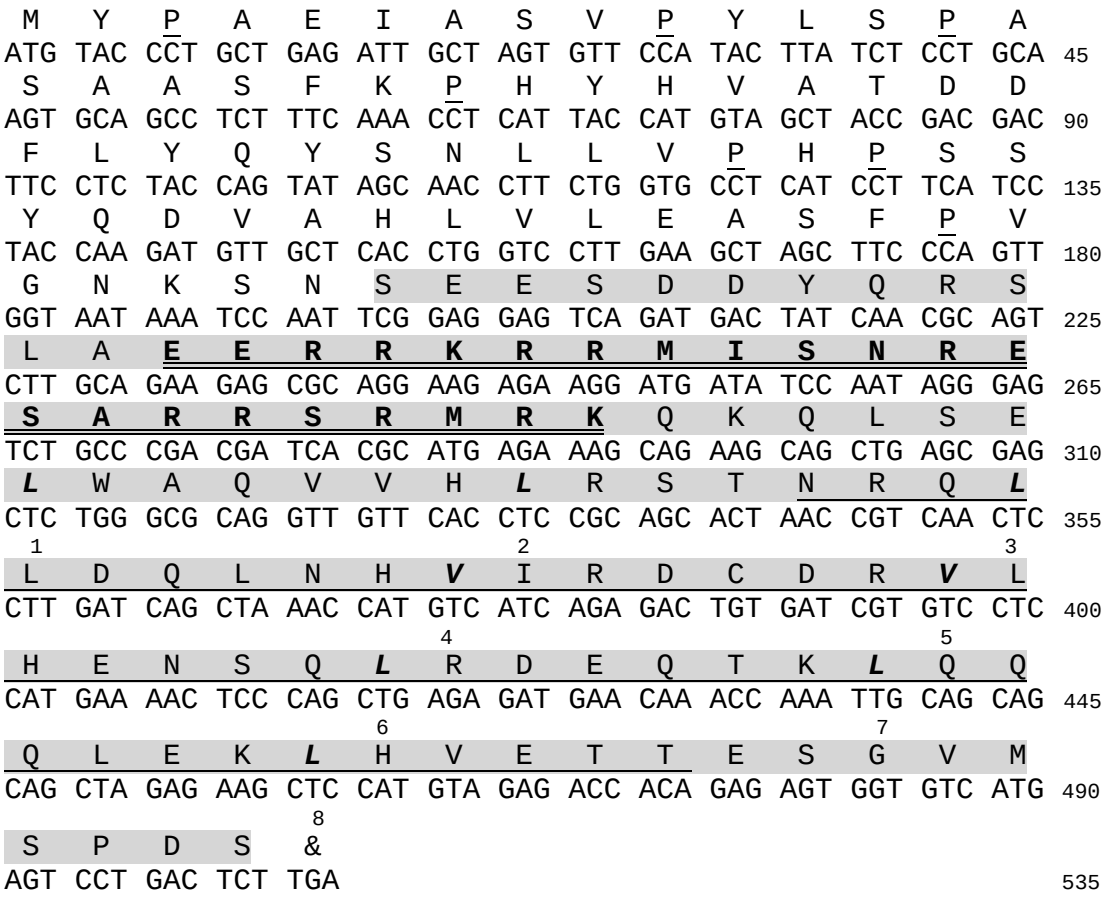

Fig. 1. Sequence of the *SCbZIP1* cDNA and predicted amino acid sequence. Amino acids corresponding to the expressed protein fragment SCbZIP1 (66-170) are highlighted in gray. The basic region is shown in bold type with double underlining. The Leu and Val of the putative zipper domain are numbered and shown in italics. The region with single underlining corresponds to the putative coiled coil region. The prolines of the putative trans-activation domain are underlined.

that allowed the rapid, efficient purification of relatively thermo-stable bZIPs with 90% purity. Fig. 3 shows that the putative SCbZIP1 protein fragment appeared almost as a single major band after the various purification steps (lane 7).

3.2. MALDI-TOF analysis

To determine the molecular weight and identity of the recombinant protein, a MALDI-TOF analysis was done using undigested and trypsin-digested protein. The primary sequence of SCbZIP1 was analyzed using the program PeptideMass (http://ca.expasy.org/tools/peptide-mass.html) to obtain the theoretical peptide masses expected from trypsin digestion, and the predicted masses were compared with the experimental data. The molecular mass was determined to be 12,525 Da, which is close to the theoretical molecular mass calculated from the primary amino acid sequence cloned (12,529 Da). The molecular weight determined by SDS–PAGE (~14.7 kDa) was larger than obtained by MALDI-TOF and the discrepancy between these values maybe reflects technical differences between the methods. The mass spectrum resulting from MALDI-TOF analysis of the digested protein is shown in Fig. 4A. Three of the expected peptides resulting from the trypsin digestion of SCbZIP1 were clearly detected in their monoprotonated form: VLHENSQLR at *m/z* 1095.5, QLLDQLNHVIR at *m/z* 1348.8 and LHVETTESGVMSPDS at *m/z* 1579.1 (Fig. 4A).

