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Transcriptome analysis shows that alkalinity affects metabolism in the roots of *Mesembryanthemum crystallinum*

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Abstract

Mesembryanthemum crystallinum is a model halophyte that switches from C_3 photosynthesis to Crassulacean acid metabolism (CAM) upon extreme abiotic stresses. This study aimed to investigate alkalinity-induced root transcriptome profiling in *M. crystallinum*. *M. crystallinum* seedlings were treated with 50 mM sodium bicarbonate ($NaHCO_3$; pH 7.5) and 90 mM $NaHCO_3$ (pH 9.5) for 7 d, respectively. Alkalinity-induced differentially expressed genes (DEGs) were identified and annotated. Functional enrichment analysis was performed for DEGs. The expression of genes related to response to stress and CAM were analyzed and compared. Comparing with control, 50 and 90 mM $NaHCO_3$ treatments induced 4 027 and 25 403 DEGs in *M. crystallinum* roots, respectively. Among these DEGs, 832 and 131 DEGs were consistently upregulated and downregulated by both stresses, respectively. These genes were associated with multiple biological processes related to response to abiotic stresses. Alkaline stress upregulated genes encoding heat shock proteins and ethylene-related genes, but downregulated genes encoding glutathione S-transferases. Also, genes that encode phosphoenolpyruvate carboxylases, phosphoenolpyruvate carboxylase kinase 1, and malate dehydrogenases related to malate accumulation were upregulated by alkalinity. This study indicated that alkaline stress affected the genes related to stress responses, metabolism, and malate accumulation in the roots of *M. crystallinum*.

Keywords: alkalinity tolerance, Crassulacean acid metabolism, DEGs, ice plant, *Mesembryanthemum crystallinum*, $NaHCO_3$, phosphoenolpyruvate carboxylase.

Introduction

Soil alkalinity (pH > 7.5) is a major abiotic stress and one of the leading constraints to plant distribution and productivity. Approximately 4.34×10^8 ha of land in more than 100 countries of the world was affected by high salinity in 2000, which covers about 6% of the total area in the world (Food and Nations 2000). The area of alkalized land is higher than that caused by salt deposition and is gradually increasing owing to climate change, improper cultivation, and irrational use of resources (Food and Nations 2000, Bai *et al*. 2016, Okur and Örçen 2020, Wang *et al*. 2020). High alkalinity changes enzyme activity, organic biomass, and the micro-etiology in soil, and then

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Abbreviations: ALDH7B4 - aldehyde dehydrogenase family 7 member B4; CAM - Crassulacean acid metabolism; DEGs - differentially expressed genes; ERF114 - ethylene-responsive transcription factor 114; *GO* - gene ontology; GST - glutathione S-transferase; HSP - heat shock protein; *KEGG* - Kyoto encyclopedia of genes and genomes; MDH - malate dehydrogenase; MKT1P - potassium channel protein gene; PEPC - phosphoenolpyruvate carboxylase; PPCK1 - phosphoenolpyruvate carboxylase kinase 1; PRX - peroxidase; SAG - senescence-associated protein.
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affects crop growth and productivity and the effect of fertilization (Semenov *et al*. 2018, Shi *et al*. 2018, Wang *et al*. 2018, Wu *et al*. 2018).

pH is an important indicator of soil health and most plants take up nutrients from the soil only in a moderate pH range (6.0 - 7.5) (South 2019). Soil with pH above 7.5 is considered alkaline (Bai *et al.* 2016) and may reduce seed germination, suppress photosynthesis, prevent seedling establishment, hamper root elongation, and inhibit the growth of most plants (Yu *et al*. 2019, Adams *et al*. 2020). During soil alkalization, soil pH increases owing to the constant accumulation of alkaline salt, including sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), among others. The increase of soil alkalinity may reduce the availability of iron, manganese, and phosphorus, and hamper the uptake of important nutritional elements, which directly or indirectly associate with plant metabolic reactions and physiological processes (Zhang *et al*. 2014, 2017; Wei *et al*. 2015, Shi *et al*. 2018). Accordingly, fertilization amendments aiming to improve the absorption of nutrients and plant breeding programs focusing on cultivating crops with high resistance to alkalinity both are effective solutions to improve the productivity of the soil.

Options for increasing crop yield and performance include cultivating crops with high resistance to abiotic stresses, including salinity and alkalinity (Ma 2019, Dixit *et al*. 2020). *Mesembryanthemum crystallinum* (ice plant) is a C_3 - Crassulacean acid metabolism (CAM) intermediate plant from Africa, Sinai, and south Europe, and naturalized in North America, Australia, China, and other countries. *M. crystallinum* enjoys great popularity because it has a high tolerance to abiotic stress (Bohnert and Cushman 2000) and its medicinal properties (anti-diabetic, anti-hyperglycemia, and immunomodulatory) (Choi *et al*. 2017, Zhang *et al*. 2019, Lin *et al*. 2020). The leaves and shoots of ice plant are covered with epidermal bladder cells, while its facultative structural characteristics facilitate water storage and response to various conditions, such as drought, low temperature, heavy metals, and salinity (Sunagawa *et al*. 2010, Kholodova *et al*. 2011, Oh *et al*. 2015, Chiang *et al*. 2016, Zhang *et al*. 2018). The stomata of CAM plant leaves remain closed during the daytime to reduce evapotranspiration and open at night to collect carbon dioxide (CO_2) under stressed conditions. On the other hand, the stomata of C_3 and C_4 plant leaves remain open in the day and shut in the dark under normal conditions. Studies have shown that *M. crystallinum* switches from C_3 photosynthesis to CAM, which is accelerated by salinity and drought (Oh *et al.* 2015, Kuźniak *et al.* 2016, Guan *et al.* 2020). There is evidence showing that *M. crystallinum* has a high tolerance to stresses including salinity and drought (Chiang *et al*. 2016, He *et al*. 2020, Kong *et al*. 2020). A high stress tolerance in *M. crystallinum* makes it a powerful resource for the study of the adaptive mechanism to different abiotic stresses (Tsukagoshi *et al.* 2015).

Some research studies indicated that the CAM-related enzymes, including phosphoenolpyruvate carboxylase isoform 1 (PEPC1) and phosphoenolpyruvate carboxylase kinase 1 (PPCK1), were upregulated in the guard cells in *M. crystallinum*, and the subunits of the V-ATPase were increased in the *M. crystallinum* leaves (Kong *et al.* 2020). Besides, some studies have shown that alkalinity decreases the expression of cell cycle-related genes and genes related to the element uptake and the metabolisms of ethylene, auxin, and riboflavin (Li *et al*. 2015, Hsieh and Waters 2016, Yu *et al*. 2017). However, the mechanisms that mediate the response and adaptation to alkaline stress in *M. crystallinum* has not been reported yet.

To explore the molecular mechanisms of alkalinity tolerance in *M. crystallinum*, we investigated the transcriptomic changes in *M. crystallinum* roots in response to alkaline stress. The difference in the transcriptome in *M. crystallinum* root to low (pH 7.5) and high (pH 9.5) alkalinity was identified by using the RNA-seq analysis. Genes related to responses to abiotic stresses and CAM metabolism were identified and described. The transcriptome profiling of alkalinity-induced changes to genes related to CAM and stress responses in *M. crystallinum* may provide new insights into alkaline tolerance in this plant. This study shows new perspectives and methods to improve the productivity of crops on alkaline soil.

Materials and methods

Plants and growth conditions: *Mesembryanthemum crystallinum* L. inbred line ZB, stored in the germplasm bank of Taizhou Academy of Agricultural Sciences (Taizhou, China), was used as materials in this study. *M. crystallinum* seeds were germinated under routine glasshouse conditions (day/night temperatures of 25/15°C, relative humidities of 70/90%, a 14-h photoperiod, and an irradiance of 350 μmol m^{-2} s^{-1}. Two-leaf seedlings were transplanted into black plastic containers containing 3 500 cm^3 of 1/2 Hoagland's solution (pH 6.5) and were maintained in a light incubator. The nutrient solution was changed every week.

For alkaline stress, *M. crystallinum* seedlings (4-week-old) were treated with 50 mM (pH 7.5, low alkalinity) and 90 mM (pH 9.5, high alkalinity) $NaHCO_3$ in 1/2 Hoagland's solution. Four-week old seedlings exposed to 1/2 Hoagland's solution were used as controls. All the experiments were performed in triplicate and sustained for 7 d under the aforementioned conditions. At the end of the experiment, the root samples were collected, cut into small pieces, frozen in liquid nitrogen for 5 - 10 min, and then stored at -70°C before RNA extraction.

