

## RESEARCH ARTICLE

# Isolation, characterization and expression analysis of novel water deficit stress-responsive *DEEPER ROOTING 1 (DRO1)* gene from drought-tolerant *Erianthus arundinaceus*

P. Swathik Clarancia<sup>a</sup>, M. Naveenarani<sup>a</sup>, R. Valarmathi<sup>a</sup>, G. S. Suresha<sup>a</sup>, G. Hemaprabha<sup>a</sup>, Bakshi Ram<sup>a</sup> and C. Appunu<sup>a\*</sup>

<sup>a</sup>ICAR-Sugarcane Breeding Institute, Coimbatore 641007, Tamil Nadu, India

\*Corresponding author: Email: cappunu@gmail.com

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### Abstract

Sugarcane (*Saccharum* spp.) is an important food, fodder and energy crop in India. Its production and productivity are mainly constrained due to water deficit stress during different growth stages. Of the root system architecture traits, deep rooting helps plants to avoid drought-induced stress by foraging moisture from deeper layers of soil. Hence, in this study, a novel drought-responsive *DEEPER ROOTING 1 (DRO1)* gene cloned from drought-tolerant *Erianthus arundinaceus* was characterized. The open reading frame of this gene is 765 bp that encodes for a single polypeptide of 254 amino acids. *In silico* analysis of DRO1 protein using bioinformatics tools revealed its size of 28.91 kDa with theoretical pI 5.39, instability index 67.57, aliphatic index 69.92 and GRAVY of -0.86. Subcellular localization by LocTree3 tool suggested that DRO1 protein expression is localized in the nucleus. The phylogenetic tree exhibited that DRO1 from *Erianthus arundinaceus* is closely associated with that of *Sorghum bicolor* and *Triticum aestivum*. Protein interaction network analysis showed DRO1 association with WOX11, which promotes the development of crown roots and ARL1 (Adventitious rootless1) required for adventitious root formation. Quantitative gene expression analysis indicated that the DRO1 gene is differentially upregulated in root tissue of *E. arundinaceus* and *Saccharum* spp. commercial hybrid under water deficit stress conditions. *EaDRO1* gene can be a novel source for developing drought stress tolerant genotypes through genetic engineering approach.

**Keywords:** *Erianthus arundinaceus*; Differential expression; Drought stress; *DRO* gene

### Introduction

Drought is a serious problem for sustainable sugarcane production. Sugarcane, being a twelve months duration crop, faces moisture stress at one or multiple growth phases namely germination, formative, grand growth and maturity phases. The intensity and magnitude of drought stress is in the rise in the recent years. Under field conditions, drought stress is further complicated with other environmental stresses due to which crops attain only about 25% of their potential yield (Boyer 1982). Moisture stress during the crop growth period accounts for about 30-70% loss

in productivity. In India, 65% of the area under sugarcane is rainfed and is inescapably linked to the vagaries of the monsoon. Therefore, strategies for the genetic enhancement of drought avoidance/tolerance in crop plants are essential for food security. One of the root architectural traits directly linked with drought tolerance is deep rooting of crop plants (Uga et al. 2013) that helps to extract water from deep soil layers (Kondo et al. 2000, 2003). Many studies have suggested that a deep root system helps plants to avoid drought stress by extracting water from deep soil layers (Yoshida

et al. 1982; Fukai et al. 1995; Gowda et al. 2011). *E. arundinaceus*, a wild relative of sugarcane produces deeper roots than commercial sugarcane varieties (Valarmathi et al. 2018). The deep root system in *E. arundinaceus* may contribute to its drought tolerance through enhanced water uptake (Price et al. 1999). Therefore, to ensure drought tolerance in commercial variety, the introduction of genes responsible for deep rooting is considered as one of the most promising breeding strategies (Gowda et al. 2011). (*DRO1*) is known to be responsible for deeper root development in rice and is highly induced in roots by drought stress (Uga et al. 2013). So far, *DEEPER ROOTING* gene (*DRO1*) has not been characterized in the *Saccharum* complex. In this study, a novel *DRO1* gene was cloned, characterized from drought-tolerant *E. arundinaceus*, a wild relative of sugarcane and its expression response to drought stress conditions was determined.

