

Identification of new ABA- and MEJA-activated sugarcane *bZIP* genes by data mining in the SUCEST database

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Abstract Sugarcane is generally propagated by cuttings of the stalk containing one or more lateral buds, which will develop into a new plant. The transition from the dormant into the active stage constitutes a complex phenomenon characterized by changes in accumulation of phytohormones and several other physiological aspects. Abscisic

acid (ABA) and methyl-jasmonate (MeJA) are major signaling molecules, which influence plant development and stress responses. These plant regulators modulate gene expression with the participation of many transcriptional factors. Basic leucine zipper proteins (bZIPs) form a large family of transcriptional factors involved in a variety of plant physiological processes, such as development and responses to stress. Query sequences consisting of full-length protein sequence of each of the *Arabidopsis* bZIP families were utilized to screen the sugarcane EST database (SUCEST) and 86 sugarcane assembled sequences (SAS) coding for bZIPs were identified. cDNA arrays and RNA-gel blots were used to study the expression of these sugarcane *bZIP* genes during early plantlet development and in response to ABA and MeJA. Six *bZIP* genes were found to be differentially expressed during development. ABA and MeJA modulated the expression of eight sugarcane *bZIP* genes. Our findings provide novel insights into the expression of this large protein family of transcriptional factors in sugarcane.

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Introduction

Development and responses to environmental stimulus are based on the cellular capacity for differential gene expression and are often modulated by transcription factors acting as switches in regulatory cascades. Transcription factors form an intricate network of protein–protein and/or protein–DNA interactions controlling the expression of the genome (Riechmann et al. 2000; Riechmann and Ratcliffe 2000).

The rice genome contains 1,611 transcriptional factors (TFs) that were grouped in 37 gene families (Xiong et al. 2005). This number is similar in the *Arabidopsis* genome, which codes for at least 1,500 TFs, which accounts for 5% of the estimated total number of genes (*Arabidopsis* Genome Initiative 2000). It is interesting to note that although several gene subfamilies exist in these two plant genomes, no families were found to be lineage-specific (Xiong et al. 2005). The basic leucine zipper proteins (bZIPs) are a family of transcriptional factors spread throughout the plant kingdom. Members of the basic leucine zipper (bZIP) family of transcriptional regulators are characterized by a conserved region rich in basic amino acid residues that binds to the target DNA and also contains the nuclear localization signal (NLS). 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 (Hurst 1995; Schlögl et al. 2004). In addition to the basic DNA binding motif and the leucine zipper region, bZIPs may contain other protein motifs, such as proline rich domains with transcriptional activation activities (Jakoby et al. 2002), and others that are involved in the interaction with other regulatory proteins (Holm et al. 2001). These conserved motifs and the alignments of the basic region were used to classify the members of this family of proteins into ten different groups (Jakoby et al. 2002). This TF family seems to participate in the regulation of many processes, including pathogen defense, light and stress signaling, seed maturation and development. Genomes from *Arabidopsis* and rice encode around 80–90 putative bZIP proteins, and only a few are known to be involved in plant development (Chuang et al. 1999; Izawa et al. 1994; Kim et al. 2004; Nieva et al. 2005; Walsh et al. 1998, 1999; Yin et al. 1997).

Phytohormones regulate and integrate plant development, overall growth and reproduction. Abscisic acid (ABA) and methyl-jasmonate (MeJA) are two of these phytohormones, which have important roles in plant development and plant response to biotic and abiotic stress (Rakwal and Komatsu 2004). ABA modulates the expression of a wide array of genes during seed development and germination (Seki et al. 2002), and in responses to various adverse environmental conditions (Shinozaki and Yamaguchi-Shinozaki 1996). Many ABA-induced genes contain conserved, ABA-responsive *cis*-acting elements named ABREs (ABA-responsive element) in their promoter region (Grill and Himmelbach 1998; Suzuki et al. 2003). The ABRE elements of these genes are targets for many plant bZIPs (Guiltinan et al. 1990; Uno et al. 2000; Kang et al. 2002; Suzuki et al. 2003). Some of these transcriptional activators are

themselves induced by ABA and/or stress treatments (Nakagawa et al. 1996; Nieva et al. 2005). *ABI5* for example, regulates the expression of some *LEA* genes mainly expressed during seeds development, and is also regulated by ABA (Finkelstein and Lynch 2000; Suzuki et al. 2003).

Jasmonates signaling molecules affect a variety of plant process including fruit ripening, senescence, and production of viable pollen, root growth and defenses against insects, pathogens and environmental stress, such as cold, salinity and drought (Creelman and Mullet 1997; Farmer and Ryan 1992; Sasaki et al. 2001). Although the biological function of several jasmonate-responsive genes is not known yet, some up-regulated genes are involved in cell-wall formation, secondary metabolism, protection against stress, and even in jasmonate biosynthesis, while down-regulated genes are involved in photosynthesis and light harvesting complex II (Cheong and Choi 2003; Creelman and Mullet 1997). Several *cis*-acting elements responsible for gene activation have been identified in the promoter regions of jasmonate-responsive genes, including G-box sequences that are target of bZIP transcriptional factors (Wasternack and Hause 2002). Plants respond to pathogen attack by induction of various defense responses, including the biosynthesis of protective secondary metabolites. In *Catharanthus roseus*, MeJA induces the gene *Str*, which contains a functional G-box (CACGTG) *cis*-regulatory sequence (Ouwerkerk and Memelink 1999) and encodes a strictosidine synthase involved in the biosynthesis of alkaloids (van der Fits et al. 2000). GBF proteins from *C. roseus* were shown to modulate the expression of the *Str* gene, suggesting a functional role for bZIPs in the MeJA regulation of the plant secondary metabolism (Sibénil et al. 2001).

