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Transcriptomic and lipidomic analysis reveals the salt-adapted in Salicornia europaea

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Abstract

Halophytes have evolved unique strategies to cope with salinity. *Salicornia europaea*, a succulent euhalophyte plant, show optimal reproduction under 200 mM NaCl. However, the role of lipid metabolism caused by salinity in *S. europaea* is not fully understood. To gain insight into this, we conducted a combined lipidomic and transcriptomic analysis to investigate the molecular basis of lipid metabolism in response to 0 and 200 mM NaCl. GO and KEGG analysis revealed that salt treatment mainly affected glycosylphosphatidylinositol (GPI) anchored biosynthesis, secondary metabolite synthesis, plant hormone signal transduction, peroxisome, and glycosphingoipid biosynthesis. We identifited 485 lipid compounds, 27 of which showed altered abundances under salt treatment. Notably, lysophosphatidylcholine (LPC) and phytosphingosine-1-phosphate (S1P) significantly increased, while polyunsaturated monogalactosyldiacylglycerols (PA), lysophosphatidylinositol (LPI), phytoceramides with hydroxylated fatty acyl (PhytoCer-OHFA), phyto-glucosylceramide (Phyto-GluCer), sphingosine (Sph), and phytosphingosine (PhytoSph) markedly decreased. Additionally, the increase in DBI index was mostly phospholipids and sphingolipids, indicating that the lipid saturation was reduced, and the fluidity was higher, which may be related to the strong salt resistance of *S. europaea.* By comparing the average carbon chain length (ACL), it can be seen that the ACL coefficient of S1P is significantly reduced with 200 mM NaCl concentration, so we speculate that S1P may be related to the salt resistance.

Key Message

Transcriptomic, physiological, and lipidomic analysis revealed *S. europaea* may adapt to the salt environment by altering the synthesis of phenylpropanoids and the synthesis of diterpenoids.

Introduction

Soil salinity is a major abiotic stress factor that negatively affects agricultural crop yield (Devkar et al. 2020; Kim et al. 2021a; Yang et al. 2023; Zhu 2016). Excess soil salinity inhibits the water absorption of plant root systems, and the water expands from the bubbles to reduce the expansion pressure, thereby causing lack of osmotic adjustment (Devkar et al. 2020). The salt level causes water deficit and ion stress to induce cell membrane instability, control of essential enzymes, overproduction of reactive oxygen (ROS) and decrease in nutrient supply (Dal Santo et al. 2012; Hasegawa 2000; Yang et al. 2019). However, salt-tolerant plants, or halophytes, grow well under salted soil conditions (Guo et al. 2020a; b). Improvement of photosynthesis and accumulation of photosynthetic products could be enhanced by the improvement of the genes involved in photosynthesis and carbohydrate metabolism (Guo et al. 2020a). Therefore, the deciphering mechanisms of chloride plants in salt tolerance provides a molecular basis for better use of chlorides (Guo et al. 2020b).

As a selective barrier to isolate living cells and their environments, the quality membrane is composed of all these components of salt stress (Lv et al. 2021). The responses of plants to salinity were membrane potential, permeability, membrane binding enzyme activity and lipid composition (Guo et al. 2019; Lv et

al. 2021). Maintenance of membrane stability and integrity under salt conditions is one of the important salt adaptation strategies for many salt-tolerant plants (Lv et al. 2021). Notably, lipid reformation and triglyceride accumulation play an important role in plant development (Zhang et al. 2022). Lipid is a large group of compounds not soluble in water but soluble in organic solvents (Abdelrahman et al. 2021; Buffagni et al. 2022; Fahy et al. 2005; Fahy et al. 2009; Hou et al. 2016). The study revealed that lipid compounds are involved in cell development and differentiation, affecting intracellular mass transport and signal transduction (Hou et al. 2016). In addition, abnormal lipid metabolism is also closely related to the development of many diseases, including cardiovascular disease, obesity, diabetes, neurodegenerative diseases, and cancer (Hou et al. 2016). In 2003, the National Institutes of Health launched the Lipid Metabolites and Metabolic Pathways Research Strategy (LIPID MAPS) project, which established the LMSD database. In this database, lipids are systematically classified into eight categories, namely fatty acyl, glycerolipids, glycerophospholipids, sphingolipids, cholesterol, polyisoprenols, glycolipids, and polyketides.

Salicornia europaea is an euhalophyte plant that is widely distributed in coastal and inland salons, which showed optimal growth 200–400 mM NaCl (Cardenas-Perez et al. 2022a; Hrynkiewicz et al. 2019; Lv et al. 2012; Lv et al. 2021; Nakahara et al. 2015; Rehman et al. 2020). It is one of the most euhalophyte plant species and can withstand about 1000 mM NaCl (Flowers and Colmer 2008). SePSS is involved in the salt tolerance of plants by regulating phosphatidylserine (PS) and phosphatidylethanolamine (PE) levels to help regulate the stability of plasma membrane and maintain the K^+/Na^+ homeostasis under salt stress (Lv et al. 2021). *S. europaea* has various therapeutic effects including antioxidant, anti-inflammatory, anti-diabetic, anti-cell toxicity and anti-obesity effects (Kim et al. 2021b). The unique morphological structure of *S. europaea* is the assimilative branch formed by the regressive leaves, which are able to accumulate Na^+ and Cl^-. *S. europaea* has a high salt content of 45%, and also needs to absorb salt during growth to promote its growth (Hrynkiewicz et al. 2019). *S. europaea* allows salt to collect in vesicles in the cells to prevent salt dispersion while ensuring that it can absorb water properly (Hrynkiewicz et al. 2019; Nakahara et al. 2015). *S. europaea* mainly contains flavonoids, pigments and chromogenic ketones, higher hydrocarbons and total fatty acids, alkaloids and other components. Studies on the pharmacological activity of *S. europaea* sinensis have shown that it has antifungal, antitumor activity, anti-inflammatory activity, antiviral activity, and antioxidant effects, as well as hypoglycemic and hypotensive effects (Hrynkiewicz et al. 2019). The seeds of *S. europaea* are also a good raw material for pressing seed oil and making cake meal, the above-ground parts of its plants can be used as fodder, and the young stems and shoots of the plants can be made into cold dishes and other food. At present, the development of *S. europaea* is mainly focused on making medicine, vegetables and oil. In the development and utilization of *S. europaea*, the main ways of utilization at present are desalination, salt soil agriculture, and development of salt tolerance genes. Therefore, *S. europaea* has a strong economic value as well as ecological value. At present, although osmoregulation has been recognized as an important physiological adaptation in saline plants with high salt tolerance, little is known about the mechanisms of response to salt at the lipidomic level. In this study, we carried out lipid

