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RESEARCH ARTICLE

Isolation and characterization of drought responsive Aldehyde dehydrogenase (ALDH) gene from drought tolerant wild relative of sugarcane, *Erianthus arundinaceus*

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Abstract
Aldehyde dehydrogenases are oxidising enzymes that play essential role in response to drought stress. Sugarcane, an important high biomass producing commercial crop and its productivity is influenced by drought stress. A drought responsive Aldehyde dehydrogenase *(ALDH)* gene was isolated from *Erianthus arundinaceus* and characterized based on *in silico* analysis to understand about its physicochemical properties, phylogeny and structure. The isolated *EaALDH* gene is 1527 bp encoding for protein of length 508 amino acids. The in *silico* study ascertained physiochemical properties of ALDH protein viz., molecular weight 54.19 kDa, theoretical pI 6.42, aliphatic index 94.27, grand average of hydropathicity (GRAVY) 0.146 and instability index 32.95. The subcellular localization analysis predicted the protein to be localized in peroxisome. The protein-protein association network showed the networking of ALDH with other ALDH family members. Real time quantitative polymerase chain reaction indicated higher expression *ALDH* gene in *E. arundinaceus* compared to *Saccharum* spp. hybrid under low water conditions. This study characterized *EaALDH* gene involved in drought stress response and highlighted its importance in incorporating *EaALDH* in sugarcane improvement programmes.

Keywords: *Erianthus arundinaceus;* Water deficit stress; ALDH gene

Introduction

Sugarcane production is significantly affected by drought stress. Water-deficit stress is a major abiotic factor posing threat to sugarcane productivity in sugarcane producing countries (Ferreira etal. 2017). Aldehyde dehydrogenases (*ALDHs*) are found to play significant role across prokaryotic and eukaryotic systems. These ALDH group of enzymes are well represented and play essential role in various biochemical, catabolic, metabolic and functional pathways in plants [Stiti et al. 2011]. ALDH proteins are found in different subcellular compartments (Mitsuya et al. 2009; Missihoun et al. 2011). And these proteins are known to play two major functions *viz.,* conversion of aldehydes to carboxylic acids in many catabolic and biosynthetic pathways and detoxification of reactive aldehydes and other reactive species generated under various stress conditions in plant which in turn avoids damages and maintain cell homeostasis [Tsuji et al. 2003; Kotchoni et al. 2006; Missihoun et al. 2018]. *ALDHs* share numerous aldehyde substrates and hold higher homology in plants and animals (Brocker et al. 2013). *ALDHs* participate in the formation of osmolytes *viz.,* β-alanine betaine, γ-aminobutyric, glycine betaine, and also might involve in carnitine biosynthesis (Tylichova etal. 2010). Expression analysis in cassava roots showed the involvement of *ALDH* in glycolysis/gluconeogenesis (Yang et al. 2011). In addition to these processes, *ALDHs* are also found to play crucial role in catabolic, metabolic and stress signalling pathways (Tola et al. 2021).

Expression of *ALDH* genes is differential in plant tissues (Tsuji et al. 2003; Missihoun et al. 2011).

Similarly, *ALDH* gene members show differential expression pattern during heat and cold stress (Jiang et al. 2019). In rice, *OsALDH* genes had elevated levels of expression during drought stress (Gao and Han 2009). Many overexpression studies of *ALDH* genes have been carried out in plants. Overexpression of *ALDH* gene members in plants enhances tolerance to abiotic stresses *viz.,* drought, salinity and oxidative stress (Rodrigues et al. 2006). Transgenic Arabidopsis plants, overexpressing *ALDH3I1* and *ALDH7B4* combated drought and salt stress conditions by reducing lipid peroxidation (Kotchoni et al. 2006). Overexpression of *ALDH3* in arabidopsis, improved tolerance against various stresses like heavy metals, methyl viologen (MV), NaCl, dehydration, and H_2O_2 with lower levels of reactive aldehydes derived from lipid peroxidation (Sunkar etal. 2003). Transgenic Arabidopsis plants overexpressing *ALDH2B7* exhibited higher survival ratio under drought stress treatment (Rasheed et al. 2018).