3.3. Circular dichroism

The structural integrity and self-association of SCbZIP1 (66-170) were analyzed by far-UV CD spectroscopy. SCbZIP1 was found to be approximately 33% alpha-helical based on the CD ellipticity minimum at 222 nm (Fig. 4B). The helical content of SCbZIP1 (66-170) corresponded to about 45 amino acid residues in contrast to the 56 residues predicted by primary sequence analysis of the corresponding region. This suggested that the SCbZIP1 leucine zipper domain may not be fully structured or may present some conformational fluctuations, as previously proposed for other eukaryotic bZIP transcription factors [29, V. H. Moreau, unpublished observation]. The thermal denaturation of SCbZIP1 (66-170) was accompanied by a decrease

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Consensus    DERELKR RRKQSNRESARRSRLRKQAE EEL   V  L  EN  LR EL  L        EN  L
                                          abcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
SCbZIP1      SLAEERRKRRMISNRESARRSRMRKQKQLSELWAQVVHLRSTNRQLLDQLNHVIRDCDRVLHENSQLRDE
O2Cl         MPTEERVRKRKESNRESARRSRYRKAAHLKELEDQVEQLKAENSCLLRRLAALNQKYNEANVDNRVLRAD
O2           KMPTEERVRKKESNRESARRSRYRKAAHLKELEDQVAQLKAENSCLLRRIAALNQKYNDANVDNRVLRAD
PcCPRF-2     DPSDAKRVRRMLSNRESARRSRRRKQAHMTELETQVSQLRVENSSLLKRLTDISQRYNDAAVDNRVLKAD
ZmOCSBF-1    AADTHRREKRRLSNRESARRSRLRKQQHLDELVQEVARLQADNARVAARARDIASQYTRVEQENTVLRAR
PcCPRF-1     NDRDLKRERRKQSNRESARRSRLRKQAEAEELAIKVDSLTAENMALKAEINRLTLTAEKLTNDNSRLLEV
NtTAF-1      NERELKREKRKQSNRESARRSRLRKQAEAEELAIRVQSLTAENMTLKSEINKLMENSEKLKLENAALMER
ZmGBF-1      DERELKREKRKQSNRESARRSRLRKQAETEELATQVESLAAENTSLRSEIGRLTESSEKLRLENSALMVK
AtGBF-2      NEKEVKREKRKQSNRESARRSRLRKQAETEQLSVKVDALVAENMSLRSKLGQLNNESEKLRLENEAILDQ
ATGBF-3      NERELKRERRKQSNRESARRSRLRKQAETEELARKVEALTAENMALRSELNQLNEKSDKLRGANATLLDK
ZmEmBP-1a    DEREIKRERRKQSNRESARRSRLRKQQECEELARKVADLTTENSALRAELDNLKKACQDMEAENSRLLGG
TaEmBP-1a    DERELKRERRKQSNRESARRSRLRKQQECEELAQKVSELTAANGTLRSELDQLKKDCKTMETENKQLMGK
AtGBOXF-1    DERELKRQKRKQSNRESARRSRLRKQAECEQLQQRVESLSNENQSLRDELQRLSSECDKLKSENNSIQDE
PcCPRF-3     DERELKRQRRKQSNRESARRSRLRKQAKSDELQERLDNLSKENRILRKNLQRISEACAEVTSENHSIKEE
PcCPRF-4a    DERELKRQKRKQSNRESARRSRLRKQAECDELAQRAEALKEENASLRAELSRFRTEYEKIVAQNEVLKEK
PcCPRF-4b    DERELKRQRRKQSNRESARRSRLRKQAECDELAQRAEVLQEENASLRAELGRARSEYEKALAQNAILKEK
OsbZIP-1a    DERELKRQRRKQSNRESARRSRLRKQAECEELAQRAEVLKQENTSLRDEVNRIRKEYDELLSKNSSLKEK
TaHBPA       DERELKKQKRKLSNRESARRSRLRKQAECEELGQRAEALKSENSSLRIELDRIKKEYEELLSKNTSLKAK
TaHBP-1a     DEREVKKQKRKQSNRESARRSRLRKQAEWEEVASRADLLKQENSSLKEELKQLQEKCDNLTSENTSLHEK
                                                  1      2      3      4      5      6
                    <------------------><----><------------------------------------>
(A)                       basic region      hinge              leucine zipper
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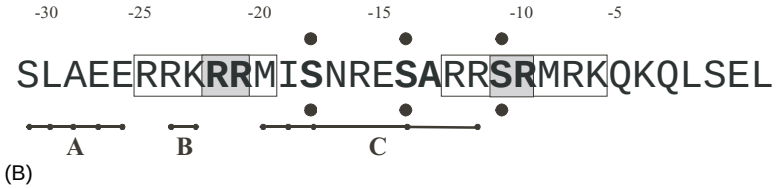

(B)

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                          -25     -20     -15     -10     -5
EmBP-1a      DEREIKRERRKQSNRESARRSRLRKQQECEEL      group 1
CPRF-1       NDRDLKRERRKQSNRESARRSRLRKQAEAEEL      group 1
TAF-1        NERELKREKRKQSNRESARRSRLRKQAEAEEL      group 1
CPRF-3       DERELKRQRRKQSNRESARRSRLRKQAKSDEL      group 1
OCSBF-1      AADTHRREKRRLSNRESARRSRLRKQQHLDEL      group 1
CPRF-2       DPSDAKRVRRMLSNRESARRSRRRKQAHMTEL      group 2
O2           KMPTEERVRKKESNRESARRSRYRKAAHLKEL      group 2
SCbZIP1      SLAEERRKRRMISNRESARRSRMRKQKQLSEL      group 2
TGA1a        SKPVEKVLRRLAQNREAARKSRLRKKAYVQQL      group 3
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- Bold letters in gray indicate the amino acids that could be the major determinants of plant bZIP DNA binding specificity.

