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Identification of cold-responsive genes in energycane for their use in genetic diversity analysis and future functional marker development

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ABSTRACT

Breeding for cold tolerance in sugarcane will allow its cultivation as a dedicated biomass crop in cold environments. Development of functional markers to facilitate marker-assisted breeding requires identification of cold stress tolerance genes. Using suppression subtractive hybridization, 465 cold-responsive genes were isolated from the cold-tolerant energycane Ho02-144. Predicted gene interactions network indicated several associated pathways that may coordinately regulate cold tolerance responses in energycane. Expression analysis of a select set of genes, representing signaling and transcription factors, genes involved in polyamine and antioxidant biosynthesis, protein degradation and in the repair of damaged proteins in the cytosol, showed their time-dependent regulation under cold-stress. Comparative expression profiles of these genes between Ho02-144 and a cold-sensitive clone (L79-1002) showed that almost all genes were induced immediately upon imposition of cold stress and maintained their expression in Ho02-144 whereas they were either downregulated or their upregulation was very low in L79-1002. Simple sequence repeat markers derived from 260 cold-responsive genes showed allelic diversity among the cold-sensitive commercial hybrids that were distinct from the *Saccharum spontaneum* clones. Future efforts will target sequence polymorphism information of these genes in our ongoing QTL and association mapping studies to identify functional markers associated with cold tolerance in sugar/energycane.

1. Introduction

Under the current scenario of increasing insecurity of the fossil fuel and the growing demand for alternative sources of bioenegy, development of an alternative viable bioenergy industry will require a stable and an abundant supply of biomass. Sugarcane, a significant component of the economy in many countries in the tropics and subtropics as a sugar source, is considered as a promising source of ligno-cellulose for ethanol production because of its high biomass production per unit area. Sugarcane, a C4 grass, produces the world's greatest crop tonnage with a yield potential of 177 t dry matter/ha/year [1]. Sugarcane annual production per hectare (39 t/ha of dry stalks and trash) compares favorably to other high-yielding bioenergy crops such as *Miscanthus* (29.6 t/ha), switchgrass (10.4 t/ha) and maize (total grain plus stover, 17.6 t/ha) [2]. Sugarcane with high fiber and little sugar is referred to as fibercane or dubbed popularly as 'energycane'. Energycane to a breeder implies to early generation sugarcane hybrids such as F_1's, BC_1's with high vigor (high biomass yields = 30+ dry Mt/ha), high fiber (18–24%), moderate soluble solids (Brix = 8–14%) and good ratooning i.e., perennial growth and consistent yields over 5–6 annual harvests.

Sugarcane stalks, after crushed and extracted for juice, are usually burned in the sugarcane factories to generate steam and electrical energy. These cellulosic bagasses could be used as the source of second generation ethanol after undergoing fermentation. With the development of cellulosic ethanol industry the ethanol output from sugarcane is expected to increase by 40–50% i.e., from the current 7500 to 13,000 l/ha [1]. However, for energycane to be a viable biofuel crop it should have the ability to grow in marginal lands and in areas that are not suitable for commercial sugarcane cultivation, which indicates that a sustained growth of energycane industry will depend on the development of high biomass-yielding, stress-tolerant cultivars adapted to poor soil conditions.

Sugarcane grows well in the tropics or in subtropical areas where the climate is moderated by surrounding water masses. The optimum temperature for growth is about 35 °C. Any temperature near freezing (chilling temperature) is cold enough to produce crop

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injury or to suppress growth and yield by affecting the developmental and physiological processes. There is little plant growth at temperatures as high as 20 °C and there may be tissue injury at temperatures below 15 °C [3]; this is because most sugarcanes have not developed strategies to avoid the devastating consequences of cold to the cells [4], and because selection of commercial germplasm is being maximized for sucrose genes, which are mostly derived from the cold-sensitive *S. officinarum* genome. However, there is evidence that sugarcane varieties differ in their sensitivity to cold [5]. A few hybrids, especially those with alleles from specific germplasm of cold-hardy *Saccharum spontaneum*, showed increased tolerance to cold stress. For example, unselected F_1 populations of cultivar x *S. spontaneum* crosses possessed adequate levels of freeze tolerance, but in the subsequent backcross breeding, the cold tolerance trait disappeared [6] due to heavy selection on sugar content. Because *S. spontaneum* is well adapted to harsh climatic conditions [7,8] and is high in fiber content, its germplasm is being increasingly introgressed recently into breeding lines of Louisiana, Brazil, Barbados and Australia with an aim to transfer genes for cold-tolerance and high fiber content into energycanes [9].

Identification of the genes/alleles for cold tolerance will have a great potential in energycane breeding programs. Several molecular approaches, such as suppression subtractive hybridization (SSH), cDNA microarrays, qRT-PCR and SUCEST data mining have been used to identify and study the expression of the genes in response to cold [10,11] and water deficit stress [12,13]. Nogueira et al. [10], using a high-density filter arrays containing sugarcane ESTs, identified 33 genes that were induced by low temperature (4 °C) stress. RNA gel-blot analysis identified SsNAC23, out of 26 non-redundant genes encoding NAC domain proteins, to be strongly induced by chilling stress in sugarcane, in addition to water stress and herbivory [11]. All of these studies used commercial sugarcanes that were bred for sugar. The present investigation is a first report where a cold-tolerant energycane clone was used to mine cold-regulated genes with a long term goal of developing functional markers to facilitate breeding of cold tolerant energycane cultivars that would allow their cultivation in more temperate climates in northern latitudes.

2. Materials and methods

2.1. Plant material, cold treatment, and physiological analysis

Two energycane clones, L79-1002 (cold sensitive; released by USDA-ARS, Houma, LA) and (Ho02-144, a cold tolerant energycane candidate, due to be released soon), were used in this study. One-month-old greenhouse-grown first-stubble plants were exposed to a temperature of 0 °C for a week inside a growth chamber (Shel Lab, Cornelius, OR), which was maintained at 14 h/10 h day/night cycle. Young fully expanded leaf samples were harvested in liquid nitrogen from both clones after 24 h, 48 h, 72 h, and 1 wk of cold stress and stored at −80 °C for RNA isolation. Leaf tissues collected before subjecting the plants to cold stress (0 h) served as the control.

2.2. Chlorophyll and cell membrane stability analysis

Total chlorophyll content was extracted from one fully expanded leaf per plant (three plants each, representing biological replicates) of control and cold-stressed plants of L79-1002 and Ho02-144 with 80% acetone twice. The chlorophyll a and b content were measured spectrophotometrically following the method described by Baisakh et al. [14] to determine the extent of bleaching and chlorophyll loss.

The membrane stability index (MSI) was determined according to Sairam et al. [15]. Leaf samples (100 mg) from control and cold-stressed plants (two plants each from L79-1002 and Ho02-144) were heated in 10 ml of double-distilled water at 40 °C for 30 min in a water bath, and the electrical conductivity of the solution (C_1) was recorded with multi parameter PCSTestr-35 (Eutech Instruments Pvt. Ltd., Singapore). Again the leaves were boiled at 100 °C on a boiling water bath for 10 min, and its conductivity (C_2) was measured. The MSI was calculated as $[1 - (C1/C2)] \times 100$.

