The FBH family of bHLH transcription factors controls ACC synthase expression in sugarcane

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Abstract

Ethylene is a phytohormone involved in the regulation of several aspects of plant development and in responses to biotic and abiotic stress. The effects of exogenous application of ethylene to sugarcane plants are well characterized as growth inhibition of immature internodes and stimulation of sucrose accumulation. However, the molecular network underlying the control of ethylene biosynthesis in sugarcane remains largely unknown. The chemical reaction catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is an important rate-limiting step that regulates ethylene production in plants. In this work, using a yeast one-hybrid approach, we identified three basic helix-loop-helix (bHLH) transcription factors, homologs of Arabidopsis FBH (FLOWERING BHLH), that bind to the promoter of ScACS2 (Sugarcane ACS2), a sugarcane type 3 ACS isozyme gene. Protein–protein interaction assays showed that sugarcane FBH1 (ScFBH1), ScFBH2, and ScFBH3 form homo- and heterodimers in the nucleus. Gene expression analysis revealed that ScFBHs and ScACS2 transcripts are more abundant in maturing internodes during afternoon and night. In addition, Arabidopsis functional analysis demonstrated that FBH controls ethylene production by regulating transcript levels of ACS7, a homolog of ScACS2. These results indicate that ScFBHs transcriptionally regulate ethylene biosynthesis in maturing internodes of sugarcane.

Keywords: ACC synthase, bHLH, ethylene biosynthesis, FBH, sucrose, sugarcane maturation, transcriptional regulation.

Introduction

Sugarcane (Saccharum spp.) is a monocot that accumulates large quantities of sucrose in the culm, reaching concentrations of up to 650 mM (Welbaum and Meinzer, 1990) or 18% of fresh weight (Inman-Bamber et al., 2011). Sugarcane culm maturation is a continuous process of sucrose accumulation that forms a concentration gradient from the younger and immature internodes at the top to the older and mature internodes at the base (Moore, 1995; Rae et al., 2005). Exogenous application of the plant hormone ethylene has a dramatic effect on sugarcane maturation by inducing sucrose accumulation in young internodes and inhibiting their growth (Chong et al., 2010; Cunha et al., 2017). Transcriptomic analysis has also identified ethylene as a potential regulator of sugar storage in the internodes (Papini-Terzi et al., 2009; Cunha et al., 2017).

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Since sucrose is the main product of sugarcane, understanding the molecular network involved in ethylene biosynthesis that affects sucrose accumulation should be very informative.

Ethylene synthesis starts with the formation of S-adenosylmethionine (SAM) from methionine through the action of SAM synthase. SAM is the substrate of ACC synthase (ACS) in the formation of 1-aminocyclopropane-1-carboxylic acid (ACC), which is converted into ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). The reactions catalyzed by ACS and ACO are the two rate-limiting steps of ethylene synthesis (Yang and Hoffman, 1984; Alexander and Grierson, 2002; Van de Poel and Van Der Straeten, 2014). ACS is encoded by a multigene family in all plants investigated (Harpaz-Saad et al., 2012). Arabidopsis has eight functional (ACS2, 4–9, and 11) and one non-functional (ACS1) ACS enzymes, which can form 25 different combinations of functional homo- and heterodimers (Tsuchisaka et al., 2009). The ACS enzymes share high similarities in their catalytic domains and are classified into three groups based on the presence of regulatory features in their non-catalytic C-terminal regions. The type 1 ACS has a calcium-dependent protein kinase (CDPK) and three mitogen-activated protein kinase (MAPK) phosphorylation sites; the type 2 possesses only a single CDPK phosphorylation site; and the type 3 does not have any predicted phosphorylation sites (Harpaz-Saad et al., 2012; Booker and DeLong, 2015). ACS is a cytosolic enzyme with a short half-life (Bleecker and Theologis, 2004). Ethylene itself controls ACS2 expression during tomato fruit ripening (Barry et al., 2000).

Transcriptional and post-translational regulation of ACS is a key mechanism in controlling ethylene levels. Different ACS members have unique and overlapping spatiotemporal expression patterns, which are modulated by hormones, developmental stages, and biotic and abiotic stresses (Tsuchisaka and Theologis, 2004; Harpaz-Saad et al., 2012). Brassinosteroids and cytokinin promote ethylene synthesis by inducing ACS expression and regulating protein stability (Yi et al., 1999; Hansen et al., 2009). Auxin induces the expression of seven functional ACS genes (ACS2, 4–8, and 11) and affects their spatial expression patterns in Arabidopsis (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004). Ethylene itself controls ACS and ACO expression during tomato fruit ripening (Barry et al., 2000).

Although the developmental and environmental cues that lead to changes in ethylene production are well described, the list of transcriptional regulators that function downstream of these stimuli is still somewhat limited. For instance, the MADS-box transcription factors MaMADS5 and RIN regulate MaACS1 and LeACS2 during fruit ripening in banana and tomato, respectively (Ito et al., 2008; Martel et al., 2011; Roy Choudhury et al., 2012). The tomato and tobacco ethylene response factors LeERF2/TERF2 are positive regulators in the feedback loop of ethylene production (Zhang et al., 2009). The Arabidopsis WRKY33 activates ACS2 and ACS6 expression in response to pathogen invasion (Li et al., 2012).

Recently, Yin et al. (2015) reported PhFBH4, a member of subgroup 16 of the bHLH transcription factor family, as a regulator of PhACS1 at flower senescence in petunia. Subgroup 16 of the bHLH family in Arabidopsis comprises FLOWERING BHLH 1 (FBH1), FBH2, FBH3, and FBH4, a group of transcription factors that are preferentially expressed in vascular tissues, bind to the E-box cis-element (CANNTG) to activate CONSTANS (CO) transcription, and consequently promote flowering. FBH1 was also implicated as a mediator of circadian clock responses to temperature changes by regulating CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) expression (Nagel et al., 2014). Transcription factors controlling ethylene synthesis genes in monocots, including sugarcane, are still largely unknown.