## Materials and methods

### *Plant growth and treatments*

The genetically pure material of *E. arundinaceus* clone Bethuadahari was obtained from field Gene Bank maintained at ICAR-Sugarcane Breeding Institute, Coimbatore. Single bud setts of *E. arundinaceus* and a commercial sugarcane 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. Drought stress was given to ninety days old plants by withholding water for ten days. Root samples were collected at 1, 3, 5, 7 and 10 days of stress and placed in liquid nitrogen and stored at -80°C until use. Root tissues collected from plants that received regular irrigation served as a control.

### *RNA extraction and cDNA conversion*

Trizol method (Chomzynski and Mackey 1995) was followed for the isolation of RNA from treated

and control samples. Genomic DNA was removed from total RNA by treating with DNase I (Thermo Fisher Scientific, USA). Treated RNA was used as a template to synthesize cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

### *Cloning of DEEPER ROOTING 1 (DRO1) gene from E. arundinaceus*

*DRO1* gene sequences of cereal crops were retrieved from NCBI GenBank. Primers were designed from the conserved region of annotated *DRO1* gene from *Oryza sativa* (BAN59748; BAN59747; DI244710; DI244711; DI244722; DI244724; HV4381681; HV438167; HV438154; HV438166; HV438155), *Triticum aestivum* (QBP14670; QBP14671; QBP14672) and *Sorghum bicolor* (DI244720; HV438164). Polymerase chain reaction (PCR) amplification was carried out using forward primer 5'-ATGAAGATTTTCAGTTGGGTAGCCAACAAGATCG-3' and reverse primer 5'-TTAGTTTGAGATATCCCCATTATGGTACTGAGGTTGC-3' with cDNA extracted from the root of *E. arundinaceus* as a template. PCR reaction was performed with 5 min of denaturation at 94°C, followed by 35 cycles of 45 seconds of denaturation at 94°C, 30 seconds of annealing at 62.5°C and extension of 1 min at 72°C with a final extension of 10 min at 72°C. PCR products were analyzed using 1% agarose gel, and the expected size of DNA fragment of *DRO1* was eluted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). Eluted DNA was ligated into TA cloning vector pTZ57R/T (55 ng/L) and transformed into *Escherichia coli* strain DH5 $\alpha$ . Recombinant plasmids containing *DRO1* gene were isolated using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA) and sequenced by Sanger method at South Campus, Delhi University, New Delhi. *EaDRO1* gene

sequence was verified and analyzed by BLAST (Basic Local Alignment Search Tool).