cluster and transcriptional analysis in *S. europaea*. Based on our results, we assumed some potential actions of lipids in plant salt adaptation.

Materials and methods

Plant materials and growth conditions

Seeds of *S. europaea* were collected from Salt Lake of Yuncheng, Shanxi Province, China. Seeds were sterilized by 1.5% NaClO for 15 min, washed three times with sterile water, placed at 4°C for 2–4 days and then planted on the small pots of soil at 23°C under 100–120 μmol photons·m^{-2}·s^{-1} with a 16 h/8 h light/dark photoperiod in the growth room.

Measurement of physiological and biochemical indicators

In all assays, seeds of *S. europaea* were surface-sterilized and sown on ½ Hoagland's solution medium with or without 200 mM NaCl. Three replicates were used for each treatment. Physiological and biochemical indicators were measured using corresponding assay kits following the manufacturer's protocols. In detail, the Chlorophyll Assay Kit (Solarbio, Cat#BC0990), Plant Soluble Sugar Content Assay Kit (Solarbio, Cat#BC0030), and Malondialdehyde (MDA) Assay Kit (Solarbio, Cat#BC0020) were used for subsequent spectrophotometric.

Bioinformatics analysis of transcriptome data

DESeq2 was performed to analyze differential expression between sample groups (Love et al. 2014). The screening conditions for differential genes were $\log_2$ Fold Change ≥ 1 and FDR < 0.05. Pathway significant enrichment analysis was based on the pathway in the KEGG database. The pathway that is significantly enriched in DEGs compared with the genome-wide background is found through hypergeometric test, and the result of KEGG enrichment is presented in the form of a scatter plot. The GO Term significant enrichment analysis was based on the GO Term in the GO database, and the hypergeometric test was used to find out GO Terms that were significantly enriched in DEGs compared with the whole genome background.

UPLC-QTOF/MS-based lipidomics and statistical analysis

To precisely measure lipidome-wide alterations under salinity, we performed an Exion UPLC-QTRAP 6500 PLUS (Sciex) liquid mass spectrometer in electrospray ionization (ESI) mode for all analyses. Lipids were extracted from the samples using a modified Bligh/Dyer extraction method (two extractions) with the addition of appropriate internal standards (Lam et al. 2014). Various polar lipids were separated by NP-HPLC using a Phenomenex Luna 3 μm silica column (inner diameter 150x2.0 mm) with the following conditions: mobile phase A (chloroform:methanol:ammonia 89.5:10:0.5), B (chloroform:methanol:ammonia:water 55:39:0.5:5.5). The gradient of mobile phase A was maintained from 95% for 5 min, then linearly reduced to 60% for 4 min within 7 min, and then further reduced to 30% for 15 min. The initial gradient was maintained for 5 min. The comparative analysis of various polar

lipids was performed Lam et al. (2014). The quantification of lipid substances was performed by adding internal standards.

The lipidomic data were analyzed using Principal Component Analysis (PCA), Orthogonal Partial Least Squares (OPLS-DA), plotting heat maps, and other methods, and the Q-test was used to test for outliers, and one-way ANOVA t-test was used for significance of differences. Data results were plotted and labeled for significant differences using R language software and Origin 8.0 software. The mobility of membrane lipids can be measured mainly by the membrane lipid double-bond index (DBI) and the carbon chain length (ACL). DBI is the average number of fatty acid chain double bonds in glycerolipid molecules. A high DBI index indicates a decrease in lipid saturation, the presence of higher unsaturated membrane lipids and higher mobility, while a decrease in DBI indicates an increase in lipid saturation. DBI = [Σ(N × mol% lipid)] /100, N is the total number of double bonds in both fatty acid chains of each glycerolipid molecule. We calculated the DBI values for 27 lipids, which can reflect the interconversion between lipids. The carbon chain length of lipid molecules is also an index reflecting the mobility of membrane lipids, the shorter the carbon chain the higher the mobility of membrane lipids, thus increasing plant resistance. ACL = [Σ(N × mol% fatty acid)] / 100, where N is the number of carbon atoms per lipid molecule, i.e. the average number of carbon atoms in the carbon chain of a lipid molecule.

Quantitative real-time PCR analysis

Total RNA was extracted with Trizol from seedlings. Quantitative real-time PCR was performed as described by (Yang et al. 2019). Primer sequences used in the study was shown in Supplementary Table S6.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean ± SE. The data were statistically analyzed as described by (Yang et al. 2023).

Results

Phenotypes of *Salicornia europaea* with or without salt treatment

To investigate the adaptation of *S. europaea* to salinity, we applied 200 mM NaCl for 10 days and eight weeks, and control groups (medium without salt) were also included. As showcased in Fig. 1a, *S. europaea* plants grew well under 200 mM NaCl, stems are stunted in the absence of salt. Additionally, the other parameters such as water content were also significantly enhanced during 200 mM NaCl treatment (Fig. 1b). These results suggest that *S. europaea* is salt-demanding for growth and is best grown at 200 mM NaCl concentration.