Modern sugarcane cultivars are interspecific hybrids between *Saccharum officinarum* and *Saccharum spontaneum* with highly complex polyploid and aneuploid genomes (Grivet and Arruda, 2002; Souza et al., 2011). In this context, functional genomics studies are challenging and therefore scarce. Although some transcriptomic and proteomic studies have been performed, the molecular networks controlling physiological processes in sugarcane, such as ethylene synthesis, are not well characterized. In this study, we aimed to identify transcription factors that control the expression of ScACS2, a sugarcane type 3 ACS isozyme. We identified three bHLH transcription factors (ScFBH1, ScFBH2, and ScFBH3), which are phylogenetically related to Arabidopsis FBHs, dimerize in cell nucleus, and bind to the ScACS2 promoter, activating its expression. We also found that ScACS2 and ScFBHs are more abundantly expressed in maturing internodes during the afternoon and night. Functional studies using Arabidopsis as a model support our hypothesis of which FBHs are transcriptional regulators of ACS2 in sugarcane. Our results provide the molecular link between the circadian clock and internode maturation through the regulation of ScACS2 in sugarcane.

### Materials and methods

#### Phylogenetic analysis

To investigate the phylogenetic relationship of sugarcane ScACS2 (NCBI accession ADZ96244.1), we retrieved ACS-related protein sequences from sorghum (*Sorghum bicolor*), maize (*Zea mays*), foxtail millet (*Setaria italica*), and switchgrass (*Panicum virgatum*) databases available on Phytozome (www.phytozome.net) and performed the alignment using the WebPRANK global alignment tool (Löytynoja and Goldman, 2010) with default parameters. Arabidopsis and tomato ACS were included to guide the classification of subgroups (Supplementary
Table S1 at JXB online). The phylogenetic tree was constructed in MEGA6 (Tamura et al., 2013) software using the neighbor-joining method with 1000 bootstrap replicates. Orthologous groups were deduced by a bidirectional best hit approach. To classify sugarcane FBH proteins, the bHLH domains of Arabidopsis FBH1 (At1g35460), FBH2 (At4g91980), FBH3 (At1g51140), FBH4 (At2g52280), ArthbHLH128 (At1g05805), ArthbHLH129 (At2g31430), AMS (At2g16910), GL3 (At5g41315), ICE1 (At3g26744), PIF3 (At1g9530), PIF4 (At2g34310), SPT (At4g36930), and TT8 (At4g09820), and sorghum Sb02g028300, Sb03g04286, and Sb07g027810 proteins were aligned, and the phylogenetic tree was constructed as described.

Promoter isolation and validation
To isolate the ScACS2 gene promoter (accession MF383332) without a sugarcane reference genome, we aligned the 1.5 kb promoter region of sorghum and maize ScACS2 putative orthologous genes (locus ID Sb03g03070 and GRMZM2G163015, respectively) and identified conserved regions to design a forward primer for PCR amplification. The reverse primer was designed inside the conserved regions to design a forward primer for PCR amplification. To isolate the ScACS2 promoter sequence was cloned into the pHGWFS7 vector (Karimi et al., 2002) and transformed into in vitro grown sugarcane leaves and calli by particle bombardment following previously defined parameters (Gallo-Meagher and Irvine, 1993). Histochemical GUS staining was performed according to the method described by Jefferson et al. (1987). The samples were then transferred to ethanol 70% to remove chlorophyll.

Promoter in silico analysis
To identify putative conserved regulatory motifs in the ScACS2 promoter, its sequence was compared with sequences of orthologous promoters of Sorghum bicolor (Sb03g03070), Zea mays (GRMZM2G163015), Setaria italica (S001369m), and Panicum virgatum (Pavirv00004519m). Promoter sequences were retrieved by selecting a 1 kb region upstream of the translational start site using Phytozome genomic data and alignments were performed by using BlastN, with the recommended parameters described by Kaplinsky et al. (2002), LAGAN (Brudno et al., 2003), and MULAN (Ovcharenko et al., 2005) algorithms. Conserved promoter elements present in at least three different species, including sugarcane, were considered in the search for putative transcription factor binding sites (TFBS). We identified putative TFBS using the PlantPAN 2.0 database (Chow et al., 2016), and the resulting cis-element sequences were manually double-checked against original references; elements containing inconsistencies were discarded.

Yeast one-hybrid assay
Yeast one-hybrid (Y1H) assays were performed using the Matchmaker Gold Yeast One-Hybrid System (Clontech, USA). The 1 kb region of the ScACS2 promoter (upstream from the translational start site) was cloned into the pAbAi vector and transformed into the yeast Y1HGold strain to generate the bait reporter strain. The minimal inhibitory concentration of α-amanitin (AbaA) and auto-activation tests were performed. To produce cDNA libraries, total RNA was extracted from leaf +1 (the highest unfolded leaf with a visible dewlap) and culm of sugarcane plants (cultivar RB885156) using an RNeasy Plant Mini kit (Qiagen, USA). Each cDNA library was prepared with a pool of RNA samples from five different plants according to the manufacturer’s instructions (Clontech, USA). Both libraries were screened, and after three rounds of re-streaking each colony, the positive clones were sequenced and the putative function of the proteins was determined by BLAST searches on the NCBI database.

Y1H confirmation
Complete coding sequences of ScFBH1 (accession MF383333), ScFBH2 (accession MF383334), and ScFBH3 (accession MF383335) were cloned into the NdeI and EcoRI sites of the pGADT7 vector fused in-frame with the Gal4 activation domain (AD). The ScFBH1 and ScFBH3 coding sequences were codon-optimized for yeast (Saccharomyces cerevisiae) usage (accessions MF383336 and MF383337). Plasmids were transformed into the yeast strain containing the ScACS2 promoter by the lithium acetate/PEG–mediated method (Gietz and Woods, 2000). Transformed yeast bait cells were plated on to SD/-Leu and SD/-Leu/AbA200 media and incubated for 5 days at 30 °C.

bHLH domain three-dimensional modeling
The three-dimensional homology models of the bHLH domain of Arabidopsis and sugarcane FBHs were generated with the SWISS-MODEL workspace (www.swissmodel.expasy.org; Biasini et al., 2014), based on sequence alignment with the 4ATK structure deposited on Protein Data Bank (www.rcsb.org). The quality of the homology models was assessed by QMEAN6 Z-scores (0 ≤ |Z-score| < 1) and a Ramachandran plot considering more than 90% of residues in the most favored regions. The models were superimposed and visualized using Chimera 1.11.2 software (www.rbvi.ucsf.edu/chimera; Pettersen et al., 2004).