### **Bioinformatics analysis**

The CDS sequence of *DRO1* was translated using the ExPASy translate tool (<https://web.expasy.org/translate/>). The cDNA and protein sequence of DRO1 were blasted against nucleotide collection (nr/nt) and non-redundant protein sequences (nr). Physio-chemical properties of EaDRO1 protein were predicted using the ProtParam tool (<https://web.expasy.org/protparam/>). Conserved motifs in DNA and protein were discovered using Motif Discovery tool of MEME Suite 5.1.1 (<http://meme-suite.org/tools/meme>). Multiple sequence alignment (MSA) of EaDRO1 with DRO1 protein sequences of other plant species was carried out in CLC Genomics Workbench 12. Phylogenetic analysis of DRO1 proteins was performed using MEGA X version 10.1. MUSCLE alignment was used to align the sequences with gap open set to -2.90, hydrophobicity multiplier to 1.20, and minimum diagonal length ( $\lambda$ ) to 24. UPGMA clustering method was used. The phylogenetic tree was generated using the neighbor-joining method with 1000 bootstrap replicates. The pair-wise deletion was used for gaps/missing data treatment. Subcellular localization and gene ontology terms were predicted using LocTree3 (<https://roslab.org/services/loctree3/>). Phosphorylation sites were predicted using the NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>). Secondary structures were predicted by *PSIPRED* 4.0 (<http://bioinf.cs.ucl.ac.uk/psipred/>), GOR IV ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=/NPSA/npsa\\_gor4.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html)) with output width set to 70. Hierarchical Neural Network (HNN) ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=/NPSA/npsa\\_hnn.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html)) with output width set to 70, and SOPMA (Self-Optimized Prediction Method with Alignment) ([\[pl?page=/NPSA/npsa\\\_sopma.html\]\(https://npsa-prabi.ibcp.fr/cgi-bin/npsa\_automat.pl?page=/NPSA/npsa\_sopma.html\)\) with the number of conformational states set to four \(Helix, Sheet, Turn, coil\), similarity threshold to eight and window width to 17. Three-dimensional structure of EaDRO1 was predicted using Phyre2 web server in the intensive mode \(<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>\). The protein structure was further evaluated using ProSA \(<https://prosa.services.came.sbg.ac.at/prosa.php>\) and ERRAT \(<https://servicesn.mbi.ucla.edu/ERRAT/>\) Chiron \(<https://dokhlab.med.psu.edu/chiron>\) web servers \(Protein-Protein interaction was studied using STRING database \(<https://string-db.org>\)\).](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.</a></p>
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### **Relative expression analysis**

For quantitative expression analysis, specific qRT-PCR primers were designed using IDT-primer quest tool. The primer pair, F-CGAGTGCTTCAAGACGGTGG and R-ATGGGGCGAAGAAAGGTGTC was used to perform qRT-PCR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control (Dharshini et al. 2016). To perform qRT-PCR experiments, the cDNA converted using total RNA isolated from root samples of drought-stressed and control plants were used as a template. qRT-PCR was performed on a Step-One Plus Real-Time PCR system (Applied Biosystems, Canada) by following the method explained elsewhere (Anunanthini et al. 2019; Manoj et al. 2019; AswinNarayan et al. 2019; Dharshini et al. 2020a, 2020b; Swathik Clarancia et al. 2020). Each reaction was carried out in triplicates. In brief, each qRT-PCR reaction consists of 50 ng cDNA, 2.5 pmol primers, 12.5  $\mu$ l of 2X MESAGREEN Master Mix (Eurogentec Belgium) and the final volume was made up to 25  $\mu$ l with sterile water (Dharshini et al. 2018). qRT-PCR reaction conditions were as follows: denaturation for 10 min at 95°C followed by annealing and extension at 1 min for 60°C (40 cycles). The fold change of the target gene was

**Table 1.** Physio-chemical properties of EaDRO1 protein

Protein	Number of amino acids	Molecular weight (kilodalton)	Theoretical pI	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
EaDRO1	254	28.91	5.39	67.57	69.92	-0.860

determined using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen 2001).