(C)

Fig. 2. Sequence comparisons of plant bZIPs. (A) Alignment of bZIP regions including up to the first six heptad repeats of the leucine zipper domain. Only 19 of the 43 aligned sequences are shown in the figure. The consensus sequence is shown on the top of the alignment. The heptads are depicted according to Lupas [45]. Partially conserved amino acids are highlighted in gray. Amino acids conserved throughout all sequences are shown in bold letters. The "d" positions of the Leu or other hydrophobic residues are numbered. (B) Schematic depiction of the basic region of SCbZIP1. The open boxes contain the bipartite nuclear localization signal (BR-A left, BR-B right); the grey highlighted boxes and bold case letters indicate the amino acids in contact with DNA; the black points (●) below and above the letters indicate residues involved in the putative regulation of DNA binding/nuclear shuttling by phosphorylation; black dots and lines below sequence identify residues indispensable for DNA binding. (C) Sequence comparison between different classes of plant bZIPs. Bold letters in gray indicate the amino acids that could be the determinants of DNA-binding specificity.

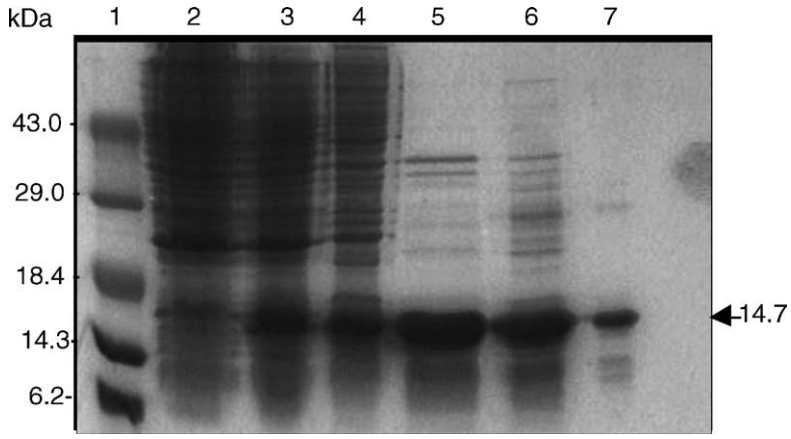


Fig. 3. Expression and purification of SCbZIP1 (60-170). Fractions from the different purification steps were subjected to SDS–PAGE in 15% gel and stained with Coomassie blue. Lane 1: low molecular mass protein markers (Gibco-BRL, USA); lane 2: non-induced *E. coli* extract; lane 3: lactose-induced *E. coli* extract; lane 4: soluble fraction of lactose-induced *E. coli* extract; lane 5: soluble, lactose-induced *E. coli* extract, heated to 80 °C; lane 6: same extract after 80% ammonium sulfate precipitation; lane 7: purified protein after reverse phase chromatography (peak fraction). The arrow indicates the band corresponding to SCbZIP1 (66-170).

in the negative signal minimum at 222 nm, and displayed a cooperative transition that was dependent on the protein concentration in the range between 8 and 80 μM, as predicted by the law of mass action for dissociation processes (Fig. 4B). At the melting temperature, SCbZIP1 (66-170) showed a linear loss of the negative CD signal. This process is characteristic of leucine zippers and has been attributed to fraying of the extremities of the zipper [29–31].

The formation of coiled-coil structures was assessed using the COILS-Prediction of Coiled Coil Regions in Proteins program (http://www.ch.embnet.org/software/COILS_form.html). The result showed that SCbZIP1 contained at least six repetitions of amino acids with a high probability of forming coiled-coils in the leucine zipper domain, which is a common characteristic of bZIP transcriptional factors (Fig. 1).

3.4. Protein phosphorylation

The phosphorylation of transcription factors is a common modification which can regulate their functional activities, including multimerization, compartmentalization or DNA binding [6,32–34]. Fig. 5 shows the results of the phosphorylation of SCbZIP1 (66-170) with different protein kinases and sugarcane nuclear extract. With the exception of PKA (lane 1), all the other kinases and the nuclear extract were capable of phosphorylating the recombinant protein SCbZIP1 (66-170) (lanes 2-6). Although SCbZIP1 (66-170) was not phosphorylated by PKA, we found at least four putative theoretical sites for phosphorylation by this kinase using the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) (data not shown). PKC was the most efficient of the kinases