RNA extraction and high-throughput sequencing: The total RNA was extracted from *M. crystallinum* root using the *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantification of RNA were assessed using the *NanoDrop 2000* spectrophotometer (*Thermo*

Fischer Scientific, Waltham, MA, USA) and *Agilent 2100* bioanalyzer (*Agilent Technologies*, Santa Clara, CA, USA). The enrichment of poly (A) + mRNA was performed using the immobilized oligo (dT) (*Invitrogen*). The enriched mRNA was fragmented, synthesized into the first- and double-strand cDNA, and purified using the *AMPure XP* beads (*Beckman Coulter*, CA, USA). For the preparation of DNA libraries, DNA fragments were end-repaired, adenylated, ligated to adapters, and amplified using PCR. The quality of DNA libraries was assessed using the *Qubit2.0* fluorometer (*Invitrogen*, Carlsbad, CA, USA) and *Agilent 2100* bioanalyzer. Root transcriptome was analyzed using the *Illumina HiSeq 4000* sequencing platform (pair-end 2 × 150 bp).

Data processing and annotation of unique genes: The raw sequence data (fastQ format) were obtained and qualified using the *FastQC* program (*version 0.11.5*, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The adaptor reads, duplicated reads, and low-quality reads (with unknown bases >10% and over half of sequences with Q < 10 reads) were filtered out. The *de novo* assembly of clean reads was performed using the *Trinity* (Plymouth, NH, USA) software (Grabherr *et al.* 2011). The annotation of obtained unigenes was performed by blasting the assembled sequences against the databases including *NCBI* non-redundant (*NR*), *Swiss-Prot*, Kyoto Encyclopedia of Genes and Genomes (*KEGG*), Clusters of Orthologous Groups (*COG*), euKaryotic Ortholog Groups (*KOG*), Gene Ontology (*GO*), and Protein family (*Pfam*). The threshold for reliable annotation is set at e-value ≤ 1e-5. The coding sequence of unigene was identified using the *TransDecoder* in the *Trinity* software.

Analysis of differentially expressed genes (DEGs): The FPKM (expected number of fragments per kb per million reads) value of each unigene was calculated using the *Bowtie* software (http://bowtie-bio.sourceforge.net/index.shtml) and the *RSEM* software. The DEGs in *M. crystallinum* root between control and stressed conditions were identified using the *DESeq* package (*Bioconductor*, http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). The significant DEGs were screened out using the criteria of fold discovery rate (FDR) < 0.05 (Benjamini-Hochberg adjustment) and $|\log_2(\text{fold change, FC})| \geq 1$. DEGs with FDR < 0.05 and $\log_2 FC \geq 1$ were upregulated genes, and those with FDR < 0.05 and $\log_2 FC \leq -1$ were downregulated genes. The sample clustering based on the expression profiles of DEGs was performed using the *Pheatmap* software (*version 1.0.8*, https://cran.r-project.org/web/packages/pheatmap) *R package*.

Functional enrichment analysis: The *GO* biological processes related to the alkalinity-responsive mechanism in *M. crystallinum* root was analyzed using the *topGO* software (*version 2.32.0*, http://www.bioconductor.org/packages/release/bioc/html/topGO.html). *KEGG* pathways related to DEGs were identified from the *KEGG* database. The significantly enriched functional themes, including the biological processes and *KEGG* pathways, were identified using the criteria of $P < 0.05$ and gene count ≥ 2.

Validation and real-time RT-PCR analysis: Eight alkalinity-responsive DEGs, including CAM-related genes and transcription factors, were randomly selected for the validation experiments using the PCR analysis. The total RNA was extracted from *M. crystallinum* roots using *Trizol* (*Invitrogen*). The reverse transcription was performed using the *SuperScript II Reverse Transcription* kit (*Invitrogen*). The PCR analysis was carried out using the *QuantiTect Sybr Green PCR Master Mix* (*Qiagen*, Hilden, Germany) and the *ABI 7500* real-time PCR system (*Applied Biosystems*, Carlsbad, CA, USA). The specific primers used for PCR analysis are shown in Table 1 Suppl. All reactions were run in triplicate following the conditions: one cycle of 95°C for 4 min and 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 1 min. The relative expressions of genes were analyzed with normalization to the internal reference gene *polyubiquitin* (Cushman *et al.* 2008) using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis: All data were expressed as the means ± standard deviations. The *GraphPad Prism* software (*version 8.3.0*, San Diego, USA) was used for the statistical analysis. The differences in the expressions of genes across groups were analyzed using the one-way *ANOVA* with Holm-Sidak correction. The difference with $P < 0.05$ was considered as statistically significant.

Results

The RNA-seq sequencing generated 248.11 million clean reads (74.43 GB) after removing adaptor sequences and low-quality reads (Table 2 Suppl.). The *de novo* assembly produced 246 253 transcripts and 129 649 unigenes. Most transcripts (62.41%) and unigenes (77.17%) were 300 - 1 000 bp in length (Fig. 1*A* Suppl.). Besides, the percentage of mapped reads in genes is shown (Fig. 1*B* Suppl.). These results showed that the sequencing quality was high and that the sequencing depth was sufficient for transcriptome coverage. The assembled sequences were blasted against databases and 86 532 annotated genes were obtained with the criterion of e-value ≤ 10^{-5} (Table 1).

Table 1. The function annotation of unigene in *Mesembryanthemum crystallinum* (ice plant) root transcriptome in seven databases.

Database	Unigene number	300 - 1000 bp	≥ 1000 bp
COG	33 067	22 856	10 211
GO	49 185	33 548	15 637
KEGG	24 116	16 903	7 213
KOG	51 567	35 182	16 385
Pfam	53 916	34 301	19 615
Swissprot	50 775	31 817	18 958
NR	83 481	57 967	25 514
All	86 532	60 844	25 688