## Results and Discussion

### *DEEPER ROOTING 1 (EaDRO1) gene isolation and bioinformatic analysis*

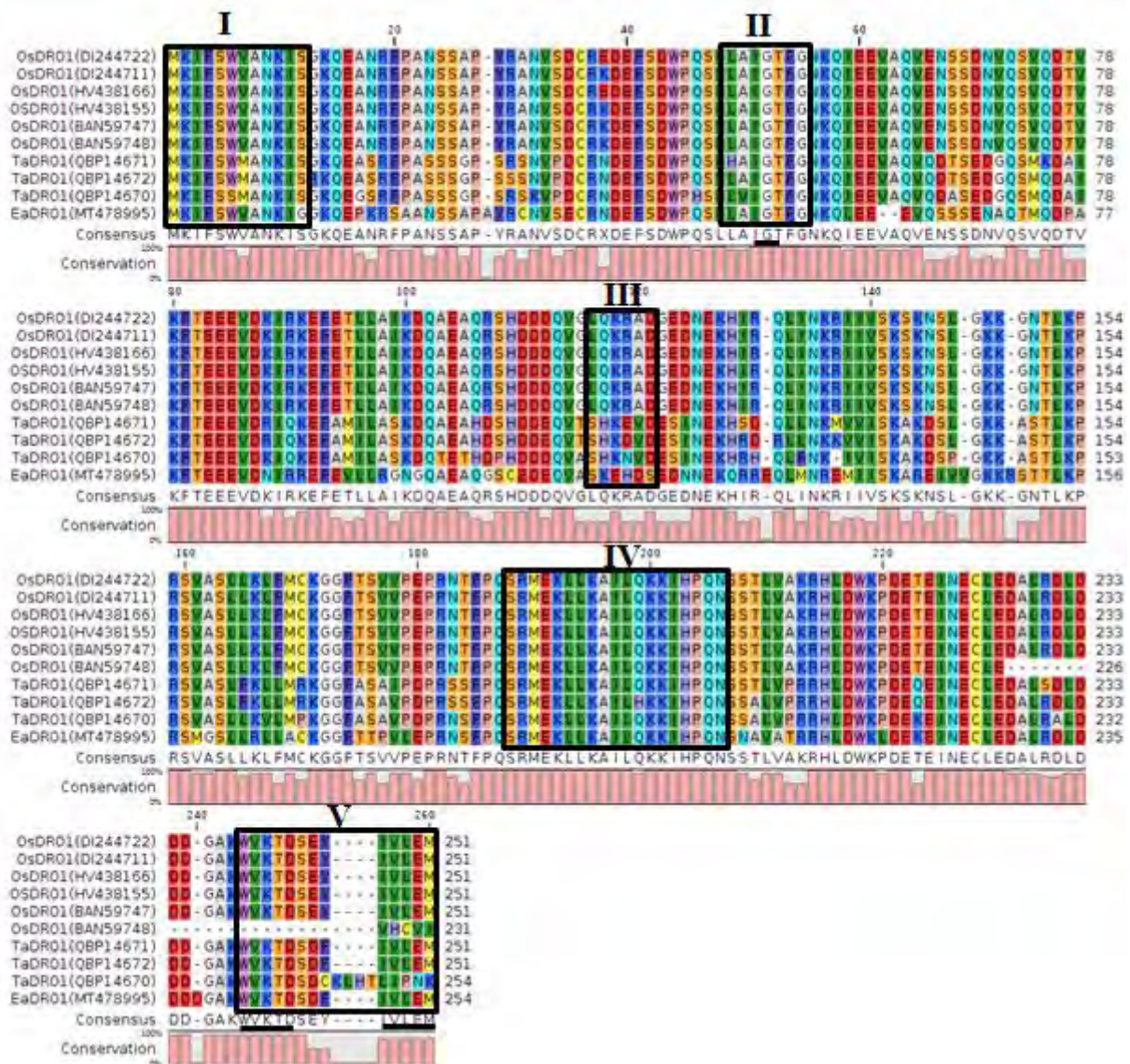
Sugarcane is a high water demanding crop due to higher accumulation of biomass. Drought is the major constraint in crops to realize higher productivity. Therefore, strategies for the genetic enhancement of drought resistance in sugarcane are essential for sustainable production. Many studies have suggested that a deep root system help plants avoid drought stress by mining water from deep soil layers (Yoshida et al. 1982; Fukai et al. 1995; Gowda et al. 2011). Role of the *DEEPER ROOTING 1* gene (*DRO1*) in development of long roots is well characterized in rice (Uga et al. 2013; Kondo et al. 2003) and in maize (Kondo et al. 2000). A wild relative of sugarcane *E. arundinaceus* produces longer and deeper roots compared to commercial varieties (Valarmathi et al. 2018). However, so far, deeper rooting gene is not reported in the *Saccharum* complex. For the first time, this study isolated and characterized *DEEP ROOTING 1* gene (*EaDRO1*) from drought-tolerant *E. arundinaceus* clone Bethuadahari, a wild relative of *Saccharum* spp. A complete coding region of *DRO1* gene sequence was submitted to the NCBI GenBank database under the accession number MT478995. Sequence analysis of *EaDRO1* revealed that the length of the ORF is 765 nucleotides which encode a protein of 254 amino acids. Coding sequence analysis revealed that *EaDRO1* shared homology

of the deeper rooting gene from *Sorghum bicolor* 92.54%, *Zea mays* 79.80% and *Triticum aestivum* 76.03%. Protein sequence analysis *EaDRO1* showed an identity of 86.43% with *S. bicolor*, 73.31% with *Z. mays* and 66.02% with *T. aestivum* subsp. *tibeticum*.