The vital roles of *ALDHs* in drought and combination of stress like wounding and salt stress in arabidopsis have also been elucidated (Zhao et al. 2017). Role of *ALDH* is reported in aroma of fragrant rice cultivars like jasmine and basmati (Sakthivel et al. 2009). In rice, *ALDH* also assists in maintaining the seed viability by aldehyde detoxification formed from lipid peroxidation during oxidative and abiotic stresses (Shin et al. 2009). Thus the involvement of *ALDHs* in various biological and stress signalling mechanisms underlines its importance in modulation of essential pathways especially which involve in stress tolerance. *Erianthus arundinaceus* possess drought tolerance characteristics besides tolerance to other abiotic stresses naturally and also related to sugarcane genetically (Augustine et al. 2015a; Anunanthini et al. 2019; Manoj et al. 2019; Narayan et al. 2019; Clarancia et al. 2020; Peter et al. 2020; Vignesh et al. 2021). Hence, in this study, we have isolated *ALDH* gene from *E.arundinaceus* and performed *in silico* characterization to understand its putative biochemical properties, function, conservation, phylogeny, subcellular localization and protein interaction networks. We have also carried out comparative expression analysis of *ALDH* gene under drought stress conditions in *E. arundinaceus* and commercial *Saccharum* hybrid.

Materials and Methods

Plant material and drought condition

Single bud setts of *E. arundinaceus* clone Bethuadahari and commercial *Saccharum* hybrid Co 86032 were planted in 18-inch pots containing soil mixture (1 Soil: 1 Sand: 1 Farmyard manure) and seedlings were raised under glasshouse at 28°C ± 2°C and drought stress was given as explained previously (Clarancia et al. 2020; Peter et al. 2020). Root samples were taken after 1, 5, and 10 days of stress, then frozen in liquid nitrogen at -80°C until use. Root tissues collected from plants that received regular irrigation served as a control.

RNA extraction and cDNA conversion

The isolation of RNA was carried out using Trizol method (Chomzynski and Mackey 1995). The extracted RNA was treated with DNase I (Thermo Fisher Scientific, USA) to remove genomic DNA. DNase treated RNA was used to synthesize cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

Cloning of Aldehyde dehydrogenase (ALDH) gene from E. arundinaceus

The annotated gene sequences of aldehyde dehyrogenases were retrieved from NCBI Genbank. *Sorghum bicolor* (AB084898.1), *Zea mays* (KJ004510.1), Oryza sativa (AF162665.1) and *Hordeum vulgare* (AB055519.1) were used for primer designing. Polymerase chain reaction (PCR) was performed using the

forward primer 5'-ATATATGGGGGCCTTCGC GAAGGAGCACCAGTTCCT-3' and reverse primer 5'- TTAACCAAAATTGATTCCCTGAG CTAGAG G -3' on cDNA template extracted from *E. arundinaceus.* PCR was carried out with 5 minutes of denaturation at 94°C, followed by 45 seconds of denaturation (35 cycles) at 94°C, 30 seconds of annealing at 62.5°C. The extension of 1 minute and final extension of 10 minutes was carried out at 72°C. On a 1% agarose gel, the PCR fragments were analysed, and the DNA fragment of predicted size was eluted using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). The eluted DNA fragment was ligated into TA cloning vector pTZ57R/T (55 ng/L) and transformation was carried out using *Escherichia coli* strain DH5α. Plasmids containing recombinant *EaALDH* gene were extracted using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA) and sequenced using Sanger sequencing method at South Campus, Delhi University, New Delhi. The sequenced *EaALDH* was analysed and verified using BLAST (Basic Local Alignment Search Tool). The verified *EaALDH* gene sequence was submitted to NCBI GenBank.

