



RNA-Seq De Novo Assembly of Red Pitaya (*Hylocereus polyrhizus*) Roots and Differential Transcriptome Analysis in Response to Salt Stress

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Received: 5 November 2018 / Accepted: 7 January 2019 / Published online: 4 February 2019
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Abstract

Pitaya (*Hylocereus polyrhizus*) is a highly valued functional food that is widely planted in Southeast Asia. However, with increased soil salinization in cultivation areas, a better understanding of the molecular mechanisms associated with salt stress responses in red pitaya is necessary. Herein, RNA-Seq was used to de novo assemble and characterize the transcriptomic profiles of red pitaya roots in response to salt stress. A total of 73,589 transcripts were obtained and the average sequence length was 1308 bp. From these transcripts, 26,878 unique transcripts were successfully matched to 13,519 Swiss-prot proteins. Gene ontology (GO) annotations showed that within the categories of molecular function, biological function, and cell component, catalytic activity (GO:0003824), metabolic process (GO:0008152), and cell part (GO:0044464) were the most enriched, respectively. 2624 transcripts were significantly differentially expressed among three time points (3 h, 7 h, and 30 h) following exposure to 450 mM NaCl. Furthermore, 261 genes were up-regulated and 61 down-regulated in all three of the time points. Glycolysis/gluconeogenesis was one of the most significantly modulated pathways. The findings presented herein provide further insight into salt stress responses in pitaya and will provide a valuable resource for future functional studies examining salt adaptations.

Keywords Pitaya · Salt stress · RNA-Seq · De novo · Transcriptome

Abbreviations

FC Fold change
GO Gene ontology

FDR False Discovery Rate
CDS Coding sequence
DEG Differentially expressed gene

Communicated by: Yuan Qin

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12042-019-09217-3>) contains supplementary material, which is available to authorized users.

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QTL	Quantitative trait loci
ABA	Absciscic acid
pfkA	6-phosphofructokinase 1
PGK	Phosphoglycerate kinase
GOT1	Aspartate aminotransferase
ALDO	Fructose-bisphosphate aldolase, class I
SQLE	Squalene monooxygenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
qRT-PCR	Quantitative real-time PCR
ncRNAs	Non-coding RNAs
TEs	Transposable elements
<i>RAS1</i>	<i>Salt 1</i>
<i>SKC1</i>	<i>Streptomyces lividans</i> K ⁺ channel
<i>Nax1</i>	<i>Na⁺ exclusion 1</i>
<i>Nax2</i>	<i>Na⁺ exclusion 2</i>
<i>P4H</i>	Prolyl 4-hydroxylase
<i>HKT</i>	High-affinity K ⁺ transporter
<i>CCCH-ZFP</i>	CCCH zinc finger protein

Introduction

Salt tolerance is an important quantitative trait (Gu et al. 2018a, b; Quesada et al. 2002), with approximately 7% of the world's total land area affected by salt (Ghassemi et al. 1995; Szabolcs and Salinisation 1994) and these areas are on the rise due to irrigation or land clearing (FAO Statistics). Soil salt limits agricultural production by affecting plant growth, limiting crop yields, and reducing crop quality (Yang 2008). Thus, improved crop salt tolerance is needed to improve growth in areas at risk of salinization.

The identification of plant genes involved in salinization responses is required to unravel the molecular basis of agronomical important traits and aid in the selection of salt-tolerant lines. Genetic and molecular studies have indicated that salt tolerance is complex in crop plants. Studies utilizing quantitative trait loci (QTL) analysis have been utilized to scan natural variations related to salt tolerance traits in *Arabidopsis* (DeRose-Wilson and Gaut 2011) and other crops (Collins et al. 2008), with genes modulating these QTLs identified. In rice, *Streptomyces lividans* K⁺ channel (*SKC1*), which encodes an HKT-type Na⁺-selective transporter involved in unloading Na⁺ from the xylem (Ren et al. 2005). In durum wheat, the Na⁺ exclusion 1 (*Nax1*) and the Na⁺ exclusion 2 (*Nax2*) function in Na⁺ exclusion (Byrt et al. 2007; Huang et al. 2006). In *Arabidopsis*, Salt 1 (*RAS1*) has been shown to be an ABA and a salt stress-inducible gene (Ren et al. 2010). Furthermore, *SOS2* ser/thr protein kinase (Qiu et al. 2003; Shi et al. 2000), the high-affinity K⁺ transporter (*HKT*) (Rus et al. 2001), and CCCH zinc finger proteins (*CCCH-ZFP*) (Mazumdar et al. 2017) have been shown to be associated with salt tolerance responses.

Red pitaya (*Hylocereus polyrhizus*) is a tropical fruit that is a highly valued functional food. This fruit is popular in Southeast Asia where soil resources are limited, thus culturing in areas with salinization is of interest. In previous studies examining pitaya, a catalase gene, *HuCAT3*, was found to be associated with abiotic stress responses to peroxide (H₂O₂), drought, cold, and salt stresses (Nie et al. 2015). Also in pitaya, a total of 309 expressed sequence tags (ESTs) and 138 unique ESTs were identified in response to drought stress (Fan et al. 2014). Herein, to better characterize red pitaya gene expression associated with salt stress responses, the root transcriptome was de novo assembled using RNA sequencing (RNA-Seq) and differential expression associated with salt stress was examined. This study provides a valuable gene resource for future functional studies pertaining to salt tolerance in pitaya.