Physio-chemical properties of *EaDRO1* protein were calculated using the ExPASy ProtParam tool and results are given in Table 1.

The molecular weight of *EaDRO1* is computed to be 28.91kDa, theoretical pI to be 5.39, instability index to be 67.57, aliphatic index to be 69.92 and grand average of hydropathicity (GRAVY) to be -0.860. The number of positively charged amino acids (Arg + Lys) was found to be 39, while negatively charged amino acids (Asp + Glu) were 45. Computation of physio-chemical parameters would assist in designing biochemical experiments for *EaDRO1* protein. SignalP prediction of *EaDRO1* showed absence of signal peptide sequence. *EaDRO1* protein is localized in the nucleus as revealed by subcellular localization prediction tool LocTree3. In Arabidopsis, *DRO1* gene is shown to be expressed predominantly in nuclei of root tip cells and is involved in auxin localization (Waite et al. 2020). This suggests that *EaDRO1* would also localize in the nuclei of root cells and influence root development.

*DRO1* and *DRO1*-like homologues belong to IGT (named for a conserved amino acid motif) gene family. The IGT gene family members are known to affect the orientation of lateral shoots. In Arabidopsis, *DRO1* is expressed predominantly in both the root vasculature and

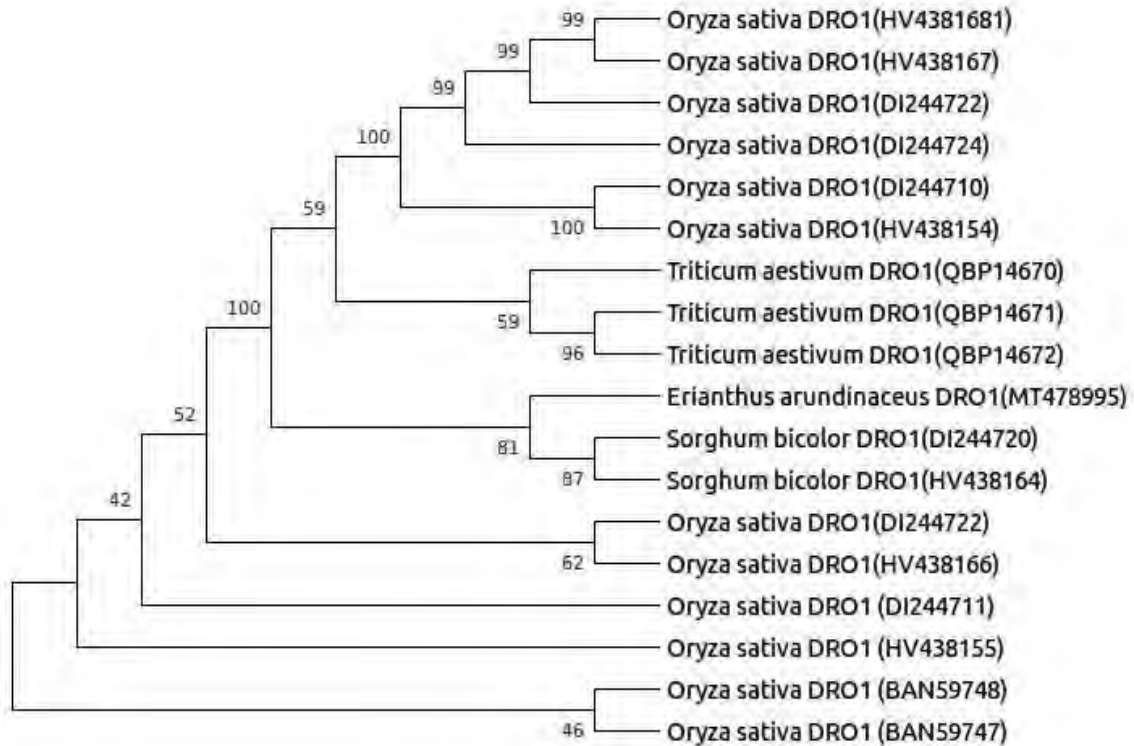


**Figure 1.** Protein sequence homology of EaDRO1 with DRO1 from other cereals. Os, *Oryza sativa*; Ta, *Triticum aestivum*; Ea, *Erianthus arundinaceus*. Domains are enclosed by black boxes represented from I-V. IGT motif in the second domain, the conserved (WVKTD) motif in the fifth domain followed EAR-like motif IVLEM are underlined by black lines in the consensus.

root tips. Overexpression of *AtDRO1* resulted in steeper lateral root angles. A C-terminal EAR-like motif present in the proteins of IGT gene family members is accountable for steeper lateral root angles which assist in drought avoidance. Furthermore, overexpression of *DRO1* in *Prunus domestica* resulted in deeper-rooting phenotypes thereby providing drought resistance (Guseman et

al. 2017). Multiple sequence alignment of DRO1 proteins showed insertions and deletions in the EaDRO1 protein sequence (Fig. 1).