Sugarcane is an important industrial crop of tropical and subtropical regions and is generally propagated by cuttings of the stalk containing one or more buds. The buds are a miniature stem with its growing point and primordia of leaves and roots. The transition from the dormant into the active stage constitutes a complex phenomenon characterized by changes in the accumulation of phytohormones and other important physiological changes (Van Dillewijn 1952).

Here we report the expression of a set of the sugarcane *bZIP* genes found in the SUCEST data base (Vettore et al. 2003) during the development and in response to ABA and MeJA. Using cDNA-arrays we found that six sugarcane *bZIP* genes were differentially expressed during plantlet development and other eight genes were modulated by ABA and MeJA. The expression profiles of *bZIPs* in specific sugarcane tissue/organs were also examined. The putative biological functions of these sugarcane transcription factors are discussed.

Materials and methods

Sugarcane growth

The sugarcane (*Saccharum* spp.) cultivar SP80-3280 was used in all experiments. Sugarcane stalk sets were cultivated in greenhouse conditions in 200 ml plastic cups containing a commercial planting mix (Plantmax, Eucatex, Brazil) plus ammonium sulfate and commercial 4-20-20 fertilizers. Twenty lateral buds were collected after 3 and 4 days and six plantlets (stem and leaves) were collected after 14 and 22 days after planting. Two replicates were done separately for each experiment. These experiments were done in two different years to increase data reliability.

Sugarcane plantlets were grown *in vitro* in MS media as described previously (Nogueira et al. 2003). Plants were kept in a growth chamber at 26°C on a 16 h/8 h day/night cycle with photon flux density of 70 $\mu\text{E m}^{-2} \text{s}^{-1}$. These plants were used for ABA and MeJA assays (see below) and three replicates were done for each treatment. One-month-old sugarcane plants cultivated in a greenhouse at the Cane Technology Center experimental station (Piracicaba, SP, Brazil) were used to evaluate gene expression in leaves, roots, and stem. Sugarcane flowers were obtained from sugarcane plants grown at Universidade Federal de São Carlos experimental station (Serra do Ouro, AL, Brazil)

ABA and MeJA assays

Sugarcane plantlets were grown *in vitro* for 4 months and then ABA was added to the medium at a final concentration of 100 μM and the leaves of the plantlets were harvested at 0, 6 and 12 h after treatment. MeJA at a final concentration of 100 μM was added to the medium and the leaves were harvested at 0, 1 and 12 h. Six plantlets were used for each time point and three replicates were done for each assay.

RNA extraction

Total RNA was isolated using the Trizol Reagent (Invitrogen, USA) and in the case of the lateral buds, a high salt precipitation (0.8 M of sodium citrate and 1.2 M NaCl) step was added according to the manufacturer's instructions, due to the high polysaccharide content.

Data mining of sugarcane bZIPs

An ordered set of *Arabidopsis* bZIP regulators was used to detect and classify sugarcane contigs encoding bZIP

transcriptional regulators. Query sequences consisting of the full-length protein sequence of each of the ten *Arabidopsis* bZIP groups (AtbZIPs) (Jakoby et al. 2002) were used to screen the SUCEST database (Vettore et al. 2003). The candidate sugarcane assembled sequences (SASs) were selected based on the level of identity of their deduced protein sequence with the bZIP protein sequences from each of the members of the ten AtbZIP groups (Jakoby et al. 2002). Moreover, the same search was performed on the *S. officinarum* DFCI Gene Index (Release 2.1), which contains an extra 7.4% sequences as compared to the SUCEST database but has 7.8% less contig sequences, probably due to different clustering algorithms. This strategy allowed us to identify 86 SASs encoding putative sugarcane bZIP transcription factors (referred to hereafter as *Saccharum* spp. bZIPs; *ScbZIPs*; Table 1S, supplementary data). The generic names were given according to the *Arabidopsis* bZIPs to provide a unique identification for each *ScbZIP* (Table 1S). To complement the putative functions of the *ScbZIP* genes, a search was carried out in the NCBI data bank (BLAST program version 2.2.10; Altschul et al. 1997) and the MATDB (Schoof et al. 2004), to find homologous genes that had been characterized in the literature.

Construction of sugarcane EST arrays

The cDNA clones were obtained from the Sugarcane EST Project (Vettore et al. 2003). Plasmid DNA was extracted and spotted on nylon Hybond-N membranes (Amersham Biosciences, Brazil) using a hand-held tool with a 96-pin printhead (V&P-Scientific, USA). Eight sets of high-density filters were produced, each one containing plasmidial DNA of the two longest cDNA clones of 86 *ScbZIP* SAS identified as described above.

Additionally, 96 ESTs representing putative house-keeping genes selected based upon their digital expression profile data (data not shown) were used as internal controls, and other 96 spots representing the empty plasmid vector (pSPORT1, Invitrogen, USA) were used in each filter as a control to assess nonspecific hybridization (Nogueira et al. 2003). Each EST was spotted at four positions (spots) on each filter to assess the reproducibility of spotting.