2.3. RNA isolation and cDNA subtraction

Total RNA was isolated from the frozen leaf tissues using Trizol (Invitrogen, Carlsbad, CA) and/or RNeasy Plant Mini Kit (Qiagen, Valencia, CA) using the manufacturer's instruction. Gel-based qualitative assay and quantitative assay in a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was performed as described earlier [16].

Seven μg of RNA extracted at four different time points of the cold stressed Ho02-144 were pooled for cDNA synthesis. cDNA subtraction was performed using the PCR-select™ cDNA subtraction kit (Clontech, Palo Alto, CA) following manufacturer's instructions except that double strand cDNA was synthesized from 20 μg of control and cold stressed RNA using the Superscript™ double-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Double stranded cDNA (5 μg) was digested with *Rsa*I. The cDNA from the cold stressed plant was ligated with two different adaptors and used as tester. Two rounds of forward subtractions were performed using cDNA from non-stressed control plant as driver. Differentially expressed upregulated genes were amplified by primary PCR with 27 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 1.5 min. The primary PCR product was enriched by secondary PCR with 12 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1.5 min.

2.4. Annealing control primer (ACP)-based cDNA synthesis and gene fishing

First-strand cDNA was synthesized from 3 μg of RNA of cold-stressed (pooled over 24 h, 48 h, 72 h, and 1 wk) and control leaf tissues of the tolerant (Ho02-144) and the sensitive (L79-1002) clones using a Gene Fishing DEG premix kit (Seegene, Rockville, MD) according to the manufacturer's instructions with minor modification as described earlier [16,17]. In brief, reverse transcription was performed for 90 min at 42 °C in a final reaction volume of 20 μl containing 4 μl of 5× reaction buffer (Promega, Madison, WI), 5 μl of 2 mM dNTP mix, 2 μl of 10 μM primer, oligo(dT)$_{15}$-ACP1 (Seegene, Rockville, MD), 0.5 μl of RNasin RNase Inhibitor (Promega, Madison, WI), and 1 μl of reverse transcriptase 200 U/μl (Promega, Madison, WI). First-strand cDNA was 5×-diluted with nuclease-free water. Second-strand cDNA synthesis was conducted at 50 °C during the first-stage PCR in a final reaction volume of 20 μl containing 3 μl (~50 ng) of the diluted first-strand cDNA, 10 μl of 2X SeeAmp ACP master mix (Seegene, Rockville, MD), 2 μl of 5 μM arbitrary ACPs (Supplementary Table 1), and 1 μl of 10 μM oligo(dT)$_{15}$-ACP2. The first-stage thermal profile was one cycle of 94 °C for 1 min, followed by 50 °C for 3 min, and 72 °C for 1 min; the second-stage thermal profile was 40 cycles of 94 °C for 40 s, followed by 65 °C for 40 s, 72 °C for 40 s, and a 5 min final extension at 72 °C. The amplified PCR products were resolved in a 2% agarose gel (Amresco, Solon, OH). The differentially expressed genes (DEGs) were identified based on their intensity or presence/absence, and were excised and extracted from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

2.5. Cloning and sequencing of differentially expressed genes

The subtracted cDNAs and the ACP-generated DEGs were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and transformed

into *Escherichia coli* DH5α cells following the method described by Ramanarao et al. [16]. Seven hundred white colonies from cDNA subtracted library and 40 colonies representing ACP-generated DEGs were confirmed positive with M13 forward and reverse primers; the plasmids were then single-pass sequenced using an ABI 3130xl sequencing platform.

2.6. Sequence processing and bioinformatics analysis

The vector sequences and the poly(A) tail were cleaned from the sequences using an in-house PERL script. The clean sequences, after excluding the exactly duplicated sequences, were assembled using the CAP3 program with default parameter settings [18]. The resulting unigenes (contigs and singlets) were functionally annotated through BLASTx and BLASTn-based [19] homology search against NCBI protein and nucleotide database (http://www.ncbi.nlm.nih.gov/) at e-value cut-off of 1e-06. The sequences were also BLASTn-searched against sugarcane EST database (SoGI) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=s_officinarum) at e-value cut-off of 1e-05. Sequence match results with significant hits, i.e. exceeding 50 nucleotides and more than 90% identity, were considered significant, extracted and parsed using in-house PERL script and MySQL database for subsequent analysis. The sugarcane unigenes were mapped against the sorghum genome (http://www.plantgdb.org/SbGDB) based on BLASTn output at 1e-05 and 60% sequence identity. The syntenic regions were depicted in the sorghum circular karyotype map using Circos (www.circos.ca).

The metabolic functions of the unigenes were determined against the KEGG pathway database (http://www.genome.jp/kegg/). Gene ontology (GO) IDs of the unigenes were retrieved from the Blast2GO (www.blast2go.org) output. The GOSlim terms for biological process, molecular function, and cellular component associated with significant BLASTx hits were assigned to sugarcane unigenes using GOSlim viewer (http://agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl) generated using GO IDs as the input with plant specific GOSlim set. An interaction network among the cold responsive sugarcane unigenes was predicted using RiceNet [20] with the homologous rice locus ID (rice cDNA database, Release 7.0, http://rice.plantbiology.msu.edu/) at cut off value of 1e−05. The network was imported into cytoscape for visualization and clustering.

2.7. Transcript profiling of differentially expressed genes

The expression pattern of 24 expressed sequence tags (ESTs), identified by cDNA subtraction and ACP, was validated by semi-quantitative and quantitative reverse transcription polymerase chain reaction (sq/qRT-PCR) following the method described earlier [16,17]. Gene-specific primers (Supplementary Table 1) were designed using Primer3Plus web resource (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and were synthesized by Integrated DNA technologies (IDT Inc, Coralville, IA). First-strand cDNA was synthesized from 1 μg of the total RNA isolated from leaf tissues from the control and cold-stressed plants of both L79-1002 and Ho02-144 at 24 h, 48 h, 72 h, and 1 wk using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). One μl of the first-strand cDNA was amplified using DEG-specific primers with the thermal profile as described earlier [16]. The amplification products were separated on 2% agarose (Amresco, Solon, OH).

The relative abundance of the genes was determined using qRT-PCR. qRT-PCR was carried out in triplicate (three biological replications corresponding to cDNAs prepared from three plants each of H002-144 and L79-1002) and the results were expressed as means ± standard error. The PCR was conducted in a 20 μl final reaction volume containing 2 μl of 10x diluted first-strand cDNA, 10 μl of SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.2 μM of each primer in a MyiQ real-time PCR analysis system (Bio-Rad, Hercules, CA) as per Ramanarao et al. [16]. A serial dilution of 100, 10, 1, 0.1 and 0.01 ng of 1st strand cDNA was used for all genes to generate a standard curve by plotting the Ct (threshold cycle) values against log (ng) 1st strand cDNA, and to ensure that the efficiencies of the individual transcripts were equal. The mRNA expression was normalized against the *S. officinarum* elongation factor (*SoEF1α*; GenBank Acc. # EF581011) and calculated as fold-change ratio in comparison to the control (0 h) using the $2^{-\Delta\Delta Ct}$ method [21]. The absolute fold-change values of the relative mRNA abundance of the genes were used for heat map analysis and visualization using MayDay 2.13 (http://www-ps.informatik.uni-tuebingen.de/mayday/wp).

