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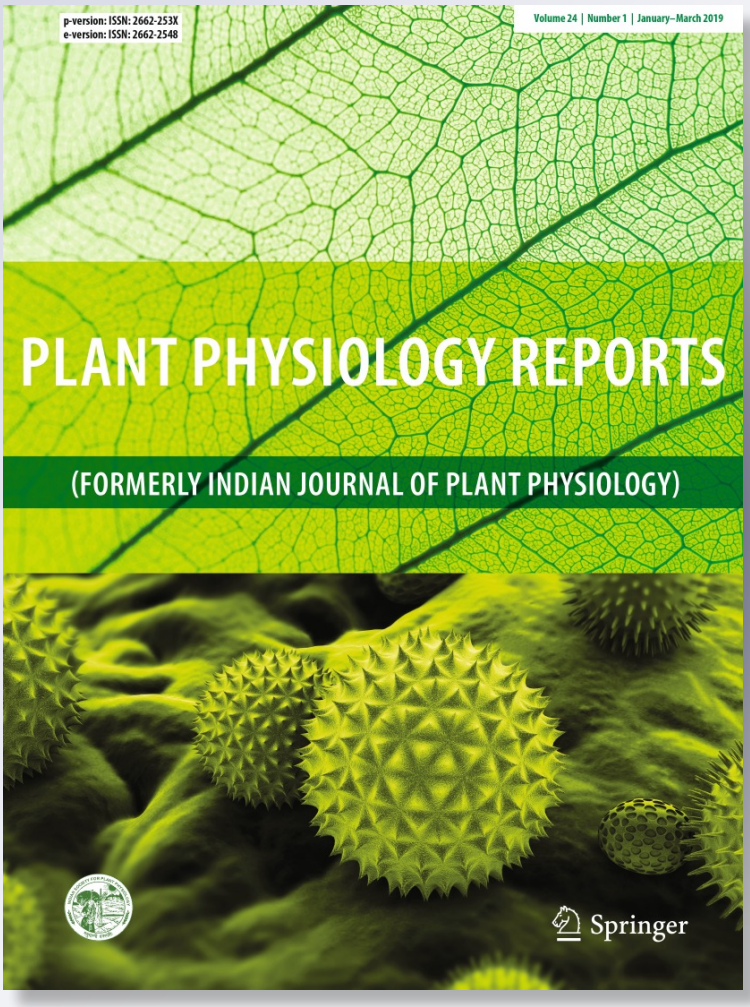

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ORIGINAL ARTICLE

Functional annotation of differentially expressed genes under salt stress in *Dichanthium annulatum*

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Abstract Soil salinity is one of the important abiotic stresses affecting plant growth and development. Halophytes can be one of the options to explore the salt tolerance potential and to identify the potential gene(s) which can be used in crop improvement programs. In view of this, the present experiment was conducted on grass halophyte, *Dichanthium annulatum*, which can tolerate soil salinity up to EC 30 dS/m (~ 300 mM NaCl) to identify the gene(s) for salt tolerance. The de novo assembly generated 267,196 transcripts and these assembled transcripts were further clustered into 188,353 transcripts. An average of 64.47% of the transcripts was functionally annotated against the *viridiplantae* databases since no genomic reference is available for *Dichanthium*. Gene ontology and pathways analysis using KAAS database identified that 48.13% transcripts were involved in molecular function, 37.21% in cellular component and 14.66% in biological processes. The annotation of these genes provides a pathway analysis for their putative functions under salt stress conditions.