Probe preparation and hybridizations

Before cDNA probe hybridization, the high-density filters were hybridized with an oligonucleotide probe corresponding to the plasmid vector used in the cDNA libraries in order to estimate DNA variations on the spots. Probe preparation and hybridization were done as described by

Nogueira et al. (2003). The oligonucleotide probe was stripped with boiling SDS and the filters were further used with cDNA probes.

cDNA probes were produced as described by Schummer et al. (1999) with slight modifications. In brief, 30 µg of total RNA was reverse transcribed with Superscript II (Invitrogen, USA) using an oligo-dT18V (3 µM) primer, with 3,000 Ci mmol⁻¹ [α -³³P]dCTP and unlabeled dATP, dGTP, and dTTP (1 mM each) for 20 min at 42°C. Unlabeled dCTP was then added to a final concentration of 1 mM, and the reaction continued for another 40 min. The cDNA probes were purified using ProbeQuant G-50 microcolumns according to the manufacturer's instructions (Amersham Biosciences, Brazil). Hybridizations were done according to Nogueira et al. (2003). Digitized images of the hybridization signals were quantified using Array Vision software (Imaging Research, Canada). To ensure that no signal remained on the membranes after stripping, each cDNA array was exposed to imaging plates for 96 h and then scanned in a phosphorimager FLA3000-G (Fujifilm, Tokyo).

cDNA array data analysis

The median value of all spot intensities obtained with the oligonucleotide probe was determined. The coefficient of variation (CV) of these median values was used to assess fluctuations in the amount of DNA between replicate filters. Only filters with CV values lower than 10% were used for subsequent analysis. After cDNA probe hybridization, the mean signal plus 1.65 standard deviations from the spots containing the empty pSPORT vector was used as a threshold to discard genes with low expression intensities (Nogueira et al. 2003), including the putative house keeping genes. These spots produced a very weak hybridization signal against the background, probably because of some degree of cross hybridization between the sugarcane cDNA and the vector sequences. Although the intensity of this background signal was very low, the mean signal plus 1.65 standard deviations from these spots was used as a threshold value to discard genes with low expression intensities after cDNA hybridization, as described by Nogueira et al. (2003). Thus, only those sugarcane ESTs displaying signal intensities above the threshold value were considered for further data analysis. In addition, to reduce the variation among replicate filters caused by differences in the experimental conditions, the average of all signal intensities obtained with the cDNA probe from each filter was set to 1 (Nogueira et al. 2003; Schummer et al. 1999). The logarithm values of the normalized signals (log₂signals) was used, since they are supposed to have a normal distribution.

In the study of gene expression during sugarcane development, the median signal of a subset of the housekeeping genes in each filter was used as a second normalization factor, because most of them were found to change their expression (data not shown). cDNA array hybridization was performed with RNA extracted 3, 4, 14, and 22 days after planting. The signal detected in the four spots of each gene on the nylon array was used in the statistical analysis. The Student's *t* test was performed using the log₂ of the signals of the four spots of each gene on each filter. Additionally, the ISER test (Drummond et al. 2005) was applied using the mean normalized signal of the four spots of each gene. In both cases, a 95% confidence level was used. The samples collected 4, 14 and 22 days after planting were compared to the sample collected 3 days after planting. The genes differentially expressed in at least two of the three samples by both tests were considered as having differential expression.

For the ABA and MeJA experiments, the median signal of all the housekeeping genes was used as normalization factor. In each comparison, *t*-tests were applied taking all log₂ signals of each gene from both replicates as input, and ISER were applied separately in each replicate hybridization. For each treatment, all genes that were selected as differentially expressed by ISER in both replicate hybridizations or selected by the *t*-test and ISER in one of the replicate hybridizations were considered as having true differential expression.

RNA-gel blot analysis

Ten micrograms of total RNA were electrophoresed on a 1% (w/v) agarose gel containing formaldehyde and transferred to a Hybond-N+filter (Amersham Biosciences, Brazil) as described by Sambrook et al. (1989). The filters were hybridized with the cDNA inserts of that up- or down-regulated ESTs labeled with α -³²P dCTP and hybridization was done at 42°C. The blots were then washed at high stringency and exposed to imaging plates. Digitized images of the RNA-blot hybridization signals were quantified using the Image Gauge software (Fujifilm, Japan).

Results

Data mining of bZIPs in the SUCEST database

The ordered non-redundant set of *Arabidopsis* bZIP transcriptional factors (Jakoby et al. 2002) was used to screen and classify the sugarcane assembled sequences (SAS) coding for bZIPs from the SUCEST project

(Vettore et al. 2003; Table 1S). To avoid the possibility of missing sugarcane bZIPs that were not found in the SUCEST project, searches were made in the DFCI *S. officinarum* Gene Index. This analysis showed that all the Gene Index TC (tentative consensus) that showed similarity with the AtbZIPs set were composed of ESTs from the SUCEST project. Each group of AtbZIPs showed a similar basic region and additional conserved motifs, characteristic of the members of each group (Jakoby et al. 2002). In order to be classified and annotated as a bZIP, the sugarcane SAS must have the characteristic domains shared by the AtbZIP groups as a result of the blastp results from the NCBI data bank (Altschul et al. 1997) and the MATDB (Schoof et al. 2004), as shown in Fig. 1. Eighty-six putative sugarcane bZIPs were found and classified in the various *Arabidopsis* bZIP groups (Jakoby et al. 2002). This number is very close to the number of bZIPs from *Arabidopsis* (76–81; for review see Riechmann et al. 2000; Jakoby et al. 2002) and the 94 putative bZIP genes found in the rice genome (Xiong et al. 2005). We also used the set of bZIPs discovered by Xiong et al. (2005) as baits, a strategy similar to that used for the *Arabidopsis* set of bZIPs (Jakoby et al. 2002), but no additional bZIPs were found in the sugarcane databases.