Electrophoretic mobility shift assay
A recombinant glutathione S-transferase (GST)–fused ScFBH2 protein was generated using the full-length ScFBH2 coding sequence cloned into the pGEX4T-1 vector (GE Healthcare Life Sciences, USA). To induce the expression of the recombinant GST and GST-ScFBH2 proteins, 0.5 mM of isopropyl β-D-1-thiogalactopyranoside was added to each bacterial culture (at OD600 ~0.3). After incubation for 20 h at 16 °C, the cell culture was collected by centrifugation and resuspended in buffer containing 20 mM HEPES-KOH, pH 7.2, 80 mM KCl, 0.1 mM EDTA, 0.1 mM PMSE, 1 mM DTT, and Pierce Phosphatase Inhibitor Mini Tablets (Thermo Scientific, USA). After sonication, the whole extracts were collected by centrifugation and used for electrophoretic shift mobility assay (EMSA).

Yeast two-hybrid assay
Yeast two-hybrid (Y2H) assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA). The coding regions of ScFBH1, ScFBH2, and ScFBH3 were cloned into pGAD7T and pGBK7T for in-frame expression with the Gal4 AD and DNA-binding domain (BD), creating all combinations of baits and preys (primers are shown in Supplementary Table S2). Cloned genes had no transcriptional activation activity in yeast cells (data not shown). Bait and prey construct pairs were then co-transformed into the yeast strain Y2H-Gold (Gietz and Woods, 2000). Yeast cells were grown on double dropout medium (DDO: SD/-Leu/-Trp) and quadruple dropout (QDO: SD/-Leu/-Trp/-His/-Ade) for 5 days at 30 °C. We tested all nine possible interactions between the three ScFBHs.
Subcellular localization and bimolecular fluorescence complementation

ScFBH1, ScFBH2, and ScFBH3, previously cloned into the pENTR/D-TOPO or pDONR221 entry vectors (Thermo Fisher, USA), were cloned into the pSITE-3CA vector for analyzing their subcellular localization, and into the pSITE-cEYFP-C1 and pSITE-nEYFP-C1 vectors for bimolecular fluorescence complementation (BiFC) (Martin et al., 2009). The construct RFP:NhH2B (Chakrabarty et al., 2007) was used as a transformation control and nuclear localization reference. The plasmids were introduced into Agrobacterium tumefaciens strain GV3101 and after being grown overnight at 28 °C, cultures were suspended in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl2, and 200 μM acetosyringone) to an OD600 of 0.5. After 3 h incubation, the Agrobacterium suspension was infiltrated into Nicotiana benthamiana leaves. Three days after infiltration, yellow fluorescent protein (YFP) and red fluorescent protein (RFP) fluorescence was detected by a Leica SP5 confocal microscope using a previously described method (Martin et al., 2009). For BiFC analysis, we tested all possible combinations between the three ScFBHs, and we tested their interaction with GST protein as a negative control.

Transient luciferase reporter assay

The ScACS2 promoter (1 kb) was cloned into the pFLASH vector (Kubota et al., 2017) to generate a luciferase reporter plasmid. The ScFBHs constructs in the pSITE-3CA vector described above were used as effectors. The 35S promoter-driven Renilla luciferase plasmid was used as a control to measure the efficiency of transient transfection (Fenske et al., 2015). Transient transfection of N. benthamiana was performed as described above. Total soluble proteins were extracted from leaf discs and luciferase activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA). The luminescence was measured using a SpectraMax M3 plate reader. Data were presented as the means ±SE obtained from three biological replicates. Significant differences of means were assessed using Tukey's test (P<0.05).

RNA extraction and quantitative RT-PCR

To quantify mRNA levels of the sugarcane ScACS2 and ScFBHs genes, the leaf +1, internode 1 (immature), and internode 5 (maturing) were harvested from 9-month-old field-grown sugarcane plants (cultivar SP803280) in summer 2013. Three biological replicates of each organ were harvested at 2 h intervals, starting 30 min after dawn (5:30 am) for a total of 12 time points during the day. The plant material was ground in liquid nitrogen and 100 mg of tissue was used for total RNA preparation using Trizol (Life Technologies, USA). RNA samples were treated with DNase I (Life Technologies, USA) and purified with the RNeasy Plant Mini Kit (Qiagen, USA). The cDNA was synthesized from 5 μg of total RNA using the SuperScript III First Strand Synthesis System (Life Technologies, USA) and diluted 10-fold. Quantitative PCRs (qPCRs) were performed using the Fast SYBR Green PCR Master Mix (Life Technologies, USA) on a Fast 7500/7500 Real-Time PCR System (Life Technologies, USA). Each reaction contained 6 μl of Fast SYBR Green Master Mix, 3 μl of H2O, 2.4 μl of each gene-specific primer pair (10 μM), and 0.6 μl of 10-fold diluted cDNA. We included negative controls (water) to confirm the absence of any contaminant. The specificity and efficiency of the SFBH1, SFBH2, SFBH3, and ScACS2 primers (see Supplementary Table S2) were assessed by constructing serial dilution standard curves and by melting curve analysis. The sugarcane GAPDH gene was used as a reference (Iskandar et al., 2004). Each data value presented is the mean ±SE derived from three biological replicates, and the value of each replicate is the mean of two technically replicated qPCR reactions.