2.8. Mining cold-responsive ESTs for microsatellite markers

The 465 cold-responsive unigenes and their matches from SoGI were searched for the presence of simple sequence repeat (SSR) motifs using MISA program [22]; criteria was set to at least five repeats for dinucleotide motifs and at least three repeats for tri, tetra, penta and hexa nucleotide motifs. Primers flanking the SSR motifs were designed as described earlier [23].

2.9. Genetic diversity of Louisiana sugarcane cultivars using cold responsive gene-derived SSR markers

Seventy clones (Supplementary Table 2) comprising of 48 Louisiana sugarcane commercial hybrids, five energycane clones, two *S. officinarum* and 15 *S. spontaneum* clones were genotyped with 12 cold responsive gene-derived SSR primers (eSSRs; Supplementary Table 3) derived from cold responsive ESTs. Two ng of genomic DNA, isolated using DNeasy plant minikit (Qiagen, Valencia, CA), was used as the template in PCR reactions in a final volume of 10 μl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 μM dNTP mix, 0.4 unit of *Taq* DNA polymerase (Promega, Madison, WI) and 0.75 μM of each primer. PCR products were resolved in a 6.5% polyacrylamide gel at 1500 V for 3 h and detected using a Li-COR Model 4300 DNA analyzer (Li-COR Biosciences, Lincoln, NE). The M13-tailed forward primers were fluorescence labeled (IRDye700 or IRDye800; IDT Inc, Coralville, IA) for multiplexing as described earlier [24].

The scoring of the gels and data analysis was done following the method described by Suman et al. [24]. Bands were scored for presence (1) and absence (0) in a binary matrix, which was subject to genetic similarity (GS) and cluster analysis among the 70 clones based on Jaccard's similarity coefficient using the unweighted pair group method with arithmetic means (UPGMA) module of the NTSYSpc version 2.2 program [25]. The estimate of principal coordinate analysis (PCoA) was also used to validate the cluster distribution of the clones.

3. Results

The effect of cold stress was apparent in the cold sensitive (L79-1002) clone with respect to the loss of chlorophyll (Fig. 1). After one week under 0 °C the chlorophyll in the sensitive clone was reduced by 37% while there was only 11% reduction in the tolerant clone Ho02-144. The membrane stability index (MSI) of Ho02-144 was reduced by 30% (60–42), whereas it was 63% (56–21) in L79-1002 after a week of exposure to cold stress. Upon transfer of the plants after a week of cold stress to the greenhouse, Ho02-144 produced new tillers after two weeks and grew normally but the sensitive clone did not produce any new tillers and showed stunted growth

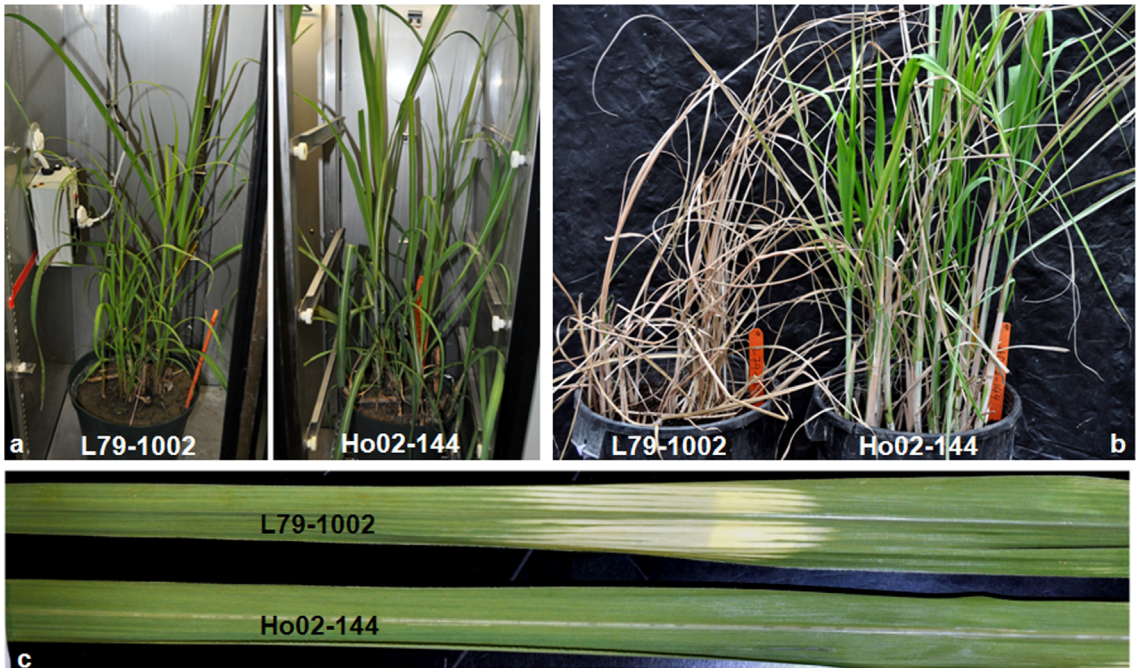

Fig. 1. Cold sensitive clone L79-1002 (left) and cold tolerant clone Ho02-144 (right) of energycane (Fig. 1a) under cold stress at 0 °C. The freeze damage is clearly visible by chlorophyll bleaching in the leaf of L79-1002 as compared to Ho02-144 (Fig. 1c); the photograph was taken after one week under cold stress. Upon transfer of the cold-stressed plants to the greenhouse Ho02-144 developed new tillers and recovered to grow normally while L79-1002 showed drying and ultimately died (Fig. 1b); the photograph was taken after a month of their transfer to the greenhouse.

inside the greenhouse. The leaves and stems of the sensitive clone withered with time leading to the death of the plant (Fig. 1).

3.1. Annotation of cold-responsive unigenes

A total of 700 white colonies representing products of cDNA subtraction and ACP-based gene fishing (Supplementary Fig. 1) were PCR-screened for size of inserts; 650 clones with insert size >200 bp were selected for plasmid extraction and sequencing. Six hundred and eighteen ESTs produced quality sequences from which twelve ESTs with insert length <50 bp were excluded. Further, six of the 606 ESTs were exact duplicates and were also excluded from further analysis. Assembly of these 600 ESTs (GenBank Acc. Nos. JZ350035–JZ350629; five ESTs matching to ribosomal and organellar genes were excluded before submission to dbEST) with insert size ranging from 56 to 848 bp (mean length = 595 bp) resulted in 58 contigs and 407 singlets, which translated to 465 unigenes (Supplementary Table 4). The longest unigene was 1546 nt.