Sugarcane bZIP genes modulated during development

Six sugarcane bZIP genes showed altered expression during sugarcane plantlet development, five being up-regulated and one down-regulated (Table 1). The expression profile of these genes was further confirmed with RNA from independent biological replicates using RNA-blot analysis (Fig. 2a). To get a further understanding of the

Table 1 Sugarcane bZIPs identified by data mining in the SUCEST database expressed during development and regulated by ABA and MEJA

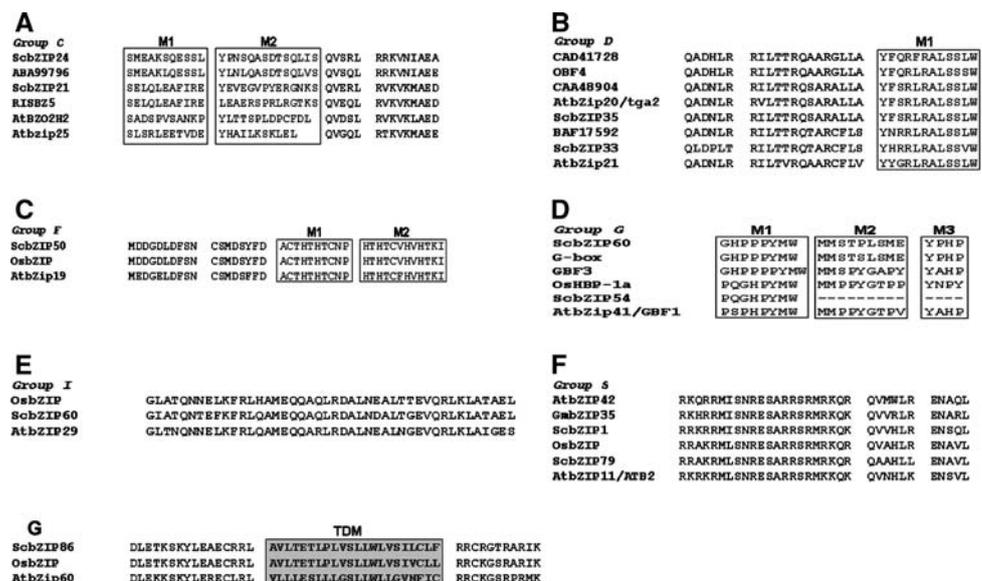
SAS ID	Name	Best hit (Ac. number)	Description	Group ^a
<i>Development</i>				
SCEQRT3020F04	ScbZIP33	BAF17592	Os bZIP	D
SCEZLB1013F09	ScbZIP35	CAA48904	OBF3.2	D
SCCART1001E06	ScbZIP54	ABF94876	OsHBP-1a	G
SCEZLR1031F04	ScbZIP60	AAA80169	mGBF1	G
SCJLRT1019E07	ScbZIP86	BAD35377	Os bZIP	–
SCSGAM1095E12 ^b	ScbZIP1	ABI34640	GmbZIP35	S
<i>ABA regulated</i>				
SCCART1003G04	ScbZIP29	CAD41728	Os bZIP	D
SCRURT2012D03	ScbZIP31	CAD41728	Os bZIP	D
SCJFLR1035B10	ScbZIP79	BAF07712	Os bZIP	S
SCCCCL4005C09	ScbZIP21	BAB39175	RISBZ5	C
SCMCST1051A01	ScbZIP24	ABA99796	Os bZIP	C
SCCCAM1003G07	ScbZIP70	AAM19114	Os bZIP	I
<i>MeJA regulated</i>				
SCJFLR1035G11	ScbZIP82	CAA44607	Ocsbf1	S
SCEPAM1053B09	ScbZIP50	BAD53586	Os bZIP	F

^a According to Fig. 1

^b This SAS has been characterized in more detail (Schlögl et al. 2004)

involvement of bZIPs in other aspects of sugarcane development, we also evaluated transcript accumulation in different tissues. Figure 2b shows the results from RNA blot analysis in leaves, flowers, stems and roots. *ScbZIP33* were up-regulated during development and was also expressed in stems. *ScbZIP1* was also up-regulated and it was expressed only in lateral bud and flowers. *ScbZIP35*

Fig. 1 Conserved plant bZIP motifs. The squares indicate the motifs identified by Jakoby et al. (2002). M1 motif 1, M2 motif 2, M3 motif 3, and TDM transmembrane domain (Iwata and Koizumi 2005). a–g representation ScbZIP groups that were modulated during bud development and phytohormone treatments. Other domains shown in the figure are suggested in this work