In silico analysis

The *EaALDH* CDS sequence was translated using ExPASY translate tool (https://web.expasy.org/translate/). The CDS and protein sequences verified by blasting against nucleotide collection (nr/nt) and non-redundant protein sequences (nr) using NCBI BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov)). Physiochemical analysis of EaALDH protein was carried out using ProtParam tool (https://web.expasy.org/protparam/). The conserved domains of EaALDH were identified using MOTIF Search webserver [MOTIF: Searching Protein Sequence Motifs (genome.jp)]. Multiple sequence alignment of EaALDH with aldehyde dehydrogenase sequences from other crop species *(S. bicolor, O. sativa, Z. mays, S. italica, Triticuma estivum and H. vulgare)* was carried using CLC Genomics Workbench 12. Phylogenetic analysis of ALDH proteins of various plant species was performed using MEGA X version 10.1. Multiple sequence alignment was carried out using MUSCLE algorithm with gap open set to -2.90, hydrophobicity multiplier to 1.20, and minimum diagonal length (λ) to 24. UPGMA clustering method was used. Neighbor-joining method with 1000 bootstrap replicates was used for phylogenetic tree construction. Gaps/missing data treatment was given using pair-wise deletion method. Subcellular localization and gene ontology terms were carried out using LocTree3 (https://rostlab.org/services/loctree3/). The NetPhos 3.1 Server (http://www.cbs.dtu.dk/ services/NetPhos/) was used to predict phosphorylation sites. GOR IV (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) and Hierarchical Neural Network (HNN) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html) were used secondary structure prediction with output width set to 70. SOPMA (Self - Optimized Prediction Method with Alignment) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl? page=/ NPSA/npsa_sopma.html) with the number of conformational states set to four (Helix, Sheet, Turn, coil), similarity threshold to eight and window width to 17 was also used for secondary structure prediction. Phyre2 web server was used for the prediction of three-dimensional structure of EaALDH in the intensive mode (http://www.sbg.bio.ic.ac.uk/~ phyre2/html/ page. cgi?id=index). ProSA (https://prosa.services.came.sbg.ac.at/prosa.php), ERRAT (https://servicesn.mbi.ucla.edu/ERRAT /), and Chiron (https://dokhlab.med.psu.edu/chiron) webservers were used to further examine the predicted protein structure. The STRING database was used for Protein-Protein interaction analysis (https://string-db.org).

Quantitative relative gene expression analysis

For quantitative *ALDH* gene expression analysis, specific qRT-PCR primers were designed using IDT-primer quest tool. The qRT-PCR was performed using the primer pair F- CCTTGGAGTTGTGGGTGTTAT and R- AGTCGTTGGAGCACCTTTC, with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal control. The quantitative gene expression analysis was performed as explained elsewhere (Dharshini et al. 2016; Manoj et al. 2019; Narayan et al. 2019; Clarancia et al. 2020; Dharshini et al. 2020).

Results and Discussion

EaALDH gene cloning

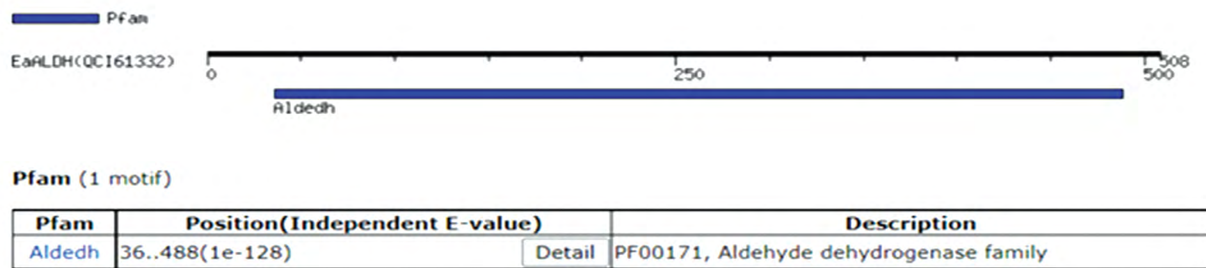
The *EaALDH* gene cloned from drought tolerant *E. arundinaceus* was 1527 bp and submitted to NCBI GenBank with an accession number QCI61332.1.