For the Arabidopsis experiment, plants overexpressing the Arabidopsis homologs FHB1 (35S:AtFHB1 #24–53) and FHB4 (35S:FHB4 #21–6), as well as the fhb quadruple mutant (35S:amiRFBH1–2, fhb2–1, fhb3–1, and 35S:amiRFBH4–3 #2–5), were previously obtained and characterized (Ito et al., 2012; Kimmonth-Schultze et al., 2016). Seedlings were grown on plates containing 1×Linsmaier and Skoog (LS; Caisson, USA) medium, pH 5.8, and 1% sucrose (w/v) under long-day (LD) conditions (16 h light/8 h dark, at 22 °C) for 2 weeks, and harvested at 3 h intervals from 1 h to 22 h after the onset of light. To quantify mRNA levels of the Arabidopsis ACS2, ACS4, ACS6, ACS7, and ACS8 genes, total RNA was isolated from seedlings using an illustration RNAprep Mini kit (GE Healthcare, USA) and treated with a Turbo DNA-free kit (Thermo Fisher, USA) to eliminate remaining DNA in the samples. To synthesize cDNA, 2 μg of total RNA was reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad, USA). cDNA was diluted 3-fold and 2 μl samples were used for qPCRs. The reactions were performed using a Bio-Rad MyQ real-time detection system. Buffer composition details can be found in Ito et al. (2012). Negative controls (water) were included. The ACS primer sequences used for qPCR were previously described (Li et al., 2012). Serine/threonine-protein phosphatase (PP2A) was used as an internal control for normalization (Hong et al., 2010). Each value presented was calculated as described above for the sugarcane experiment.

Arabidopsis hypocotyl and root length measurement

All hypocotyl and root measurement assays were performed with seedlings (Col-0, fhb quadruple mutant #2–5, 35S:FHB1 #24–53, and 35S:FHB4 #21–6) grown on 0.5% LS (Caisson, USA) medium containing 1% sucrose (w/v), 0.8% agar (Fisher, USA), pH 5.8, with 10 μM AgNO3 (PhytoTechnology, USA) or 100 μM AgNO3 (Sigma, USA). The plates were chilled at 4 °C in the dark for 3 days to synchronize germination and then moved to 22 °C under LD conditions for 10 days and DD (continuous dark) for 4 days in a vertical position. Light was provided by full-spectrum white fluorescent bulbs with a fluence rate of 60–90 μmol m–2 s–1. Hypocotyl and root length was measured using ImageJ software (Schneider et al., 2012). The data were analyzed by ANOVA followed by comparison of mean values using Tukey's test. Data are presented as mean ±SD.

Ethylene measurements

For ethylene measurements, four Arabidopsis seedlings of each genotype (Col-0, fhb quadruple mutant #2–5, 35S:FHB1 #24–53, and 35S:FHB4 #21–6) were grown in LD conditions on 0.5× Murashige and Skoog (Sigma, USA) medium containing 1% sucrose (w/v) and 0.8% agar (Fisher, USA), pH 5.8, inside flasks capped with rubber serum stoppers. After 2 weeks, the ethylene concentration was measured using a gas chromatograph equipped with a flame ionization detector (HP-6890) as described by Arraes et al. (2015). Each value reported represents the average of four biological replicates ±SD. The data were analyzed by ANOVA followed by comparison of mean values using Tukey's test. Data are presented as mean ±SD. The total ethylene production was expressed as μl h–1.

Results

The ScACS2 promoter is transcriptionally active and contains multiple transcription factor binding sites

As a first step to identify the transcription factors able to bind to the ScACS2 promoter, we confirmed the transcriptional activity of the promoter by transient transformation of a promScACS2::GUS construct into in vitro-grown sugarcane leaves and calli by particle bombardment. Histological staining showed several GUS foci on sugarcane leaves and calli transformed with the PUBI::GUS (positive control) and promScACS2::GUS constructs (Supplementary Fig. S1), confirming the transcriptional activity of the isolated ScACS2 promoter sequence. No GUS foci were observed on negative controls.

The ACS phylogenetic analysis revealed that ScACS2 clustered with Arabidopsis ACS7 and other putative type 3 ACSs.
from different species (Supplementary Fig. S2). This result is consistent with previous ACS phylogenies (Gallie and Young, 2004; Booker and DeLong, 2015). To identify conserved cis-elements present in the ScACS2 promoter, we compared its sequence with the promoter sequences of orthologous ACS genes from sorghum, maize, foxtail millet, and switchgrass identified by the phylogenetic analysis. Several conserved regions were found, suggesting the presence of common regulatory cis-elements on type 3 ACS gene promoters among different grasses (Supplementary Fig. S3). Within these conserved promoter sequences, we found putative TFBS functionally involved in physiological processes associated with ethylene. These TFBS are involved with plant development (root hair and endosperm-specific expression elements), hormone signaling (BZR1-binding site), response to light (GATA and SORLIP elements), and response to stresses such as dehydration (MYB2 binding site), hypoxia (ANAERO1 element), and pathogen attack (WRKY binding sites). We also identified putative bHLH transcription factors binding sites (E-boxes) and ARF and Golden2-like binding sites (Supplementary Table S3).

As we identified a transcriptionally active promoter sequence of the ScACS2 gene, we next aimed to identify potential transcription factors that bind to the sequence using Y1H screening. We prepared two Gal4 AD fused cDNA libraries derived from sugarcane leaf and culm to screen proteins that can bind to the 1 kb region of the ScACS2 promoter. Approximately 360,000 clones were screened and 11 different clones retained their ability to grow in selective media (-Leu/+AbA200) after being re-streaked for three generations (see Supplementary Table S4 for proteins identified in the screening). Not surprisingly, the Y1H screening identified proteins able to bind to the ScACS2 promoter, including proteins similar to ARF, Golden2-like, and three different bHLH domain-containing transcription factors. As these bHLHs shared high levels of similarity with Arabidopsis bHLHs named as FBHs, we named the three different sugarcane bHLH transcription factors ScFBH1, ScFBH2, and ScFBH3.