All 465 unigenes matched to the sequences in sugarcane gene index (SoGI, release 3.0) at 1e-05 cutoff value with ≥60% alignment in similarity. Blast search with NCBI protein and nucleotide database assigned putative functions to 426 unigenes and 39 did not have any hit, which represent sequences in the untranslated regions, non-coding RNAs or sequences specific to sugarcane. Confirmation of sugarcane sequence similarity to sorghum was established by significant similarity of 341 unigenes to sorghum gene indices (release 9.0) distributed over the 10 sorghum chromosomes (Fig. 2a and b).

Interrogation of the cold-responsive unigenes against KEGG database revealed that 145 ESTs were involved in diverse metabolic pathways. Most of the pathways were involved in the metabolism and biosynthetic processes; maximum numbers of ESTs were involved in amino acid metabolism (Fig. 3a). Twenty four and 23 ESTs were involved in arginine and proline, and cysteine and methionine metabolism, respectively. Gene ontology (GO) analysis of the ESTs showed that the GOslim terms for biological process, molecular function and cellular component were assigned to 39.3%, 36% and 24.69% of the ESTs, respectively. GO enrichment analysis indicated that most ESTs had catalytic activity under cellular and biosynthetic processes and were represented mostly in the plastid (Fig. 3b). Maximum number of ESTs belonged to transcription factor category involved in nucleotide binding activity. The results obtained with KEGG pathway analysis and GOslim terms assignments were concurrent to each other and represent the diversity of the expressed genes of sugarcane under cold stress.

3.2. Network analysis

Two hundred sixteen energycane cold-responsive unigenes that shared significant similarity with the rice gene models were used for construction of a gene network. The gene interaction network established with 70 genes after removing ribosomal genes and elongation factors, uncovered several associated pathways that may regulate and coordinate complex genomic responses and control cold tolerance in energycane (Supplementary Table 5). Further, genes were clustered into 11 classes within the resulting network based on their biological function: kinases regulating cell signaling, protein/nucleic acid binding regulatory transcription factors, genes involved in abiotic stress response, carbohydrate and protein metabolism, plant development, photosynthetic reactions, transport functions and vitamin biosynthesis, in addition to proteins of unknown functions. Different clusters of genes with specific functions interacting with each other suggested their role in diverse biological processes in energycane.

3.3. Temporal expression of cold-responsive genes in tolerant and sensitive cultivars under cold stress

Expression pattern of 24 ESTs representing members of different biosynthetic pathways, transcription factors and signaling genes (Fig. 4) were analyzed at different time points in the cold tolerant cv. Ho02-144 and sensitive cv. L79-1002 by (semi)quantitative

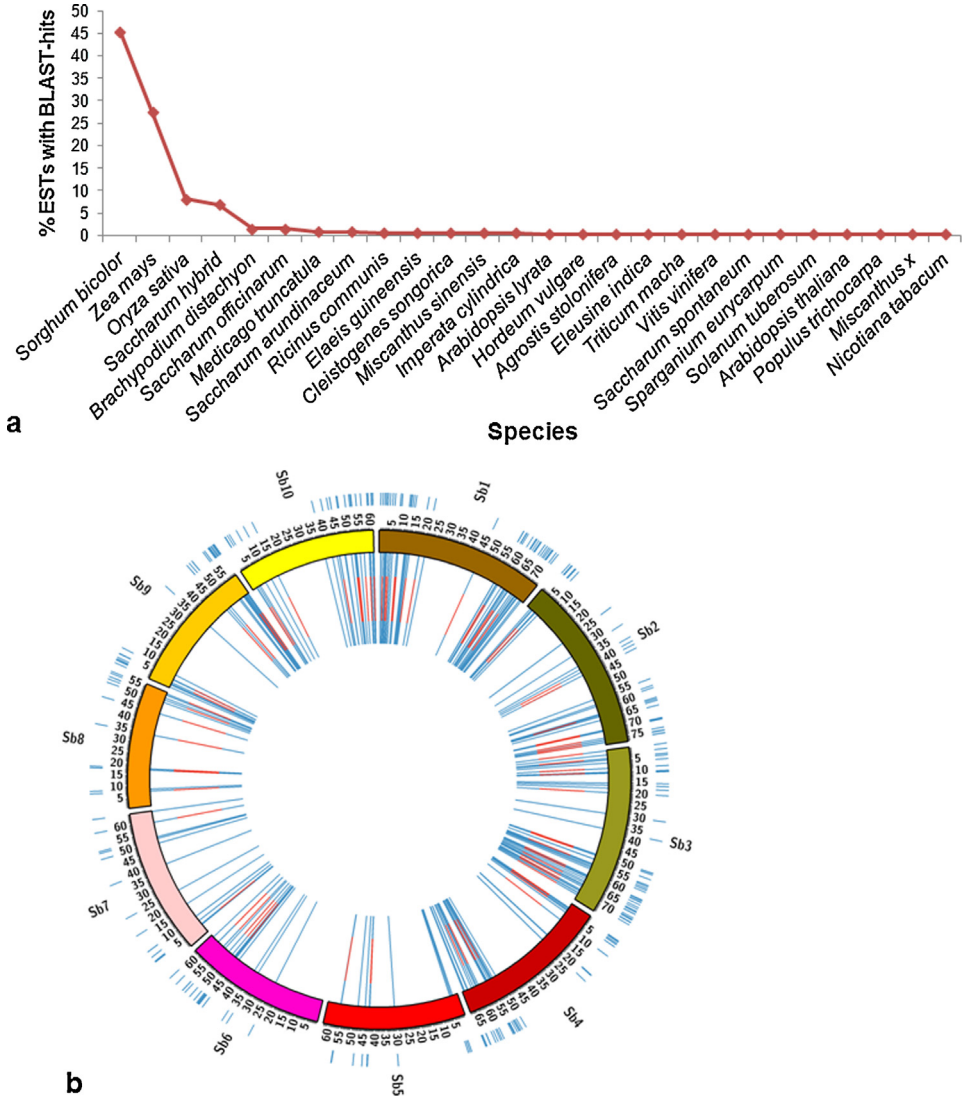

Fig. 2. Sequence similarity of energycane unigenes against different species (a) and against Sorghum transcriptome (b). Blue lines in (b) represent syntenic region; red lines superimposed over blue lines represent cold-responsive unigenes that were identified with SSR motifs (For interpretation of reference to color in this figure legend, the reader is referred to the web version of this article.)