Keywords Salt stress · Halophytes · *Dichanthium* · Gene · Salt tolerance

Introduction

Soil salinity is one of the major problems worldwide, especially in countries where irrigation is an essential input for agriculture. Due to non-availability of good quality water, farmers are forced to use saline water for irrigation (Barrett-Lennard and Setter 2010). Since soil salinity reduces the soil fertility and hence crop yield, there is an urgent requirement for other alternates. One of such approaches can be increasing crop production dramatically by improving plant productivity under stress conditions. But the genetic diversity for stress tolerance within traditional crops is too narrow to achieve this goal immediately (Colmer et al. 2006). Therefore, the most feasible approach is identification of stress tolerance genes in extremophiles and then introducing them into traditional crops. Halophytes, the plants growing under extreme saline conditions, could be the best choice to identify salt tolerance genes. Halophytes grow in a wide variety of saline habitats, from coastal regions, salt marshes and mudflats, to inland deserts, salt flats and steppes. Halophytes have evolved a range of adaptations to tolerate seawater and survive under higher concentrations of salts up to EC 50 dS/m (~ 500 mM NaCl) (Kumar et al. 2018). These include adjustment of internal water relations through ion compartmentation in cell vacuoles, accumulation of compatible organic solutes, succulence, and salt-secreting glands and bladders etc. (Flowers and Colmer 2008; Shabala 2013; Agarwal et al. 2013). Optimal halophyte growth is achieved at a concentration of around 50 mM NaCl for monocots and between 100 and 200 mM for dicots (Glenn et al. 1999; Flowers et al. 2014). Few reports are available using comparative proteomics and other molecular technologies with the goal of identifying new salt-responsive genes or proteins in halophytes (Pang et al. 2010; Wang

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et al. 2013). Although much useful information related to salt tolerance has been accumulated from the analysis of salt-stressed glycophytes such as *Arabidopsis thaliana*, the specific regulatory mechanisms that enable halophytes to survive in extremely saline habits have yet to be completely elucidated. *D. annulatum* is a species of grass commonly used as forage for livestock and also known as marvel grass or Hindi grass. It is native to tropical Asia, the Middle East and parts of Africa. In general, it is a perennial grass often with stolons. The root system goes no deeper than one meter. Plants can be diploid, tetraploid or hexaploid. It is tolerant of varied soil conditions, including soils high in clay and sand, poorly drained soils, and soils that are somewhat alkaline and saline. It forms a turf that can stand up to grazing pressure. Hence, in the present study, we have taken the grass halophyte, *Dichanthium annulatum*, which can tolerate soil salinity up to EC 30 dS/m ($\sim$ 300 mM NaCl) to exploit the de-novo RNA-seq technology for functional annotation and gene ontology under salinity stress. To our knowledge, this is the first study conducted to understand the molecular basis of salinity tolerance of grass halophytes, *D. annulatum,* using the RNA-Seq approach.

Materials and methods

Plant material

Dichanthium annulatum plants were raised through root cuttings in pots filled with sandy and moist soil in a screen house at Division of Crop Improvement, ICAR—Central Soil Salinity Research Institute, Karnal (29°43′N, 76°58′E, and 245 m above the mean sea level), Haryana, India in 4 replications. The soil salinity in pots was created by initially using natural soil of EC 15.92 dS/m (150 mM NaCl) brought from the highly saline experimental farm of CSSRI at Nain, Panipat, Haryana. Further salinity level of EC 30 dSm^{-1} ($\sim$ 300 mM NaCl) was maintained by irrigation with saline water. The saline treatment was maintained in three pots with one set of control plants irrigated with normal tap water.

RNA library preparation

RNA was isolated from leaves using standard Trizol protocol. RNA sequencing libraries were prepared with Illumina-compatible NEBNext® Ultra™ Directional RNA Library Prep Kit (New England BioLabs, MA, USA). Total RNA at final concentration of 100 ng to 1 μg was taken for mRNA isolation, fragmentation and priming. Fragmented and primed mRNA was further subjected to first strand synthesis in the presence of Actinomycin D (Gibco, life technologies, CA, USA) followed by second strand synthesis. The double-stranded cDNA was purified using HighPrep magnetic beads (Magbio Genomics Inc, USA). Purified cDNA was end-repaired, adenylated and ligated to Illumina multiplex barcode adapters as per NEBNext® Ultra™ Directional RNA Library Prep Kit protocol.

Adapter-ligated cDNA was purified using HighPrep beads and was subjected to 15 cycles of Indexing-PCR (37 °C for 15 min followed by denaturation at 98 °C for 30 s, cycling (98 °C for 10 s, 65 °C for 75 s) and 65 °C for 5 min) to enrich the adapter-ligated fragments. The final PCR product (sequencing library) was purified with HighPrep beads, followed by library quality control check. Illumina-compatible sequencing library was quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and its fragment size distribution was analysed on Agilent 2200 Tapestation.