Next, we determined the subcellular localization of ScFBH proteins. Fluorescence signals from chimeric ScFBH proteins fused to YFP were analyzed by confocal microscopy in transiently transformed N. benthamiana epidermal cells (Fig. 1B). In all cases, strong YFP signals concentrated in the cell nucleus, as confirmed by the co-expression of N. benthamiana histone H2B fused to RFP. This result showed that ScFBH1, ScFBH2, and ScFBH3 were localized in the nucleus, supporting their role as transcription factors.

**ScFBHs are closely related to Arabidopsis FBHs**

Our phylogenetic analysis revealed that ScFBHs belong to subfamily 16 of the bHLH transcription factor family (according to the classification of Toledo-Ortiz et al., 2003), the same subfamily as the Arabidopsis FBHs (Fig. 2A). ScFBH1, ScFBH2, and ScFBH3 contain 361, 385, and 424 amino acids, respectively, and one bHLH domain on their C-terminal regions. Amino acid identities within the bHLH domains between sugarcane and Arabidopsis FBHs ranged from 72.5% to 96.1%. Superposition of three-dimensional models of the bHLH domains of sugarcane and Arabidopsis FBHs demonstrates structure and sequence conservation in the bHLH motifs involved in DNA binding (Fig. 2B), including Glu-13 and Arg-17 residues (Fig. 2C), which are crucial to the E-box binding (Fisher and Goding, 1992; Shimizu et al., 1997). Ito et al. (2012) determined that Arabidopsis FBHs bind to E-box (CANNTG) elements present on the CO promoter. The 1 kb region of the ScACS2 promoter contains six E-boxes (Fig. 2D). We confirmed the direct binding of ScFBH2 to the E-box elements present in the ScACS2 promoter by EMSA (Fig. 2E). A mutated version of the E-box used as a competitor did not disturb the ability of ScFBH2 to bind to the native sequence, as expected. The high conservation of the DNA-binding domain between Arabidopsis FBHs and ScFBHs, as well as the EMSA results, provide clear evidences that ScFBHs interact with the ScACS2 promoter by binding to E-box elements.

**ScFBHs form homo- and heterodimers to activate ScACS2 expression**

The bHLH transcription factors usually function as homodimers or heterodimers (Heim et al., 2003). Our three-dimensional homology modeling predicted the dimerization of ScFBHs (Fig. 2B). Therefore, we investigated the physical interaction between ScFBHs using the Y2H system. The ScFBH genes were cloned into the pGADT7 and pGBK7 vectors to generate translational fusions with the Gal4 AD and BD. Auto-activation of any BD constructs was tested before conducting the Y2H assays, and it was not detected (data not shown). The AD and BD vector constructs were co-transformed into yeast; all nine possible combinations between the hybrid proteins were tested. Yeast grew in all tested interactions and in the positive control, while no growth was observed in the negative control (Fig. 3A). Few colonies grew in plates transformed with AD-ScFBH3 fusion; we attribute this to the cell toxicity presented by this protein in bacteria and yeast cells (data not shown).
In order to confirm the Y2H observations, we performed a BiFC assay. The genes ScFBH1, ScFBH2, and ScFBH3 were cloned into pSITE-cEYFP-C1 (which contains a split eYFP C-terminal fragment) and pSITE-nEYFP-C1 (which contains a split eYFP N-terminal fragment) and transiently transformed into N. benthamiana epidermal cells by Agrobacterium infiltration. Again, in all nine possible combinations among the three tested chimeric proteins, we detected strong YFP fluorescence in the nucleus of transformed cells, where the nuclear reference RFP-NbH2B protein was also detected (Fig. 3B). No YFP signals were detected in cells co-expressing ScFBH1, ScFBH2, or ScFBH3 fused to the N-terminal fragment of YFP and GST fused to the C-terminal YFP fragment. The Y2H and BiFC results demonstrated that ScFBH1, ScFBH2, and ScFBH3...
form all possible homo- and heterodimer combinations in vivo and that such interactions specifically occur in the nucleus.

To investigate whether the binding of homo- and heterodimers of ScFBHs to the ScACS2 promoter is important for the transcriptional regulation of ScACS2, we utilized the ScACS2 promoter-controlled luciferase reporter system. When ScFBH1, ScFBH2, and ScFBH3 were transiently expressed in *N. benthamiana* epidermal cells, ScFBH2 significantly increased the activity of the luciferase reporter controlled by the ScACS2 promoter (Fig. 4). All heterodimer combinations of ScFBHs significantly induced ScACS2 promoter activity (Fig. 4). Together with the protein interaction assays, these results suggest that ScFBH proteins associate as dimers with the ScACS2 promoter to induce the transcription of ScACS2.

**ScFBHs and ScACS2 are expressed higher in maturing internodes**

Next, we measured the mRNA levels of sugarcane *ScFBH* and *ScACS2* in different plant organs throughout the day in order to analyze their spatiotemporal expression patterns. In sugarcane, the younger, immature internodes are located in the top part of the plant, whereas the oldest and mature internodes, with higher sucrose content, are close to the roots. Our qPCR analysis demonstrated that the expression patterns of both ScFBHs and ScACS2 overlap in sugarcane organs. Low levels of ScACS2 mRNA were detected in the leaf and in the immature internode, and the expression level was significantly higher in the maturing internode (Fig. 5A). We found similar results for *ScFBH1*, *ScFBH2*, and *ScFBH3*, that is, lower expression in the leaf and higher expression in the culm, reaching the highest levels in the maturing internode (Fig. 5A).

The overall daily expression patterns of *ScFBH* were similar throughout the day (Fig. 5B–D). In the leaf, *ScFBH* expression levels peaked 2.5 hours after dawn, then reduced during the daytime, and increased again during the night (Fig. 5B). In the internodes, the expression levels of *ScFBH* were low at dawn, and increased during the day (Fig. 5C and D). We observed the same pattern for *ScACS2* in maturing internode: its expression increased during the day, reaching the highest levels in the afternoon/evening (Fig. 5D).