RT-PCR. A general trend was observed with respect to the expression of all the selected genes under cold stress in the tolerant clone Ho02-144 (Fig. 4a and b, Supplementary Table 6). Although large variation was observed in the expression of a few genes among the biological replicates, the tolerant clone still maintained higher level of gene expression compared to the sensitive clone (Supplementary Table 6). Genes in the tolerant clone responded early to cold stress with higher accumulation of their transcripts within 24 h of stress compared to the control. High expression level was maintained through 72 h in most of the genes except for a few transcription factors such as PHD (plant homeodomain transcription factor, CS_176) and DUSP4 (dual specificity phosphatase 4, CS_209). The highest expression of majority of the genes was observed either after 24 h or 72 h of exposure to 0 °C in tolerant clone whereas it was at 48 h in the sensitive clone. Interestingly, three ESTs, CS_83, CS_103, and CS_107 representing transcription factor genes, ethylene binding factor (EBF), zinc finger protein (ZFP), and auxin responsive factor (ARF), respectively showed the highest accumulation of their transcript under cold in the tolerant clone, especially after 24 h. The ESTs, CS_7, CS_126, and CS_153 coding for ATP-dependent zinc metalloprotease (FTSH), heat shock factor (HSF), and NADH subunit 2 (ND2), respectively showed gradual increase in their transcript accumulation with the highest level at 72 h of cold stress (Fig. 5). The expression of HSF was consistently high at all time points in Ho02-144. On the other hand, the transcripts of almost all these genes were detectable to a varying degree in the sensitive clone under control condition, but were downregulated at 24 h after cold stress. However, a majority of the genes showed upregulation in their expression after 48 h of cold stress; however, the fold change in their transcript accumulation was much lower than those of the tolerant clone.

3.4. Molecular markers development based on sugarcane ESTs

One hundred seventy nine cold responsive ESTs were identified to contain SSR motifs; 124 ESTs were with perfect SSRs (with single motif repeats) and eight were complex with two or more SSRs separated by ≤ 100 bp. Among the SSR motifs, trinucelotide repeats were the highest (164) followed by tetra (15), di (7), hexa (4) and penta (1). Among the trinucleotide motifs, TGC/GCA type was with highest occurrence followed by AGA/TCT and AAG/CTT in that order (Fig. 5). The eSSRs derived from eleven unigenes (Supplementary

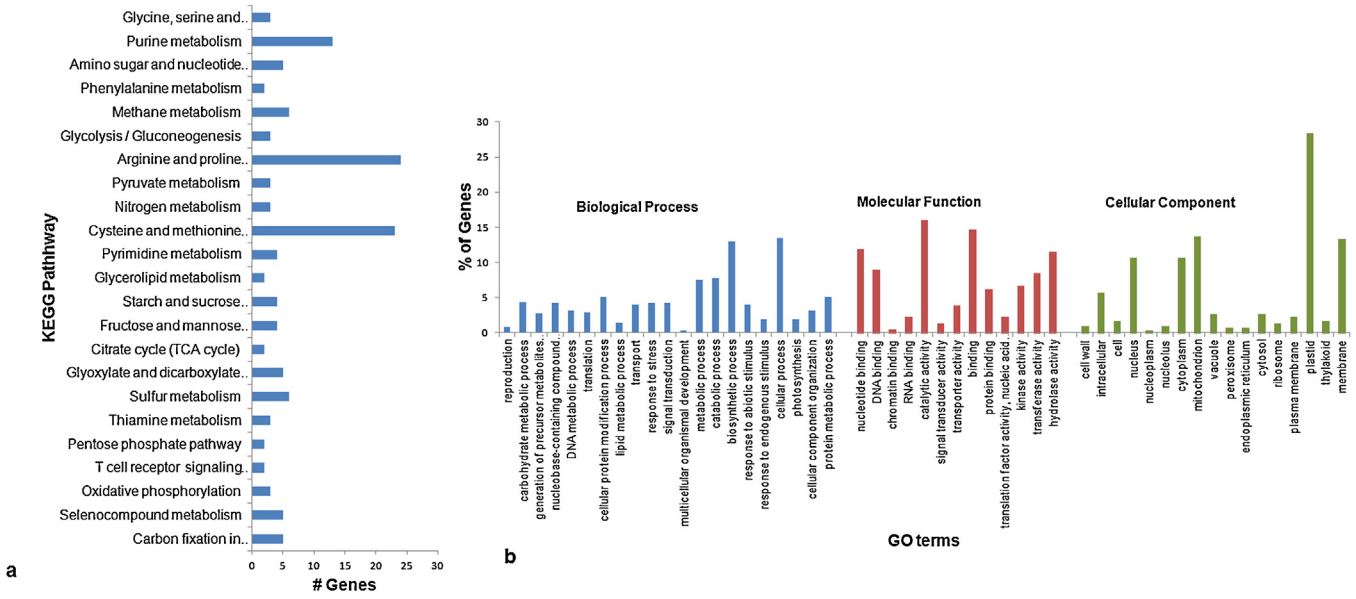

Fig. 3. Pathway analysis (a) and gene ontology (b) of the energycane cold-responsive unigenes.

Table 3) were used to genotype 53 sugarcane and energycane clones along with their progenitors.

3.5. Cold-responsive unigenes-derived eSSR-based genetic diversity among sugarcane clones

Twelve eSSRs, derived from the cold-responsive unigenes, generated 170 polymorphic alleles among 70 clones used for genotyping. Genetic diversity among the clones was analyzed by both UPGMA cluster and principal coordinate analyses (PCoA). The dendrogram obtained from the cluster analysis (Fig. 6) revealed that a large genetic diversity existed among the cultivated clones and their ancestral parents. In general, commercial sugarcane hybrids displayed a narrower range of genetic diversity in comparison to the *S. spontaneum* clones. At 0.47 genetic similarity (GS) coefficient, the clones (excluding Coimbatore) were grouped into two major clusters: Cluster I included the 53 sugarcane hybrids and two *S. officinarum* genotypes, whereas Cluster II consisted of the 14 *S. spontaneum* genotypes (Fig. 6). Lowest pairwise GS value of 0.40 was observed between Coimbatore (#57) and all other clones analyzed. All the energycanes, Ho02-113, Ho02-144, Ho02-147 (#67) and L79-1002 (#69) were grouped together in a subcluster along with SES234BF1 (#68). Ho02-144 (#66) was closest to its siblings Ho02-113(#65) and Ho02-147 (#67) with more than 85% GS. Highest GS value of 0.88 was noted between the energycanes Ho02-113 and Ho02-144.

Results of the principal coordinate analysis (PCoA) supported the cluster analysis. The ancestral *S. spontaneum* clones were clearly separated from the cultivated sugarcane clones and the two clones of *S. officinarum* (Supplementary Fig. 3). Three coordinates cumulatively explained 27.8% of the total variation (coordinate 1, 2, 3 explaining 23.7%, 1.4% and 2.7%, respectively).

4. Discussion

Sugarcane is a tropical plant better suited to grow within the temperature range of 32 °C to 38 °C. Low temperature reduces yield, affects bud sprouting and quality of sugarcane. Complexity of the genome and lack of a draft genome sequence in sugarcane make forward genetics difficult to identify genes associated with traits of agronomic interest. SSH has been preferred over other popular low-scale RNA imaging techniques as cDNAs can be subtracted to identify genes regulated under a specific stress. In the present study, SSH was used to magnify the transcriptome of sugarcane under freezing temperature with an objective to identify the cold stress responsive genes and their putative interaction network. As expected, all of the cold responsive ESTs showed marked similarity with genes of *Sorghum bicolor* and were distributed over all 10 chromosomes (Fig. 2b).