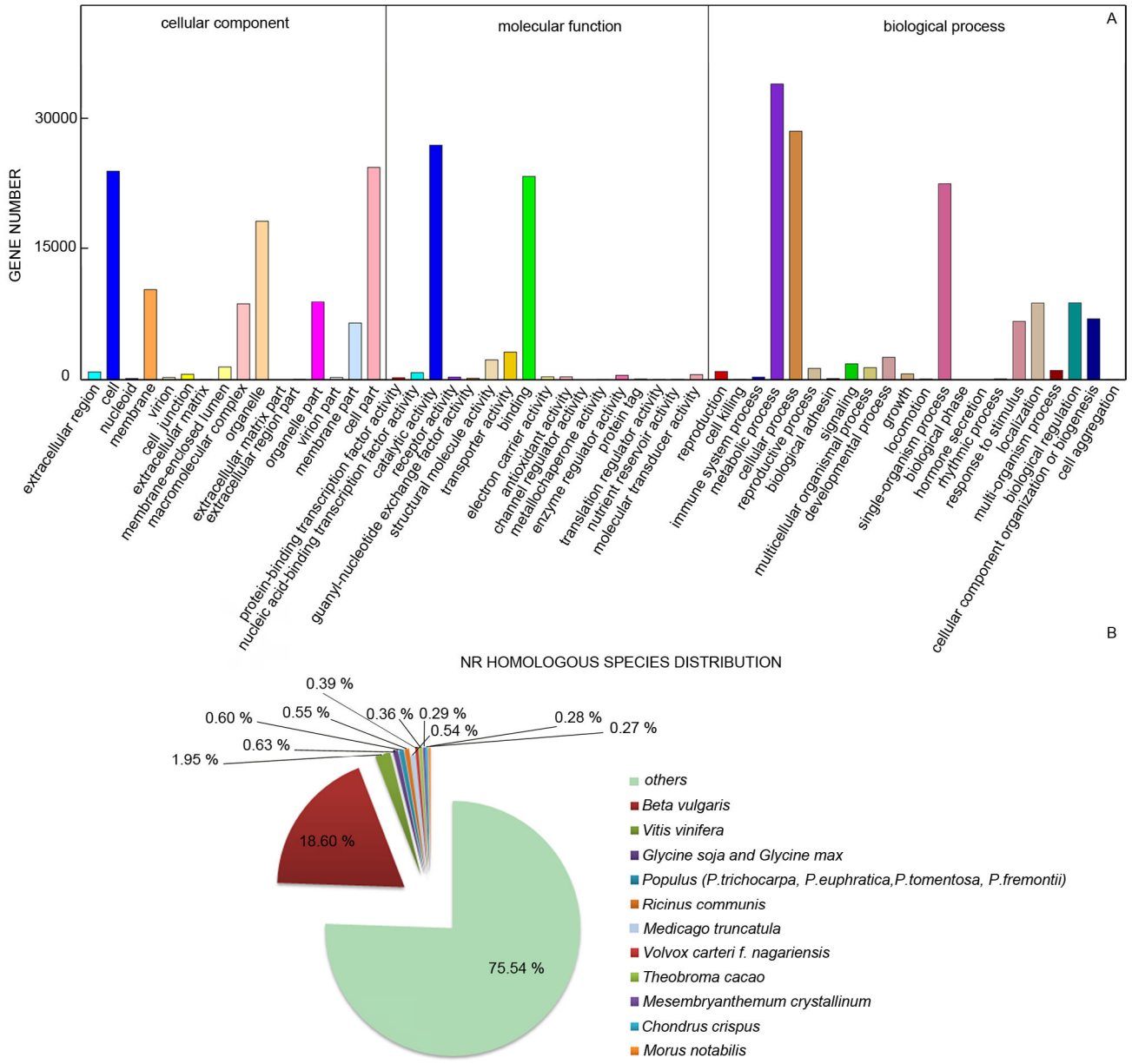

Fig. 1. Annotation of the unigenes in the root transcriptome of *M. crystallinum*: *A* - the gene ontology (*GO*) functional categories (cellular component, molecular function, and biological process) related to the annotated unigenes in *M. crystallinum* root transcriptome; *B* - the result of the homologous species distribution annotated in the *NCBI* non-redundant (*NR*) database. A large part of the *M. crystallinum* root transcriptome could not be annotated due to the lack of genomes of the reference and homologous species.

The functional annotation of 86 532 annotated unigenes showed that they were associated with multiple biological processes, cellular components, and molecular functions. Unigenes were associated with biological processes related to “response to stimulus” (6 644 unigenes) and “metabolic process” (34 007 unigenes). Most genes have the molecular functions of “catalytic activity” (26 976 unigenes), “binding” (23 344 unigenes), and “transporter activity” (3 137 unigenes; Fig. 1*A*). The analysis of homologous species distribution in NR showed that most genes (56.34%, 48 574/86 532) were annotated to unknown species, 18.60% (16 091 unigenes) and 1.95% (1 686 unigenes) of unigenes mapped to the genome of *Beta vulgaris* and *Vitis vinifera*, respectively (Fig. 1*B*). Only 0.63% of unigenes mapped to the known genome of *M. crystallinum*.

The RNA-seq analysis showed that the root transcriptome of 4-week-old *M. crystallinum* seedlings was changed after being treated with 50 mM (pH 7.5, low alkalinity) and 90 mM $NaHCO_3$ (pH 9.5, high alkalinity) for 7 d (Fig. 2). The M-*versus*-A plots indicating the DEGs in response to 50 mM and 90 mM $NaHCO_3$

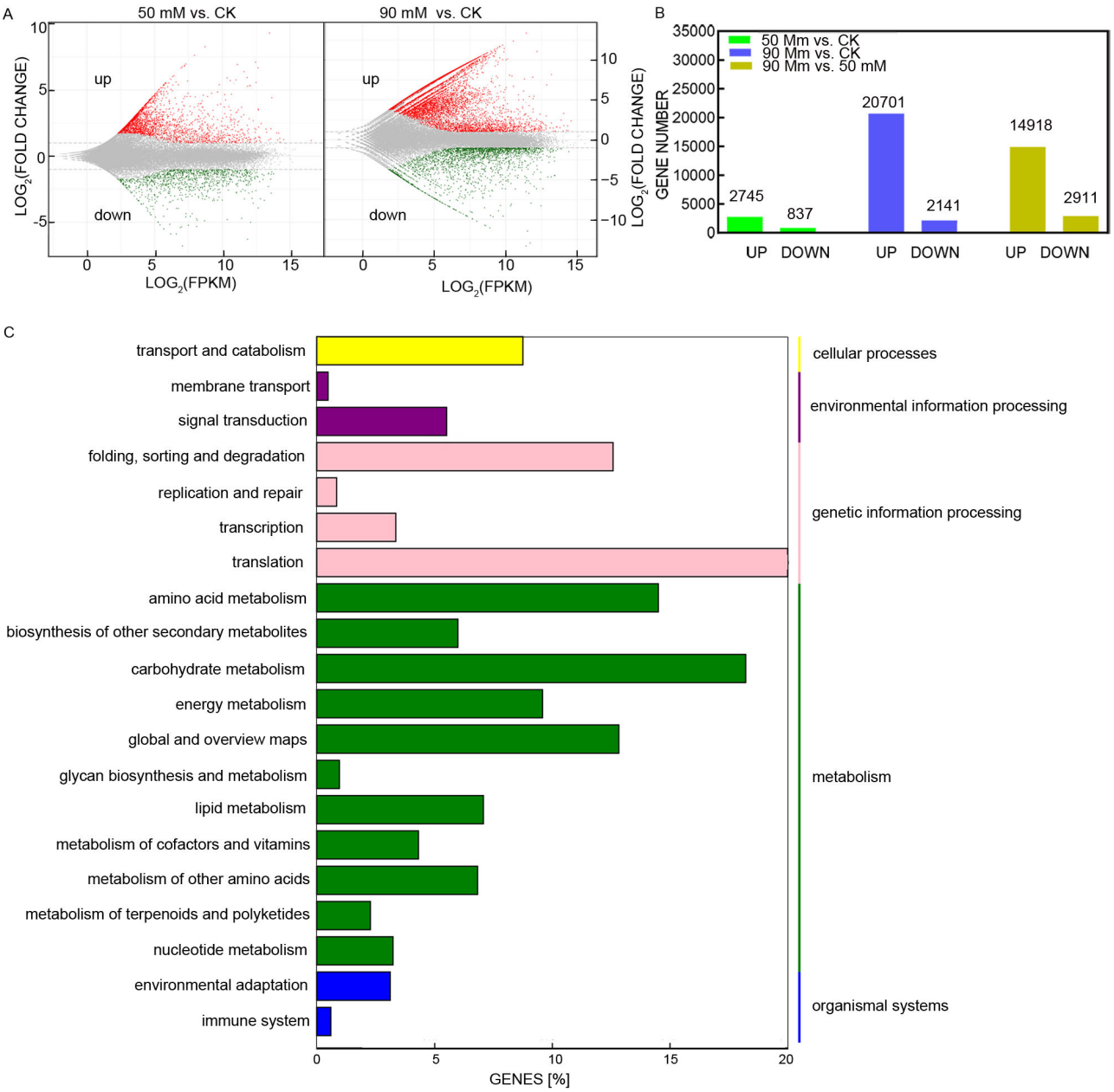

Fig. 2. The statistical summary and functional enrichment analysis of the differentially expressed genes (DEGs) in *M. crystallinum* root in response to alkaline stresses. *A* - The M-*versus*-A (MA) plots of DEGs in response to 50 and 90 mM $NaHCO_3$ treatments compared with control, respectively. The significant upregulation and downregulation were identified with the criteria of false discovery rate (FDR) ≤ 0.05 and |log2(fold change, FC)| ≥ 1. *B* - The statistical number of the annotated DEGs in response to treatments. The value above the column indicates the gene number involved in each column. *C* - The results of the Kyoto encyclopedia of genes and genomes (*KEGG*) pathway enrichment for the DEGs in response to alkaline stresses. This is a representative picture of the DEGs induced by the 90 mM $NaHCO_3$ treatment.

treatments are shown in Fig. 2*A*. A total of 4 027 and 25 403 unigenes were differentially expressed in the root of *M. crystallinum* after being treated with 50 and 90 mM $NaHCO_3$ for 7 d, respectively. A total of 3 582 DEGs (88.95%, 2 745 upregulated and 837 downregulated genes) and 22 842 DEGs (89.92%, 20 701 upregulated and 2 141 downregulated genes) were annotated in at least one of the seven databases listed in Table 1 (Fig. 2*B*). Also, 17 829 genes were differentially expressed between the 50 mM and 90 mM groups, including 14 918 upregulated genes and 2 911 downregulated genes (Fig. 2*B*). The sample clustering based on the expression profiles of DEGs is shown in Fig. 2 Suppl.