De novo transcriptome sequencing

Sequencing for 150 bp length paired-end reads was performed in an Illumina HiSeq sequencer (Genotypic Technologies, Bangalore) to produce on an average of 45.01 million raw sequencing reads. The reads were processed for quality assessment and low-quality filtering before the assembly. The raw data generated was checked for the quality using FastQC.

Processed reads were assembled using graph-based approach by Trinity program. Trinity combines the overlapping reads of a given length and quality into longer contig sequences without gaps. The characteristic properties, including N50 length, average length, maximum length, and minimum length of the assembled contigs were calculated. The clustering of assembled transcripts based on sequence similarity was performed using CD-HIT-EST with 95% similarity between the sequences, which reduces the redundancy without exclusion of sequence diversity that is used for further transcript annotation and differential expression analysis.

Functional annotation and gene ontology

Clustered transcripts were annotated using homology approach to assign functional annotation using BLAST tool against "*viridiplantae*" data from the UniProt database. Transcripts were assigned with a homolog protein from other organisms at E-value less than e-5 and minimum similarity greater than 30%. Pathway analysis was done by using KAAS Server. *Oryza sativa japonica*, *Zea mays*, *Musa acuminata* and *Dendrobium officinale* were considered as reference organisms for pathway identification having more than 60% similarity with the experimental halophytic plant *D. annulatum.*

Results and discussion

De novo sequence assembly of transcriptome

About 41.41 million reads from *Dichanthium* were generated and used for the downstream analysis in the present experiment. Among all raw reads, on an average of 96.65% of high-quality data (having a Phred-like quality score > q30) was retained for every sample. The high-quality RNA-seq reads were de novo assembled into transcripts using Trinity, as the reference genomic sequence is not available for *Dichanthium*. In absence of reference genome, the transcriptome coverage efficiency has been assessed by relating the unique genes with the closest available transcriptome in de novo sequencing (Délano-Frier et al. 2011; Parchman et al. 2010). The Trinity assembly of high quality reads resulted in 267,196 transcripts and further clustering resulted in 188,353 transcripts with an average length of 864 bp and N50 of 1100 bp. Since the shorter sequences may lack a characterized protein domain or may be too short to show sequence matches, resulting in false negative results, the contigs which were less than 300 bp in length were excluded. Compared with the other EST sequencing technologies, the RNA-Seq provides assembled and annotated high quality reads and is being used in a number of plants such as *Spartina* (Ferreira de Carvalho et al. 2013; Gedye et al. 2010), *Cynodon* (Hu et al. 2015), *Sorghum* (Dugas et al. 2011) and some others. Various scientific approaches are being used to study the expression pattern of genes under stress environment. To reveal a variety of molecular responses against abiotic stress in non-model plants on a transcriptomic scale, NGS is regarded as the best method even when complete genome sequences are absent.

GO annotation

Since the fully-sequenced reference genome of *D. annulatum* is not available, hence the clustered high-quality reads were blasted against the *viridiplantae* database. Of the 188,353 clustered transcripts, 64.47%transcripts (121,446) were annotated and remaining 35.52% (66,907) transcripts could not be annotated due to lack of information for *Dichanthium* in the NCBI database. The percentage of annotated transcripts obtained in present study is within the range of annotation percentage reported in other species e.g. 64% in *Artemia* (De Vos et al. 2019), 61% in sugar beet (Lv et al. 2018), 82% in *Amaranthus* (Délano-Frier et al. 2011), 68% in *Spartina* (Ferreira de Carvalho et al. 2013) and *Cicer* (Garg et al. 2011). Similarly, 35% of unique transcripts obtained in *D. annulatum* are in accordance with the other reports as 8% unique transcripts in Maize (Vega-Arreguín et al. 2009), 7% in Ginseng and *Amaranthus* (Délano-Frier et al. 2011; Sun et al. 2010), 13% in *Spartina pectinata* (Gedye et al. 2010) and 35% in moth bean (Tiwari et al. 2018). The top BLAST hits for *D. annulatum* showed 49.25% of sequence similarity to *Z. mays* followed by *Dichanthelium oligosanthes* (14.36%), *O. sativa subsp. Japonica* (12.77%), *S. bicolor* (6.46%), *H. vulgare subsp. Vulgare* (3.05%), *Setaria italic* (2.41%), *Aegilops tauschii* (1.80%), *Saccharum hybrid cultivar R570* (1.13%) and *Arundo donax* (1.11%) as presented in Fig. 1.