Functional annotation of the genes indicated that a complex interplay of genes was implicated in the cold stress response of energycane. Differential expression patterns of the genes, representing different levels of stress cascade i.e., perception, relay and response, between the tolerant and sensitive clones indicated their possible role in cold stress tolerance mechanism.

4.1. Cold regulation of amino acid metabolism

In the present study the transcript coding for S-adenosylmethionine decarboxylase (SAMDC) was highly accumulated under cold stress, which indicated that polyamines play pivotal roles in cold stress response of sugarcane. SAMDC was found upregulated in Arabidopsis under cold stress [26]. Polyamines (PA) such as putrescine and spermidine are widely known for their association with abiotic stress tolerance, particularly cold stress in many crop plants [27], where increase in spermidine catabolism is suggested [26]. In most crops, PA level is the discriminating standard between cold tolerant and sensitive cultivars. Although the exact molecular mechanism of their role in stress tolerance is still elusive, it is suggested that polyamines add stability to cellular function under stress by binding to membrane, cellular compartments, and other proteins.

4.2. Transcriptional regulation and cold stress

All of the nine transcription factors selected for expression analysis showed upregulation under cold stress in the tolerant energycane clone. Auxin response factors (ARFs) showed very high induction of their expression under cold stress. Although, ARF is known to be involved in cell expansion, cell division, morphogenesis and gravitropic responses [28], it was shown to be upregulated in Arabidopsis during late time point of cold stress [29]. The

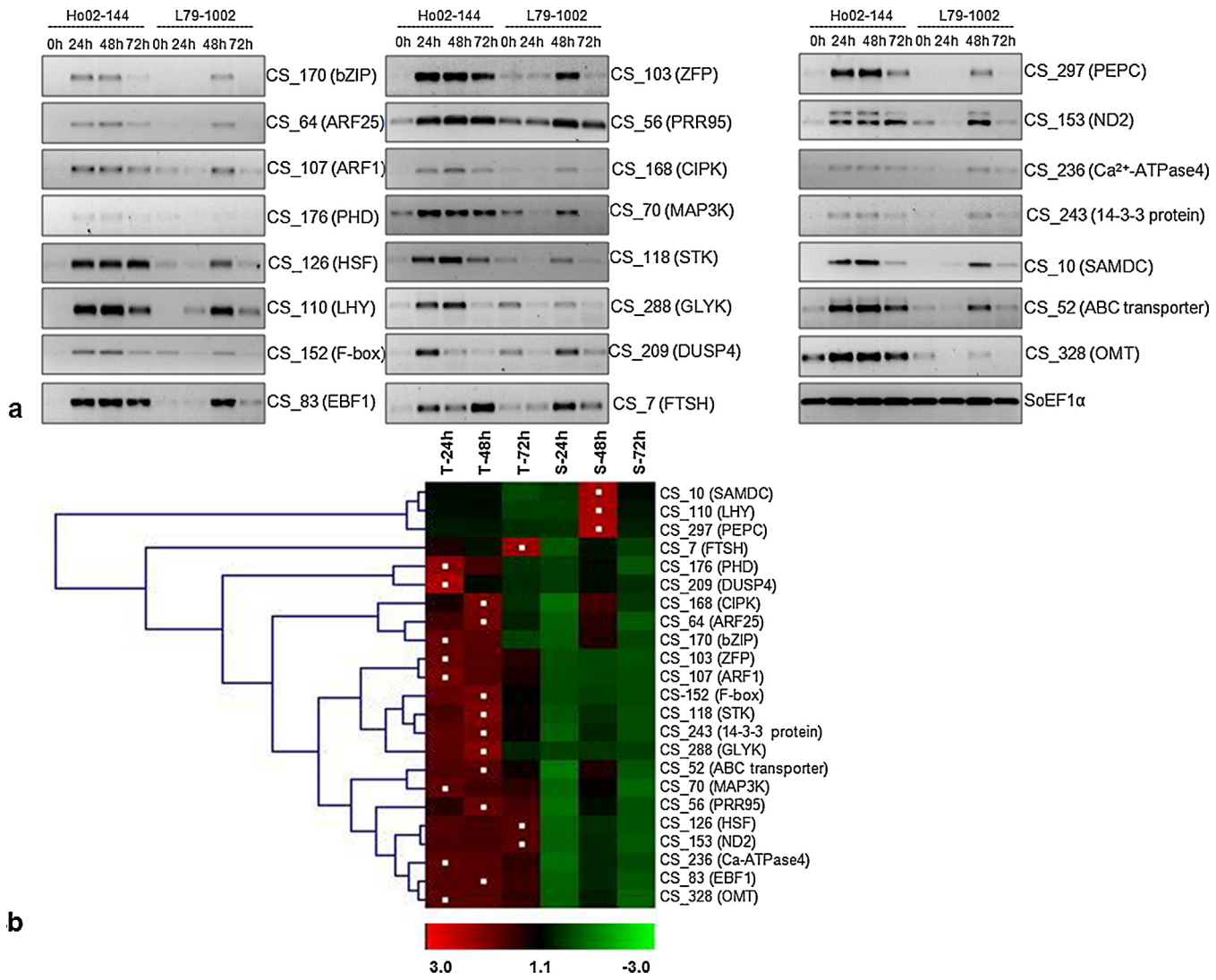

Fig. 4. Temporal expression pattern of 23 selected cold-responsive unigenes in cold-tolerant (Ho02-144) and cold-sensitive (L79-1002) clone using end-point PCR (a) and heat map derived from the qRT-PCR data (b). The white dots in (b) represent the time points at which the genes had highest accumulation of their transcripts. Total RNA was extracted from the leaves of sugarcane cvs. Ho02-144 and L79-1002 after exposure to 0 °C for 0, 24, 48 and 72 h. PCR was performed using cDNA from control and cold treated plants. Elongation factor 1α (SoEF1α) was used as loading control and reference gene. bZIP = basic leucine zipper domain containing transcription factor; ARF25 = auxin response factor binding protein 25; ARF1 = auxin response factor binding protein 1; PHD = plant homeodomain transcription factor; HSF = heat shock transcription factor; LHY = late elongated hypocotyl; F-box = F-box (ore9); EBF1 = ethylene binding factor 1; ZFP = zinc finger protein (ccch domain containing); PRR95 = two-component response regulator PRR95; CIPK = CBL-interacting protein kinase1; MAP3K = mitogen activated protein kinase kinase kinase; STK = serine threonine protein kinase (NIMA-related kinase 6); GLYK = D-glycerate 3-kinase; DUSP4 = dual specificity phosphatase 4; FTSH = filamentation temperature-sensitive H (ATP-dependent zinc metallo-protease); PEPC = phosphoenolpyruvate carboxylase; ND2 = NADH dehydrogenase subunit 2; Ca^{2+}-ATPase 4 = calcium-transporting ATPase 4 (endoplasmic reticulum type); SAMDC = S-adenosylmethionine decarboxylase; ABC transporter = ATP binding cassette transporter; OMT = 2-oxoglutarate malate translocator.

transcription factor, basic leucine zipper (bZIP) was found to be highly upregulated in early stages of both cold tolerant and sensitive energycanes, but with higher fold increase in the tolerant clone. Microarray experiments with Arabidopsis also demonstrated late induction of bZIP transcription factors under cold stress [29].