Four insertions and two deletions were prominent in EaDRO1 compared to DRO1 of other cereal crops. DRO1-like homologs harbour five distinct domains which are conserved and variable in



**Figure 2.** Phylogenetic relationships of EaDRO1 protein with cereal crops *O. sativa*, *T. aestivum*, and *S. bicolor*: The tree was constructed with 1000 bootstrap replicates.

length (Ashraf et al. 2019). All five domains were present in EaDRO1 (Fig. 1). Presence of the IGT motif was identified in the second domain region of EaDRO1. The EAR-like motif of five amino acids IVLEM in the fifth domain of DRO1-like homologs is present in EaDRO1. Newly reported conserved amino acids for the distinction of DRO-like proteins (WVKTD) is also identified in EaDRO1 in the fifth domain region at the C-terminus as in other orthologs. Presence of C-terminal EAR-like motif (IVLEM) in EaDRO1 protein proposes the plausible role of *EaDRO1* in the ecotypic phenotypes of *E. arundinaceus* to tolerate drought. Phylogenetic analysis showed EaDRO1 is closely related to *S. bicolor* (Fig. 2). Phosphorylation site prediction of EaDRO1 showed 17 serine residues, 1 tyrosine residue and 6 threonine residues as putative sites for phosphorylation.

### **Motifs, secondary and three dimensional structure analysis in EaDRO1**

Conserved motifs were predicted using the MEME Suite. There are three different conserved motifs in *EaDRO1* gene and protein. The motifs are RAGAAGCAGCTCAR, GGMDCWAGTNMAGA, GCGGG, and GCAACGABDAG. The consensus of motifs present in protein are FRCNVLECRN, WPQNLNAIGTRGN, and DGFKWV. Secondary structure prediction was carried to probe the structural information of EaDRO1 protein. Secondary structure predicted for *Triticum aestivum* DRO1 (TaDRO1) protein showed maximum percentage of alpha-helix to be 49.80 %, extended strand to be 9.96 %, beta-turn to be 3.59 % and random coil to be 4.18 %. Similarly, the secondary structure prediction of EaDRO1 protein showed alpha-

**Table 2.** Analysis of secondary structures of EaDRO1 protein

Server name	$\alpha$ -Helix		Extended-strand		Random coil	
	No. of residues	Percentage (%)	No. of residues	Percentage (%)	No. of residues	Percentage (%)
GOR	124	48.8	26	10.24	104	40.94
HNN	93	36.1	21	8.27	140	55.12
SOPMA	93	36.1	20	7.87	128	50.39

helix to be 48.82 %, extended strand to be 10.24 %, beta-turn to be 5.12 %, and random coil to be 55.12 % (Table 2).