The similarity distribution of *Dichanthium* sequences *w.r.t.* reference showed that 55.07% of transcripts had a similarity of more than 80%. Based on sequence similarity, annotated transcripts were categorized into three ontologies i.e. biological processes (BP), cellular component (CC) and molecular function (MF). Among these ontologies, BP (1945 terms) was most abundant in terms of different categories identified followed by MF (1486 terms) and CC (522 terms). In the biological processes, genes involved in transcription (3309), transcription regulation (3138), metabolic process (1948), carbohydrate metabolic process (1490), translation (1163) signal transduction (1118), defense response (726), response to oxidative stress (448), response to salt stress (190) and sodium ion transport (53) were highly represented. The salt stress-responsive transcripts observed under biological processes include dehydration responsive element binding protein (DREB; DN58174_c6_g4_i3), Myb family transcription factor EFM (DN72104_c1_g7_i1), auxin response factor (DN26188_c0_g3_i1), AP2-EREBP-type transcription factor (DN56877_c4_g3_i1), chaperonin-like RbcX protein (DN60403_c1_g1_i4), glutathione peroxidase (DN64844_c1_g7_i1), L-ascorbate peroxidase (DN61491_c0_g6_i3), peroxidase (DN64999_c3_g1_i2), catalase (DN68555_c1_g1_i4), putative methionine sulfoxide reductase (DN62432_c2_g3_i3) etc. It is already established that salt stress leads to the overproduction of reactive oxygen species (ROS) which are toxic to plant growth and metabolism. To scavenge the ROS system, plants adopt various mechanism such as activation of antioxidative enzymes i.e. catalase, peroxidase, ascorbate peroxidase etc.(Acosta-Motos et al. 2017; Das and Roychoudhury 2014; Yoshida et al. 2014). In the present study, different transcripts that belong to various antioxidative enzymes were found to be expressed which may detoxify the ROS components.

Within the molecular function ontology, ATP binding, DNA binding, ADP binding, metal ion binding, protein kinase activity, zinc ion binding, solute/proton antiporter activity, calcium ion binding etc. were highly represented. Some transcripts that are involved in the molecular function ontology are LRR receptor-like serine/threonine-protein kinase (DN47453_c0_g1_i1), asparagine synthetase