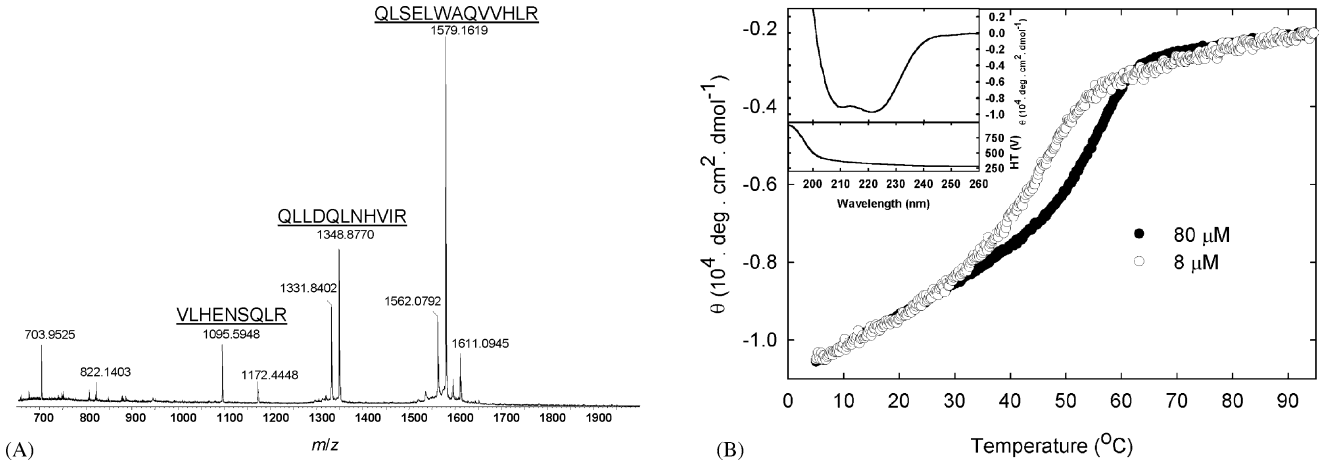


Fig. 4. MALDI-TOF and circular dichroism analysis of the recombinant protein SCbZIP1 (66-170). (A) The masses of monoisotopic peaks with a relative intensity greater than 5% of the most intense peak in the spectrum were used for comparison to a theoretical digestion of the protein by trypsin. The peaks that fitted the theoretical peptide masses are shown, together with the sequences of the peptides. (B) Thermal denaturation of 8 μM (open symbols) or 80 μM (closed symbols) SCbZIP1 (66-170) was followed by the decrease in the negative ellipticity band at 222 nm as described in Section 2. The inset shows the far-UV CD spectrum of 8 μM SCbZIP1 (66-170) at 10 °C and the horizontal transmittance (HT) compensation in the photomutiplier. The raw ellipticity values were corrected to absolute ellipticity; and the CD spectra for 8 and 80 μM SCbZIP1 (66-170) at 10 °C were superimposed.

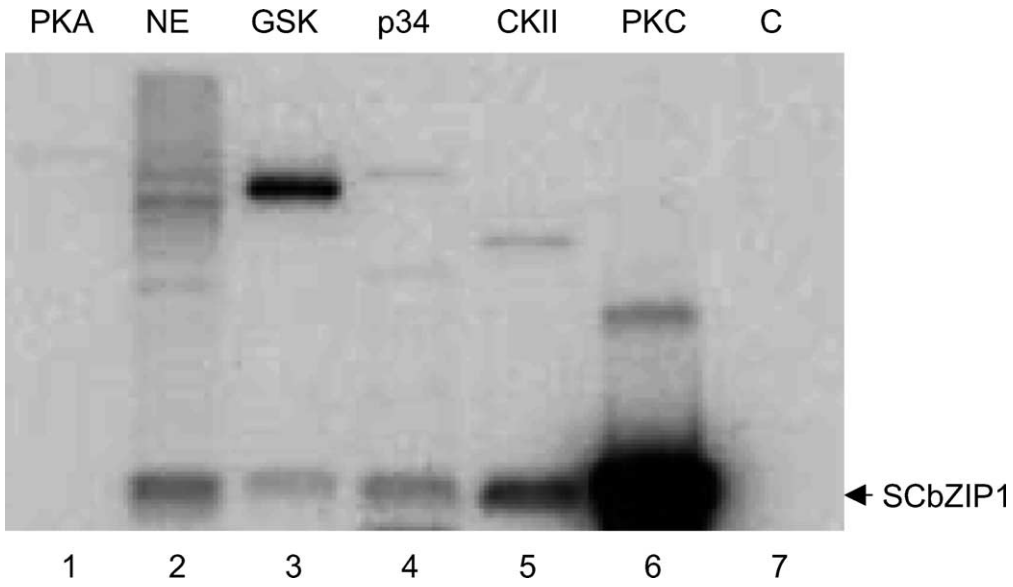


Fig. 5. In vitro phosphorylation of the recombinant SCbZIP1 (66-170). Phosphorylation reactions were done as described in Section 2. Radiolabeled phospho-proteins were separated by SDS–PAGE 15% gels. The gel was dried and analyzed by autoradiography. Lane 1: PKA, bovine protein kinase A; lane 2: NE, sugarcane leaf nuclear extract; lane 3: GSK, human protein kinase α-3 glycogen synthase; lane 4: p34, human cyclin B-dependent protein kinase; lane 5: CKII, *Zea mays* casein protein kinase type II; lane 6: PKC, rat protein kinase C; lane 7: C, negative control reaction without the protein CbZIP1. The arrowhead indicates the position of the phosphorylated SCbZIP1 (66-170).

in phosphorylating SCbZIP1 (66-170) and produced a band of high intensity (Fig. 5, lane 6). This could be due to the presence of two putative PKC sites in the basic region of SCbZIP1 (RMISNRE and NRESARRS) and a third site in the second heptad repeat (LRSTNRQ) (Fig. 2A and B).