In TaDRO1 and EaDRO1 secondary structures, there was dominance of random coils followed by alpha-helices and beta strands. Lesser percentage of beta strands compared to alpha-helices is indicative of the default preference of alpha-helix structures in secondary conformations of proteins to maintain stability. The percentage of random coils was higher than other secondary structure elements.

Three-dimensional structure of EaDRO1 protein was modelled using Phyre2 web server in the

by EaDRO1 protein *in vivo* to increase thermal stability under drought conditions (Ruggiero et al. 2019). Three-dimensional structure of EaDRO1 protein was energy minimized using Chiron web server. After energy minimization, clashes in the protein structure were minimized and submitted to Protein Model Database (PMDB). The PMDB id for the EaDRO1 model is PM0083216. The energy minimized structure was subjected to Ramachandran plot analysis for geometrical validation. Sterically allowed and disallowed regions in the protein structure can be studied based on Ramachandran plot analysis (Table 3).

In EaDRO1 protein structure, 205 (81.3%)

**Table 3.** Stereo-chemical analysis of EaDRO1 protein

Protein	Ramachandran Plot				
	Favoured region (%)	Allowed region (%)	Outlier region (%)	ERRAT	ProSA
EaDRO1	81.3	13.5	5.2	49.05	-3.58

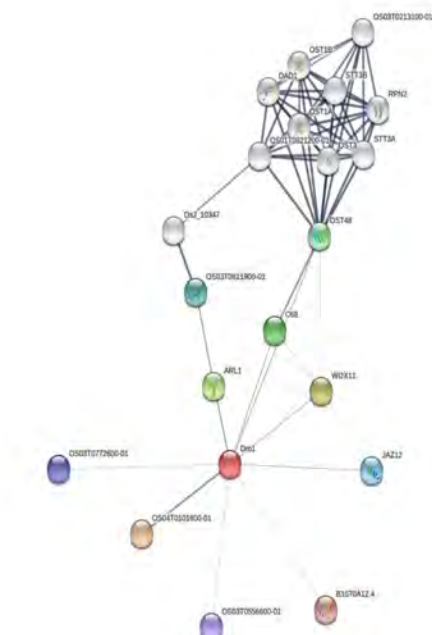
intensive mode. Multiple templates were used to model the structure. The multi-template modelled structure of EaDRO1 depicted the presence of alpha helices and larger loop regions. Loop regions are inversely correlated with stability (Nagi and Regan 1997). Secondary structure of EaDRO1 protein has longer loop regions. This suggests ad hoc loop optimization mechanisms to be adopted

amino acid residues were present in the favoured region, 34 (13.5%) residues were present in the allowed region and 13 (5.2%) were present in the disallowed region (outlier region). Therefore, more than 90% of residues of EaDRO1 protein had allowed conformations that in turn indicated the stability of the protein. ProSA web server predicted

Z-Score of -3.58 which fell within the region of experimentally determined DRO1 structures by NMR spectroscopy and X-ray crystallography. The quality factor of the structure predicted by ERRAT web server was 49.05 suggesting further refinement in the predicted EaDRO1 protein structure.

### Protein-protein association of DRO1 protein

A network of proteins, functionally associated with DRO1 was created using STRING database (Fig. 3).



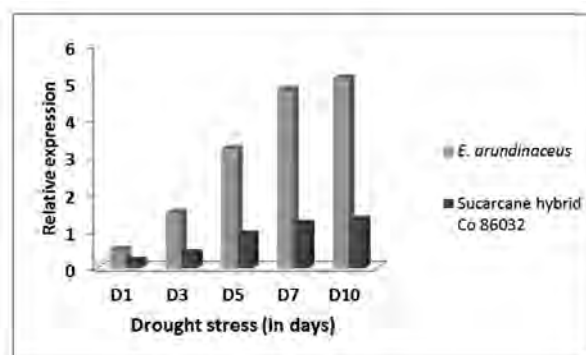
**Figure 3.** DRO1 protein interaction analysis. Each sphere (termed as node) represents all the proteins produced by a single, protein-coding gene locus. Colored nodes represent query protein and first shell of interactors. White nodes represent second shell of interactors. Empty and filled modes represent proteins of unknown and known/predicted 3D structures respectively. Edges/connecting lines represent protein-protein associations.