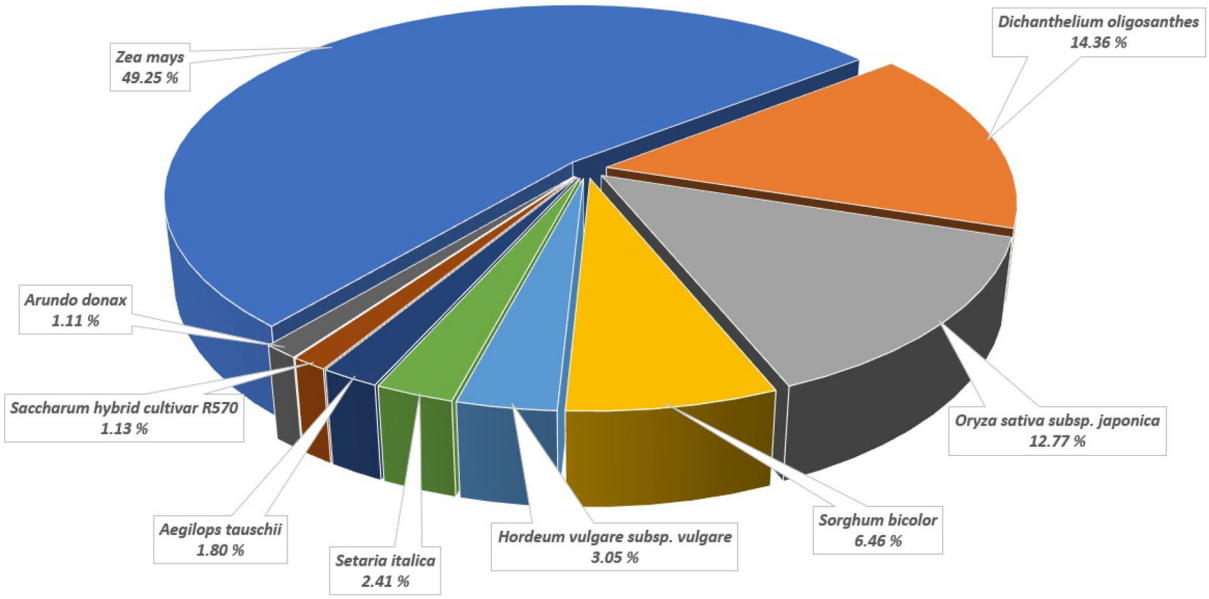

Fig. 1 Species distribution of the top BLAST hits for the *D. annulatum*

(DN61786_c1_g1_i2), salt overly sensitive 1 (SOS1, DN44879_c0_g1_i1), SOS2 (DN68935_c1_g2_i4) sodium/hydrogen exchanger (DN68364_c0_g12_i7), cation/H^+ antiporter (DN61714_c4_g5_i1), K^+ efflux antiporter 6-like isoform X2 (DN72421_c2_g4_i1), plasma membrane Na^+/H^+ transporter (DN73047_c3_g5_i3), calcium-binding EF-hand family protein (DN68613_c2_g4_i13), calcineurin B-like7 (DN65265_c0_g3_i1), calmodulin-like protein 1 (DN69677_c0_g1_i2). It has been reported in earlier studies that these above-mentioned transcripts are involved in salt tolerance mechanism (de Barajas-Lopez et al. 2018; Himabindu et al. 2016). de Barajas-Lopez et al. (2018) reported that protein kinases are involved in the salt stress and showed that SOS2 (Salt Overly Sensitive 2), SnRK3s (a.k.a. calcineurin B-like protein-interacting protein kinases, CIPKs) are Ser/Thr protein kinase acting in the SOS pathway, providing salt tolerance. Likewise, many reports in the literature showed that sodium/hydrogen exchanger, cation/H^+ antiporter and K^+ efflux antiporter 6-like isoform X2 are involved in ion homeostasis (Palavalasa et al. 2017).

Cellular component ontology mainly represents genes involved in integral component of the membrane (24,131), nucleus (7293), cytoplasm (2735), plasma membrane (2098), chloroplast (1441) and intracellular (1382). Likewise, Hu et al. (2015) reported the RNA-seq for gene identification in bermudagrass (*Cynodon dactylon*) under salt stress and found the 40,483 unigenes which they categorized into similarly three ontologies with 47 functional groups. At the cellular level, 22.9% of transcripts represent the integral component of the membrane which involves the membrane transporters such as Potassium transporter (DN64455_c0_g1_i4), Zinc transporter (DN62616_c2_g1_i2), bidirectional sugar transporter SWEET (DN66098_c1_g5_i21), transmembrane amino acid transporter family protein (DN69618_c4_g5_i2), ABC transporter (DN56431_c0_g2_i2), V-type proton ATPase subunit C (DN71221_c2_g5_i1). In cytosol of plant cell, a high ratio of K^+/Na^+ is an important factor for maintaining ion homeostasis under salt stress. It is reported that in high soil salinity, Na^+ may inhibit the potassium transporters by competing with the K^+ for influx into plant roots (Assaha et al. 2017).

Most abundant 10 terms from each functional ontology of biological functions, molecular functions and cellular components are represented as doughnut chart in Fig. 2 and some important genes/proteins involved mainly in salt stress tolerance mechanisms are summarized in Table 1.

KEGG pathway analysis

The pathway analysis is an important part for functional study of genes. Recently, a number of studies have been reported on pathway analysis in salt stress (Naika et al. 2013; Zhang et al. 2018). To identify the active biological pathways in *Dichanthium*, 27,431 unique transcripts were annotated against the KAAS server. From unique pathways identified, protein processing in the endoplasmic reticulum (1274 transcripts) was the most abundant pathway and anthocyanin biosynthesis (2 transcripts) was the least in terms of the number of homologous transcripts. Along with these pathways, we identified some other important pathways *i.e.* starch and sucrose metabolism (964 transcripts), RNA transport (947 transcripts), MAPK signalling pathway (763 transcripts), glutathione metabolism (697