In silico analysis using bioinformatic tools

The translated protein of *EaALDH* gene is of 508 amino acids in length. The nucleotide sequence blast showed that homology of *EaALDH* with other plant *ALDH* sequences. *EaALDH* gene shared identity of 96.80% and query coverage 100% with *S.bicolor;* identity of 88.34 % and query coverage of 99% with *O. sativa* and identity of 97% and query coverage of 88.41% with *T.aestivum*. Similarly, *EaALDH* protein shared identity of 97.25% and query coverage 100% with *S. bicolor;* identity of 92.14 % and query coverage of 100% with *O. sativa* and identity of 90.77% and query coverage of 100% with *T.aestivum.* Physicochemical property prediction of EaALDH showed its molecular weight to be 54.19 kDa (Table 1). Computation of theoretical pI assist in fabrication of biochemical experiments. Theoretical pI was computed to be 6.42 which is less than 7 showing the acidic nature. Negatively charged residues (Aspartic acid + Glutamic amino acid) were calculated to be 44 in EaALDH polypeptide. And also, positively charged residues (Arginine + Lysine) were estimated to be 42 in EaALDH protein. Presence of more negatively charged amino acids than the postively charged amino acids indicates the intracellularic nature of EaALDH (Cedano et al. 1997). Aliphatic index refers to the volume occupied by the aliphatic side chains. Thermophilic proteins possess comparatively higher aliphatic index than mesophilic proteins. The calculated aliphatic index of EaALDH protein is 94.27. Higher aliphatic index of EaALDH protein suggests the higher thermostability of the protein (Ikai 1980). The instability index for stable proteins is lower than 40 (Guruprasad et al. 1990). EaALDH was computed to have instability index (II) of 32.95 suggesting that protein structure is stable. The Grand average of hydropathicity (GRAVY) value was predicted to be 0.146 suggesting the hydrophobic nature of the protein. The conserved function domain analysis using MOTIF Search webserver showed the presence of specific pfam domain PF00171to aldehyde dehydrogenase family (Fig. 1). Subcellular localization prediction using LocTree3 tool predicted localization of EaALDH at peroxisomes which suggests

Table 1. Physio-chemical properties of EaALDH protein

Protein	Number of amino acids	Molecular weight (kilodalton)	Theoretical pI	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
EaALDH	508	54.19	6.42	32.95	94.27	0.146



Pfam	Position(Independent E-value)	Description
Aldedh	36..488(1e-128) Detail	PF00171, Aldehyde dehydrogenase family

Figure 1. Motif analysis of EaALDH protein

Figure 2. Multiple sequence alignment of ALDH proteins

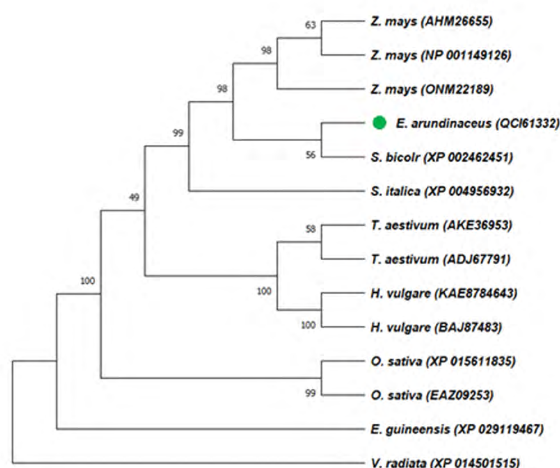


Figure 3. Phylogenetic analysis of ALDH proteins

the role of EaALDH in neutralizing reactive species. Multiple sequence alignment revealed the strong conservation of EaALDH and other ALDH proteins (Fig. 2). Phylogenetic analysis of EaALDH proteins showed the evolutionary relationships with other plant species. EaALDH was closely related to *S. bicolor* followed by *Z. mays* and *S.italica* (Fig. 3).

Secondary and three-dimensional structure analysis in EaALDH

The secondary structure prediction of EaALDH protein was carried out using GOR, HNN and SOPMA prediction servers. The percentage of alpha helices predicted by GOR, HNN and SOPMA were 28.54, 38.39 and 43.50 respectively. Similarly, percentage of extended strands predicted were 21.65, 17.52 and 17.32 by GOR, HNN and SOPMA respectively. And also, 49.80, 44.09 and 34.84 were percentage of random coils predicted by GOR, HNN and SOPMA respectively. Secondary structure prediction suggested the dominance of random coils followed by alpha helices and extended strands (Table 2). The predicted three-dimensional structure of EaALDH by Phyre2 webserver harboured sixteen alpha helices and nineteen beta strands connected by loops (Fig. 4). The modelled structure was energy minimized using Chiron webserver to remove steric clashes. The energy minimized structure was studied using Ramchandran plot (Supplementary Fig. 1) which showed 95.7% of residues in favoured region, 3.0% of residues in allowed region and 1.4% of residues in outlier region. ProSA analysis (Supplementary Fig. 2) of EaALDH structure gave Z score of -9.98 which fell in the region of experimentally determined structures indicating