Results

Sample Sequencing and De Novo Assembly

To investigate salt tolerance in red pitaya, seedlings were treated with 300, 450, or 600 mM NaCl for 1 week (Fig. 1a). In the seedling treated with 600 mM NaCl, most of the roots experienced rot and the seedlings looked wilted. However, at the lower salt concentrations, the seedling grew well (Fig. 1a), thus showing that a short-term NaCl concentration of 450 mM NaCl can be tolerated. During drought and salt stress, some plants showed an increase in soluble sugar contents to confer tolerance (Mark et al. 2012), thus levels were examined herein. Under 450 mM NaCl concentration, the soluble sugar

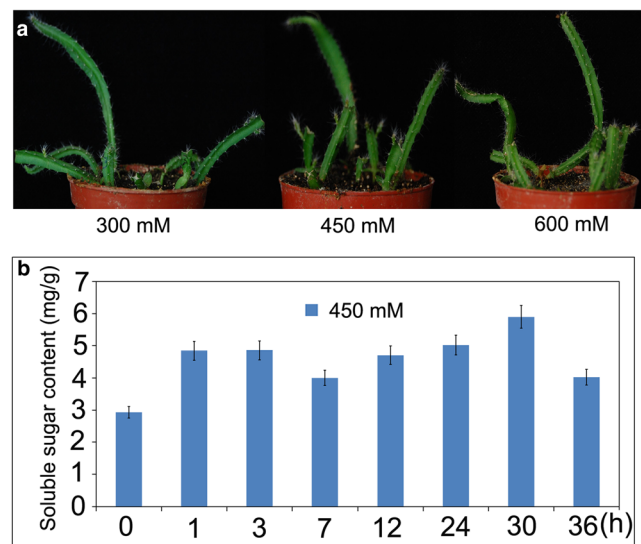


Fig. 1 Plant growth and soluble sugar content in red pitaya seedlings after salt stress. **a** Plant growth in different salt concentrations for 1 week. **b** Soluble sugar content in seedling treated with 450 mM NaCl

content increased at all of the time points, but with the lowest elevation at 7 h and the highest at 30 h (Fig. 1b).

To identify genes associated with salt stress responses in the roots, samples were treated with 450 mM NaCl and collected at four time points (0 h, 3 h, 7 h, and 30 h) for RNA-Seq, with the 0 h sample used as a control. Using the Illumina HiSeq2500 platform, an average of 23,306,060 paired-end raw reads with an average length of 150 bp were generated from each of the 12 root sample datasets (20,225,559–25,097,895 reads; three replicates for each time point). In the de novo transcriptome, 73,589 total transcripts, with a total sequence length of 96.3 Mb, were obtained. The average sequence length was 1308 bp, with a range of 200 bp to 22,390 bp, and the N50 length was 1883 bp (Table 1).

Sanger Sequencing and qRT-PCR Validations

To confirm the sequence assembly, nine transcripts were randomly selected and sequenced. The average CDS length was found to be 1179 bp \pm 228 bp, with a range of 771–1662 bp. The amplified CDS regions showed a 99–100% identity to their associated red pitaya transcripts (Table 2). To validate the RNA-Seq expressional data, 12 contigs (genes) were randomly selected from the transcriptome and examined via qRT-PCR. The qRT-PCR results showed fold changes and time associated patterns that were consistent with the RNA-Seq dataset (Fig. 2). These findings confirm the reliability of the obtained data.

Table 1 De novo assembly and annotation metrics for the pitaya transcriptome

Item	Statistic value
Total sequences	73,589
Total bases	96,259,213
Min sequence length	224
Max sequence length	22,390
Average sequence length	1308.07
Median sequence length	909.00
N25 length	3011
N50 length	1883
N75 length	1011
N90 length	598
N95 length	470
As	29.15%
Ts	29.08%
Gs	21.08%
Cs	20.69%
(A + T)s	58.23%
(G + C)s	41.77%
Ns	0.00%

Functional Annotations

A total of 26,878 unique transcripts were successfully mapped to 13,519 protein sequences in Swiss-Prot, with the sequences associated with 960 species. The species with the most sequences in common was *Arabidopsis thaliana* (5473 genes), followed by *Mus musculus* (616 genes), *Oryza sativa subsp. japonica* (608 genes), and *Caenorhabditis elegans* (597 genes). Similar results were seen when performing a query against the NCBI refseq RNA database, with 24,569 transcripts successfully mapped to 16,060 genes (Supplementary Tables 2 and 3).

GO annotations were obtained using the PANTHER database (Fig. 3). Within the categories of molecular functions, biological functions, and cell component, the most enriched terms were associated with catalytic activity (GO:0003824), metabolic process (GO:0008152), and cell part (GO:0044464), respectively (Supplementary Table 4). Pathway analysis was performed using the KEGG database, with 405 pathways associated with the obtained annotated DEGs. The most enriched pathways were associated with metabolic pathways (926 genes), biosynthesis of secondary metabolites (407 genes), and biosynthesis of antibiotics (231 genes; Fig. 4 and Supplementary Table 5).

Identifying DEGs Associated with Salt Stress Responses

When comparing the experimental group that was treated with 450 mM NaCl to the control, a total of 2624 significant DEGs were identified at 3 h, 7 h, and 30 h after the start of the treatment. Of these, 540 genes were up-regulated at 3 h post-treatment, 445 genes at 7 h, and 994 at 30 h. Down-regulated genes were also identified at 3 h (549 genes), 7 h (257 genes), and 30 h (869 genes) post-treatment (Table 3 and Supplementary Table 6). The DEG numbers were correlated with the soluble sugar content levels, with the highest levels in both noted at 30 h. Interestingly, 261 genes were shown to be up-regulated across all three of the time points, while 61 genes were down-regulated (Fig. 5 and Table 4).