As protein-protein association data for *E. arundinaceus* is not present in STRING database, DRO1 protein association was studied for annotated *Oryza sativa* in the database. In the

network created, DRO1 showed associations with WOX11 which promotes the development of crown roots by regulating the genes essential for crown root development and hormone-responsive genes involved in cytokinin and auxin signalling (Zhao et al. 2009). DRO1 also associates with ARL1 (Adventitious rootless1) which is required for adventitious root formation in rice (Liu et al. 2005; Coudert et al. 2011) and JAZ12 which might act on the initial response of Jasmonic acid (JA) - regulated gene expression toward drought tolerance (Sasaki-Sekimoto et al. 2014). DRO1 is found to interact directly or indirectly with numerous other proteins in the network. These interactions indicate that manipulation of *DRO1*, *WOX11*, *ARL1* and *JAZ12* genes could improve the root system architecture in sugarcane.

### EaDRO1 is responsive to water deficit stress conditions

Comparative quantitative expression analysis of *DRO1* gene was carried out in *E. arundinaceus* and *Saccharum* spp. hybrid Co 86032 under water deficit stress conditions (Fig. 4).



**Figure 4.** Quantitative *DRO1* transcript analysis in roots of *E. arundinaceus* and sugarcane hybrid Co 86032 during water deficit stress condition.

No significant difference was noticed in *DRO1* expression level in both genotypes under normally irrigated conditions. The *DRO1* expression level was higher in *E. arundinaceus* compared to that of commercial sugarcane hybrid Co 86032 under



stress condition. Moreover, a gradual increase in the accumulation of *EaDRO1* transcripts in line with the progression of drought stress was recorded. Expression of *DRO1* gene in *Erianthus* was 3 to 5 fold higher when compared to the commercial variety during stress. In light of higher expression of *DRO1* gene in deeper-rooting genotypes (Uga et al. 2013), *DRO1* from *E. arundinaceus* could play an important role in avoiding stress. The higher expression of *DRO1* gene in *E. arundinaceus* highlights its vital role in the root architecture under water deficit stress for enabling the plant to survive under abiotic stress condition as noticed in other crops (Uga et al. 2013). Hence, overexpression of *EaDRO1* gene through genetic engineering approach in sugarcane would help in enhancing the root architecture thereby tolerating drought stress.

### Conclusion

In this study, a novel drought-responsive gene *DRO1* was isolated from drought-tolerant wild relative of sugarcane namely *Erianthus arundinaceus*. Physio-chemical parameters, secondary structure elements, tertiary structure, conserved motifs, subcellular localization, phosphorylation sites and phylogeny of *DRO1* were studied using bioinformatics tools. *In silico* analysis helped in understanding biochemical properties, the stability of protein and nuclear localization of *EaDRO1* protein. Presence of C-terminal EAR-like motif (IVLEM) in *EaDRO1* protein highlights its role in drought tolerance of *E. arundinaceus*. Protein interaction network revealed *EaDRO1* association with *WOX11*, which promotes the development of crown roots, *ARL1* (Adventitious rootless1) required for adventitious root formation and *JAZ12* that modulates the gene expression towards drought-resistance. Expression analysis revealed that *DRO1* gene is highly up-regulated in *E. arundinaceus* compared to *Saccharum* hybrid during drought conditions.

Hence, *EaDRO1* gene could be a novel source for developing drought stress tolerant genotypes in the sugarcane improvement programme via genetic engineering. Furthermore, *WOX11*, *ARL1* and *JAZ12* genes could also be exploited along with *EaDRO1* for developing drought-resistant sugarcane by enhancing root system architecture and regulating gene expression to withstand water deficit stress conditions.

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