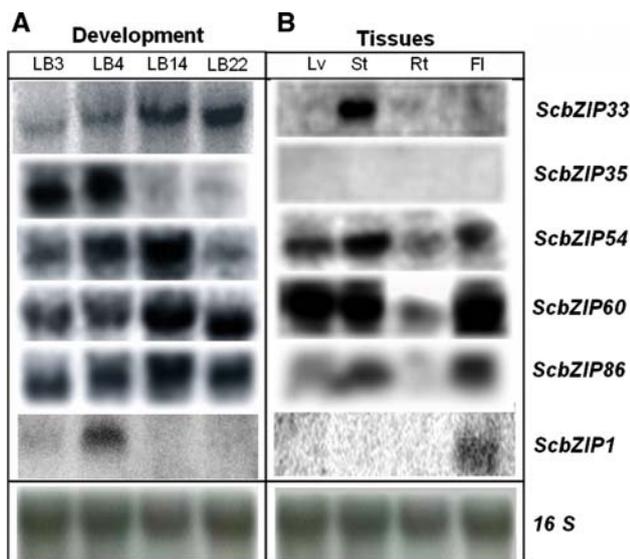


Fig. 2 Expression profile of sugarcane *bZIP* genes. **a** RNA gel blots showing gene expression during sugarcane early development. *LB3*–*22* Days of lateral bud development. **b** RNA gel blots showing gene expression in different sugarcane tissues. *Lv* leaves, *St* stem, *Rt* roots and *Fl* flowers. In **a** and **b**, each *lane* was loaded with 10 μ g of total RNA. The 16S hybridization signal is shown *below* the hybridization signal to confirm equal loading

was expressed in lateral buds and was down-regulated during bud germination, showing high levels of expression at three and four days after planting. *ScbZIP54*, *ScbZIP60*, and *ScbZIP86* were up-regulated and had similarly high levels of expression in all stages of plantlets development, although the level of the transcripts from *ScbZIP54* decreased at 22 days. *ScbZIP54* and *ScbZIP60* were expressed in all sugarcane organs with similar levels, except in roots, where lower levels were observed. *ScbZIP86* was highly expressed in flowers, stems and weakly in leaves.

Sugarcane *bZIP* genes modulated by ABA and MeJA

The cDNA arrays used previously were hybridized with RNA from plantlets treated with ABA and MeJA. The expression profiles of eight SASs was altered in response to ABA and MeJA treatments (Table 1). None of them corresponded to the six SASs modulated during plantlet development. Most of the ABA- and MeJA-responsive sugarcane *bZIPs* showed similarity with rice *bZIPs* that have not been characterized. RNA-blot analyses with four selected genes showed good agreement with the cDNA array data, confirming that these *bZIP* genes are responsive to ABA and MeJA (Fig. 3).

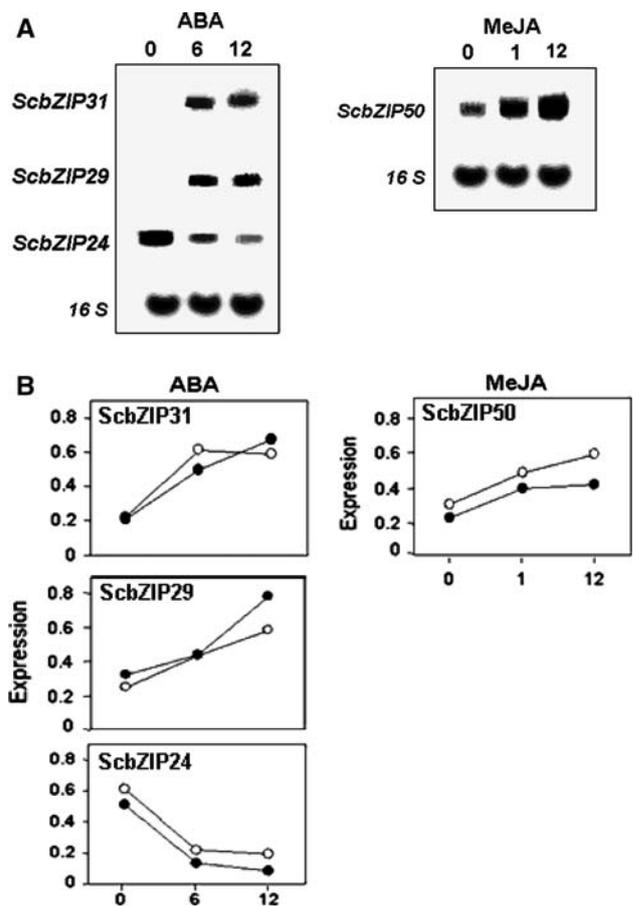


Fig. 3 Expression profile of ABA and MeJA modulated sugarcane *bZIP* assembled-sequences. **a** RNA gel blots hybridized with the genes indicated on the *top* of the gel pictures. Each *lane* was loaded with 10 μ g of total RNA isolated from sugarcane plants after 0, 6 and 12 h after ABA treatment, and after 0, 1 and 12 h after MeJA treatments. The signal corresponding to the 16S ribosomal probe is shown *below* the hybridization signal to confirm equal loading. **b** Expression levels observed in the RNA blots are shown in **a** (closed circles) and cDNA arrays (open circles)