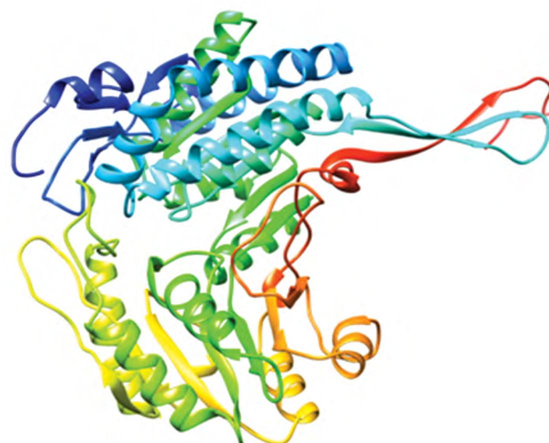
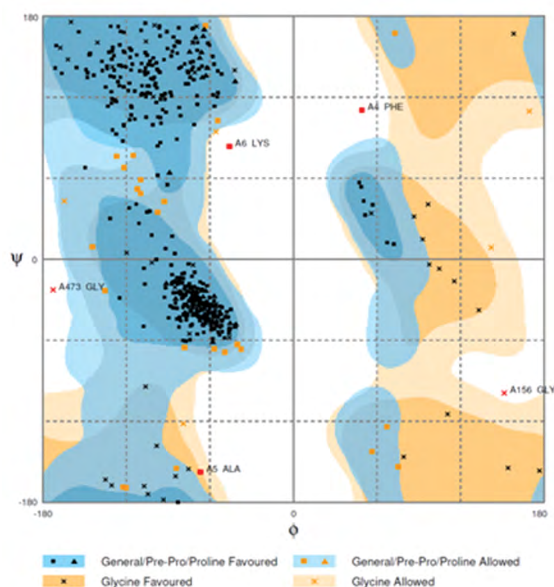


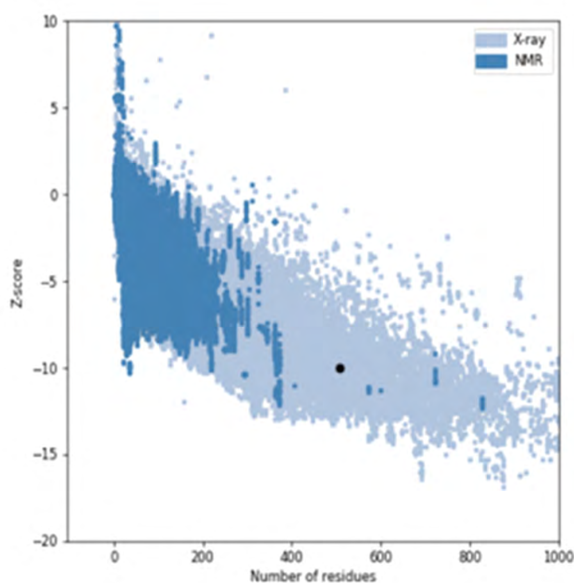
Figure 4. Three-dimensional structure of EaALDH protein

Table 2. Analysis of secondary structures of EaALDH protein

Server name	α-Helix		Extended-strand		Random coil	
	No. of residues	Percentage (%)	No. of residues	Percentage (%)	No. of residues	Percentage (%)
GOR	145	28.54	110	21.65	253	49.80
HNN	195	38.39	89	17.52	224	44.09
SOPMA	221	43.50	88	17.32	177	34.84



Supplementary Figure 1. Ramachandran plot analysis of three-dimensional structure of EaALDH protein

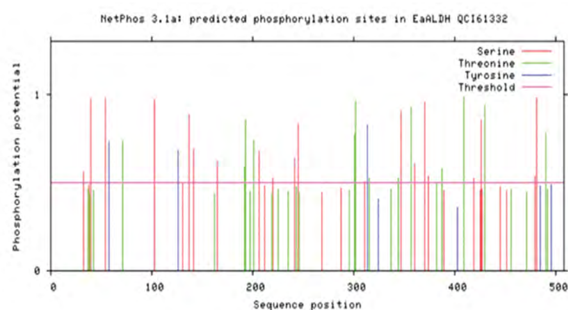


Supplementary Figure 2. ProSA analysis of three-dimensional structure of EaALDH protein

Table 3. Stereo-chemical analysis of EaALDH protein

Protein	Favoured region (%)	Allowed region (%)	Outlier region (%)	ERRAT	ProSA
EaDRO1	93.3	5.7	1.0	89.86	-9.98

that the model is reliable (Table 3). The overall quality factor computed by ERRAT was 89.86. The phosphorylation site residues such as tyrosine, threonine and serine of EaALDH were predicted using NetPhosserver (Supplementary Fig. 3). Six tyrosine residues, 21 serine residues and 13 threonine residues, totally 40 phosphorylation site residues were predicted in EaALDH protein.