Physiological and biochemical indexes of *Salicornia europaea* under salinity

Prior to these experiments, the conditions of 200 mM NaCl treatment were examined by phenotype and biochemical evaluation, and the seriousness of the stress treatment condition was assured. We measured

total soluble sugar levels of control and 200 mM NaCl-treated in *S. europaea* (Fig. 1c). Total soluble sugar contents were significantly higher in 200 mM NaCl-treated compared to the controls. The high level of total soluble sugars should provide sufficient levels of raw material and explain why plants treated with 200 mM NaCl are larger than those of control plants.

The efficiency of photosynthesis is related to chlorophyll contents. Then, the control and NaCl treatment were measured. The chlorophyll content in the blade of NaCl treatment was significantly higher than the control. Compared control plants, chlorophyll a, chlorophyll b and total chlorophyll content 40.1, 53.1, and 45.6%, respectively (Fig. 1d). Meanwhile, NaCl-treated *S. europaea* plants also showed lower MDA content than control plants (Fig. 1e). These results indicate that the cell membrane integrity in NaCl-treated S. europaea plants is much higher than in control plants.

Analysis of differentially expressed genes under salinity and KEGG pathway classification

To investigate the differences in gene expression between control and 200 mM NaCl, differential gene expression (DEGs) analysis was performed using DEseq2. In this study, a total of 77.15 Gb of data were measured using the DNBSEQ platform. After assembly and redundancy, 81,629 unigene were obtained, with a total length of 109,393,660 bp. The Clean reads of each sample reached more than 6.3 Gb. The percentage of Q30 in each sample was 92.38% and above, indicating that the sequencing quality was good enough for subsequent analysis (Table S2). The clean reads were compared to the assembled *S. europaea* gene set using Bowtie2 (v2.2.5) (Langmead and Salzberg 2012), followed by RSEM to calculate the expression levels of genes and transcripts to obtain the comparison results (Li and Dewey 2011). Based on the no-reference genome of *S. europaea*, the filtered high-quality clean reads were required to be assembled using Trinity (v2.0.6), and then the transcripts were clustered and de-redundant using Tgicl to obtain the unigene. The average length of All-unigene was 1340bp, and the total length was 109,393,660bp. the length of assembled unigene was mainly concentrated in 200bp-300bp, 300bp-400bp and ≥3000bp. the GC content was around 40% of the sequencing index (Table S3).

A total of 61,061 unigene were annotated and their functions were determined after matching *S. europaea* transcriptome with the NR database, and the distribution of similarity in the matching results reflects the degree of matching of *S. europaea* unigene sequences with known genomic species. All unigene of *S. europaea* compared to the NR database had high homology with sugar beet, quinoa and spinach, which belong to the same family of quinoa. Specifically, the highest homology was found with sugar beet (*Beta vulgaris subsp. vulgaris*), with 22,294 unigene matches to sugar beet (36.51%); the next highest homology was found with quinoa (*Chenopodium quinoa*), with 20,225 unigene matches to quinoa (33.12%); A total of 13,245 unigene pairs were found with spinach (*Spinacia oleracea*), accounting for 21.69% of the total number of unigene pairs. There are also some homologies with other species such as *Rhodamnia argentea* (Fig. S2).

In order to explore the genes differentially expressed in *S. europaea* under salinity, this study was conducted to compare the 200 mM (SE2) and 0 mM (SE1). 200 mM NaCl treatment compared with control, with 1257 up-regulated genes and 635 down-regulated genes (Table S4). The significantly

differentially expressed genes were annotated in the GO database and found to be annotated into 34 subcategories, as shown in Fig. 3. They were annotated into 3 groups, including cytoarchitecture, intracellular, and protein-containing complexes.

The KEGG pathway analysis of significantly differentially expressed genes provides a more visual picture of their expression in various metabolic pathways. As shown in Fig. 2, they were annotated into 19 subcategories. The highest enrichment of differential genes was found in metabolic branches with 311 genes, which were annotated into 11 groups, including global and general overview maps, carbohydrate metabolism, biosynthesis of other secondary metabolites, glycan biosynthesis and metabolism, lipid metabolism, metabolism of terpenoids and polyketides, metabolism of other amino acids, amino acid metabolism, energy metabolism, nucleotide metabolism, metabolism of cofactors and vitamins In cellular processes there is only one subcategory: transport and catabolism; in environmental information processing there are two subcategories: signal transduction and membrane transport; in genetic information processing there are four subcategories: folding, sorting and degradation, translation, transcription, replication and repair. To elucidate the main biological pathways affected by salt treatment in *S. europaea*, gene enrichment analysis of differentially expressed genes was performed based on KEGG analysis to identify significantly enriched biological pathways. The results of KEGG pathway enrichment analysis of differentially expressed genes are shown in Table S5, involving a total of 44 KEGG Pathways. these metabolic pathways may be closely related to the molecular mechanism of salt acclimation in *S. europaea*.