The *GO* functional categories and *KEGG* pathways related to DEGs were identified to investigate the biological functions associated with the DEGs in *M. crystallinum*

root in response to alkaline stresses. We observed that the DEGs upon high and low alkaline stresses were predominantly enriched in biological process categories of "metabolic process" and "response to stimulus" with the major molecular functions of "catalytic activity", "binding", and "transporter activity" (Fig. 3 Suppl.). *KEGG* pathway enrichment analysis showed that most DEGs were primarily involved in the metabolism of carbohydrates, amino acids, and lipids (Fig. 2*C*). Also, some DEGs were associated with pathways related to genetic information processing, including translation, replication, and repair. Functional annotation for DEGs between the 50 mM and 90 mM groups showed they were associated with similar functional categories to DEGs induced by both (Fig. 4 Suppl.).

To illustrate the molecular responses in *M. crystallinum* root in response to gradually increased alkaline stress, we analyzed the common DEGs under both 50 and 90 mM $NaHCO_3$ conditions. The Venn diagram analysis showed that 2 053 upregulated genes and 498 downregulated genes were common for high and low alkaline stresses (Fig. 3*A*), 832 of which were upregulated and 75 were downregulated by high alkalinity (90 mM) compared with low alkalinity (50 mM; Fig. 3*B*,*C*). Moreover, 13 583 DEGs were specifically upregulated by 90 mM $NaHCO_3$ (Fig. 3*B*). Among the common 498 downregulated genes, 14 were upregulated and 131 were downregulated by the comparison between high and low alkalinity (Fig. 3*D*,*E*). Also, 1 438 genes were consistently downregulated by high alkalinity in comparison with control and low alkalinity (Fig. 3*E*). These upregulated (n = 2 053) and downregulated genes (n = 498) were differentially expressed under both alkaline conditions. Those DEGs that were consistently upregulated (n = 832, Table 3 Suppl.) and downregulated (n = 131, Table 4 Suppl.) induced by both high and low alkalinities may play crucial roles in response to alkaline stresses.

Among the 832 upregulated and 131 downregulated genes that were consistently changed in response to high and low alkaline stresses, 29 upregulated DEGs and 14 downregulated DEGs were associated with the biological processes related to response to abiotic and biotic stresses based on the annotation in the *GO* database (Table 3 Suppl. and Table 4 Suppl.), including "response to salt stress" (GO:0009651), "response to water deprivation" (GO:0009414), and "response to stress" (GO:0006950). The expression patterns of the 43 DEGs in the root samples sequenced are shown in Fig. 4. Among these genes, five genes encoding heat shock proteins (HSPs; including *HSP90*, *HSP70B*, and *HSF8*), four genes encoding senescence-associated protein (SAG, including *SAG24*, *SAG12*, and *SAG39*), glutathione S-transferase T2 gene (*GSTT2*), and two ethylene-related genes (ethylene-responsive transcription factor 114, *ERF114*; and aldehyde dehydrogenase family 7 member B4, *ALDH7B4*) were gradually upregulated by 50 and 90 mM $NaHCO_3$ in *M. crystallinum* root. Three genes encoding GSTs (including *GSTU7* and *GST*) were gradually downregulated (Fig. 4). The upregulated genes *HSP90* and *ALDH7B4* and the two downregulated genes including potassium channel protein gene (*MKT1P*, up) and *LOC104781435* (encodes the E3 ubiquitin-protein ligase SDIR1, down) were involved in "response to salt stress" (GO:0009651; Fig. 4 Suppl.).

Among consistently changed DEGs upon alkaline stresses, 30 upregulated genes were associated with metabolic process (GO: 0008152; Fig. 5*A*), including one fructose-bisphosphate aldolase, *LOC104898474*; one phosphatidate phosphatase encoding gene *PAH1*; and one ABC transporter subfamily member *LOC104891628*; and two genes encoding polyadenylate-binding protein 2, *AT3G25610*) and three downregulated genes (including three ABC members *LOC104898459*, *ABCB15*, and *LOC104898462*. Besides, CAM-related genes were identified from the genes responsive to high alkalinity, including seven genes encoding PEPC1, 2, and PEPC-like proteins, two genes encoding PPCK1 protein, and 24 genes encoding malate dehydrogenases (MDHs), including MDH2, MDH, and 3-isopropylmalate dehydrogenase 1 (IMDH1; Fig. 5*B*). Most genes were upregulated by alkalinity as compared with control (Fig. 5*B*). The two MDH-7/-6 were increased by low alkalinity (50 mM $NaHCO_3$, pH 7.5 for 7d), but were downregulated by high alkalinity (90 mM $NaHCO_3$, pH 9.5 for 7d) compared with control (Fig. 5*B*). These results suggested that the alkalinity treatments may generally promote CAM and change metabolism processes in *M. crystallinum* root.

The expressions of eight alkalinity-responsive DEGs in *M. crystallinum* root were verified by the PCR analysis (Fig. 6). The results showed that the expressions of seven genes were significantly upregulated or downregulated by alkaline stress. Genes including WRKY family transcription factor 28 (*WRKY28*), ethylene-responsive transcription factor 114 (*ERF114*), bidirectional sugar transporter SWEET1 (*SWEET1*), *PEPC1*, and cysteine-rich repeat secretory protein 4 (*CRRSP4*), were significantly upregulated by low and/or high alkaline stress in *M. crystallinum* root (Fig. 6*A*), and genes encoding E3 ubiquitin-protein ligase SDIR1 and glutathione S-transferase U7 (*GSTU7*) were downregulated by alkaline stress (Fig. 6*B*).

Discussion

M. crystallinum is known as a model halophyte. Few reports have been focused on its response to alkalinity and other abiotic stress (Tsukagoshi *et al.* 2015, Kong *et al*. 2020). Transcriptome analysis is a powerful tool to investigate the molecular mechanism of plant growth under stressed conditions (Byun *et al*. 2018). Here, we analyzed the transcriptome of *M. crystallinum* upon exposure to two alkalinity-stressed conditions (50 mM $NaHCO_3$, pH 7.5; and 90 mM $NaHCO_3$, pH 9.5). Our study showed that alkalinity changed transcriptome in *M. crystallinum* root. The genes associated with senescence (including *SAG24*, *SAG39*, and *SAG12*) and ethylene (including *ERF114* and *ALDH7B4*), and genes encoding HSP proteins were gradually upregulated by alkaline stress. However, genes encoding ABC transporters, peroxidases, and GSTs