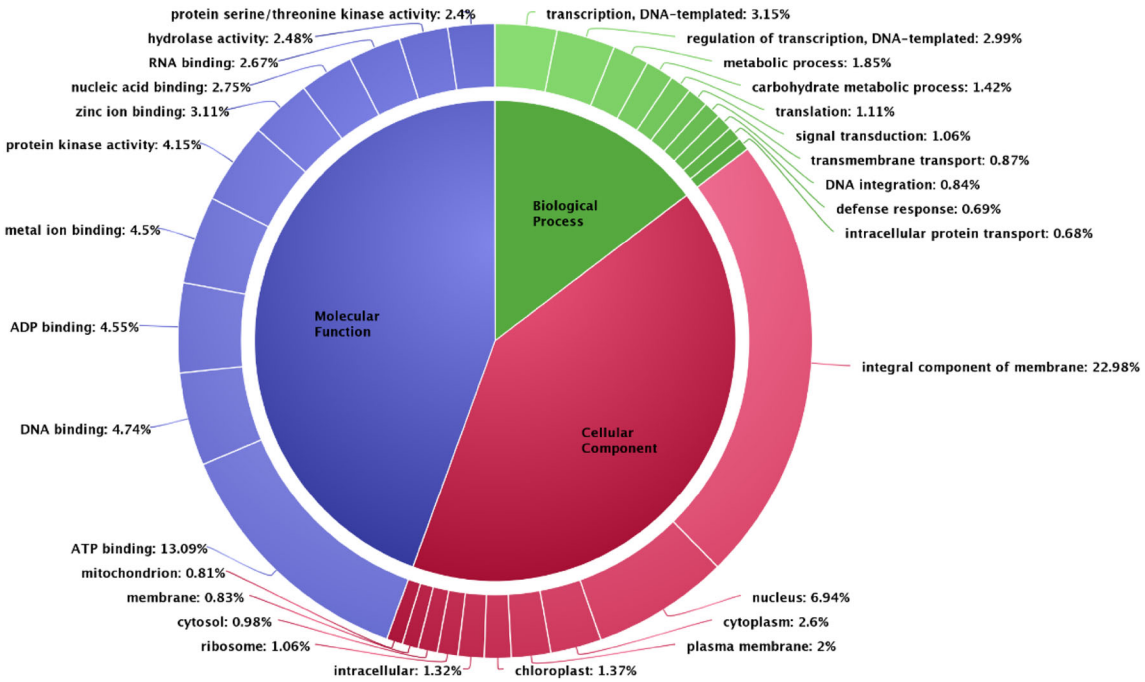

Fig. 2 Frequency of top 10 abundant GO terms under biological process, molecular function and cellular component categories in *D. annulatum*

transcripts) which may have a major role in salt stress tolerance.

In plants, Endoplasmic reticulum (ER) is highly vulnerable to stress conditions where assembling and folding of proteins take place. During salt stress, misfolded or unfolded proteins can accumulate which may activate various signalling pathways (Deng et al. 2013). Liu et al. (2016) reported about 220 up-regulated transcripts between seed-type and fibre-type *Cannabis sativa* in salt stress and identified the DEGs that were involved in endoplasmic reticulum protein processing pathway. The top 10 unique pathways identified for all transcripts in *Dichanthium* are presented in Fig. 3. Most of the expressed transcripts are involved in glycolysis, purine metabolism, RNA transport, endocytosis, starch and sucrose metabolism, plant hormone signal transduction etc. Comparative proteomics of *Thellungiella* leaves identified 209 salt-responsive proteins (Wang et al. 2013). Functional classification of these proteins into 16 categories indicated that the majority are involved in carbohydrate metabolism, followed by those involved in energy production and conversion, and then those involved in the transport of inorganic ions. Pathway analysis revealed that most of the proteins are involved in starch and sucrose metabolism, carbon fixation, photosynthesis, and glycolysis. Of these processes, the most affected were starch and sucrose metabolism, which might be pivotal for salt tolerance. In our studies also, one of the responsive pathways to salt tolerance includes starch and sucrose metabolism including glycolysis. The complete mechanism of these transcripts can be revealed by studying the differential gene expression and validation by Real-time PCR to design a schematic flowchart for salt tolerance mechanism in grass halophyte, *D. annulatum*.