To confirm the accuracy of gene sequences obtained through transcriptome sequencing and the trustworthiness of the differential expression profile of *S. europaea*, 15 significantly expressed differentially expressed genes which involved lipid metabolism and ion transport, were selected for expression assessment and analysis with the help of fluorescence real-time quantitative PCR (Fig. 3). These genes, such as *CL10701.Contig3_All* (cytochrome P450 710A11-like), *CL11255.Contig1_All* (beta-galactosidase), *Unigene11029_All* (sugar beet subspecies glucan endo-1,3-β-glucosidase), *CL3676.Contig6_All* (quinoa xyloglucan endo-glucanase), *CL3151.Contig4_All* (peanut gibberellin regulatory protein 11), *CL7273.Contig2_All* (cassava growth factor ABP19a), *CL9234.Contig2_All* (sugar beet subspecies peroxidase 40) and *CL7300.Contig2* (quinoa acyl hydrolase) were all found to be differentially expressed genes whose expression increased with 200 mM NaCl concentration. *CL1006. Contig2_All* (non-specific phospholipase C3-like), *CL10914.Contig2_All* (fatty acyl-ACP thioesterase B), *CL146.Contig16_All* (spinach cyclic nucleotide-gated ion channel 1), *Unigene17718_All* (quinoa-like calmodulin-binding protein 60A), *CL1253.Contig5_All* (*S. europaea* Ca^{2+}/H^{+}), *CL1253.Contig3_All* (*S. europaea* Ca^{2+}/H^{+}) and *CL4125.Contig_All* (quinoa-like metal tolerance protein C4) were decreased with salt treatment. The GO functions of these 15 differentially expressed genes suggest that they are mainly involved in substance metabolism, signal transduction, and hormone regulation. The amount of variation of all 15 differentially expressed genes measured was in agreement with the transcriptome sequencing results.

Expression of key genes associated with Na^{+} segregation and plasma membrance stability

Many mechanisms have evolved in plants as responses to high salinity, such as excessive Na^+ reduction in the cytoplasm (Lv et al. 2012). These genes included *SeSOS1* and *SeNHX1*, encoding a tonoplast Na^+/H^+ antiporter (Cardenas-Perez et al. 2022a; Lv et al. 2012); *SeVHA-A*, encoding catalytic subunit A of the vacuolar H^+-ATPase (Lv et al. 2012); *SeVP1* and *SeVP2*, encoding the vacuolar H^+-PPase (Lv et al. 2012); and *SePSS*, encoding phosphatidylserine synthase (Lv et al. 2021). The transcript amount of *SeSOS1*, *SeNHX1*, *SeVHA-A*, *SeVP1*, and *SePSS* increased significantly with 200 mM NaCl (Fig. 4).

Lipidomic analysis revealed dynamic changes in lipid compounds in salt level

To study the effect of salinity on the changes of intracellular lipid content, we extracted lipids from *S. europaea* leaves and analyzed them. A total of 485 differential metabolites were detected in the metabolome analysis, it includes 2 glycerides: diglycerides (DAG) and triglycerides (TAG); 1 free fatty acid (FFA); 1 sterol ester (SE); 2 galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG); 7 phospholipids: phosphatidylcholines (PC), phosphatidylserines (PS), phosphatidylethanolamines (PE), phosphatidylinositols (PI), phosphatidylglycerols (PG), phosphatidic acid (PA) and cardiolipins (CL); 4 lysophospholipids: lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidic acid (LPA) and lysophosphatidylinositol (LPI); 8 sphingolipids: ceramide (Cer), phytoceramide (PhytoCer), glucosylceramide (GluCer), phytoglucosylceramide (Phyto-GluCer), hydroxylated fatty acyl phytoceramide (PhytoCer-OHFA), plant sphingosine (PhytoSph), phytosphingosine-1-phosphate (t-S1P), and glycosyl inositol phosphoceramide (GIPC) (Fig. 6). In order to determine the detailed lipid data, multivariate statistical *analysis* was performed for the samples, that is, the *principal component analysis* (PCA), *orthogonal partial least squares*-discriminate *analysis* (OPLS-DA) and heatmap analysis (Fig. 5). The plant cell membrane is the medium for sensing and transmitting external signals, perception *and* transmission *of* stress *signals are* important aspects *of the plant* response to environment stress, so it is essential for maintaining proper permeability and fluidity in the cell membrane. Among the 27 major lipid components detected in this experiment, sterol esters, phospholipids, galactolipids and lysophospholipids were the main components of membrane lipids. Therefore, the subsequent focus of this study was to analyze the contents of sterol esters, phospholipids, galactolipids, and lysophospholipids, which are closely related to membrane stability, changes in the ratios of DGDG/MGDG and PC/PE.

When compared to the control, the relative levels of the TAG [48:0 (16:0), 50:0 (16:0), 50:0 (18:0), and 52:0 (18:0)], LPC (16:0, 16:1, and 18:2), t-S1P (t18:1 and t18:0), and GIPC (t18:0/h26:0 and d18:0/h26:1) were significantly increased by 200 mM NaCl treatment in *S. europaea*. When compared to the control, the relative levels of the MGDG [34:3 (18:1/16:2), 36:6 (21:5/15:1), 36:6 (15:0/21:6), 36:5 (18:2/18:3), and 36:4 (18:3/18:1)], PA (32:1, 32:0, 34:3, 34:2, 36:6, 36:5, 36:4, 36:2, 36:1, 38:6, 38:3, 38:1, 40:3, and 40:1), LPI (16:2), and Sph (t18:1, t18:0, d18:0, and d20:0) were significantly decreased by 200 mM NaCl treatment in *S. europaea* (Fig. 7).

DBI index and ACL index analysis

The fluidity of membrane lipids can be reflected in membrane lipid double bond index (DBI) and average carbon chain length (ACL). The DBI index can reflect the saturation of plant lipids. A high DBI index indicates a decrease in lipid saturation, the presence of higher unsaturated membrane lipids and higher fluidity, and a lower DBI indicates an increase in lipid saturation. The DBI index of diphosphatidyl glycerol (CL) was the highest, indicating that the saturation of CL was the lowest, followed by galactolipids MGDG and DGDG, and the DBI index of these three lipids was decreased slightly under salt treatment, probably due to the fact that the double bond index of the three lipids had the greatest effect on membrane fluidity. The double bond index DBI of TAG was extremely significantly reduced at 200 mM salt concentration compared to control, the DBI index of the remaining lipids increased at 200 mM containing LPA, LPC, LPE, LPS and Sph, especially for GIPC (Table 1). The increased DBI index was mostly phospholipids and sphingolipids, indicating that the lipid saturation was reduced, and it had higher mobility, which could resist high salt stress and increase the resistance of *S. europaea*.