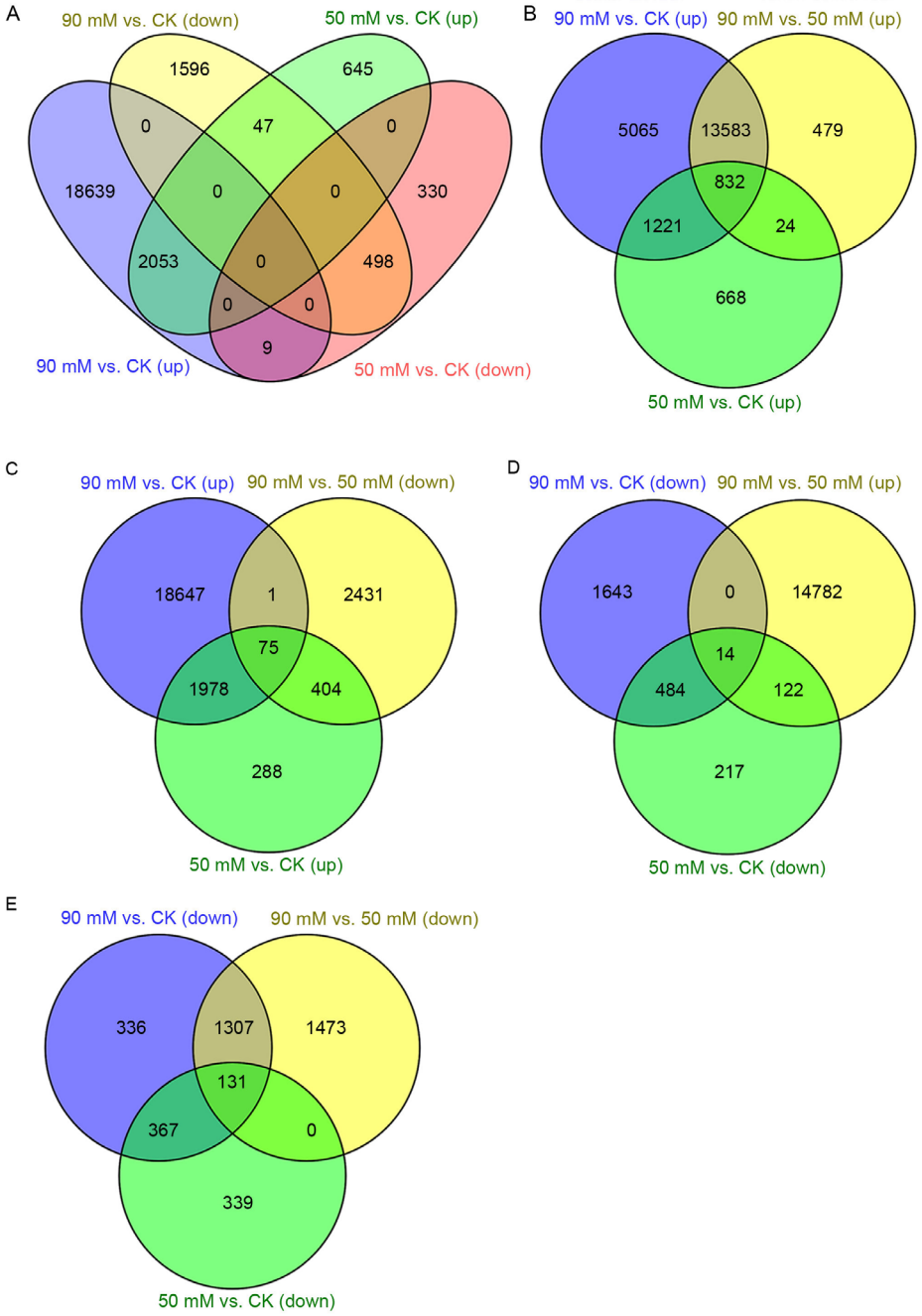

Fig. 3. Venn diagram analysis of the differentially expressed genes (DEGs) in *M. crystallinum* root in response to alkaline stresses. *A* - The Venn diagram indicating the common DEGs under both alkaline stresses (low alkaline stress: 50 mM $NaHCO_3$, pH 7.5, for 7 d; and high alkaline stress: 90 mM $NaHCO_3$, pH 9.5, for 7 d). *B* to *E* - The Venn diagrams showing the upregulated and downregulated DEGs common to different conditions: 50 mM $NaHCO_3$ *vs*. control (CK), 90 mM $NaHCO_3$ *vs*. CK, and 90 mM *vs*. 50 mM $NaHCO_3$.

were generally decreased in *M. crystallinum* root upon alkaline stresses. Also, we found that genes related to CAM, including *MDH*, *MDH2*, *PEPC1*, *PEPC2*, and *PPCK1*, were generally upregulated in *M. crystallinum* root upon alkaline stresses. These results confirmed that the alkalinity-responsive mechanisms in *M. crystallinum* root were related to senescence, phytohormone regulation, metabolic pathways, and CAM.

M. crystallinum is native to the Namibian desert in the south of Africa and has been introduced to the southeast coast of China for decades. Studies have shown that *M. crystallinum* has an adaptive mechanism of switching from C_3 to CAM photosynthesis to enhance stress tolerance and the efficiency of water usage (Bohnert and Cushman 2000). Moderate salinity stress may be optimum for its growth (Ostrem *et al*. 1987, Oh *et al*. 2015, Tsukagoshi

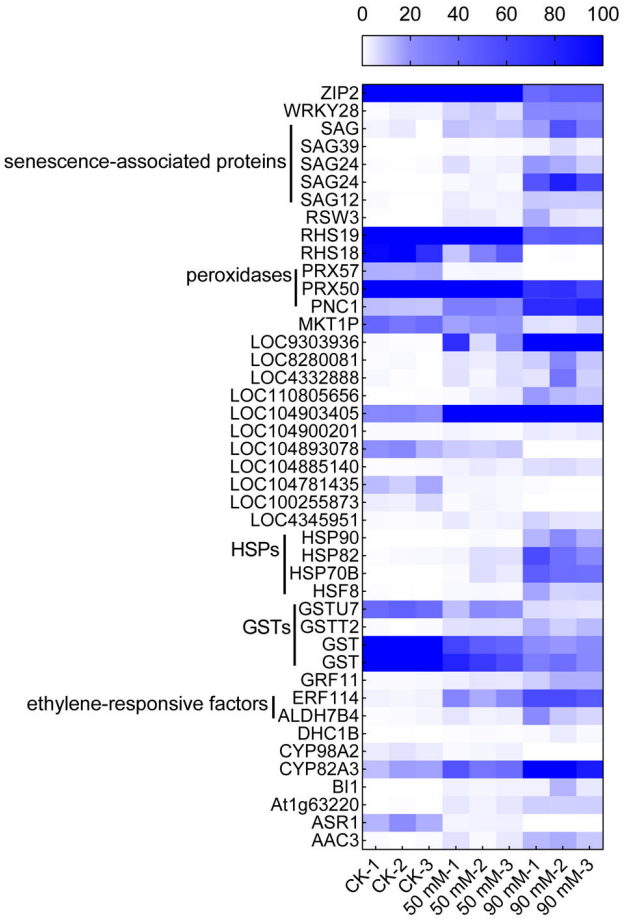

Fig. 4. The heatmap shows the expression patterns of the 43 differentially expressed genes related to stress responses in *M. crystallinum* root. *The blue shades* represent the expressions (reads count) of the corresponding genes. AAC3 - ADP/ATP carrier 3, ALDH7B4 - aldehyde dehydrogenase family 7 member B4, ASR1 - abscisic stress-ripening protein 1, CYP82A3 - cytochrome P450 82A3, CYP98A2 - cytochrome P450 98A2, DHC1B - cytoplasmic dynein 2 heavy chain, ERF114 - ethylene-responsive transcription factor 114, GRF11 - general regulatory factor 11, GST - glutathione S-transferase, HSF8 - heat shock factor 8, HSP - heat shock protein, MKT1P - potassium channel protein gene, PNC1 - cationic peroxidase 1, PRX - peroxidase, RHS19 - root hair specific 19, RSW3 - glycosyl hydrolases family 31, SAG - senescence-associated protein, SDIR1 - E3 ubiquitin-protein ligase SDIR1, SWEET1 - bidirectional sugar transporter SWEET1, WRKY28 - WRKY family transcription factor 28, ZIP2 - ZIP zinc transporter 2.

et al. 2015, Barkla *et al*. 2016). The transition from C_3 to CAM photosynthesis could be accelerated by the abiotic stresses of salinity and drought (Bohnert and Cushman 2000, Oh *et al*. 2015, Kuźniak *et al*. 2016, Guan *et al*. 2020). The salt-induced stress-adaptive mechanisms in *M. crystallinum* are based on the phenomenon of "osmotic interference" by which accumulated inorganic salts are released into the soil (Vivrette and Muller 1977). In our present study, we have found that CAM-related genes contributing to malate accumulation were differentially expressed in the root of *M. crystallinum* upon exposure to alkaline stress (50 mM and 90 mM $NaHCO_3$), including *MDH*, *MDH2*, *PEPC1*, and *PPCK1*, and most of them were upregulated by alkaline stress. These findings indicated that alkaline stress promoted CAM in *M. crystallinum* root.

The CAM-related enzymes including *PEPC1* and *PPCK1* were upregulated in *M. crystallinum* during the C_3 - CAM transition (Kong *et al.* 2020). In the C_4 pathway, PEPC promotes the saccharide metabolism through the carboxylation of phosphoenolpyruvate to oxaloacetate, which is then converted to malate by MDHs or decarboxylated to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Kustka *et al*. 2014, Yamamoto *et al*. 2015, Ewe *et al*. 2018). *PEPC* and *PPCK*s are often increased in plant in the context of abiotic and biotic stresses, such as alkaline, drought, viral infection, and others (Doubnerová and Ryšlavá 2011, Liu *et al*. 2017, Zou *et al*. 2021, Taticharoen *et al*. 2023). Our present study showed that both low and high alkalinities upregulated the expressions of the *PEPC1*, *PEPC2*, and *PPCK1* genes compared with control, without changing the expression of genes encoding enzymes related to the CAM pathway during the day, which might result in accumulated malate in the roots of *M. crystallinum*. Malate functions as a vacuolar osmolyte. We found that the low alkalinity had higher efficacy in upregulating *MDH* and *PEPC1*, while high alkalinity resulted in higher expressions of genes encoding PEPCs, PPCK1, and multiple transcripts of the *MDH* gene. These results showed that both alkaline stress enhanced malate accumulation by promoting enzymes related to CAM in *M. crystallinum* root.