Abiotic stress affects cellular activities by damaging important proteins. Protein degrading machinery was also activated in the tolerant clone of energycane as a survival to cold stress. The upregulation of EIN3-binding F-box protein under cold stress, as observed in the present study, suggested that it was targeting the proteins involved in ethylene response pathways for degradation by 26S proteasome. EIN3-binding F-box protein mutant had high accumulation of EIN3 and growth inhibition phenotype [30]. Up regulation of EIN3-F box protein (Ore9) promoted degradation of EIN3 and thus delayed senescence under cold stress [31]. Similarly, diverse classes of kinases and phosphatases, which played important role from sensing the stress to initiation of the responses, were found to be activated under cold stress. The serine/threonine kinase, upon activation by osmotic stress, could phosphorylate a phosphatidylinositol transfer protein which in turn can upregulate the activities of phosphatidylinositol 3-kinase and 4-kinase in sugarcane [32].

Abscisic acid (ABA) has been implicated in cold stress response of many plants [33]. Induction and regulation of expression of genes in both ABA-dependent and ABA-independent signaling pathways under cold stress are well established [34]. However, none of the 465 cold-responsive unigenes identified in the subtraction/ACP library of the energycane clone Ho02-144 showed similarity to ABA-pathway genes. But a few genes analyzed in the present study were shown to be directly or indirectly associated with the ABA level in plants in response to cold stress. For example, putrescine was shown to regulate cold-stress response in plants in ABA-dependent mechanism by modulating ABA biosynthesis at

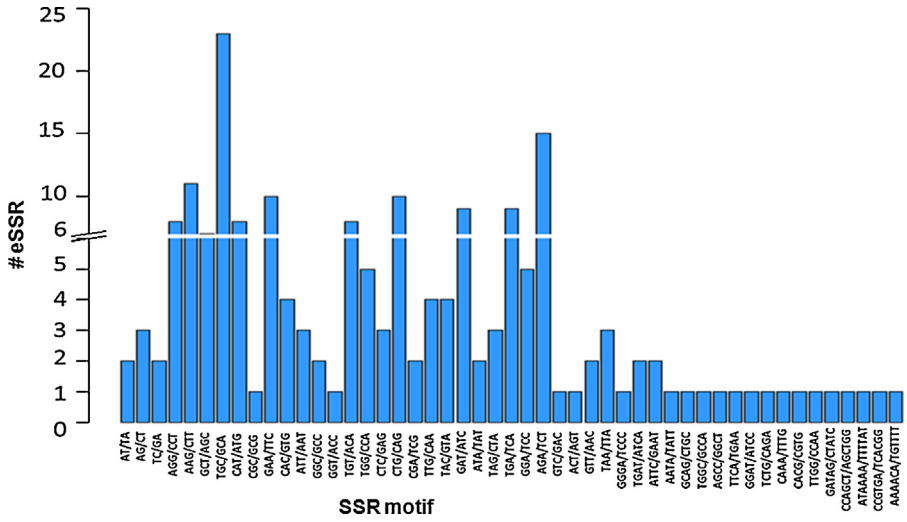

Fig. 5. Distribution of simple sequence repeat (SSR) motifs in the SSR-containing unigenes of energycane Ho02-144.

the transcriptional level [26,35]. Auxin response factor 2 (ARF2) was shown to be an ABA-responsive regulator due to the presence of a reverse ABF/ABRE binding *cis*-element [36]. The expression of ARF2 was increased by 2- to 5-fold following treatment of Arabidopsis seedlings with 30 μM ABA [36]; the overexpression of ARF in response to cold stress in the present study provides an indirect evidence of the involvement of ABA in cold stress response of sugarcane. Similarly, bZIP transcription factor is also known to bind to ABA-responsive element of the stress responsive genes [29], which again indicates that ABA is involved in cold stress tolerance mechanism of energycane. Semiquantitative RT-PCR of an ABA-responsive element binding factor (ABF) showed its overexpression in the tolerant energycane clone under cold, which suggested its positive role in cold stress adaptation (Supplementary Fig. 2). Therefore, it is possible that ABA-pathway genes were represented in the subtraction library but were not captured with the present low-scale sequencing effort and thus needs further investigation to study their important roles in the cold stress response of energycane. On the other hand, expression of an ABA-independent gene encoding dehydration responsive binding protein (DREB1A) was upregulated in the tolerant clone whereas it showed down-regulation in sensitive clone upon exposure to cold stress in a time-dependent manner (Supplementary Fig. 2).

4.3. Genes involved in the photosynthetic machinery

Chloroplast survival is synonymous to cell and plant survival from the reactive oxygen species during abiotic stress. FtsH gene coding for an ATP-dependent zinc metalloprotease was highly upregulated by cold stress in both tolerant and sensitive