Discussion

Data mining of ScbZIPs

A total of 86 sugarcane putative genes encoding *bZIP* factors (Table 1S) were identified in sugarcane using the ordered set of *Arabidopsis* *bZIP* factors described by Jakoby et al. (2002). The ScbZIPs presented at least one conserved motif shared by other plant *bZIPs* (Fig. 1) (Jakoby et al. 2002). The sequence similarity in the basic region and the conservation of other specific group motifs probably reflect similar functional characteristics that could help to infer the putative function of the sugarcane *bZIP* proteins.

bZIPs involved in sugarcane development

Sugarcane stalks contain one or more buds and these buds can develop into mature plants, since they have a miniature stem with primordial leaves and roots. The expression profiles of 86 sugarcane *bZIP* showed that five genes were up-regulated and one was down-regulated during the development of sugarcane plantlets (Fig. 2; Table 1). These genes were classified into different *Arabidopsis* bZIP groups, with two grouped in the D group (ScbZIP33 and 35), two in the G group (ScbZIP54 and 60), one belonged to Group S (ScbZIP1) and one (ScbZIP86) to the group that did not fit any of the other *Arabidopsis* groups (AtbZIP60, 62 and 72) (Jakoby et al. 2002).

ScbZIP33 best hit in the NCBI database is an unknown bZIP from rice, but it also presented high similarity (74%) to *Liguleless2* (Harper and Freeling 1996). This maize gene encodes a bZIP transcription factor that is expressed ubiquitously throughout the leaf primordia development and that participates in establishing the blade/sheath boundary of the leaf (Harper and Freeling 1996). Moreover, it is also involved in the transition from vegetative to the reproductive shoot apex (Walsh and Freeling 1999). The expression of *ScbZIP33* was higher during the initial phase of leaf development, and in addition it presents conserved motifs (Fig. 1), suggesting a role similar to the *Liguleless2* gene during leaf primordial development. *ScbZIP33* was also expressed in the sugarcane stem (Fig. 2), indicating that this bZIP might also be involved in other functions yet to be elucidated.

ScbZIP35 was expressed in the early stages of development and was detected only in the lateral buds (Fig. 2). ScbZIP35 bZIP showed 97 and 95% of similarity with OBF3.2 and OBF3.1 from maize (Foley et al. 1993), respectively and 92% to HBP-1b from wheat (Mikami et al. 1994). OBFs and HBP have highly conserved basic regions, and similar DNA-binding properties (Foley et al. 1993). HBP-1b specifically binds to the ACGT core sequences in the promoter region of the histone genes (Hex box, GGTGACGTGGC). These data indicate that ScbZIP35 might act controlling the expression of sugarcane histone genes that are expressed in lateral buds during sugarcane plantlet development. Interestingly, both ScbZIP33 and ScbZIP35 belong to Group D, which contains genes that participate in plant development. The bZIP proteins from D group that are involved in development are PAN, which controls floral organ in *Arabidopsis* (Chuang et al. 1999) and *Liguleless2* (Harper and Freeling 1996; Walsh and Freeling 1999).

ScbZIP54 was also up-regulated and presented the highest expression in 2-week-old sugarcane plantlets, then dropped after 3 weeks of plant development. It was ubiquitously expressed in all evaluated sugarcane tissues

indicating that this SASs could participate in other processes in sugarcane (Fig. 2). ScbZIP54 has high sequence similarity to HBP-1a, a wheat bZIP involved in the transcriptional regulation of histone genes in a cell cycle-dependent way (Tabata et al. 1991). These results suggest that ScbZIP54 might act regulating histone genes during sugarcane development.

ScbZIP1 belongs to group S and this is the largest bZIP group in *Arabidopsis* but only *ATBZIP11/ATB2* has been analyzed in detail. As proposed for ATB2, several group S bZIPs might thus be involved in balancing carbohydrate demand and supply. However, data derived from monocot and dicot species suggest that homologues of group S bZIPs are also transcriptionally activated after stress treatment or are specifically expressed in defined parts of the flower (Jakoby et al. 2002). *ScbZIP1* is expressed in lateral bud and flower and can participate in the flower and bud development.

ScbZIP60 showed higher levels of expression at 2 and 3 weeks after planting and was expressed in all organs evaluated (Fig. 2). This sugarcane bZIP shares sequence similarity to the maize GBF1 (Fig. 1), which is induced by hypoxia and seems to be involved in the activation of *Adh1* (de Vetten and Ferl 1995). These findings indicate that ScbZIP60 is involved in the late stages of bud germination and it might also participate in other stress responses. ScbZIP54 and ScbZIP60 belong to group G, which is composed by proteins that bind to G-box promoter sequences (ACGT core) and are supposed to regulate light responsive genes (Armstrong et al. 1992). Other GBFs-like from this group (ROM1 and 2) might regulate genes encoding storage proteins during seed maturation (Chern et al. 1996). The conservation of the group motifs (1, 2 and 3) and in the amino acid sequences in the basic region (Fig. 1d) highlights the putative roles of these sugarcane TFs in the regulation of light-induced genes from sugarcane.