As roots are in direct contact with soil and immediately respond to high alkalinity, the growth and development of roots are majorly affected by alkalinity, as evident from the changes in expressions of related genes (Li *et al*. 2015, Hsieh and Waters 2016). The influence of soil alkalinity on root growth is a complicated process involved by multiple genes and complex genetic regulatory networks. Many studies have shown that alkaline stress decreases the expression of cell cycle-related genes, including *CYCA2;1*, *CYCB1;1*, *CYCB3;1*, *CYCD1;1*, and *CYCD2;1*, which are thought to be the positive regulators of the cell cycle in root development (Wakeel and Gan 2018, Khan *et al*. 2019). The expressions of genes related to ethylene and auxin metabolism are also changed by alkaline stress (Li *et al*. 2015, Yu *et al*. 2017, Wakeel and Gan 2018). Upon exposure to alkaline stress, plants may modify auxin distribution, plasma membrane H^+-ATPase activity, and microfilament stabilization, which results in shorter roots (Ma *et al*. 2015, Yu *et al*. 2017, Wakeel and Gan 2018). However, the upregulation of two ethylene-responsive factors *ERF114* and *ALDH7B4* was also reported in other crops, including rice, *Arabidopsis*, and soybean (Gil-Monreal *et al*. 2017, Gautam *et al*. 2020, Shen *et al*. 2020). Also, the upregulated *AtERF114* gene was confirmed in *phb3* mutant *Arabidopsis* seedlings with shorter roots compared with wild-type seedlings (Kong *et al.* 2018). The upregulations of these two genes were associated with increased resistance to abiotic stresses

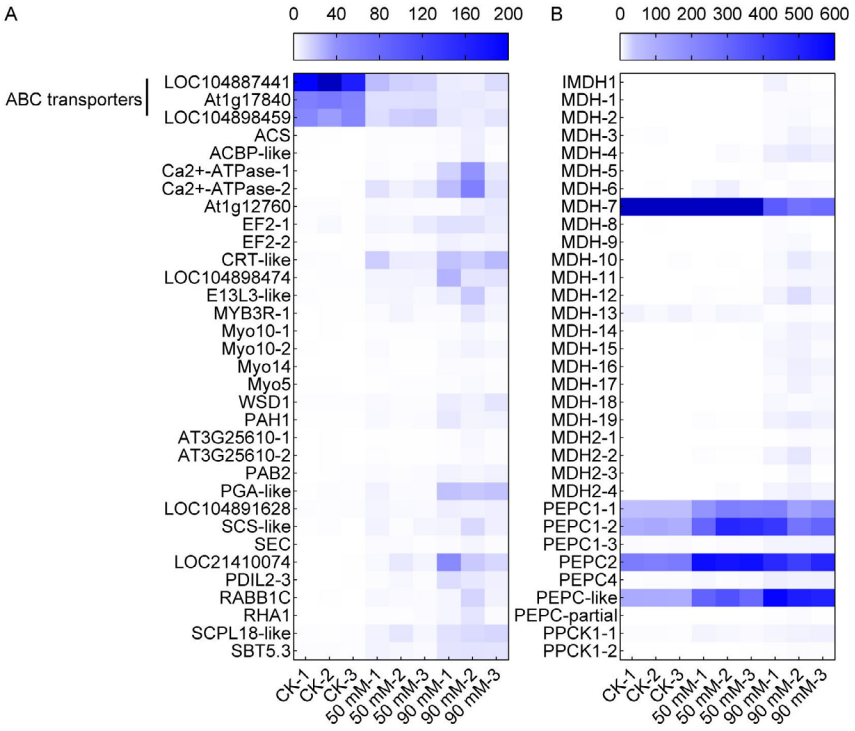

Fig. 5. The heatmaps show the expression profiles of the differentially expressed genes related to metabolism (*A*) and Crassulacean acid metabolism (CAM; *B*) in *M. crystallinum* root. *The blue shades* represent the expression levels (count of reads) of genes. ACBP - acyl-CoA-binding domain-containing protein, ACS - acetyl-coenzyme A synthetase, Ca^{2+}-ATPase - calcium-transporting ATPase, CRT - 3-hydroxybutyryl-CoA dehydratase, E13L3 - glucan endo-1,3-beta-glucosidase, EF2 - elongation factor 2, MDH - malate dehydrogenase, Myo - myosin, MYB3R-1 - Myb domain protein 3R-1, PAB2 - polyadenylate-binding protein 2, PAH1 - phosphatidate phosphatase 1, PDIL2-3 - protein disulfide-isomerase 2-3, PEPC - phosphoenolpyruvate carboxylase, PPCK1 - phosphoenolpyruvate carboxylase kinase 1, PGA - polygalacturonase, RABB1C - RAB GTPase homolog B1C, RHA1 - Ras-related protein RABF2a, SBT5.3 - subtilisin-like protease 5.3, SCS - succinyl-CoA ligase, SCPL18 - serine carboxypeptidase-like 18, SEC - UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase, WSD1 - O-acyltransferase.

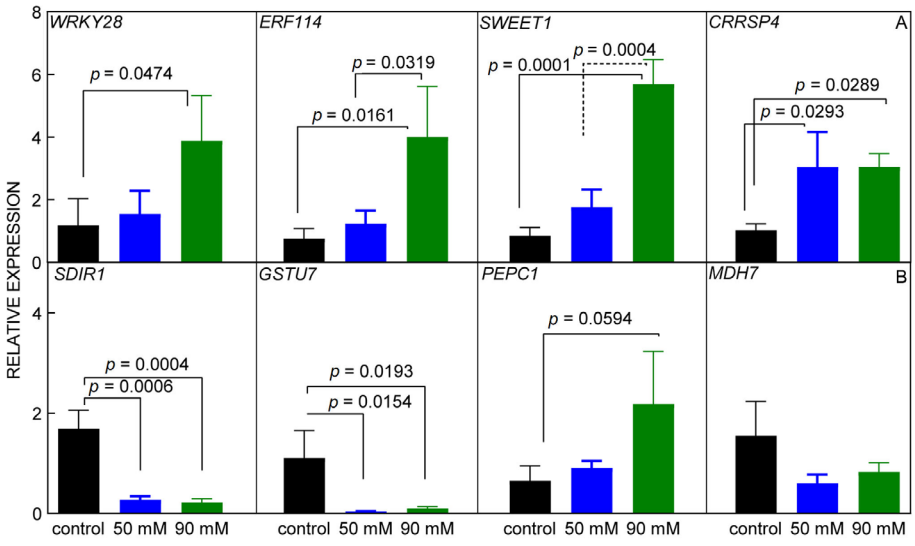

Fig. 6. The expression of eight genes differentially expressed in *M. crystallinum* root in response to alkaline stress. *A* and *B* - the relative expressions of the eight genes were determined by PCR analysis. The differences in the expressions of genes across groups were analyzed using the one-way *ANOVA* with Holm-Sidak correction. Means ± SDs, $n = 3$. PEPC1 - phosphoenolpyruvate carboxylase 1, CRRSP4 - cysteine-rich repeat secretory protein 4, MDH7 - malate dehydrogenase 7, GSTU7 - glutathione S-transferase U7, ERF114 - ethylene-responsive transcription factor 114, SDIR1 - E3 ubiquitin-protein ligase SDIR1, SWEET1 - bidirectional sugar transporter SWEET1, WRKY28 - WRKY family transcription factor 28.

in plants (Gil-Monreal *et al*. 2017, Gautam *et al*. 2020). The changed expression profiles of auxin- and ethylene-related genes may show the inhibition of cell division and development and *M. crystallinum* root growth.