The average carbon chain length of lipid molecules, ACL, is also an index reflecting the fluidity of membrane lipids. The shorter the carbon chain, the higher the fluidity of membrane lipids, thus increasing the resistance of plants. By comparing the average carbon chain lengths, the longest carbon chain was CL, which reached an average of 72 carbon atoms, there was no difference between *S. europaea* treated with 200 mM NaCl (Table 2). The shortest lipid carbon chains in the treated *S. europaea* were both phospholipids and sphingolipids, and the shortest carbon chains were Shp and S1P, which reached an average of 6 carbon atoms, the significant changes with salt treatment indicating that Shp and S1P were related to the resistance of *S. europaea* (Table 2). The average carbon chain length (ACL) of Sph lipids and PhytoCer were significantly increased at 200 mM salt concentration, but the GluCer and S1P were significantly decreased. These results suggested that sphingolipids might be responsible for changing membrane fluidity through the carbon chain length of membrane lipids in salt-treated plants. The DBI index of sphingolipids was significantly increased, and the ACL index was decreased in salt-treated plants, which may have the greatest influence on membrane fluidity.

Discussion

The purpose of this study is to elucidate the function and regulation of the reaction to salinity of *Salicornia europaea*. The *S. europaea* is one of the ideal model plants for exploring the salt tolerance mechanism of saline plants, because of its strong salt tolerance, short transmission time, high seed production and high agronomic value (Araus et al. 2021; Calone et al. 2022; Cardenas-Perez et al. 2020; Cardenas-Perez et al. 2022b; Feng et al. 2015; Furtado et al. 2019; Guo et al. 2020a; Lv et al. 2021; Yuan et al. 2016). *S. europaea* showed different growth regulation at different salt concentrations, the phenotype showed severe phenotypic defects in the absence of salt treatment, which were manifested by smaller plant growth, less branching and slow growth and development to complete the life history (Fig. 1a). The growth of *S. europaea* was the best at 200 mM NaCl concentration, slightly inhibited at 400 mM NaCl concentration, and showed significant growth inhibition and dwarf plants at 600 mM NaCl concentration (Fig. S1).

Transcriptome sequencing can help to analyze the complex physiological and developmental relationships among genes and to identify genes in response to salinity, therefore, we performed transcriptome analysis of *S. europaea* under 200 mM NaCl treatment. GO classification showed that salt treatment mainly affected the functions of *S. europaea* such as cytoarchitecture, catalytic activity, binding, cellular processes, and metabolic processes (Fig. 2). The differential genes were enriched in metal ion binding, hydrogen peroxide catabolic process, response to oxidative stress, peroxidase activity, nitric oxide biosynthesis process and other biological functions under salt treatment, and salt hordeum adapted to the salt environment by altering or regulating these functions to live long in the salt environment. Differential genes under salt treatment were expressed in higher numbers in metabolic processes such as global and general overview maps, carbohydrate metabolism, biosynthesis of other secondary metabolites, mainly enriched in glycosylphosphatidylinositol (GPI) anchored biosynthesis, phenylpropanoid biosynthesis, diterpenoid biosynthesis, phytohormone signaling, peroxisome, MAPK signaling pathway and other pathways. This implies that the gene expression in the above pathways is altered during long-term adaptation of salt environments in *S. europaea*, which enables the evolution of molecular mechanisms for adaptation to salt environments.

Based on KEGG enrichment results, both plant MAPK signaling and phytohormone pathways were enriched for up- and down-regulated transcripts under salt treatment. Plants use antioxidant enzymes to protect cellular and subcellular systems from salt damage caused by ROS (Foyer et al. 1991). Among the transcriptome results in *S. europaea*, *CL10202.Contig1_All*, *CL10202.Contig2_All*, *CL5442.Contig1_All*, and *CL5442.Contig2_All* were annotated as copper and zinc family superoxide dismutases, and their expression gradually increased with salt concentration. The expression of *Unigene17660_All gene* (a homolog of spinach peroxidase), which was gradually up-regulated after NaCl treatment but decreased in concentration after 800 mM NaCl treatment (Ma et al. 2013). Presumably, salt stress induced peroxidase up-regulation to protect *S. europaea* in response to salt stress.

Calcium ions (Ca^{2+}) play an important role as second messengers in plants in response to various abiotic stresses, and Ca^{2+}/H^{+} exchangers (CAXs) are an important part of this process (Zhao et al. 2009a; Zhao et al. 2009b). Many stresses induce an increase in cytoplasmic free Ca^{2+} concentration in plant cells, generating signals transduced along various signaling pathways (Zhao et al. 2009a; Zhao et al. 2009b). A putative Ca^{2+}/H^{+} exchange gene (*SeCAX3*) from *S. europaea*, was transformed into a calcium-sensitive yeast strain, the transformed cells were able to grow in the presence of 200 mM Ca^{2+} (Zhang et al. 2015). In the present study, we identified seven Ca^{2+}/H^{+} reverse transporter proteins, including *CL1253.Contig1_All*, *CL1253.Contig2_All*, *CL1253.Contig3_All*, which were differentially expressed under salt stress versus control differentially expressed under treatment and down-regulated expression.