ScbZIP86 was expressed in all stages of the sugarcane development, with high levels 14 days after planting. This sugarcane bZIP showed similarity with a rice bZIP with unknown function. ScbZIP86 showed high sequence identity and the presence of a putative transmembrane domain (TMD) with AtbZIP60 (Fig. 1g), which modulates the expression of genes involved in protein quality control in the endoplasmic reticulum from *Arabidopsis* (Iwata and Koizumi 2005). This process has been implicated in pathogen response, seed development and normal growth conditions (Vitale and Ceriotti 2004; Iwata and Koizumi 2005). *AtbZIP60* was induced by agents that induce the endoplasmic reticulum (ER) stress response. AtbZIP60 did not fit in any other *Arabidopsis* group, and Jakoby et al. (2002) did not show any putative domain in this group, but here we showed that there are some other plant bZIPs that

present similar motifs to AtbZIP60, including ScbZIP86 and a rice bZIP (Fig. 1g). Our results indicate that ScbZIP86 are involved in seedling, stem and flower development during sugarcane growing and could regulate gene expression by a mechanism similar to the observed for AtbZIP60.

It is worth to mention that the number of *bZIP* modulated during sugarcane development may be underestimated because most of the transcriptional factors presented a very low expression level and could be lost during the data analysis, or they could be expressed at different conditions than those analyzed here. In the same way, the potential for cross-hybridization has to be considered in studies with gene families using cDNA arrays containing ESTs. Girke et al. (2000) performed a series of hybridizations with fragments of the *FAD2* gene having the same length but different nucleotide identities. They found that decreasing identity to 90% reduced the hybridization signal to 50%, while a further decrease to 80% almost abolished the signal. In the case of the *ScbZIPs* modulated during the development, we only found high level of sequence identity (93%) between *ScbZIP54* and *ScbZIP55*. While *ScbZIP54* passed the statistical tests, *ScbZIP55* signal in the different spots on the nylon cDNA arrays showed a high variation and the gene was not selected by our statistical approach. This variation probably reflects differences intrinsic to the technique, such as the amount of DNA of each gene on the nylon arrays. There was only another case of sequence similarity above 80% among the *bZIPs* modulated by hormones and it will be discussed below.

Hormonal control of *bZIP* expression

Many aspects of plant development and stress responses are mediated by phytohormones such as ABA and MeJA (Cheong and Choi 2003; Devoto and Turner 2003; Himmelbach et al. 2003). Transcription factors play a key role in mediating the action of these phytohormones (Himmelbach et al. 2003). In sugarcane, eight *bZIP* genes were modulated by ABA or MeJA treatments. ABA induced the expression of two genes and repressed other four, while MeJA induced two genes (Fig. 3; Table 1). The six *bZIPs* encoded by genes modulated by ABA belong to different groups: two belong to group C (*ScbZIP21* and *ScbZIP24*), two to group D (*ScbZIP29* and *ScbZIP31*), one to group I (*ScbZIP70*) and one to group S (*ScbZIP79*). The two genes induced by MEJA encode proteins that belong to group F (*ScbZIP50*) and S (*ScbZIP82*).

ScbZIP29 and *SbZIP31* were up-regulated by ABA (Table 1; Fig. 3), both had as best hits in the NCBI an unknown *OsbZIP* and the *AtbZIP57* (OBF4/TGA4) protein. These *bZIPs* belong to D group and members from this

group are involved in defense against pathogens. Salicylic acid (SA) modulates expression of PR-genes (pathogen related genes), and TGA-like factors regulate the PR-expression genes by binding to *as-1 cis* elements (Zhou et al. 2000). *AtbZIP57* (TGA4/OBF4) interacts with other factors involved in ethylene response and members of this group might act integrating different systemic signals during stress response (Singh et al. 2002; Knight and Knight 2001). In this sense, *ScbZIP29* and *ScbZIP31* could participate in the signaling integration of stress in sugarcane.

ScbZIP21 and *ScbZIP24* were down-regulated by ABA (Fig. 3) and have similarity to *RISBZ5* (Onodera et al. 2001) and to an uncharacterized *bZIP* from rice, respectively. *RISBZ5* was found expressed in late stages of rice seed developing (Onodera et al. 2001). The *ScbZIP21* best hit at MATDB was of BZO2H2/AtbZIP9, a *bZIP* from the *Arabidopsis* C group. This group is supposed to be involved in seed-specific expression. *ScbZIP24* is very close to *AtbZIP25*, a member from group C that was recently shown to be involved in seed-specific gene regulation (Lara et al. 2003). We can not rule out that *ScbZIP21* and *ScbZIP24* could also modulate seed-specific gene expression since this organ was not evaluated in this work, but there are evidences that some *bZIPs* TFs are modulate by ABA during seed maturation (Finkelstein and Lynch 2000).

ScbZIP70 and *ScbZIP79* were both down-regulated by ABA (Table 1). *ScbZIP70* has similarity with an unknown *bZIP* from rice and *ScbZIP79* is similar to ATB2/AtbZIP11 (Rook et al. 1998). These two *ScbZIP* belong to group I and S, respectively. Members of group I are suggested to be involved in vascular development and regulate genes from gibberellin biosynthesis (Jakoby et al. 2002). Group S is composed by plant *bZIPs* that might be involved in carbohydrate balance and supply and direct evidences came from de ATB2/AtbZIP11 analysis (Rook et al. 1998). Gibberellins biosynthesis and carbohydrates dynamics are known to be involved in the control of several aspects of plant development (Swain and Singh 2005; Rolland et al. 2006), and our findings are in line with the role of *bZIPs* in sugarcane development.