**FBH overexpression in Arabidopsis induces typical ethylene-related phenotypes, increased ACS7 expression, and ethylene production**

As it is technically difficult to genetically study the function of genes in sugarcane, we decided to use Arabidopsis to assess whether FBH transcription factors regulate ACS gene transcription. We hypothesized that if Arabidopsis FBHs are also involved in *ACS* transcriptional regulation, overexpression and loss-of-function of *FBH* would change the expression levels of the *ACS* gene and potentially ethylene production. To assess this possibility, we first analyzed the hypocotyl and root length of *FBH1* and *FBH4* overexpressors and of the *fbh* quadruple mutant. Ethylene and ACC exposure can stimulate hypocotyl elongation (Smalle et al., 1997) and inhibit root growth (Ruzicka et al., 2007; Ivanchenko et al., 2008).
in light-grown seedlings. In dark-grown seedlings, ethylene inhibits hypocotyl and root elongation, exaggerates tightening of the apical hook, and induces swelling of the hypocotyl, in what is known as the triple-response phenotype (Guzmán and Ecker, 1990). Light-grown 35S:FBH1 and 35S:FBH4 seedlings exhibited significantly longer hypocotyls compared with wild-type seedlings (Fig. 6A), a phenotype that resembled the constitutive ethylene response mutant ctr1 (Smalle et al., 1997), and 35S:FBH4 was defective in root growth (Fig. 6B and C). We also investigated the phenotypes of etiolated

**Fig. 3.** Homo- and heterodimerization of ScFBH1, ScFBH2, and ScFBH3. (A) Physical interaction between ScFBH1, ScFBH2, and ScFBH3 analyzed by Y2H assay. ScFBH1, ScFBH2, and ScFBH3 were cloned into pGADT7 and pGBK7 to generate AD and BD fusions. The Y2H-Gold yeast strain was co-transformed with all possible bait and prey combinations. The ability of yeast cells to grow on QDO (SD-Leu/-Trp/-His/-Ade) indicated positive interactions. Yeast cells transformed with AD-T + BD53 and AD-T + BD-LAM were included as positive and negative controls, respectively. (B) BiFC visualization of sugarcane bHLH interactions in tobacco leaf cells. ScFBH1, ScFBH2, and ScFBH3 were fused with the N- and C-termini of YFP and all nine possible interactions were tested. The interactions between ScFBHs fused with N-terminal YFP and GST fused with C-terminal YFP were tested as negative controls. The RFP:NbH2B construct (Chakrabarty et al. 2007) was co-transformed as a control of transformation efficiency and nuclear location. Bars=10 μm. (This figure is available in color at JXB online.)
The amino acid alignment of the bHLH domains showed a high level of similarity between Arabidopsis FBHs and sugarcane ScFBHs. Both species’ FBHs possess conserved Glu-13 and Arg-17 residues (Fig. 2C), which are involved in the interaction with the E-box consensus CANNTG (Shimizu et al., 1997; Toledo-Ortiz et al., 2003). E-boxes exist on the ScACS2 promoter (Fig. 2D) and are conserved among the promoters of homologous type 3 ACS genes from other grasses (Supplementary Table S3). EMSA analysis showed that ScFBH2 directly binds to E-boxes (CAGTTG) present in the ScACS2 promoter (Fig. 2E). Yin et al. (2015), meanwhile, found that the petunia homolog PhFBH4 is up-regulated during flower senescence and binds to the CACGTTG motif of the PhACS1 promoter to activate its expression. This finding is similar to our findings, indicating that the molecular link between FBHs and ACS might be conserved widely in both monocots and eudicots. Consistent with their role as transcriptional activators, all three ScFBH proteins are targeted to the nucleus (Fig. 1B).

The analysis of ethylene production confirmed the differences observed in the phenotypic evaluation and ACS gene expression. Overexpression of FBH4, which produced the strongest ethylene-related phenotypes and induced higher levels of ACS7, also produced more amounts of ethylene (Fig. 7B).

Discussion

Plant bHLH transcription factors comprise a large family, and are involved in the transcriptional control of various biological processes (Toledo-Ortiz et al., 2003). Our Y1H screening identified three related bHLH transcription factors that bind to the ScACS2 promoter (Fig. 1A and Supplementary Table S4) and act as transcriptional activators (Fig. 4). Phylogenetic analysis demonstrated that these proteins belong to subfamily 16 of the bHLH superfamily (Fig. 2A), to which four FBHs of Arabidopsis belong. Arabidopsis FBHs are transcriptional activators of CO and regulate photoperiodic flowering time. Ito et al. (2012) demonstrated that FBH1, FBH2, FBH3, and FBH4, by binding to the E-boxes, act in a redundant fashion on the CO promoter to activate its expression. FBH homologs identified in poplar and rice exhibited similar functions when overexpressed in Arabidopsis (Ito et al., 2012; Song et al., 2013). The amino acid alignment of the bHLH domains showed a high level of similarity between Arabidopsis FBHs and sugarcane ScFBHs. Both species’ FBHs possess conserved Glu-13 and Arg-17 residues (Fig. 2C), which are involved in the interaction with the E-box consensus CANNTG (Shimizu et al., 1997; Toledo-Ortiz et al., 2003). E-boxes exist on the ScACS2 promoter (Fig. 2D) and are conserved among the promoters of homologous type 3 ACS genes from other grasses (Supplementary Table S3). EMSA analysis showed that ScFBH2 directly binds to E-boxes (CAGTTG) present in the ScACS2 promoter (Fig. 2E). Yin et al. (2015), meanwhile, found that the petunia homolog PhFBH4 is up-regulated during flower senescence and binds to the CACGTTG motif of the PhACS1 promoter to activate its expression. This finding is similar to our findings, indicating that the molecular link between FBHs and ACS might be conserved widely in both monocots and eudicots. Consistent with their role as transcriptional activators, all three ScFBH proteins are targeted to the nucleus (Fig. 1B).