Functional Annotations of DEGs Associated with Salt Stress Responses

A total of 1617 DEGs were successfully mapped to 1284 protein sequences in Swiss-Prot, while 1007 DEGs were uncharacterized transcripts (Supplementary Table 7). The DEGs found to be in common with all three of the time points in samples exposed to 450 mM NaCl were further analyzed using GO and KEGG analyses. GO analysis revealed that the DEGs were mainly associated with catalytic activity (GO:0003824), metabolic process (GO:0008152), and cell

Table 2 CDS amplification and sequencing results for assembly validation

GeneID	CDS length in RNA-Seq (bp)	Sequencing length (bp)	Sequencing cover (%)	Identity (%)
Contig26610	897	897	100	100
Contig46534	1143	1143	100	99
Contig44278	1218	1218	100	100
Contig25524	1026	981	95	100
Contig57906	771	771	100	100
Contig29387	1206	1206	100	99
Contig 5479	1512	1512	100	100
Contig30436	1662	1662	100	99
Contig12207	1183	1182	100	99

part (GO: 0044464) in the categories of molecular function, biological process, and cellular component (Fig. 6).

KEGG pathway analysis showed that the DEGs associated with salt stress were involved in 86 pathways, and they were mainly enriched in secondary metabolite, carbohydrate, and amino acid metabolic pathways (Supplementary Table 8). The secondary metabolite pathway was comprised of 15 essential pathways and contained 33 DEGs. Of those, pathways associated with secondary metabolite biosynthesis were the most prevalent and contained 18 DEGs (Supplementary Table 9). Among the 18 DEGs, 15 were up-regulated and 3 were down-regulated. The down-regulated DEGs included Contig31365 (PORA), Contig6213 (TYRA2), and Contig13954 (COMT1; Supplementary Table 10). The carbohydrate metabolic pathway was comprised of 13 essential pathways and contained 33 DEGs, with the glycolysis/gluconeogenesis pathway being the most significant pathway of the 13 and containing 8 up-regulated DEGs (Supplementary Tables 11 and 12). The amino acid metabolic pathway was comprised of 9 essential

pathways and contained 23 DEGs, with the biosynthesis of amino acids pathway being the most significant of the 9 and containing 10 DEGs (Supplementary Table 13). Among the 10 DEGs, 9 DEGs were up-regulated and 1 DEG was down-regulated, Contig6213 (TYRA2; Supplementary Table 14).

When comparing DEGs associated with secondary metabolite biosynthesis, glycolysis/gluconeogenesis, and amino acid biosynthesis, 5 up-regulated genes were found to be in common. These genes included Contig25524, Contig7784, Contig29387, Contig29388, and Contig9566, which encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 6-phosphofructokinase 1 (pfkA), phosphoglycerate kinase (PGK), PGK, and fructose-bisphosphate aldolase, class I (ALDO), respectively.

Discussion

Salt exposure activates a complex response that inhibits plant growth and other physiological processes (Albacete et al. 2008; Naeem et al. 2012; Radwan et al. 2015). When compared with crop plants, few genomic studies have focused on stress response molecular markers in the economically important pitaya plant. When examining stress responses, RNA-Seq analysis is an effective strategy compared to EST or microarray analyses. Herein, de novo transcriptome sequencing and assembly was performed using RNA-Seq and differential expression associated with red pitaya salt stress responses was identified. These identified DEGs were then annotated and further examined via GO and KEGG analyses. The findings presented herein will provide valuable insights into the molecular mechanisms of salt stress responses in red pitaya.

The de novo assembly identified a total of 73,589 transcripts (Table 1), with 26,878 (36.5%) unique transcripts functionally characterized and annotated (Supplementary Tables 2 and 3). In a previous study utilizing RNA-Seq, 122,677 transcripts associated with red pitaya pulp coloration were identified, with approximately 99.99% of all of the transcripts annotated (Hua et al. 2016). However, the annotation rate herein