Supplementary Figure 3. Phosphorylation site prediction of EaALDH protein

Protein-protein association of **ALDH** *protein*

Protein-protein interaction network of *S. bicolor* ALDH protein was constructed using STRING database as no annotation was available for *E.*

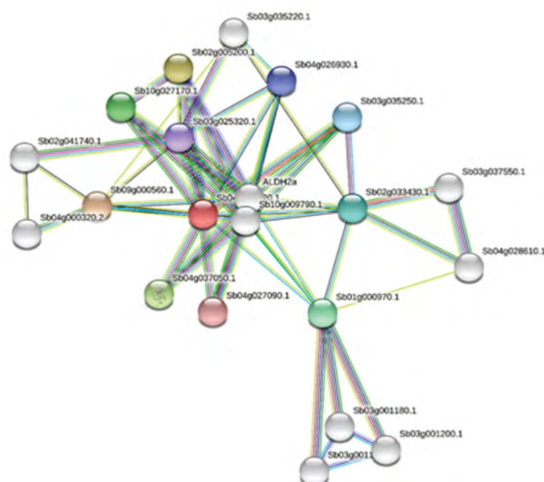


Figure 5. Protein-Protein interaction network of ALDH proteins.

arundinaceus. The network showed the interaction of ALDH protein with other family members of ALDH (ALDH2a) (Fig. 5).

Differential expression of EaALDH under drought stress

Comparative quantitative expression analysis of *ALDH1* gene was carried out in *E. arundinaceus* and *Saccharum* spp. hybrid Co 86032 under drought conditions (Fig. 6). Expression of ALDH was not altered under normal irrigated conditions in both genotypes. However, ALDH expression was comparatively higher in *E. arundinaceus* than commercial sugarcane hybrid Co 86032 under stress conditions. And, also ALDH expression was increased along the progression of drought stress. This indicates that ALDH plays vital role in enhanced tolerance of *E. arundinaceus* compared to commercial variety. Previous studies showed that ALDH expression is variable and widespread throughout plant tissues and also developmentally regulated (Tsuji et al. 2003; Missihoun et al. 2011). Over expression of aldehyde dehydrogenase gene in various plants resulted in improved tolerance to different stresses (Sunkar et al., 2003; Kotchoni et al. 2006; Missihoun et al. 2011; Zhao et al. 2017). Thus, significant higher expression of ALDH gene in *E. arundinaceus* and *Saccharum* root tissues suggests the involvement of ALDH in root responses towards drought. Over expression of abiotic stress responsive genes from wild relative

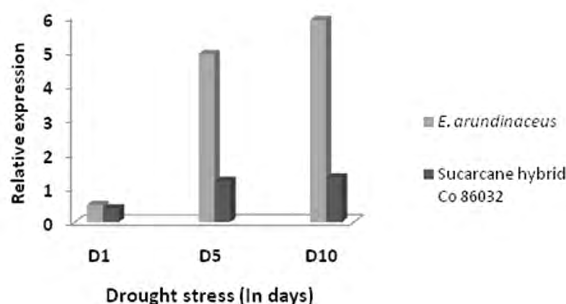


Figure 6. Quantitative expression of ALDH transcript in *E. arundianceus and Saccharum* hybrid during drought condition.

species improved the stress tolerance in commercial *Saccharum* hybrids (Augustine et al. 2015b, c; Mohanan et al. 2020, 2021; Narayan et al. 2021). However, further studies are required to support ALDH activity in roots of *E. arundinaceus* and its plausible role when transferred to commercial *Saccharum* hybrid to overcome dehydration and drought stress conditions.

Conclusion

Aldehyde dehydrogenase *(EaALDH)* gene was isolated from drought tolerant wild relative of *Saccharum* spp., *E. arundinaceus.* The isolated gene and translated gene product (protein) were analysed *in silico* for its physicochemical properties, secondary structures, subcellular localization, phosphorylation sites, conserved domains, phylogeny, three-dimensional structure and protein-protein interaction network. Differential regulation of *ALDH* gene expression was observed in drought tolerant *E. arundinaceus* and commercial sugarcane hybrid. This study suggests that developing transgenic sugarcane overexpressing *EaALDH* might assist in improving drought tolerance in sugarcane varieties.

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