It has been observed that glycophytes and halophytes might possess the same set of genes, but exhibit differential expression and in most of the instances, the difference in post-translational regulation between halophytes and glycophytes distinguishes salt-sensitive or salt-tolerant phenotypes. Salinity tolerance involves complex responses at the molecular, cellular, metabolic and physiological levels. At the molecular level, genes encoding ion transporters, transcription factors, protein kinases, and osmolytes are able to confer salinity tolerance (Kasuga et al. 1999; Tuteja 2007). Pathways such as plant hormone signalling pathway, SOS (salt overly sensitive) pathway, calcium-signaling pathway, MAPK (mitogen-activated protein kinase) signal transduction and transporters and proline metabolism also have key roles in the salinity stress tolerance (Shinde et al. 2018; Yao et al. 2018). In our studies also, the functional annotation of *Dichanthium* sequences identified the similar transcripts involved in salinity tolerance. These annotations provide a valuable resource for dynamic transcriptome changes in salt-tolerant research in halophytes. Our results provide the genome sequence information for the exploration of salt tolerance mechanisms in this species and improve our knowledge of halophyte response to salt stress. Currently, all overexpression studies to improve salt tolerance have been based on the

Table 1 Transcripts involved in salt stress tolerance in *D. annulatum*

S. no.	Transcript ID	Protein name	Pathways/functions
Biological functions			
1.	DN58174_c6_g4_i3	Dehydration responsive element binding protein 1A	Transcription
2.	DN72104_c1_g7_i1	Myb family transcription factor EFM	Transcription
3.	DN26188_c0_g3_i1	Auxin response factor	Transcription regulation
4.	DN56877_c4_g3_i1	AP2-EREBP-type transcription factor	Transcription regulation
5.	DN60403_c1_g1_i4	Chaperonin-like RbcX protein	Response to salt stress
6.	DN64844_c1_g7_i1	Glutathione peroxidase	Response to oxidative stress
7.	DN61491_c0_g6_i3	L-ascorbate peroxidase	Response to oxidative stress
8.	DN64999_c3_g1_i2	Peroxidase	Response to oxidative stress
9.	DN68555_c1_g1_i4	Catalase	Response to oxidative stress
10.	DN62432_c2_g3_i3	Putative methionine sulfoxide reductase	Response to oxidative stress
11.	DN62473_c2_g2_i3	ABA responsive element binding factor 1	Response to salt stress
12.	DN58069_c0_g1_i2	Delta-1-pyrroline-5-carboxylate synthase	Response to salt stress
13.	DN65906_c5_g1_i4	Pyrroline-5-carboxylate reductase	Response to salt stress
14.	DN69314_c2_g5_i2	Heat shock 70 kDa protein	Response to salt stress
15.	DN60567_c2_g1_i3	Type IV inositol polyphosphate 5-phosphatase 11	Response to salt stress
Molecular functions			
16.	DN47453_c0_g1_i1	LRR receptor-like serine/threonine-protein kinase	ATP binding
17.	DN61786_c1_g1_i2	Asparagine synthetase	ATP binding
18.	DN44879_c0_g1_i1	SOS1	Solute: proton antiporter activity
19.	DN68935_c1_g2_i4	SOS2	Protein kinase activity
20.	DN68364_c0_g12_i7	Sodium/hydrogen exchanger	Solute: proton antiporter activity
21.	DN61714_c4_g5_i1	Cation/H^+ antiporter	Solute: proton antiporter activity
22.	DN72421_c2_g4_i1	K^+ efflux antiporter 6-like isoform X2	Solute: proton antiporter activity
23.	DN73047_c3_g5_i3	Plasma membrane Na^+/H^+ transporter	Solute: proton antiporter activity
24.	DN68613_c2_g4_i13	Calcium-binding EF-hand family protein	Calcium ion binding
25.	DN65265_c0_g3_i1	Calcineurin B-like7	Calcium ion binding
26.	DN69677_c0_g1_i2	Calmodulin-like protein 1	Calcium ion binding
27.	DN46215_c1_g2_i2	Zinc finger family protein	Metal ion binding
28.	DN61869_c0_g2_i4	C2C2-GATA transcription factor (GATA transcription factor 22)	Zinc ion binding
29.	DN53319_c0_g3_i2	Calcium-dependent protein kinase SK5	Protein kinase activity
30.	DN46770_c0_g1_i1	Putative wall-associated receptor protein kinase family protein	Protein Kinase Activity
Cellular components			
31.	DN64455_c0_g1_i4	Potassium transporter	Integral component of membrane
32.	DN62616_c2_g1_i2	Zinc transporter	Integral component of membrane
33.	DN66098_c1_g5_i21	Bidirectional sugar transporter SWEET	Integral component of membrane
34.	DN69618_c4_g5_i2	Transmembrane amino acid transporter family protein	Integral component of membrane
35.	DN56431_c0_g2_i2	ABC transporter	Integral component of membrane
36.	DN71221_c2_g5_i1	V-type proton ATPase subunit C	Integral component of membrane
37.	DN59535_c1_g2_i2	WAT1-related protein	Plasma membrane component
38.	DN58499_c4_g1_i1	Putative wall-associated kinase	Plasma membrane component
39.	DN63257_c2_g3_i4	Reticulon-like protein	endoplasmic reticulum membrane
40.	DN66070_c0_g1_i1	Leucine aminopeptidase 2 chloroplastic	Cyoplasm