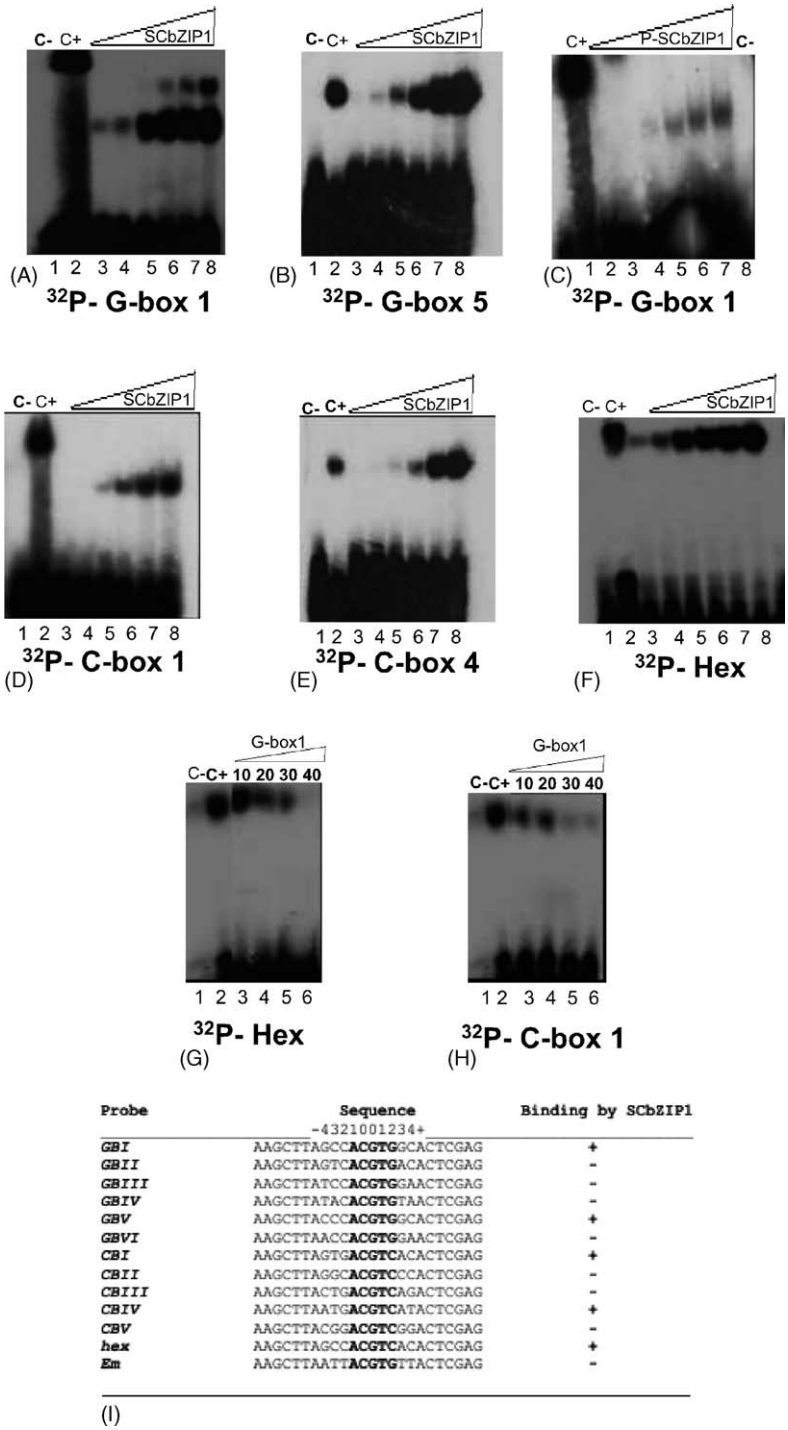
The activity of several bZIP transcription factors is modulated by CKII [4,34–36]. To investigate whether the DNA-binding activity of SCbZIP1 (66-170) could be modulated by CKII, we phosphorylated the recombinant protein and analyzed its DNA-binding capacity by EMSA using the G-box1 probe (Fig. 6). The SCbZIP1-binding affinity for the G-box1 DNA decreased strongly after phosphorylation by CKII. Similar results have also been reported for the HY5 protein from *Arabidpsis* [32]. After phosphorylation, HY5 protein showed a decreased affinity for the light-responsive G-box element of the CHS1 promoter. Opaque 2 was able to bind to the O2 DNA-binding site only in its non-phosphorylated or hypo-phosphorylated form [33]. Our results suggest that the phosphorylation of SCbZIP1 could be a post-translational modification involved in regulating the binding activity or localization of this sugarcane protein in vivo.

3.5. DNA-binding activity

Since many of the DNA sequence elements used to screen for plant bZIP proteins have a common ACGT core sequence [16], we investigated the influence of different sequences flanking the ACGT core (positions ±3 and ±4) in order to characterize the putative DNA targets for SCbZIP1. The nomenclature used to define the nucleotide positions of the dyad symmetrical ACGT-binding site followed that used for the yeast GCN4-binding site [19]. The DNA binding of the SCbZIP1 (66-170) was assayed by EMSA using six G-box sequences, five C-box element sequences [16], and a Hex [16] and an Em1d [37] sequence. Fig. 6 shows selected results of the EMSA experiments. SCbZIP1 (66-170) had a similar affinity for G-box1 and G-box5 (Fig. 6A and B). This was not surprising since the two probes had a very similar DNA sequence, with the only difference being the substitution of a G in G-box1 for a C in G-box5 at position −4 (Fig. 6I). SCbZIP1 (66-170) did not bind any of the other four G-box probes tested (data not shown, Fig. 6I). Thus, SCbZIP1 displayed a binding specificity similar to that described for the type A cauliflower nuclear G-box-binding protein [15].

Other protein–DNA-binding studies have demonstrated that plant bZIPs can also interact with C-box elements [16]. As shown here (Fig. 6D, E, I) two of the five C-box probes tested bound to SCbZIP1 (66-170). These two C-box sequences had two bases changed at positions +4 and −4 (Fig. 6I). SCbZIP1 (66-170) did not bind any of the other three C-boxes tested, although C-box3 has been shown before to bind other plant bZIPs, including Opaque 2, TAF-1, and CPRF-2 [16].