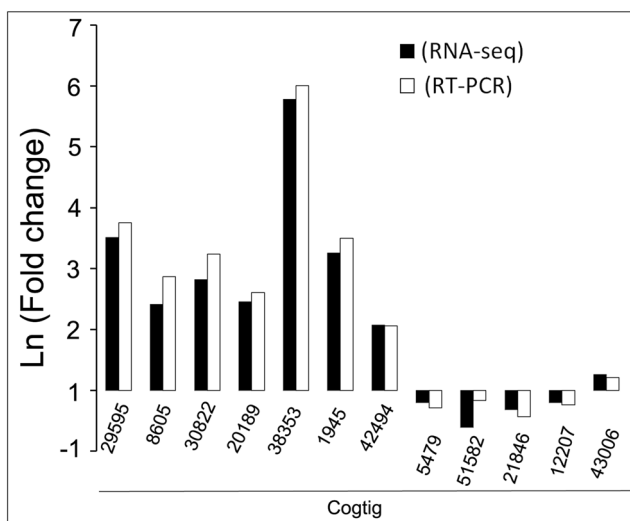
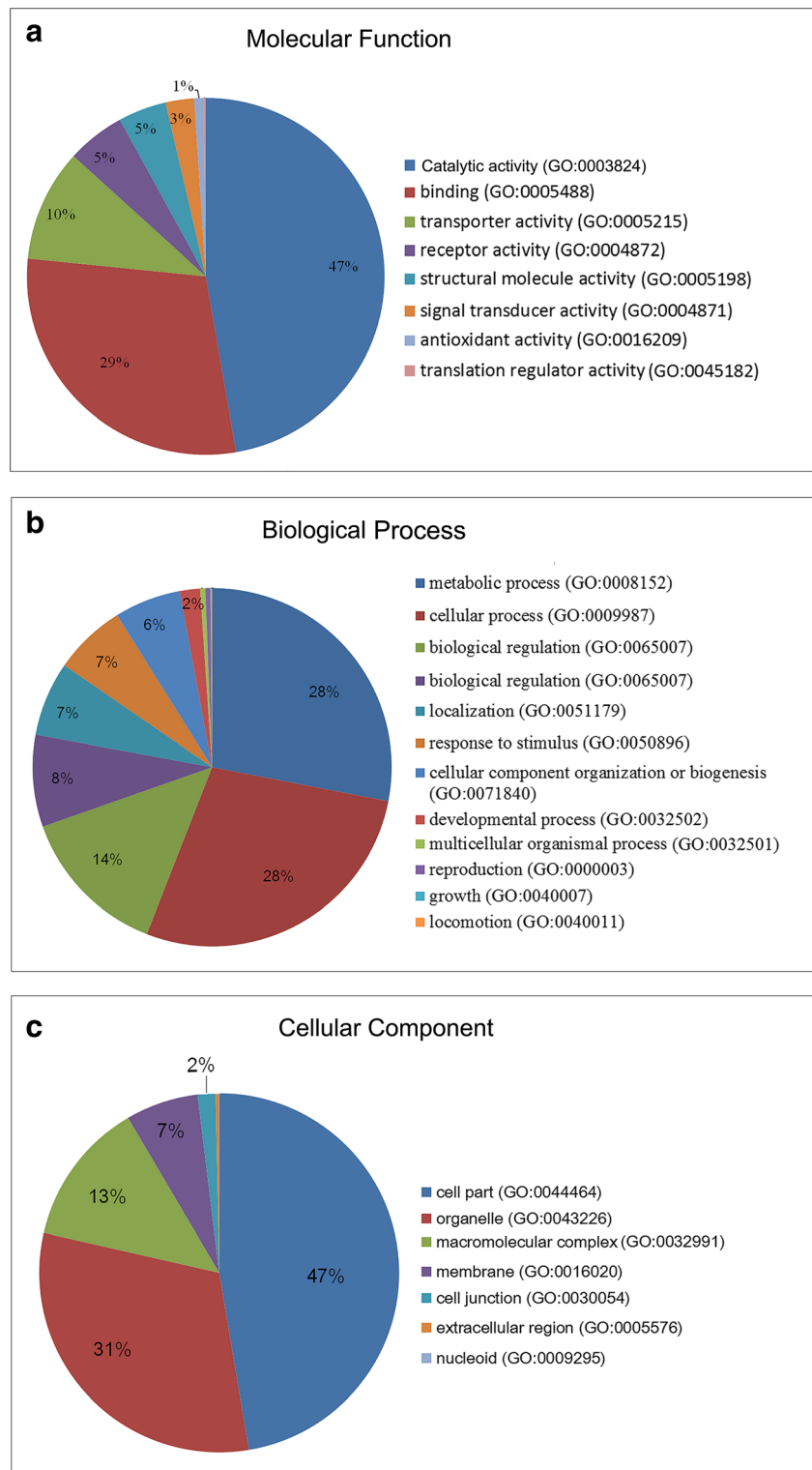


Fig. 2 RNA-Seq dataset validation by using qRT-PCR. Twelve DEG contigs (x-axis) were randomly selected from the RNA-Seq dataset and validated by qRT-PCR. The y-axis displays natural log (ln)-transformed fold changes for both datasets

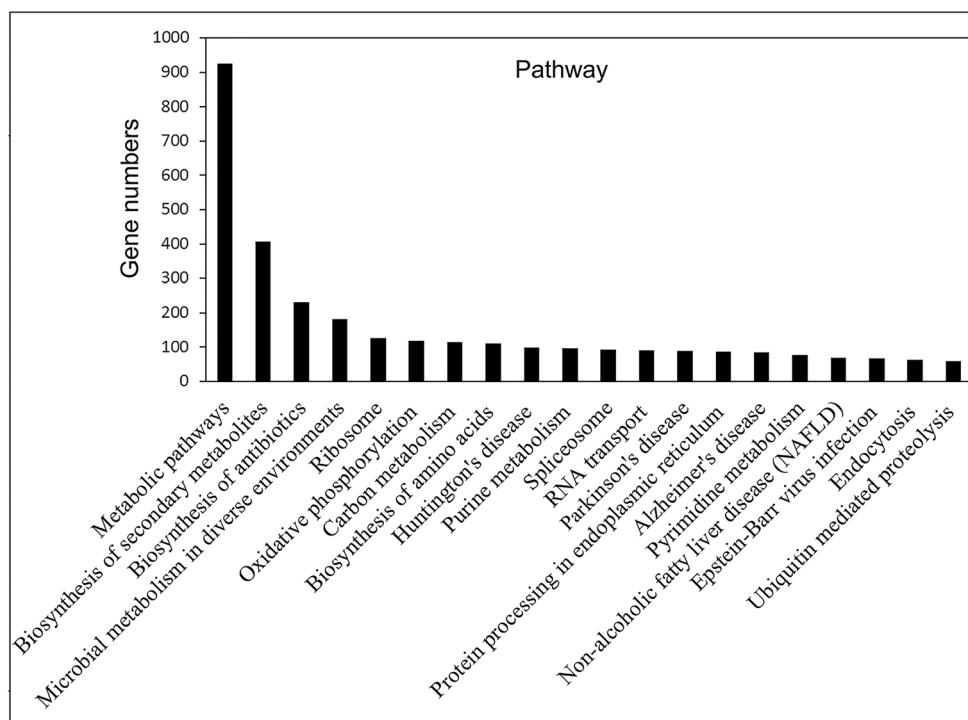
Fig. 3 GO classifications of DEGs using the PANTHER database. The obtained 13,519 annotated DEGs were classified into three GO categories: molecular function (a), biological process (b), and cellular component (c). Displayed percentages are based on gene numbers for a given GO category



was much lower, with other reports also showing much lower annotation rates. For example, only 25% to 50% of the assembled sequences in the Jamaican fruit bat (*Artibeus jamaicensis*) (Shaw et al. 2012), the carrot transcriptome (Iorizzo et al. 2011), and the European abalone (*Haliotis*

tuberculata) (Xia et al. 2015) were functionally annotated. Therefore, our data are comparable to the latter studies, with the uncharacterized transcripts possibly attributed to non-coding RNAs (ncRNAs), transcribed transposable elements (TEs), pitaya specific genes, or pseudogenes.