The bHLH transcription factors are known to form homodimers and heterodimers, and to interact with members of other transcription factor families than the bHLH
family to regulate the expression levels of target genes (Heim et al., 2003; Toledo-Ortiz et al., 2003). The three-dimensional modeling of ScFBHs and Arabidopsis FBHs predicted that they dimerize (Fig. 2B). We confirmed all nine possible combinations of interactions among sugarcane FBH proteins (ScFBH1, ScFBH2, and ScFBH3) by Y2H and BiFC assays (Fig. 3A and B). We also demonstrated that homo- and heterodimers activate the ScACS2 promoter at distinct levels in the transient luciferase reporter assay (Fig. 4). This indicates that ScFBHs might bind as dimers on the E-boxes of the ScACS2 promoter to activate its expression. Whether different combinations of ScFBH dimers

Fig. 5. Expression profiles of ScFBH1, ScFBH2, ScFBH3, and ScACS2 in different sugarcane organs and throughout the day. (A) Expression levels of ScFBH1, ScFBH2, ScFBH3, and ScACS2 in leaf +1 and immature and maturing internodes. Bars represent the mean ±SE expression values from all time points. Asterisks indicate significant differences in mean gene expression between organs (P<0.05, Tukey’s test). (B–D) Daily expression profiles of ScFBH1, ScFBH2, ScFBH3, and ScACS2 in leaf +1 (B), immature internode (C), and maturing internode (D). ScACS2 expression values are presented on the secondary y-axis. Harvesting started 0.5 h after dawn (5.30 h) and was then repeated every 2 hours. The horizontal bars above the graphs represent periods of light (white bars) and darkness (black bars). Expression values were normalized using expression of the GAPDH gene (Iskandar et al., 2004) as a reference, and the expression values were calculated by the ΔCt method (Livak and Schmittgen, 2001). Each point represents a mean ±SE (n=3). (This figure is available in color at JXB online.)
FBH transcription factors control ACS expression in sugarcane

act differently to regulate the transcription of target genes at different plant tissues and physiological conditions still needs to be investigated.

The gene expression analysis showed that \textit{ScACS2} and \textit{ScFBH} transcript levels are low in leaves and immature internodes. However, \textit{ScACS2} expression level is higher in the

\textbf{Fig. 6.} Effect of \textit{FBH} overexpression and \textit{fbh} loss of function on hypocotyl length, root length, and ACS7 expression in Arabidopsis seedlings grown under long-day (LD) conditions. (A) Hypocotyl length and (B) root length of Col-0, \textit{fbh} quadruple mutant, 3SS:FBH1, and 3SS:FBH4 seedlings. After stratification, plants were grown on Linsmaier and Skoog media containing ACC (10 µM) or AgNO₃ (100 µM) for 10 days at 22 °C under LD conditions. For each treatment, data presented are the means of 17–27 seedlings ±SD. Means were compared by ANOVA and Tukey's test (P<0.05); the same letters above each bar indicate that there was no statistically significant difference between treatments. (C) Phenotypes of 10-day-old seedlings grown with and without ACC or AgNO₃ under LD conditions. Bars=0.5 cm. (This figure is available in color at JXB online.)
maturing internodes, where the expression levels of \( \text{ScFBHs} \) are also higher. Since the maturation process in sugarcane is characterized by a gradual increase in sucrose content from the apical to the basal internodes, our data revealed that the expression of \( \text{ScACS2} \) and \( \text{ScFBHs} \) correlates with plant development and the sucrose content of the internodes (Fig. 5A). Cunha et al. (2017) found that the application of ethylene to sugarcane plants induced sucrose synthase (SuSy) activity on maturing internodes a few days after the treatment. This ethylene treatment induced a 60% increase of sucrose levels approximately 1 month after the application, when the SuSy activity reduced to the level of activity in non-treated plants. In leaves, ethylene has no effect on SuSy activity or sucrose levels. In addition, in comparison with immature internodes, ETHYLENE-INSENSITIVE3-LIKE (EIL) homologs and one ACO homolog are repressed in mature internodes (Papini-Terzi et al., 2009), indicating that there is a less active ethylene response in mature internodes; we believe that this represents a late stage of maturation. Interestingly, ethylene treatment induces \( \text{ScFBH1} \) in sugarcane internodes (Cunha et al., 2017), which could indicate a feedback regulation of \( \text{ACS} \) by ethylene through the action of \( \text{ScFBH1} \). Although the precise role of ethylene in sucrose accumulation in sugarcane needs to be studied further, our work supports the notion that ethylene acts as a modulator of the maturation process. Our findings imply that \( \text{ScFBHs} \) are a part of the network involved in the sucrose accumulation mediated by ethylene.

To determine the potential contribution of \( \text{ScFBHs} \) to the regulation of \( \text{ScACS2} \) throughout the day, we measured their daily expression patterns in leaves and immature and maturing internodes. In leaves, \( \text{ScFBHs} \) expression levels peak in the morning; however, the overall levels are very low relative to their expression levels in maturing internodes, and \( \text{ScACS2} \) expression remains constantly low throughout the day (Fig. 5B). In maturing internodes, where transcript levels of all genes are higher, the daily expression patterns of \( \text{ScACS2} \) and \( \text{ScFBHs} \) are similar. The overall expression pattern of \( \text{ScACS2} \) and \( \text{ScFBHs} \), which is lower just after dawn and increases in the course of the day, resembles the expression pattern of \( \text{FBH4} \) in LD conditions (Ito et al., 2012). The expression of \( \text{FBH4} \) and possibly \( \text{FBH1} \) has a diurnal oscillation pattern under constant light conditions, indicating the
involvement of circadian regulation (Ito et al., 2012). Circadian oscillation of ethylene synthesis has been described in Arabidopsis (Thain et al., 2004), potato (Chincinska et al., 2013), and sorghum (Finlayson et al., 1999, 2004). In Arabidopsis, the ACS6 promoter is the binding target of the core clock protein TOC1 (Huang et al., 2012), and the rhythmic production of ethylene correlates with the expression pattern of ACS8 (Thain et al., 2004). Furthermore, FBH1 and the core clock protein CCA1 reciprocally regulate each other’s transcription (Nagel et al., 2014). Taken together, these data suggest that ScFBHs may be involved in the circadian regulation of ACS expression in sugarcane and provide initial cues for further investigations.