Cell surface proteins can attach to the cell membrane through glycolipid structures called glycosylphosphatidylinositol (GPI) anchors (Fujita et al. 2009). Modification of proteins by GPI anchoring is a highly conserved post-translational modification in eukaryotes (Fujita et al. 2009). Many genes involved in the GPI biosynthetic pathway have been identified in mammalian cells, yeast and

trypanosomes (Fujita et al. 2009). In our study, the GPI biosynthetic pathway was significantly enriched in 200 mM NaCl than control groups (Table S5). GPI is involved in cellular signaling, immune responses, etc., and that GPI even has the ability to act directly as a second messenger for biological receptors (Sarnataro et al. 2017). These results suggested that the GPI plays an important role in signal transduction, plant growth and development processes and various stress responses in *S. europaea.*

Analysis of the data from the lipidomics of *S. europaea* showed that the most abundant lipid within the plant of *S. europaea* was MGDG and PC (Fig. 6). Based on the significance analysis of the data, the lipid molecules that may have the greatest effect on salt tolerance in *S. europaea* are phospholipids and sphingolipids. Phospholipids (PL) play a central role in biological systems, including the formation of lipid bilayers in cell membranes, and regulate multiple biological pathways by releasing a myriad of signaling molecules, and PI plays a crucial role in controlling plant polarity and growth. Phospholipids play a key role in plant development and function as important regulatory membrane lipids. There were 5 lipid molecules with significant variability at 200 mM salt concentration. Sphingolipids play critical roles in most major cellular biological responses, including structural integrity of membranes and formation of membrane structural domains, all major cellular signaling pathways, sphingolipid metabolism, etc. Among the lipids of higher plants, sphingolipids account for a considerable proportion, and there is a significant increase in sphingolipid content in *S. europaea* plants with increasing salt concentration, which may be a key lipid for enhancing their salt tolerance. Ceramide (Cer) is a naturally occurring compound that mediates apoptosis. Sph had a significant decrease in lipid molecules with increasing salt concentration trend. GIPC (glycosyl inositol phosphoribosylate ceramide) showed a significant increase with increasing salt concentration, indicating that GIPC lipid molecules are key lipids to enhance salt tolerance in *S. europaea* plants (Fig. 7).

The DBI index reflects the saturation of plant lipids, a high DBI index indicates a decrease in lipid saturation, the presence of higher unsaturated membrane lipids and higher mobility, and a decrease in DBI indicates an increase in lipid saturation. The highest DBI index for bisphosphatidylglycerol (CL) indicated the lowest saturation and higher membrane fluidity of CL. The increase in DBI index was mostly for phospholipids and sphingolipids, indicating that the lipids were less saturated and more mobile, which could resist high salt stress and increase the resistance of *S. europaea* plants (Table 1).

The ACL index reflects membrane lipid mobility, the shorter the carbon chain, the higher the mobility of membrane lipids, thus increasing plant resistance. The ACL index showed that the shortest carbon chains of *S. europaea* lipids treated with different salt concentrations were phospholipids and sphingolipids, among which Sph and S1P were the shortest, we believe that these two lipid molecules may be related to salt hordeum resistance (Table 2). The variability of S1P was extremely significantly reduced at a salt concentration of 200 mM, thus increasing the resistance of salt hordeum plants. The mobility of membrane lipids increased and thus the resistance of *S. europaea* plants was enhanced. This result suggests that it may be the sphingolipids that alter the membrane mobility through the length of membrane lipid carbon chains in salt-treated *S. europaea* plants. A significant increase in DBI index and a significant decrease in ACL index of sphingolipids in salt-treated salt-hornwort plants may have the

greatest effect on membrane fluidity. Undoubtedly, the mechanism of salt suitability of *S. europaea* still needs to be further illustrated.

Conclusion

Our results showed that *S. europaea* changes the cell structure, increases the stabilities of the stem and leaves, increases its antioxidant enzyme activity, lowers leaf dehydration, thereby enhancing the water holding capacity of the plant, thereby adapting to the base soil environment. *S. europaea* may adapt to the salt environment by altering metabolic processes, including the synthesis of phenylpropanoids and the synthesis of diterpenoids. Compared with the salt concentration-treated plants, the sphingolipids, which were basically significantly increased in content, may be the key lipids to enhance the salt tolerance of the plants; the phospholipids, which were significantly decreased in content, may protect the integrity of the membrane and thus improve the salt tolerance. In addition, the response of *S. europaea* to salinity can provide important insights into the action of other lipids and their salt adaptation mechanisms.

Declarations

Author contributions

Conceived and designed the experiments: LY and WL. Performed the experiments: LY, YB, JY, YG, PS, CH, YW, XG and WL. Analyzed the data: LY, YB and YG. Wrote the paper: LY. All authors reviewed the manuscript.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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Tables

Table 1 Changes in the double bond index (DBI) of lipid molecules in *S. europaea*

Lipid class	Plant species	Sodium chloride concentration	
		0 mM	200 mM
DAG	*S. europaea*	2.7391±0.0868	2.6517±0.0600
TAG	*S. europaea*	4.6444±0.2725	4.3715±0.1478 ***
FFA	*S. europaea*	0.2633±0.0162	0.2522±0.0553
SE	*S. europaea*	2.3134±0.1036	2.2135±0.0281
MGDG	*S. europaea*	5.7679±0.0155	5.7623±0.0207
DGDG	*S. europaea*	5.2148±0.0147	5.1823±0.0324
PA	*S. europaea*	2.9495±0.0226	2.9163±0.0386
PC	*S. europaea*	2.9681±0.0364	2.9042±0.0524
PE	*S. europaea*	2.6848±0.0175	2.6589±0.0162
PG	*S. europaea*	1.7627±0.0791	1.7525±0.0659
PI	*S. europaea*	2.2619±0.0269	2.2028±0.0216
PS	*S. europaea*	2.1285±0.0094	2.1189±0.0106
CL	*S. europaea*	8.7840±0.0294	8.7502±0.0274
LPA	*S. europaea*	1.2502±0.0606	1.2843±0.0404 **
LPC	*S. europaea*	1.4621±0.0149	1.4828±0.0224 **
LPE	*S. europaea*	1.679±0.03810	1.7142±0.0447 *
LPI	*S. europaea*	1.5615±0.1253	1.4840±0.1226
LPS	*S. europaea*	1.5983±0.0749	1.7366±0.0644 *
Sph	*S. europaea*	0.3820±0.1303	0.4881±0.0265 **
Cer	*S. europaea*	0.9819±0.0566	0.8905±0.0208
GluCer	*S. europaea*	1.0019±0.0005	1.0000±0.0013
PhytoCer	*S. europaea*	0.9143±0.0093	0.8768±0.0174
S1P	*S. europaea*	0.8095±0.0341	0.8039±0.0303
GIPC	*S. europaea*	0.8685±0.0121	0.8879±0.0162 ***