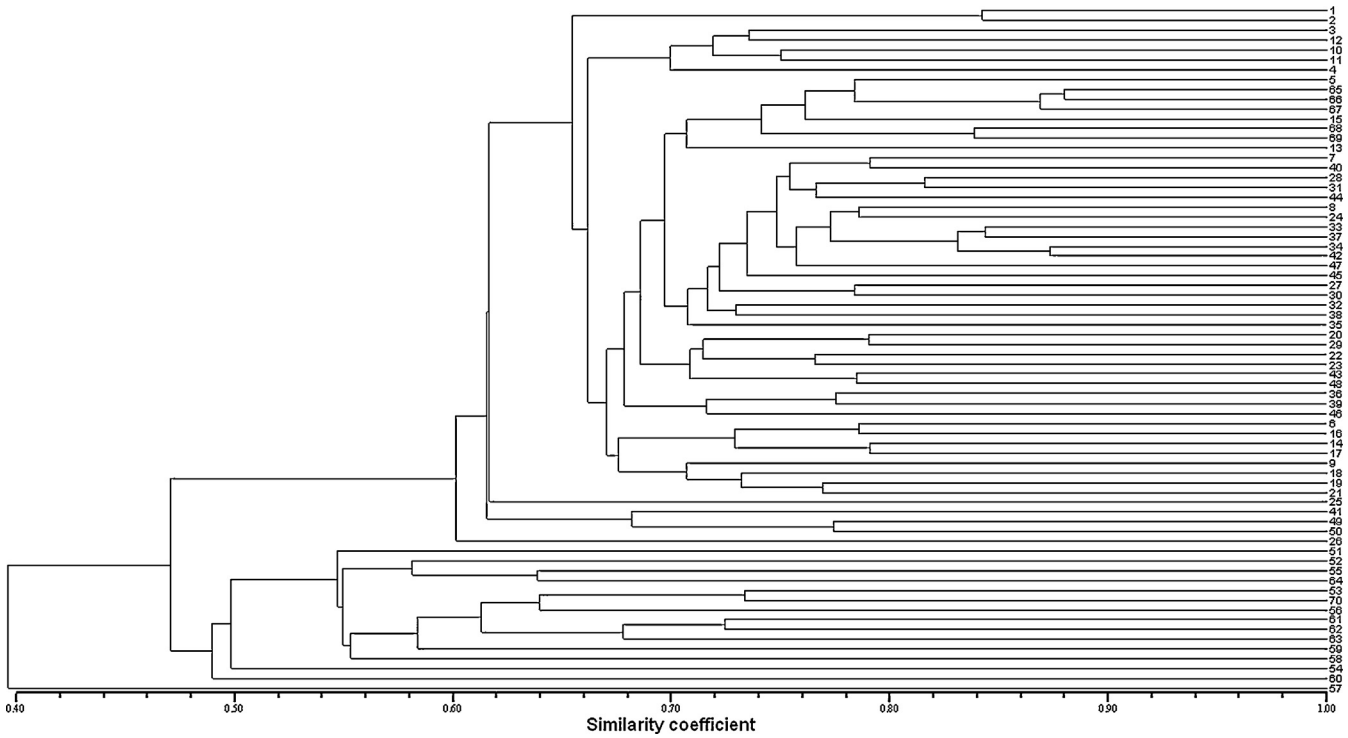

Fig. 6. Dendrogram analysis showing the cluster pattern of the sugar/energycane hybrids and their progenitors based on the allelic diversity at unigene-derived simple sequence repeat loci. 1–48 and 65–69 = *Saccharum* hybrids, 49 & 50 = *S. officinarum* clones, 51–64, 70 = *S. spontaneum* clones (details in Supplementary Table 2).

energycane, with very high expression level in the former (Fig. 4). Cold stress is known to cause destabilization of photosystem II (PSII) and reduce the photosynthesis efficacy of plants [37], which was also evident from the loss of chlorophyll in the leaves of the sensitive clone (Fig. 1). FtsH protease, localized in the thylakoid membrane, helps in degrading the destabilized D1 protein of PSII reaction center [38], thereby preventing aggregation of misfolded proteins in the cell. Increased protein degradation and misfolding during cold stress may have increased the demand of FtsH protease in the tolerant clone to enable it to maintain the protease activity to protect the photosystem and chloroplast biogenesis from stress-induced damage [39].

Similarly, genes involved in Krebs cycle and glucose and fructose metabolism were also highly induced under cold stress. These genes included phosphoenolpyruvate carboxylase (PEPC), NADP dehydrogenase subunit 2 (ND2) and D-glycerate 3-kinase (GLYK). The expression of PEPC and other photosynthesis-related genes was observed to be higher in *Miscanthus giganteus* in comparison to maize under low temperature conditions [40]. An ABC transporter (TUR2) and o-methyltransferase (OMT) genes were induced by cold stress in an aquatic plant *Spirodela polyrriza* [41] and rice [42], respectively. Similarly, Ca^{2+}-ATPase that acts as an antiporter catalyzing the active transport of Ca^{2+} from cytosol to intracellular organelle or apoplast has been implicated in cold stress response in the plants [43].

4.4. Utility of cold-responsive genes in development of markers

EST-derived microsatellite markers (eSSRs) have become markers of choice for the sugarcane scientific community for the ease of their use, their inexpensive development and amenability to highthroughput operation [44]. Because eSSRs are derived from the transcripts with a putative function, they are useful for functional diversity assay in natural populations or germplasm collections. The eSSRs derived from the sugarcane genes also had high sequence similarity with sorghum, so these can be used as anchor markers for comparative mapping and could prove useful for marker-assisted selection. Moreover, their cross transferability across different species have facilitated us tracking of the ancestral *Saccharum* species-specific alleles in the commercial sugarcane hybrids (unpublished data). In the present study, ninety-two percent of the eSSRs were observed to be polymorphic, which is higher than that reported in earlier studies with genomic and genic SSR markers of sugarcane [45,46]. The higher polymorphism could be due to high rate of slippage of the microsatellite sequences during replication and to large allelic diversity among the parental genotypes used to produce the commercial hybrids. *S. spontaneum* showed the most diversity, which further conformed to our earlier reported results [24]. On the other hand, genetic similarity value was higher among sugarcane hybrids, which indicated that only a few parental clones were involved in the development of the foundation clones through nobilization in breeding programs [47]. The eSSRs from cold-responsive genes differentiated *S. officinarum* and *S. spontaneum* with only 7% similarity to each other, which was clearly evident from the cluster analysis results (Fig. 6). Grouping of the commercial hybrids with *S. officinarum* in Cluster I was expected because most of the modern cultivars inherited the major part of the genome of *S. officinarum* during nobilization events. Similar results were also reported earlier from our lab with the use of gene-based TRAP markers [24]. Thus genic markers seem to be useful to assess genetic diversity and discriminate between different species of the *Saccharum* complex. However, the conserved sequences of the transcribed regions within the same genus accounted to low (0.20) average polymorphic information content (PIC) of the eSSR markers in the present study; this was in agreement with a previous study by Cordeiro et al. [48]. Another reason for the low PIC could be due to the low frequency of transmission of the diverged sequence differences between *S. officinarum* versus *S. spontaneum* and cultivated sugarcane varieties [49]. Nevertheless, the eSSRs could be used with gSSR and other marker systems for mapping in sugarcane.

In summary, we were able to identify genes involved in diverse biological/cellular/molecular mechanisms in sugarcane in response to cold stress by selectively enlarging its transcriptome through SSH and ACP-based library. Cold stress induced phosphatases and kinases involved in signal transduction and post-translational modification; activated different regulons through respective transcription factors, retrotransposons; upregulated biosynthesis of polyamines, and positively controlled protein degradation and ubiquitination pathways; and induced genes involved in the repair of damaged cellular proteins in the cytosol and chloroplast and genes involved in antioxidant biosynthesis for ROS scavenging in the cold-tolerant energycane clone. Early accumulation and subsequent maintenance of high messages in Ho02-144 could be the determining factor for its ability to tolerate cold for a prolong time period. A much comprehensive coexpression network established through next generation sequencing and validation through protein-protein interaction and transgenic overexpression/knock down will provide better clues to the cold stress response of sugar/energycane. Further, the interaction network with the coregulated connections between genes of interest need further validation based on experimental evidence, which will help decipher the specific biological inference among the interacting genes in sugarcane and other grasses, because many processes are conserved among different monocotyledonous species [20]. These genes would have a great potential in the engineering of sugarcane plants with higher cold tolerance that would allow the cultivation of this plant in more temperate climates. Additionally, the differentially expressing cold responsive genes will be very useful to mine for SNPs/indels for QTL and association mapping to identify functional markers associated with cold tolerance in sugar/energycane.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.07.001.

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