GSTs and PRXs are antioxidant enzymes of great importance for detoxification. These enzymes show significant roles in responses to abiotic stresses and conferring tolerance against them in plants, including heat, cold, salt, and herbicides (Singh *et al*. 2016, ul Haq *et al*. 2019). HSPs, GSTs, and PRXs have different changing profiles in plant roots in response to abiotic stresses (Singh *et al*. 2016, Kumar and Trivedi 2018, Mishra *et al*. 2018, ul Haq *et al*. 2019, Ghangal *et al*. 2020, Hasan *et al*. 2020). Ghangal *et al.* (2020) showed that the *CaGSTU4*, *CaGSTU20*, *CaGSTU21*, and *CaGSTU13* genes in *Cicer arietinum* root were decreased by desiccation, salinity, and cold stress, but were upregulated by desiccation and salinity stress in the shoot of chickpea. Tsukagoshi *et al.* (2015) showed that 'peroxidase activity'-related genes in *M. crystallinum* were downregulated by high salinity. Also, the growth of *M. crystallinum* root was inhibited by 250 and 500 mM NaCl (Tsukagoshi *et al*. 2015). However, there is less information on the association of these genes with stress-induced C_3 - CAM transition in *M. crystallinum* and other CAM plants. Our present study showed that the HSPs encoding genes were upregulated and GSTs and PRXs encoding genes were downregulated in *M. crystallinum* root upon exposure to alkaline stress. The identification of alkalinity-responsive genes may reveal that molecular responses and mechanisms in *M. crystallinum* root were associated with the deregulation of GSTs and PRXs.

Conclusions

In summary, this work of transcriptome analysis indicated that the alkalinity-responsive mechanisms in *M. crystallinum* root were mediated by many genes and multiple biological processes, including response to stimulus. High alkaline stress ($NaHCO_3$, pH 9.5) induced a higher number of DEGs (n = 25 403) in *M. crystallinum* root compared with low alkaline stress ($NaHCO_3$, pH 7.5, n = 4 027). The downregulation of auxin and ethylene-related genes were alkalinity-responsive in *M. crystallinum* root. Noticeably, we found that the transcriptions of genes related to CAM in the dark phase were increased by alkaline conditions. Alkaline stress upregulates CAM-related genes, including *MDH*, *MDH2*, *PEPC1*, and *PPCK1* to increase the accumulation of malate in *M. crystallinum* root. This study provides referential information on the mechanisms of alkaline stress-induced responses and adaptive mechanism in *M. crystallinum* root.

Availability of data and material: All data generated or analyzed during this study are included in this published article. The original sequencing data is available from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/gds/) with the accession number PRJNA683211. The microarray datasets (GSE17883, GSE46208, and GSE76613) are available from the National Center for Biotechnology Information Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

References

Adams R.P., Peña F., Wilcox E., Scow B.: The effects extreme alkaline soil on biomass and hydrocarbon yields in *Helianthus annuus* cv. Munchkin, Firecracker and Little Becka (*Asteraceae*, sunflowers). - Phytologia **102**: 143-149, 2020.

Bai L., Wang C., Zang S. *et al.*: Remote sensing of soil alkalinity and salinity in the Wuyu'er-Shuangyang River Basin, Northeast China. - Remote Sens. **8**: 163, 2016.

Barkla B.J., Vera-Estrella R., Raymond C.: Single-cell-type quantitative proteomic and ionomic analysis of epidermal bladder cells from the halophyte model plant *Mesembryanthemum crystallinum* to identify salt-responsive proteins. - BMC Plant Biol. **16**: 110, 2016.

Bohnert H.J., Cushman J.C.: The ice plant cometh: lessons in abiotic stress tolerance. - J. Plant Growth Regul. **19**: 334-346, 2000.

Byun M.Y., Cui L.H., Lee J. *et al.*: Identification of rice genes associated with enhanced cold tolerance by comparative transcriptome analysis with two transgenic rice plants overexpressing *DaCBF4* or *DaCBF7*, isolated from Antarctic flowering plant *Deschampsia antarctica*. - Front. Plant Sci. **9**: 601, 2018.

Chiang C.-P., Yim W.C., Sun Y.-H. *et al.*: Identification of ice plant (*Mesembryanthemum crystallinum* L.) microRNAs using RNA-Seq and their putative roles in high salinity responses in seedlings. - Front. Plant Sci. **7**: 1143, 2016.

Choi J.-H., Jo S.-G., Jung S.-K. *et al.*: Immunomodulatory effects of ethanol extract of germinated ice plant (*Mesembryanthemum crystallinum*). - Lab. Animal Res. **33**: 32-39, 2017.

Cushman J.C., Tillett R.L., Wood J.A. *et al.*: Large-scale mRNA expression profiling in the common ice plant, *Mesembryanthemum crystallinum*, performing C_3 photosynthesis and Crassulacean acid metabolism (CAM). - J. Exp. Bot. **59**: 1875-1894, 2008.

Dixit V.K., Misra S., Mishra S.K. *et al.*: Characterization of plant growth-promoting alkalotolerant *Alcaligenes* and *Bacillus* strains for mitigating the alkaline stress in *Zea mays*. - Anton. Leeuw. **113**: 889-905, 2020.

Doubnerová V., Ryšlavá H.: What can enzymes of C_4 photosynthesis do for C_3 plants under stress? - Plant Sci. **180**: 575-583, 2011.

Ewe D., Tachibana M., Kikutani S. *et al.*: The intracellular distribution of inorganic carbon fixing enzymes does not support the presence of a C_4 pathway in the diatom *Phaeodactylum tricornutum*. - Photosynth. Res. **137**: 263-280, 2018.

Food and Nations AOotU.: Extent and causes of salt-affected soils in participating countries. Global network on integrated soil management for sustainable use of salt-affected soils. FAO Land and Plant Nutrition Management Service, Rome 2000.

Gautam R., Meena R.K., Woch N., Kirti P.: Ectopic expression of *BrALDH7B2* gene encoding an antiquitin from *Brassica rapa* confers tolerance to abiotic stresses and improves photosynthetic performance under salt stress in tobacco. - Environ. Exp. Bot. **180**: 104223, 2020.

Ghangal R., Rajkumar M.S., Garg R., Jain M.: Genome-wide analysis of glutathione S-transferase gene family in chickpea suggests its role during seed development and abiotic stress. -