***, $p<0.001$; **, $p<0.01$; *, $p<0.05$

Table 2 Variation in the average carbon chain length (ACL) of lipid molecules in *S. europaea*

Lipid class	Plant species	Sodium chloride concentration	
		0 mM	200 mM
DAG	*S. europaea*	34.722±0.0630	34.657±0.0530
TAG	*S. europaea*	52.1096±0.123	52.019±0.0760
FFA	*S. europaea*	17.1809±0.040	17.163±0.0470
SE	*S. europaea*	18.000±0.0000	18.000±0.0000
MGDG	*S. europaea*	35.503±0.0840	35.363±0.1120
DGDG	*S. europaea*	33.6429±0.060	33.656±0.0190
PA	*S. europaea*	35.120±0.0260	35.114±0.0400
PC	*S. europaea*	28.8662±0.672	28.490±0.4180
PE	*S. europaea*	36.2739±0.062	36.197±0.0680
PG	*S. europaea*	33.5740±0.028	33.595±0.0380
PI	*S. europaea*	34.1286±0.031	34.162±0.0090
PS	*S. europaea*	38.2675±0.078	38.088±0.1090
CL	*S. europaea*	72.000±0.0000	72.000±0.0000
LPA	*S. europaea*	17.374±0.0880	17.434±0.0250
LPC	*S. europaea*	17.3068±0.049	17.352±0.0370
LPE	*S. europaea*	17.7787±0.010	17.791±0.0210
LPI	*S. europaea*	16.6209±0.129	16.687±0.0890
LPS	*S. europaea*	18.000±0.0000	18.000±0.0000
Sph	*S. europaea*	6.8756±2.3450	8.7860±0.4760 **
Cer	*S. europaea*	39.4319±0.326	39.3060±0.2960
GluCer	*S. europaea*	40.4266±0.057	40.3010±0.095 *
PhytoCer	*S. europaea*	25.7271±0.431	27.632±0.8410 **
S1P	*S. europaea*	6.7855±6.2700	4.3330±0.5150 ***
GIPC	*S. europaea*	41.0695±0.050	41.159±0.0810