We next examined the interaction of SCbZIP1 with Em1d [37] and Hex [16], two other oligonucleotide probes which also contain the ACGT core sequence but differ in their flanking regions (Fig. 6I). The Hex motif consists of half a G-box and half a C-box and could therefore be a DNA target for bZIP proteins with affinity for both G- and C-box motifs [16]. As expected, SCbZIP1 (66-170) bound with high affinity to Hex (Fig. 6F), but did not bind to the Em1d probe (data not shown).



Probe	Sequence (-4321001234+)	Binding by SCbZIP1
GBI	AAGCTTAGCC**ACGTG**GCACTCGAG	+
GBII	AAGCTTAGTC**ACGTG**ACACTCGAG	-
GBIII	AAGCTTATCC**ACGTG**GAACTCGAG	-
GBIV	AAGCTTATAC**ACGTG**TAACTCGAG	-
GBV	AAGCTTACCC**ACGTG**GCACTCGAG	+
GBVI	AAGCTTAACC**ACGTG**GAACTCGAG	-
CBI	AAGCTTAGTG**ACGTC**ACACTCGAG	+
CBII	AAGCTTAGGC**ACGTC**CCACTCGAG	-
CBIII	AAGCTTACTG**ACGTC**AGACTCGAG	-
CBIV	AAGCTTAATG**ACGTC**ATACTCGAG	+
CBV	AAGCTTACGG**ACGTC**GGACTCGAG	-
hex	AAGCTTAGCC**ACGTC**ACACTCGAG	+
Em	AAGCTTAATT**ACGTG**TTACTCGAG	-

(I)

Fig. 6. Electrophoretic mobility shift assays for the protein SCbZIP1 (66-170) with different types of ACGT core-containing DNA probes. (A–F) Proteins were incubated with the radiolabeled double-stranded DNA oligonucleotide probes indicated at the bottom of each panel (see panel I for the corresponding sequences). Increasing amounts of SCbZIP1 (66-170) were incubated with each probe: lane 1: negative control without protein (C−); lane 2: positive control, 500 ng Opaque 2 protein from *Coix* (C+); lanes 3–8: 15, 30, 70, 150, 300, or 500 ng of SCbZIP1 (66-170) protein. (C): SCbZIP1 (66-170) protein phosphorylated in vitro by CKII before EMSA. G, H: Competitive EMSA: Increasing amounts of unlabeled double-stranded DNA probe were incubated with the protein and the radiolabelled probe. I: The DNA sequences of the probes used in the EMSA experiments with SCbZIP1 (66-170). The numbering of the bases relative to the core ACGT was adapted from the nomenclature for the GCN4 transcriptional factor [19]. The probes bound by SCbZIP1 (66-170) are shown by + and those not by −.

Competitive EMSA experiments were used to further investigate the specificity of SCbZIP1 (66-170) for G-box1, Hex and C-box1. Fig. 6G and H shows the results of the competitive assay with ^{32}P-labeled Hex and C-box1 in the presence of unlabeled G-box1. The binding to Hex and C-box1 was sensitive to 40- and 30-fold excess amounts of G-box1 unlabeled probe, respectively. These results confirmed that SCbZIP1 (66-170) had greater affinity for the G-box1 and Hex probes than for C-box1.

3.6. Dimerization experiments

bZIP proteins are able to form homo and heterodimers. Based on the amphipathic nature of the alpha-helix of bZIPs, the charged or polar amino acids at positions *a*, *e*, and *g* (Fig. 2A) of one bZIP can interact with the corresponding positions on another bZIP through hydrogen bonds or hydrophilic interactions. A chemical cross-linking dimerization assay with the cross-linker BS3 was therefore used to assess the dimerization of SCbZIP1 (66-170) with itself and with two different truncated forms of Opaque 2 protein from *Coix lagrima-job* (Fig. 7).

Each of the three protein constructs tested (SCbZIP1, O2_BL and O2_L) had the ability to form dimers (Fig. 7, lanes 3, 5 and 8), as shown by the appearance of bands corresponding to double the molecular mass (29, 18, 34 kDa) of the respective monomeric forms (15, 9, 17 kDa; Fig. 7B, lanes 2, 4 and 7).

Heterodimerization of SCbZIP1 (66-170) with both O2 constructs was also observed (Fig. 7B, lanes 6 and 9). Both the O2 homodimerization and heterodimerization occurred in the same reaction medium when SCbZIP1 (66-170) and O2_L were incubated together with the cross-linker (Fig. 7, lanes 6). This could indicate that the propensity of O2_L to form a heterodimer with SCbZIP1 (66-170) was superior to that of SCbZIP1 (66-170) to form a homodimer, since only a very weak band of SCbZIP1 (66-170) dimer was seen under these conditions (Fig. 7, lane 6). In contrast, O2_BL did not form a homodimer, but could still engage in heterodimer formation with SCbZIP1 (66-170) (Fig. 7, lane 9).