Fig. 4 KEGG pathway of annotated DEGs. A display of the gene numbers (y-axis) for the top 20 most enriched pathways (x-axis)



In previous studies, many different pathways have been implicated in response to salt stress in different plants. In *Aspergillus oryzae*, the DEGs were mainly enriched in the areas of fatty acid biosynthesis and degradation, linoleic acid metabolism, arginine biosynthesis, and arginine and proline metabolism (He et al. 2018). In *Arabidopsis*, they were mainly associated with transcription factors, cell wall remodeling, ethylene biosynthesis, and signaling pathways (Shen et al. 2014). Tomato DEGs were mainly enriched in amino acid and carbohydrate metabolic pathways (Zhang et al. 2017). In this study, DEGs were involved in 86 pathways, which suggested that there are complex biological processes triggered by saline stress in pitaya. The DEGs were mainly enriched in metabolic pathways including secondary, carbohydrate, and amino acid metabolic pathways (Supplementary Table 8). These pathways appear to play an important role in pitaya salt tolerance.

Secondary metabolites, such as steroids, alkaloids, and phenylpropanoids, have been closely associated with abiotic stress tolerance (Ewas et al. 2017; Hua et al. 2016; Qiao et al. 2017; Resmi et al. 2015). In red pitaya, 15 essential pathways comprised the secondary metabolite pathway and included 33 DEGs (26 up-regulated and 7 down-regulated genes;

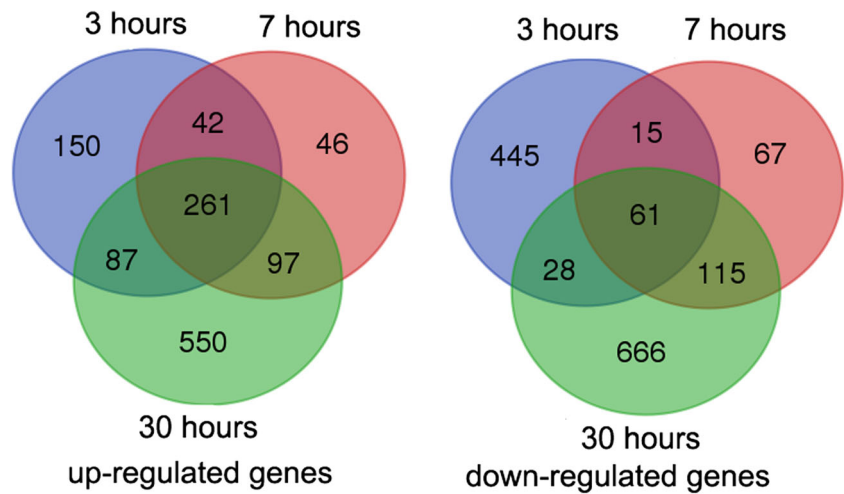
Supplementary Table 8). These pathways included the biosynthesis of isoquinoline alkaloid, sesquiterpenoid, triterpenoid, porphyrin, steroid, tropane, piperidine, pyridine alkaloid, phenylpropanoid, and chlorophyll metabolism. This finding further supports that secondary metabolites play important roles in abiotic stress tolerance.

Carbohydrate metabolism is a fundamental metabolic process in living organisms and is one of the most sensitive to salt stress in red pitaya (Table 4). The carbohydrate metabolic process category is comprised of 13 essential pathways, including glycolysis/gluconeogenesis, carbon fixation in photosynthetic organisms, fructose and mannose metabolism, and starch and sucrose metabolism. Within the glycolysis/gluconeogenesis pathway, 8 up-regulated DEGs were identified after short and continuous salt stress. Glycolysis is the process of converting glucose into pyruvate and generating small amounts of ATP (energy) and NADH (reducing power) (Berg et al. 2010). It is a central pathway that produces important precursor metabolites that are either six-carbon compounds (glucose-6-phosphate and fructose-6-phosphate) or three-carbon compounds (glycerone-phosphate, glyceraldehyde-3-phosphate, glycerate-3-phosphate, phosphoenolpyruvate, and pyruvate). Glycolysis/gluconeogenesis has been

Table 3 Differentially expressed genes in red pitaya roots exposed to salt stress

Time post-salt treatment	Down-regulated genes	Up-regulated genes	Total gene numbers
3 h	549	540	1089
7 h	488	258	756
30 h	870	995	1865

Fig. 5 Overlapping venn diagram for the DEGs sampled at different time points post-salt treatment. Numbers represent deduplicated UniProtKB IDs included in each subset



implicated in many stress responses, such as a resistance to aluminum stress in wheat (Hamilton et al. 2001) and rice (Wang et al. 2014), drought stress in ramie and rice (An et al. 2016; Lenka et al. 2011), and freezing stress in loquat defense (Xu et al. 2017).