ScbZIP82 was up-regulated by MEJA (Table 1) and shows similarity (78%) to a maize *bZIP*, OCSBF1, which probably is involved in maize leaf development (Singh et al. 1990). This *bZIP* belongs to the group S, which contains genes that are also involved in stress response (Martínez-García et al. 1998), opening the possibility that *bZIP82* might be involved in sugarcane stress responses mediated by MEJA. *ScbZIP82* has 97% identity to *ScbZIP80*, which also would be expected to be identified in our experiments, because this level of identity would favor cross-hybridization in the cDNA arrays. However, *ScbZIP80* showed high variation in the signal detected in

the nylon arrays, probably due to technical variations, and was not selected by the statistical tests. In this sense, we cannot discard that this bZIP is also modulated by MEJA.

MEJA also up-regulated *ScbZIP50*, which belongs to the F group. *ScbZIP50* is the first characterized bZIP in this group, giving a first insight in the putative role of the other bZIPs from the F group: the regulation of stress responses modulated by MEJA.

A model for the action of bZIPs and other ABA mediators in the regulation of gene expression induced by ABA involves ABA-activated kinase proteins (Nieva et al. 2005), such as the wheat pKABA1 (Droger-Laser et al. 1997; Johnson et al. 2002). This kinase activates or stabilizes bZIPs by phosphorylation (e.g., ABI5 and related genes), resulting in ABA-regulated gene expression through binding of the bZIP to the ABA responsive elements (ABRE) in the gene promoters. However, there are evidences that other *cis*-elements are necessary to regulate gene expression by ABA (e.g. DRE, drought responsive elements). Some works have shown that heterodimerization of bZIP and other transcriptional factors occur frequently. The rice TRAB1 protein, a homolog to ABI5, was shown to interact with OsVP1 activating gene expression induced by ABA (Hobo et al. 1999). OsVP1 was shown to interact with ABI5-like bZIPs altering global gene expression patterns through ABA gene modulation (Suzuki et al. 2003). We can speculate that the sugarcane bZIPs induced/repressed by ABA in our experiments could respond to ABA through an ABA-dependent pathway (Fig. 4). Probably the signaling mediated by MEJA could have a model similar to ABA, where kinases are induced by MEJA and phosphorylation active/repress bZIPs and other transcription factors that lead to the modulation of gene expression. Jasmonate (JA) is an early signaling molecule in rice and some works have shown induced-mitogen-activated protein kinase (MAPK) cascade by applied JA. MAPK cascade is a signaling pathway that link in various ways between upstream receptors and downstream targets (Jonak et al. 2002).

Our results indicate that gene regulation by ABA and MeJA in sugarcane involves the action of many groups of bZIPs. Several studies in other species show that these phytohormones control many aspects not only in plant development but also stress responses such as drought and pathogen attack.

In summary, we used mRNA profiles generated by DNA-arrays and GeneBank/MATDB searches to deduce functions of sugarcane ESTs encoding putative bZIP transcription factors. We have identified a number of sugarcane bZIPs that are expressed in particular organs, at particular developmental stages and modulated by ABA and MeJA. Our findings highlight the participation of new bZIPs in the regulation of sugarcane development and their

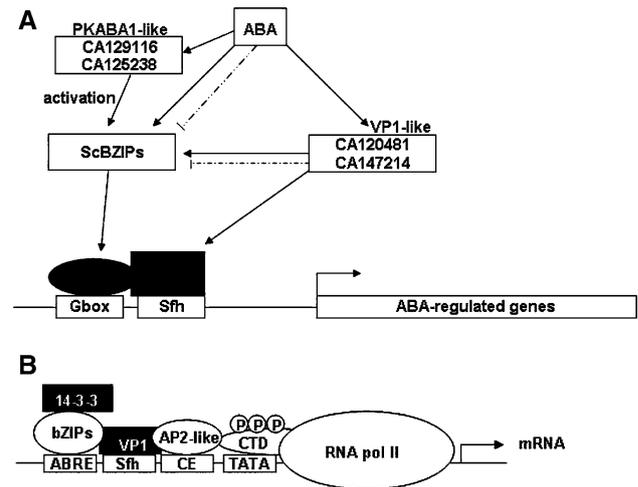


Fig. 4 Putative feed-forward regulation of ABA signaling in sugarcane. ABA could induce/repress *ScbZIPs* that are stabilized or activated (e.g., ABI5) by protein kinases also induced by ABA (e.g., PKABA1). Activated bZIPs could then regulate the gene expression by binding to G-box (e.g., ABRE) elements present in the promoter region of ABA-regulated genes. We can also speculate the induction of sugarcane VP1-like proteins that, in turn, would induce/repress bZIPs. bZIPs, via heterodimerization, modulate gene expression by binding to G-box and Sfh promoter elements. Combination of regulatory elements could positively or negatively modulate gene expression. *Black line* shows ABA induction and *dot lines* gene repression. The access number of sugarcane PKABA-like and VP1-like genes are indicated in the *rectangles*. Adapted from Suzuki et al. (2003)

putative role in the activation of genes in response to ABA and MeJA. Further functional studies of *ScbZIPs* in sugarcane will be needed to establish the physiological role of these proteins. Major goals for future research could include overexpression/gene silencing of some bZIPs in sugarcane transgenic plants.

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