3.7. SCbZIP1 mRNA expression

The expression of SCbZIP1 mRNA during plantlet development, and after treatment with ABA and exposure to cold, were analyzed by Northern blotting (Fig. 8). The transcripts were expressed in the initial stages of sugarcane plantlet development (Fig. 8A). Expression was also seen in the lateral buds and flowers, but not in any of the other tissues analyzed (Fig. 8B). These findings suggested that SCbZIP1

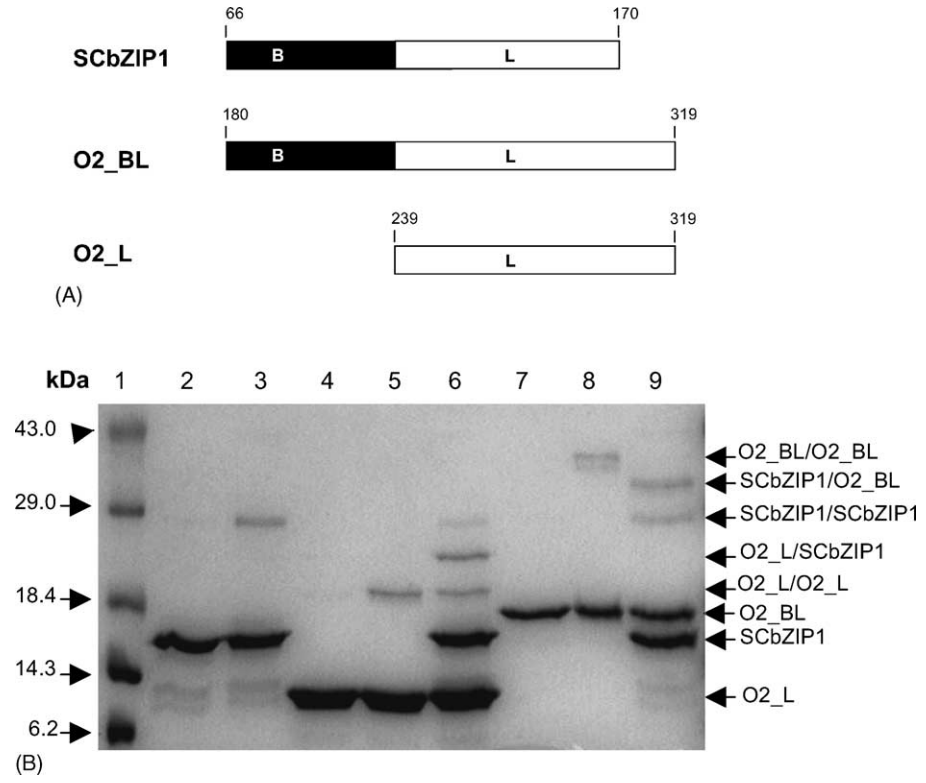


Fig. 7. SCbZIP1 homodimerization and heterodimerization with Opaque 2 from *Coix* in vitro. (A) Schematic representation of the expressed proteins. The basic region (B) is depicted in black and the leucine zipper domain (L) in white. The amino acids spanning the constructs are indicated. (B) SDS–PAGE 15% gel of the proteins or protein-mixtures subjected or not to dimerization in vitro with the chemical cross-linker BS3. Lane 1: low molecular mass protein markers; lane 2: SCbZIP1 without cross-linker; lane 3: SCbZIP1 incubated with 0.5 mM BS3; lane 4: O2_L without BS3; lane 5: O2_L with 0.5 mM BS3; lane 6: SCbZIP1 and O2_L with 0.5 mM BS3; lane 7: O2_BL without BS3; lane 8: O2_BL with 0.5 mM BS3; lane 9: O2_BL and SCbZIP1 with 0.5 mM BS3. The respective protein monomers/dimers are indicated by the arrows.

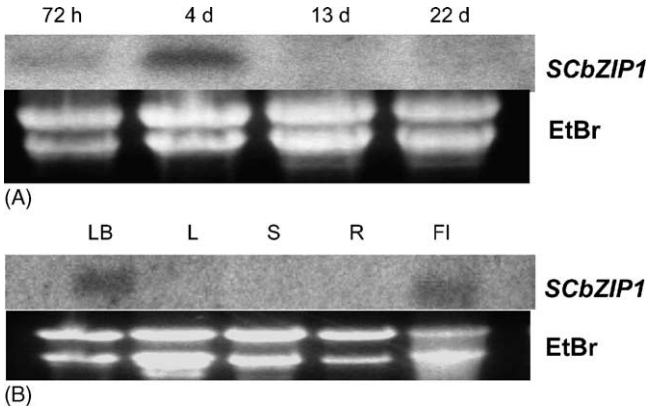


Fig. 8. Northern blot analysis of the expression of *SCbZIP1* mRNA during sugarcane plantlet development and in different plant tissues. (A) Total RNA extracted 72 h, 4, 13 or 22 days after planting. (B) Total RNA extracted from lateral bud (LB), leaves (L), steam (S), roots (R) and flowers (Fl). The total RNA stained with ethidium bromide before being transferred to the membrane is shown under each lane.

could play a role in some stages of plant and flower development. No expression of SCbZIP1 mRNA was observed in leaves from plants treated with ABA or exposed to cold (data not shown), although other plant bZIPs, such ABI5, are induced by abscisic acid or cold exposure [7,38].

4. Discussion

Amino acid sequence analysis of the SCbZIP1 cDNA clone showed that the carboxy terminus contained a long bZIP motif with eight heptad repeats. The CD spectra of SCbZIP1 revealed a α-helical pattern, with a helical content of about 33%. This relatively low helical content may be associated with conformational fluctuations previously reported for bZIP transcriptional factors [29]. Such conformational fluctuations have been proposed to be important in determining the specificity of the formation of heterodimers and, therefore, for biological function [29]. In addition, CD spectra showed that the thermal denaturation of SCbZIP1 (66-170) was dependent on the protein concentration, suggesting that a dissociation reaction occurred (Fig. 4B). The shift observed in the $t_{1/2}$ of the denaturation curves was compatible with the dissociation of a dimeric protein and corroborated the finding that SCbZIP1 (66-170) could form stable dimers in solution. Taken together, CD and MALDI-TOF results confirmed the identity of the recombinant protein and showed that SCbZIP1 (66-170) maintained its secondary structure after the purification protocol which involved denaturation by heating.