During the salt stress response in red pitaya, the carbon fixation in the photosynthetic organism pathway was the second most significant pathway. Salt stress has been reported to limit photosynthesis and CO₂ diffusion by decreasing stomatal and mesophyll conductance (Flexas et al. 2004). In photosynthetic organisms, carbon fixation has been shown to be impacted during salt stress in rice (Lee et al. 2016) and cotton (Chen et al. 2016). Collectively, these findings suggest that the

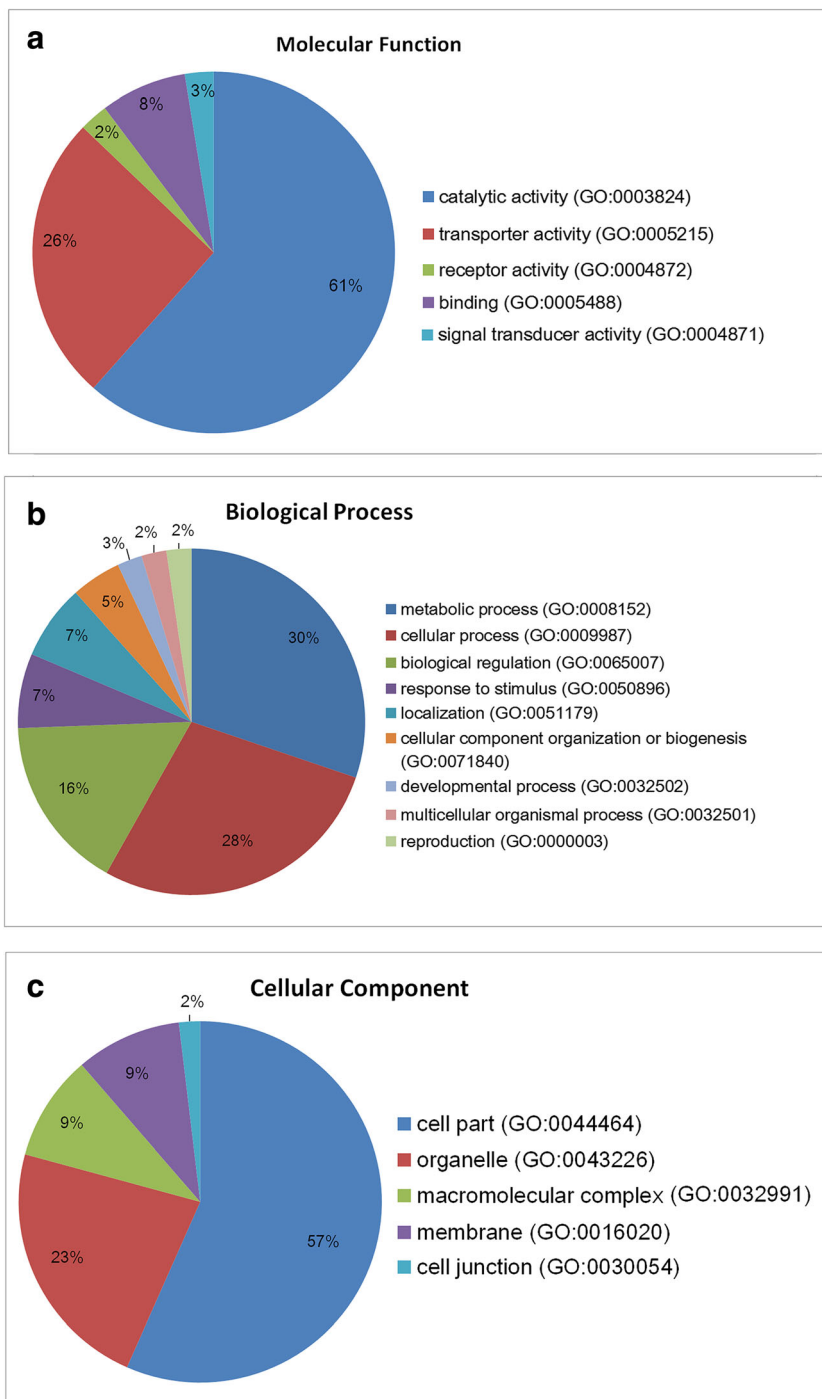
glycolysis/gluconeogenesis and carbon fixation in photosynthetic organism pathways play an important role in red pitaya salt stress tolerance.

Amino acid metabolism is crucial to the salt stress response (Zhang et al. 2017). In red pitaya, 9 biosynthetic and metabolic pathways associated with amino acid metabolism were identified following salt stress and included 9 DEGs, 8 up-regulated and 1 down-regulated. This may indicate that nitrogen distributions have been altered in response to salt stress. Two of the up-regulated genes, prolyl 4-hydroxylase (*P4H*) and aspartate aminotransferase (*GOT1*), are associated with arginine and proline metabolism (Supplementary Table 7). Proline can protect plants