Mol. Biol. Rep. **47**: 2749-2761, 2020.
Gil-Monreal M., Zabalza A., Missihoun T.D. *et al.*: Induction of the PDH bypass and upregulation of the *ALDH7B4* in plants treated with herbicides inhibiting amino acid biosynthesis. - Plant Sci. **264**: 16-28, 2017.
Grabherr M.G., Haas B.J., Yassour M. *et al.*: Full-length transcriptome assembly from RNA-Seq data without a reference genome. - Nat. Biotechnol. **29**: 644-662, 2011.
Guan Q., Tan B., Kelley T.M. *et al.*: Physiological changes in *Mesembryanthemum crystallinum* during the C_3 to CAM transition induced by salt stress. - Front. Plant Sci. **11**: 283, 2020.
Hasan M.S., Islam S., Hasan M.N. *et al.*: Genome-wide analysis and transcript profiling identify several abiotic and biotic stress-responsive glutathione S-transferase genes in soybean. - Plant Gene **23**: 100239, 2020.
He J., Chua E.L., Qin L.: Drought does not induce crassulacean acid metabolism (CAM) but regulates photosynthesis and enhances nutritional quality of *Mesembryanthemum crystallinum*. - PLoS ONE **15**: e0229897, 2020.
Hsieh E.-J., Waters B.M.: Alkaline stress and iron deficiency regulate iron uptake and riboflavin synthesis gene expression differently in root and leaf tissue: implications for iron deficiency chlorosis. - J. Exp. Bot. **67**: 5671-5685, 2016.
Khan A.R., Wakeel A., Muhammad N. *et al.*: Involvement of ethylene signaling in zinc oxide nanoparticle-mediated biochemical changes in *Arabidopsis thaliana* leaves. - Environ. Sci.-Nano **6**: 341-355, 2019.
Kholodova V., Volkov K., Abdeyeva A., Kuznetsov V.: Water status in *Mesembryanthemum crystallinum* under heavy metal stress. - Environ. Exp. Bot. **71**: 382-389, 2011.
Kong W., Yoo M.J., Zhu D. *et al.*: Molecular changes in *Mesembryanthemum crystallinum* guard cells underlying the C_3 to CAM transition. - Plant Mol. Biol. **103**: 653-667, 2020.
Kong X., Tian H., Yu Q. *et al.*: PHB3 maintains root stem cell niche identity through ROS-responsive AP2/ERF transcription factors in *Arabidopsis*. - Cell Rep. **22**: 1350-1363, 2018.
Kumar S., Trivedi P.K.: Glutathione S-transferases: role in combating abiotic stresses including arsenic detoxification in plants. - Front. Plant Sci. **9**: 751, 2018.
Kustka A.B., Milligan A.J., Zheng H. *et al.*: Low CO_2 results in a rearrangement of carbon metabolism to support C_4 photosynthetic carbon assimilation in *Thalassiosira pseudonana*. - New Phytol. **204**: 507-520, 2014.
Kuźniak E., Kornas A., Kaźmierczak A. *et al.*: Photosynthesis-related characteristics of the midrib and the interveinal lamina in leaves of the C_3–CAM intermediate plant *Mesembryanthemum crystallinum*. - Ann. Bot.-London **117**: 1141-1151, 2016.
Li J., Xu H.-H., Liu W.-C. *et al.*: Ethylene inhibits root elongation during alkaline stress through AUXIN1 and associated changes in auxin accumulation. - Plant Physiol. **168**: 1777-1791, 2015.
Lin Y.-H., Kuo W.-W., Chen I.-H. *et al.*: Uses of *Mesembryanthemum crystallinum* L. callus extract in delaying skin cell aging, nursing skin, treating and preventing skin cancer. United States Patent Application No. US 2017/0274029 A1. *Available at*: https://www.freepatentsonline.com/y2017/0274029.html, 2020.
Liu X., Li X., Zhang C. *et al.*: Phosphoenolpyruvate carboxylase regulation in *C4-PEPC*-expressing transgenic rice during early responses to drought stress. - Physiol. Plantarum **159**: 178-200, 2017.
Ma H.: Saline–alkaline resistance: physiological and ecological characteristics of sheepgrass (*Leymus chinensis*). - In: Liu G., Li X., Zhang Q. (ed.): Sheepgrass (*Leymus chinensis*): An Environmentally Friendly Native Grass for Animals. Pp. 117-137. Springer, Singapore 2019.
Ma Q., Jin K., Peng G., Xia Y.: An ENA ATPase, MaENA1, of *Metarhizium acridum* influences the Na^+-, thermo- and UV-tolerances of conidia and is involved in multiple mechanisms of stress tolerance. - Fungal Genet. Biol. **83**: 68-77, 2015.
Mishra D., Shekhar S., Singh D. *et al.*: Heat shock proteins and abiotic stress tolerance in plants. - In: Asea A., Kaur P. (ed.): Regulation of Heat Shock Protein Responses. Heat Shock Proteins. Vol. 13. Pp. 41-69. Springer, Cham 2018.
Oh D.H., Barkla B.J., Vera-Estrella R. *et al.*: Cell type-specific responses to salinity - the epidermal bladder cell transcriptome of *Mesembryanthemum crystallinum*. - New Phytol. **207**: 627-644, 2015.
Okur B., Örçen N.: Soil salinization and climate change. - In: Prasad M.N.V., Pietrzykowski M. (ed.): Climate Change and Soil Interactions. Pp. 331-350. Elsevier, Amsterdam 2020.
Ostrem J.A., Olson S.W., Schmitt J.M., Bohnert H.J.: Salt stress increases the level of translatable mRNA for phosphoenolpyruvate carboxylase in *Mesembryanthemum crystallinum*. - Plant Physiol. **84**: 1270-1275, 1987.
Semenov M., Blagodatskaya E., Stepanov A., Kuzyakov Y.: DNA-based determination of soil microbial biomass in alkaline and carbonaceous soils of semi-arid climate. - J. Arid Environ. **150**: 54-61, 2018.
Shen C., Yuan J., Qiao H. *et al.*: Transcriptomic and anatomic profiling reveal germination process of different wheat varieties in response to waterlogging stress. - BMC Genet. **21**: 93, 2020.
Shi B., Zhang J., Wang C. *et al.*: Responses of hydrolytic enzyme activities in saline-alkaline soil to mixed inorganic and organic nitrogen addition. - Sci. Rep.-UK **8**: 4543, 2018.
Singh R.K., Jaishankar J., Muthamilarasan M. *et al.*: Genome-wide analysis of heat shock proteins in C_4 model, foxtail millet identifies potential candidates for crop improvement under abiotic stress. - Sci. Rep.-UK **6**: 32641, 2016.
South D.B.: Is the recommended pH for growing hardwood seedlings wrong? - Reforesta **7**: 81-108, 2019.
Sunagawa H., Cushman J., Agarie S.: Crassulacean acid metabolism may alleviate production of reactive oxygen species in a facultative CAM plant, the common ice plant *Mesembryanthemum crystallinum* L. - Plant Prod. Sci. **13**: 256-260, 2010.
Taticharoen T., Matsumoto S., Chutteang C. *et al.*: Response and acclimatization of a CAM orchid, *Dendrobium* Sonia 'Earsakul' to drought, heat, and combined drought and heat stress. - Sci. Hortic.-Amsterdam **309**: 111661, 2023.
Tsukagoshi H., Suzuki T., Nishikawa K. *et al.*: RNA-Seq analysis of the response of the halophyte, *Mesembryanthemum crystallinum* (ice plant) to high salinity. - PLoS ONE **10**: e0118339, 2015.
ul Haq S., Khan A., Ali M. *et al.*: Heat shock proteins: dynamic biomolecules to counter plant biotic and abiotic stresses. - Int. J. Mol. Sci. **20**: 5321, 2019.
Vivrette N.J., Muller C.H.: Mechanism of invasion and dominance of coastal grassland by *Mesembryanthemum crystallinum*. - Ecol. Monogr. **47**: 301-318, 1977.
Wakeel A., Gan Y.: A model for the ethylene-mediated auxin distribution under Cr (VI) stress in *Arabidopsis thaliana*. - Plant Signal. Behav. **13**: e1473685, 2018.
Wang Y., Ji H., Hu Y. *et al.*: Different selectivity in fungal communities between manure and mineral fertilizers: a study in an alkaline soil after 30 years fertilization. - Front. Microbiol. **9**: 2613, 2018.
Wang Z., Tan W., Yang D. *et al.*: Mitigation of soil salinization

and alkalization by bacterium-induced inhibition of evaporation and salt crystallization. - Sci. Total Environ. **755**: 142511, 2020.

Wei L.-X., Lv B.-S., Wang M.-M. *et al.*: Priming effect of abscisic acid on alkaline stress tolerance in rice (*Oryza sativa* L.) seedlings. - Plant Physiol. Biochem. **90**: 50-57, 2015.

Wu B., Hou S., Peng D. *et al.*: Response of soil micro-ecology to different levels of cadmium in alkaline soil. - Ecotox. Environ. Safe. **166**: 116-122, 2018.

Yamamoto N., Sasou A., Saito Y. *et al.*: Protein and gene expression characteristics of a rice phosphoenolpyruvate carboxylase *Osppc3*; its unique role for seed cell maturation. - J. Cereal Sci. **64**: 100-108, 2015.

Yu S., Yu L., Hou Y. *et al.*: Contrasting effects of NaCl and $NaHCO_3$ stresses on seed germination, seedling growth, photosynthesis, and osmoregulators of the common bean (*Phaseolus vulgaris* L.). - Agronomy **9**: 409, 2019.

Yu Y., Duan X., Ding X. *et al.*: A novel AP2/ERF family transcription factor from *Glycine soja*, *GsERF71*, is a DNA binding protein that positively regulates alkaline stress tolerance in *Arabidopsis*. - Plant Mol. Biol. **94**: 509-530, 2017.

Zhang C., Wu W., Xin X. *et al.*: Extract of ice plant (*Mesembryanthemum crystallinum*) ameliorates hyperglycemia and modulates the gut microbiota composition in type 2 diabetic Goto-Kakizaki rats. - Food Funct. **10**: 3252-3261, 2019.

Zhang H., Liu X.-L., Zhang R.-X. *et al.*: Root damage under alkaline stress is associated with reactive oxygen species accumulation in rice (*Oryza sativa* L.). - Front. Plant Sci. **8**: 1580, 2017.

Zhang J., Wang P., Tian H. *et al.*: Identification of interior salt-tolerant bacteria from ice plant *Mesembryanthemum crystallinum* and evaluation of their promoting effects. - Symbiosis **76**: 243-252, 2018.

Zhang W.J., Niu Y., Bu S.H. *et al.*: Epistatic association mapping for alkaline and salinity tolerance traits in the soybean germination stage. - PLoS ONE **9**: e84750, 2014.

Zou C.-L., Wang Y.-B., Wang B. *et al.*: Effects of alkali stress on dry matter accumulation, root morphology, ion balance, free polyamines, and organic acids of sugar beet. - Acta Physiol. Plant. **43**: 13, 2021.