The phosphorylation of SCbZIP1 (66-170) by CKII decreased its binding affinity for G-box1 when compared to the non-phosphorylated protein (Fig. 6C). The serine residues conserved in the basic region of all GBF proteins are targets to CKII phosphorylation [4]. The importance of these conserved serines for DNA-binding activity has been studied using gel-shift assays, as in the case of a wheat bZIP transcriptional factor, HBP-1a(17) [39]. All of the serines in this protein were mutated to glutamic acids to mimic the effects of phosphorylation, and resulted in decreased DNA-binding activity, especially when the serines at the PKC sites SAR and RSR were mutated [39]. Thus, the phosphorylation of conserved serines in the basic region would be expected to cause a decrease of DNA-binding activity. Phosphorylation of the basic domain leads to the translocation of GBF to the nucleus where a putative specific phosphatase activity could dephosphorylate the serines at the NLS site, thereby allowing GBFs to bind to G-box DNA target sequences [4]. The serines in the basic region of SCbZIP1 could be regulated by phosphorylation in vivo and therefore be involved in the regulation of nucleo-cytoplasmic translocation and/or DNA binding.

Plant bZIP proteins show specificity in binding DNA sequence elements containing an ACGT core. Gel mobility shift experiments using 10 recombinant plant bZIP proteins have shown that the nucleotides flanking the ACGT core greatly influence the binding specificity and affinity [16]. Three main elements have been identified, namely, A-, C- and G-boxes and bZIPs have been classified into three groups based on a strong affinity for G-boxes (group 1), a similar affinity for G- and C-boxes (group 2), and a stronger affinity to C-boxes (group 3) [16].

The EMSA results showed that SCbZIP1 had a strong affinity for G- and C-boxes, and for Hex, an ACGT element containing G-box/C-box hybrid sites. Analysis of the sequences flanking those G-box bound by SCbZIP1 (66-170) revealed that position −3 was always occupied by a C and there was strong binding only when a G or C was present at position −4. The presence of T or A at position −4 led to a loss of binding activity (data not shown). For plant bZIPs binding to the ACGT elements there was an exclusive preference for C or A at position −3 and a preference for G or T at position −4 in the G-box elements [16]. However, SCbZIP1 (66-170) bound strongly to an ACGT element with C at position −4. This difference in binding preference could reflect sequence differences in the amino acids in the basic region (Fig. 2).

SCbZIP1 (66-170) bound with high affinity to C-box elements containing a T at −3 and an A at −4 in agreement with results obtained by others [16], who showed that most plant bZIPs can bind to DNA sequences containing the same flanking sequences. Furthermore, SCbZIP1 (66-170) was able to bind to the Hex motif, a probe with half a G-box and half a C-box. Thus, based on the classification according to affinity previously reported [16], the SCbZIP1 protein can be classified into group 2 because of its affinity for the G-, C-box and Hex probes.

Our results support the hypothesis that bZIP-binding activity could depend on the binding affinity of protein dimer subunits for ACGT half sites [16]. Although all plant bZIP proteins exhibit differential binding for the high affinity G-

and C-box elements, they all have a similar DNA-binding specificity in that they prefer almost the same flanking sequences. Fig. 2C shows a comparison of the plant bZIP protein DNA-binding basic regions. There are four strictly conserved amino acids that directly contact DNA: Arg −11, Ser −12, Ala −15 and Asn −19 [17]. Arg at −11 is essential for DNA binding because an Opaque 2 Arg to Lys point mutant at this position was unable to bind to promoter of 22 kDa zein genes [40]. Ala −15 could be the major determinant for plant bZIP DNA-binding specificity, and the presence of Ala −15 could differentiate group 3 from groups 1 and 2 [17]. It is not yet clear how bZIP proteins can discriminate between different G-box elements, but it is likely that amino acids present in BR-A (Fig. 2B), or in sequences flanking the basic region or hinge region can affect binding specificity [16,17].

The binding specificities of bZIP proteins in vivo may be regulated by a combination of heterodimerization with other bZIP proteins, accessory proteins, co-factors, chaperones and/or post-translational modification, including phosphorylation [5,16]. The *Arabidopsis* GBF and RSG form heterodimers with other proteins, a mechanism that might have evolved to generate additional functional diversity [41,42]. As shown here, SCbZIP1 (66-170) interacted with two truncated forms of Opaque 2 from *Coix*. These results indicate that SCbZIP1 may interact with other sugarcane transcriptional factors.

GBFs are involved in the regulation of gene expression during plant development. The expression of the ribulose-1, 5-bisphosphate carboxylase small subunit gene (*Rbcs2*), for instance, is regulated during tomato fruit development via a G-box within the *Rbcs2* promoter [43]. The presence of G-box elements in the light-regulated promoters of the gene *Chs* indicates that GBF proteins may participate in light-induced gene activation [44]. *SCbZIP1* mRNA was expressed during the early stages of sugarcane development and the EMSA and phosphorylation results suggested that SCbZIP1 could bind to G-box elements present in various gene promoters. The ability of SCbZIP1 (66-170) to form dimers with other bZIP transcription factors could also affect the affinity for different DNA elements and amplify the participation in various physiological processes in plant development. Future studies should clarify the function of SCbZIP1 in sugarcane development and lead to the characterization of physiologically relevant dimerization partners.

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