Table 4 Top 20 DEGs in red pitaya after salt stress

Gene ID	Log FC	Log CPM	P value	FDR	Annotation
Contig24846	9.534835	0.769307	1.34E-15	1.86E-12	sp P33079 A10A5_SOYBN
Contig7086	8.90638	1.982083	1.43E-26	1.29E-22	sp P0CC65 NU2C2_GOSBA
Contig8840	8.54971	-0.16032	2.12E-07	4.54E-05	sp Q9LYC8 GRXS6_ARATH
Contig71997	7.349401	-1.22578	1.23E-06	0.000195	sp Q42652 SUSY_BETVU
Contig57430	7.296158	-1.26977	1.94E-05	0.001938	sp Q02921 NO93_SOYBN
Contig12513	7.225206	-1.32956	1.08E-06	0.000175	sp P31843 RRPO_OENBE
Contig3435	6.973678	6.931575	2.42E-14	2.77E-11	sp Q84VY3 STAD6_ARATH
Contig18497	6.794775	6.135415	1.84E-18	5.3E-15	sp P48977 ADH_MALDO
Contig24381	6.403647	5.046958	3.46E-13	3.11E-10	sp Q02921 NO93_SOYBN
Contig60935	6.354429	2.637264	8.55E-13	6.85E-10	sp P48977 ADH_MALDO
Contig67500	-8.40874	-0.27825	2.99E-07	5.94E-05	sp P22151 GRG1_NEUCR
Contig16674	-7.93694	-0.71554	1.11E-06	0.000179	sp Q9CAT6 OCT1_ARATH
Contig4514	-7.72357	1.611954	7.03E-13	5.82E-10	sp Q2R3P9 SWT14_ORYSJ
Contig21958	-5.82964	0.657211	1.02E-12	7.97E-10	sp F8WKW1 UGT2_GARJA
Contig2134	-4.85273	2.310295	1.38E-08	4.15E-06	sp O23522 PLA14_ARATH
Contig38644	-3.66254	1.342286	7.78E-09	2.47E-06	sp D4N502 DIOX3_PAPSO
Contig44413	-3.57805	1.016638	5.9E-07	0.000105	sp O23086 ALMTA_ARATH
Contig30812	-3.30617	4.580997	1.19E-10	6.26E-08	sp P49865 NTPR_ENTHA
Contig5996	-3.24492	1.510348	5.57E-05	0.004628	sp Q9T072 BH025_ARATH
Contig69302	-3.16317	0.057528	8.16E-05	0.006256	sp Q9FJ76 WAXS5_ARATH

Fig. 6 GO analysis of common DEGs after salt stresses with 450 mM NaCl. Annotated DEGs were classified into three GO categories (molecular function (a), biological process (b), and cellular component (c)). Displayed percentages are based on gene numbers for a given GO category



during stress and aids in plant growth when exposed to salinization (de Carvalho et al. 2013; Razavizadeh and Ehsanpour 2009; Werner and Finkelstein 1995). Prolyl 4-hydroxylase gene (*P4H1*) is induced by low oxygen stress (Asif et al. 2009). *GOT1* is important in the modulation of carbon and nitrogen distributions that are needed for amino acid metabolism by catalyzing the reversible transamination of an amino group from aspartate to α -ketoglutarate to yield glutamate and oxaloacetate (Boutet et al. 2005).

Therefore, *P4H* and *GOT1* up-regulation during salt stress may have an important role in enhancing salt tolerance in red pitaya.

Further more, 5 of the up-regulated DEGs were found in all of the three key pathways (biosynthesis of secondary metabolites, glycolysis/gluconeogenesis, and biosynthesis of amino acids), namely, Contig25524 (GAPDH), Contig7784 (pfkA), Contig29387 (PGK), Contig29388 (PGK), and Contig9566 (ALDO). In plants, pfkA is a key

enzyme in glycolysis that catalyzes the conversion of fructose 6-phosphate to fructose 1,6-diphosphate; which can then be converted to phosphodihydroxy acetone and glyceraldehyde 3-phosphate, which is catalyzed by ALDO. GAPDH is an oxidative phosphorylation enzyme, which further catalyzes glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate. This step is the only oxidation reaction in glycolysis. PGK catalyzes 1,3-bisphosphoglycerate into 3-phosphoglycerate and ATP. PDC catalyzes pyruvate into acetaldehyde and CO₂. During an infection or environmental stress, such as osmotic, salt, heat, ABA, or methyl viologen stress, GAPDH has been seen to be induced (Zhang et al. 2011). Furthermore, *GAPDH* overexpression was shown to enhance salt stress tolerance in *Oryza sativa* (Zhang et al. 2011). In plants exposed to butanol toxicity, *pfkA* and pyruvate kinase (*pykA*) overexpression increased the stress tolerance (Ventura et al. 2013). *Pfk* has also been shown to play an adaptive role during an infection in *Rhizoctonia solani* (Mutuku and Nose 2012). During thermal stress in *Saccharomyces cerevisiae*, PGK was identified as a DEG following transcriptional analysis (Piper et al. 1986). In *Arabidopsis*, *PGK2* overexpression increased salt stress tolerance (Nie et al. 2015), while in transgenic tobacco, *PGK2a-P* overexpression improved yields during salt stress (Joshi et al. 2016). Moreover, *ALDO* has been associated with abiotic stress responses to heat stress (Traylor-Knowles et al. 2017), hypoxic stress (Abe et al. 2007), drought stress (Khueychai et al. 2015), and salt stress (Fan et al. 2009). These findings suggested that these genes play an important role in the salt stress response in red pitaya.

Interestingly, we found that 38.4% DEGs (1007 DEGs) were functionally uncharacterized and unannotated, which possibly attributed to ncRNAs, TEs, or pitaya specific genes (Supplementary Table 7). ncRNAs are sequences with very low or no potential for encoding protein and are functional in developmental processes or stress responses (Borah et al. 2018; Brant and Budak 2018; Zhao et al. 2016). In *Arabidopsis*, long noncoding RNA (lncRNA) *SVLKA* affected plant freezing tolerance, while overexpressing lncRNA *DRIR* enhanced drought and salt stress tolerance (Kindgren et al. 2018; Qin et al. 2017). In *Zea mays*, miR159, miR164, miR167, miR393, miR408 and miR528 were involved in root development and stress responses (Liu et al. 2012). TEs are abundant in plants that play an important role in genome and gene evolution (Vicent 2010). In plants, TEs contribute to the origin of ncRNAs and generate novel gene regulatory networks in stress response and development (Cho 2018; Wang et al. 2016, 2017). Epigenetic regulation of TEs affected gene expression (Le et al. 2015). Thus, we convinced that these uncharacterized DEGs will contribute to better understand salt stress response in pitaya.