In addition to the findings that ScFBHs bind to the ScACS2 promoter in yeast and increase ScACS2 promoter activity in N. benthamiana cells (Figs 1 and 4), the similar expression patterns of ScFBHs and ScACS2 led us to hypothesize that ScFBHs act as direct transcriptional activators of ScACS2. We also hypothesize that this regulation may be conserved in other plants besides sugarcane, since FBHs can be found in various plant genomes (Ito et al., 2012). We made use of Arabidopsis genetic resources to test this hypothesis. Ethylene and ACC production stimulate hypocotyl elongation (Smalle et al., 1997; Zhong et al., 2012; Yu et al., 2013) and inhibit root growth in light-grown Arabidopsis seedlings (Ruzicka et al., 2007; Negi et al., 2008). We observed that overexpression of both FBH1 and FBH4 induced hypocotyl elongation in LD-grown Arabidopsis seedlings; this phenotype was reversed by the application of AgNO3, an ethylene antagonist (Fig. 6A). Additionally, etiolated 35S:FBH4 seedlings had shorter hypocotyls, similar to the ethylene triple response (Supplementary Fig S4). Ethylene production and ACS7 expression were higher in plants overexpressing FBH4, especially until 16 h after the onset of light. In contrast, ethylene production was lower in the fbh mutant and ACS7 expression decreased at night (Fig. 7). Etiolated fbh mutant seedlings showed longer hypocotyls compared with wild-type plants (Supplementary Fig. S4), probably due to lower ACC production in the absence of the ACS7 activator. These results suggest that FBH4 contributes to ethylene production by inducing ACS7 expression in the afternoon and at night. The results also indicate that a similar mechanism may exist in maturing internodes of sugarcane in which ScFBHs regulate the expression of ScACS2.

Our results demonstrated that the overexpression of FBH4 in Arabidopsis caused a reduction of root length in LD-grown plants, a condition that was reverted by treatment with AgNO3, while the opposite effect was observed in plants overexpressing FBH1 (Fig. 6B). Nagel et al. (2014) have pointed out that FBH1 seems to be a dual-function transcription factor, which acts as both an activator of CO (Ito et al., 2012) and a repressor of CCA1 expression (Nagel et al., 2014). Our data show that FBH1 overexpression induces ACS7 expression during the subjective day, but may inhibit ACS7 and ACS8 at night, while FBH4 overexpression induces ACS8 at night (Supplementary Fig. SSD). Recently, Tian et al. (2015) demonstrated that overexpression of AthHLH129, another member of subfamily 16, induces increased root length on LD-grown plants, possibly implicating other members of the subfamily, such as FBH1, in root development. This may explain why we observed differences in root phenotypes and ethylene production between FBH1 and FBH4 overexpressors. We highlight that all three ScFBHs are phylogenetically closer to Arabidopsis FBH4 than FBH1 (Fig. 2A). Such complex mechanisms can also explain why the roots of FBH overexpressors seem to be insensitive to ACC treatment in etiolated seedlings (Supplementary Fig S4B).

Publications regarding the functional characterization of sugarcane transcription factors and their mechanistic regulation are still scarce, probably due to the complexity of the sugarcane genome and the instability of transgene expression (Arruda, 2012). Therefore, the use of model plants, such as Arabidopsis and tobacco, for functional studies of sugarcane genes or related genes is a common approach in sugarcane reverse genetics (Trujillo et al., 2008; Begcy et al., 2012; Cesarino et al., 2013). Our findings support the notion that the regulation of ACS may be conserved in other species in addition to sugarcane, and may utilize conserved transcriptional regulators, such as FBHs.

Here we reported that three sugarcane bHLH transcription factors, ScFBH1, ScFBH2, and ScFBH3, were isolated as transcription factors that bind to the E-boxes in the ScACS2 promoter. Our protein–protein interaction and subcellular localization analyses showed that these transcription factors form homo- and heterodimers in the nucleus. Gene expression analysis in sugarcane revealed that ScFBHs and ScACS2 are expressed differently in leaves and immature and maturing internodes, with similar expression profiles. Arabidopsis transgenic plants overexpressing FBH1 and FBH4 exhibit elongated hypocotyls, which is a typical phenotype of light-grown plants exposed to ACC or ethylene. Overexpression of FBH4, the closest homolog of ScFBHs, also induced ethylene production, probably due to the induction of the ScACS2 homolog in Arabidopsis, ACS7. Taken together, these results suggest that ScFBHs may have a similar function to that of Arabidopsis FBHs, and may directly regulate the transcription of ScACS2. Our study therefore provides a new mechanistic insight into the regulatory network of ethylene synthesis in sugarcane.

Supplementary data

Supplementary data are available at JXB online.

Table S1. IDs of proteins used in the ACS phylogenetic analysis.

Table S2. List of PCR primers used in this study.

Table S3. Putative transcription factor binding sites present in conserved sequences of the ScACS2 promoter.

Table S4. List of proteins that bind to the ScACS2 promoter identified by yeast one-hybrid screening.

Fig. S1. Transcriptional activity of the ScACS2 promoter assessed by particle bombardment in sugarcane tissues.

Fig. S2. Phylogenetic placement of ScACS2.

Fig. S3. Promoter conserved sequences in ScACS2 and homologous promoters from other grasses.

Fig. S4. Hypocotyl length of FBH overexpression and loss-of-function Arabidopsis dark-grown seedlings.

Fig. S5. Expression of Arabidopsis ACS2, 4, 6, and 8 genes in FBH overexpression and loss-of-function plants.
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