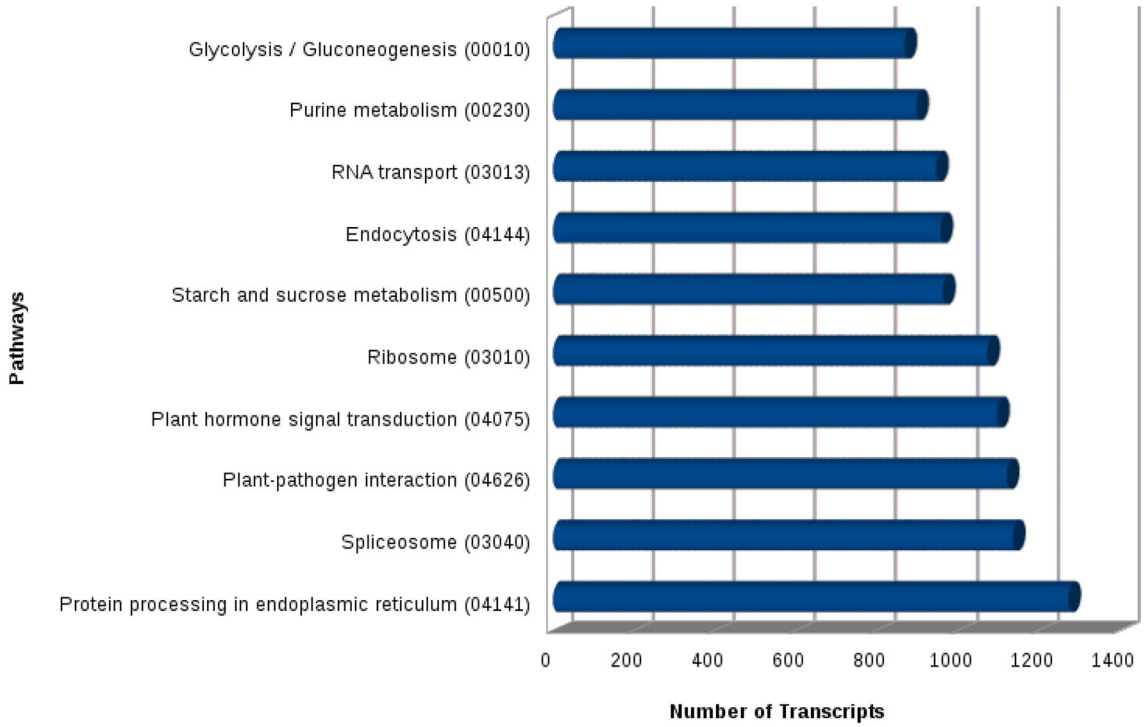

Fig. 3 Top 10 most highly represented pathways in *D. annulatum*

assumption of a glycophytic pathway for salt tolerance and since *Dichanthium* is still unexplored, the present work using genes from halophytes will certainly be helpful in future crop modelling.

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