Material and Methods

Plants and Materials

Red pitaya seeds were germinated and grown in a greenhouse under controlled conditions (14 h photoperiod, 28 °C ± 1 °C, and 60% ± 5% relative humidity). Three-month-old seedlings were treated with 300, 450, or 600 mM NaCl for 1 h–36 h. The treated seedlings were collected, the soluble sugar content was measured, and RNA was extracted from the roots.

Sample Collection and Preparation

Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's protocol. RNA quality was assessed using 1% agarose gel electrophoresis and a RNA Nano 6000 Assay Kit with an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 1.5 µg of total RNA per sample was utilized as the starting material. Sequencing libraries were generated using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, USA) according to the manufacturer's instructions, and index codes were added to attribute sequences to each sample. Finally, paired-end cluster generation and sequencing were performed using an Illumina HiSeq platform.

Quality Control

Raw data in a fastq format were processed using in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters or ploy-Ns or low quality reads from the raw data. All of the downstream analyses were based on the obtained clean high-quality data.

De Novo Transcriptome Assembly

Two programs were used to assemble the transcriptome. De novo assembly of the RNA-Seq reads was first conducted for each sample separately using the Trinity program (Haas et al. 2013) with default parameters. The program assembled the high-quality RNA-Seq reads into sequences, clustered the sequences, and then reported full-length transcripts. Next, de novo assembly of the 12 transcriptomes was performed using a contig assembly program (CAP3; <https://www.msi.umn.edu/sw/cap3-contig>) with parameters -p 99 -f 2 -o 100. Statistical analysis was performed using the NGS QC Toolkit v.2.3.3 with default parameters (Trapnell et al. 2012).

Identification of Differential Gene Expression

The TopHat program (Trapnell et al. 2012) was utilized to map the clean RNA-Seq library reads to the de novo assembled reference transcriptome. The program 'multicov' in the

bed tools was then applied to count the number of alignments for each transcript per sample. To identify differentially expressed genes (DEGs) between two datasets, a pairwise comparison was performed using the ‘run_DE_analysis.pl’ function in the trinityrnaseq-2.0.6 program (Haas et al. 2013) with the parameter setting of ‘–method edgeR’. For data to be considered high quality, a \log_2 FC (fold change) > 2, a count value for each gene in the two datasets > 20 and an adjusted p value FDR (False Discovery Rate) ≤ 0.001 had to be obtained.

Transcriptome Annotations and Classifications

The generated transcripts were queried against the Swiss-Prot protein database (<http://www.uniprot.org/>) using BLASTX and the NCBI refseq RNA database using BLASTN with an E-value cut-off of 0.00000001. Gene ontology (GO) annotations were obtained using the PANTHER database (<http://www.pantherdb.org/>) and KEGG pathway analysis was performed using KASS (KEGG Automatic Annotation Server v.2.1; http://www.genome.jp/kaas-bin/kaas_main?).

Transcriptome Assembly Validation Via Sanger Sequencing and DEG Validations Via qRT-PCR

To validate the assembly accuracy, 9 randomly selected transcripts from the de novo transcriptome were selected. PCR primers were designed using primer premier 5 (Premier Biosoft, USA) (Supplementary Table 1) and whole coding sequence (CDS) regions were amplified. PCR reactions were performed using a thermo-cycler (Sensquest, Germany) with a PCR kit (DSMix, Dongsheng Biotech, China) and red pitaya cDNA was obtained according to the manufacturer’s instructions. Amplifications were performed in a total reaction volume of 20 μ l [20 ng cDNA and 0.5 μ l of each primer (10 μ M)]. The PCR reaction protocol was as follows: an initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1–2 min, with an additional extension at 72 °C for 10 min. PCR products were sequenced and analyzed using vector NTI 9.1 software (Invitrogen corporation, USA).

To validate the identified RNA-Seq based DEGs, quantitative real-time PCR (qRT-PCR) was employed with 12 randomly selected transcripts evaluated (Supplementary Table 1). Prior to performing qRT-PCR, total RNA was extracted and cDNA was synthesized (Xia et al. 2015). The qRT-PCR reaction was performed on a Roche Light Cycler 480 Real-time PCR System (Roche, Switzerland) using SYBR Green qPCR mix (Dongsheng Biotech, China) according to the manufacturer’s instructions. The amplifications were performed in a total volume of 10 μ l [5 μ l of 2X SYBR Green Master Mix reagent (Promega, USA), 1 μ l of 1:10 diluted

cDNA, and 0.4 μ l of each primer (10 μ M)]. The thermal cycling protocol was as follows: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and an extension at 72 °C for 20 s. An additional temperature-ramping step from 95 °C to 65 °C was used to produce a melting curve. Three technical replicates were performed for each sample and *EF1 α* was used as an internal control. Expression levels were normalized to three housekeeping genes using the $\Delta\Delta$ Ct method. Expressional differences between the control and experimental samples were evaluated using a two-tailed t test.

Quantification of Soluble Sugars

The soluble sugar content was quantified as previously described (Wei et al. 2014).

Acknowledgements This project was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020500), the National Natural Science Foundation of China (31671659 and 3172384), the National Key Research and Development Program of China (2016YFC1403002), and the Guangzhou Science and Technology Project (201804010409).

Availability of Data and Materials Section All the read data were available at DDBJ database (BioSample Accession no.: SAMD00115774-SAMD00115785).

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