***, $p<0.001$; **, $p<0.01$; *, $p<0.05$

Figures

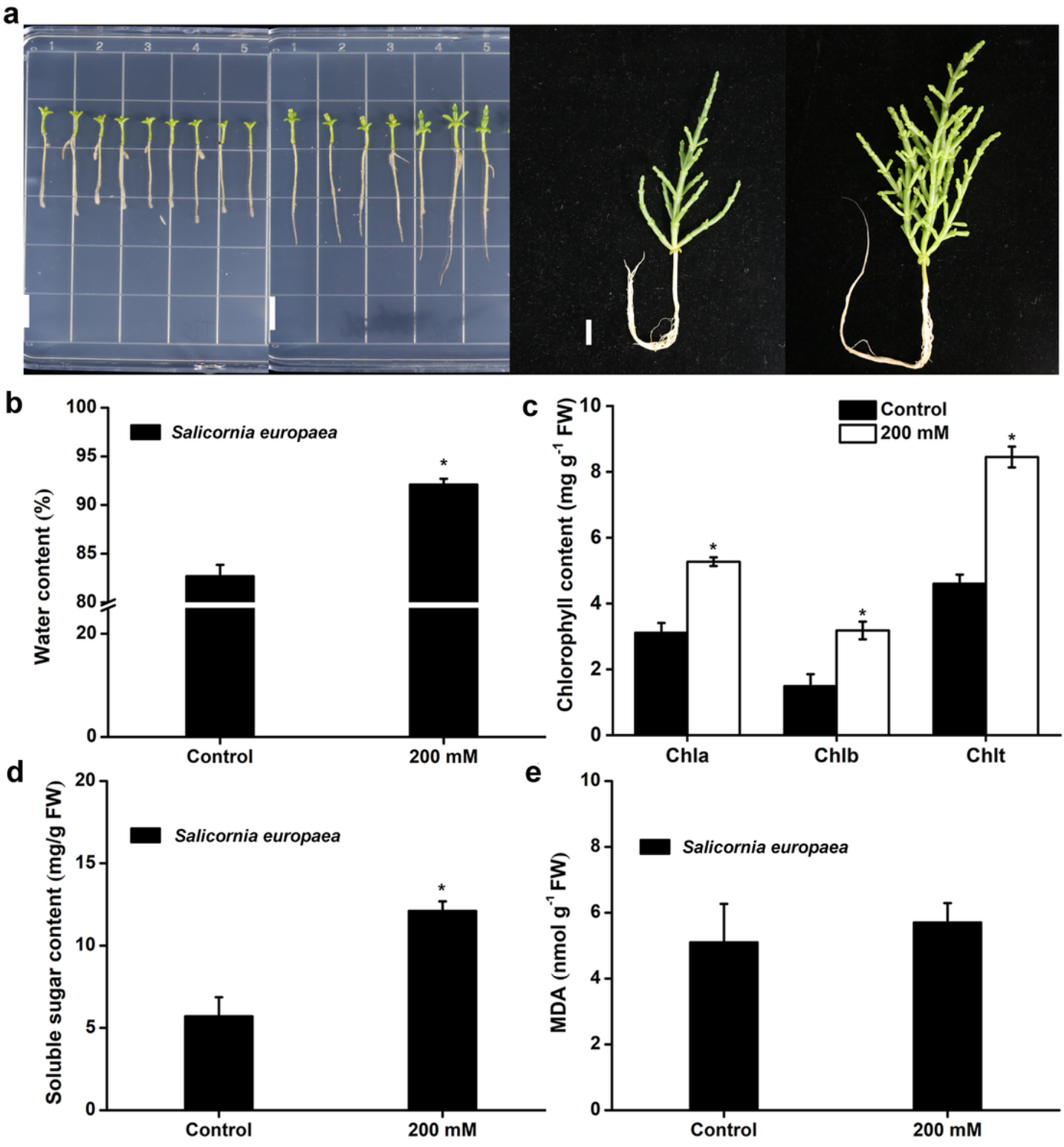


Figure 1

Physiological characteristics of salt suitability in *S. europaea*.(**a**) Phenotypes of *S. europaea* under stress of different salt concentration. From left to right, 0 mM, 200 mM NaCl treatment. (**b**-**e**) Water content,

chlorophyll content, soluble sugar content and MDA in leaves with or without 200mM NaCl treatment. Data are shown as the mean ± SD (*p < 0.05) and are derived from at least three repeats

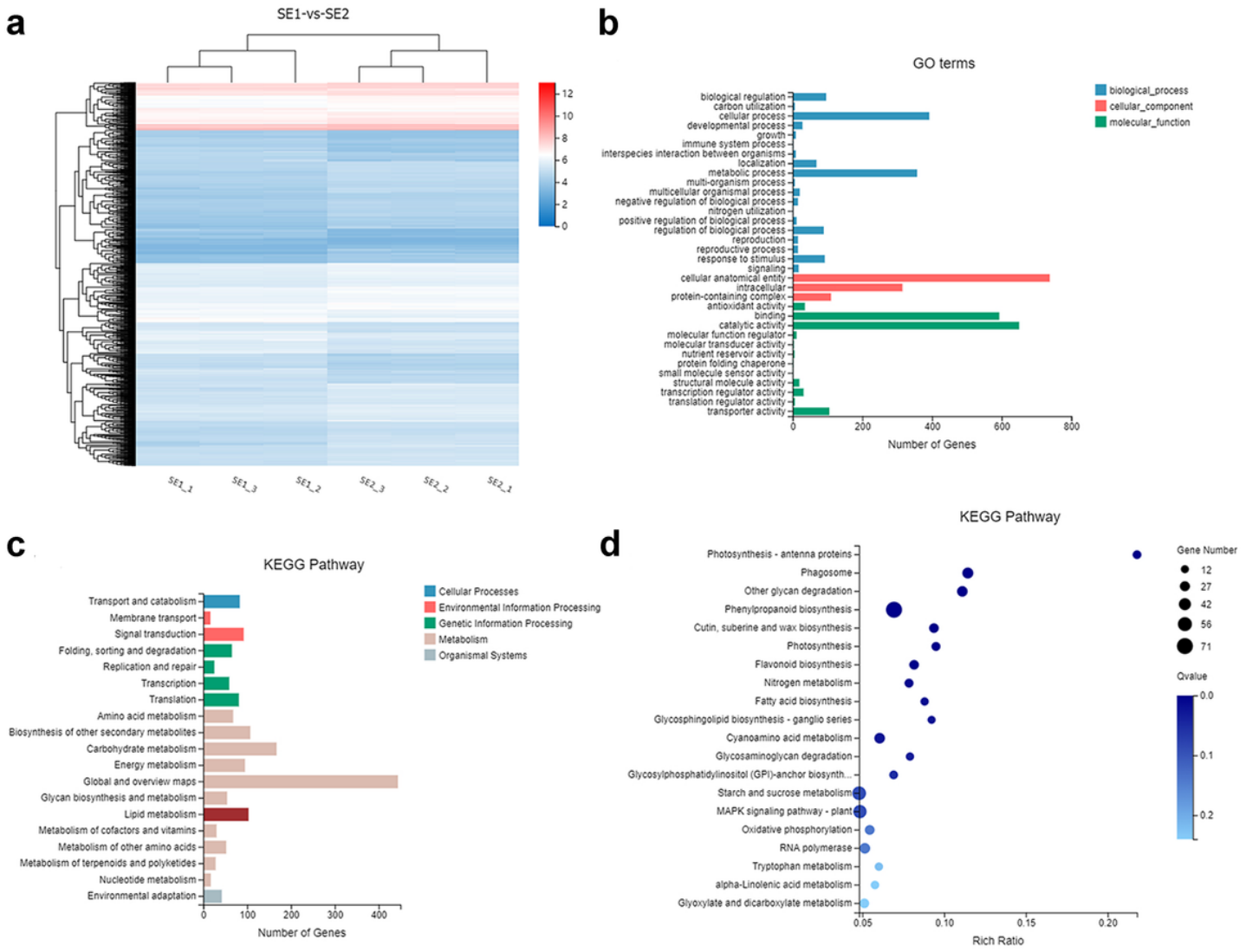


Figure 2

GO enrichment of the DEGs and KEGG pathway. (**a**) Heatmap map of differentially expressed genes with or without 200mM NaCl treatment in *S. europaea*. (**b**) GO classification of differentially expressed genes with or without 200mM NaCl treatment in *S. europaea*. (**c**) Analysis of KEGG pathway in the groups SE1 vs SE2. (**d**) GO annotation map of metabolism-related genes

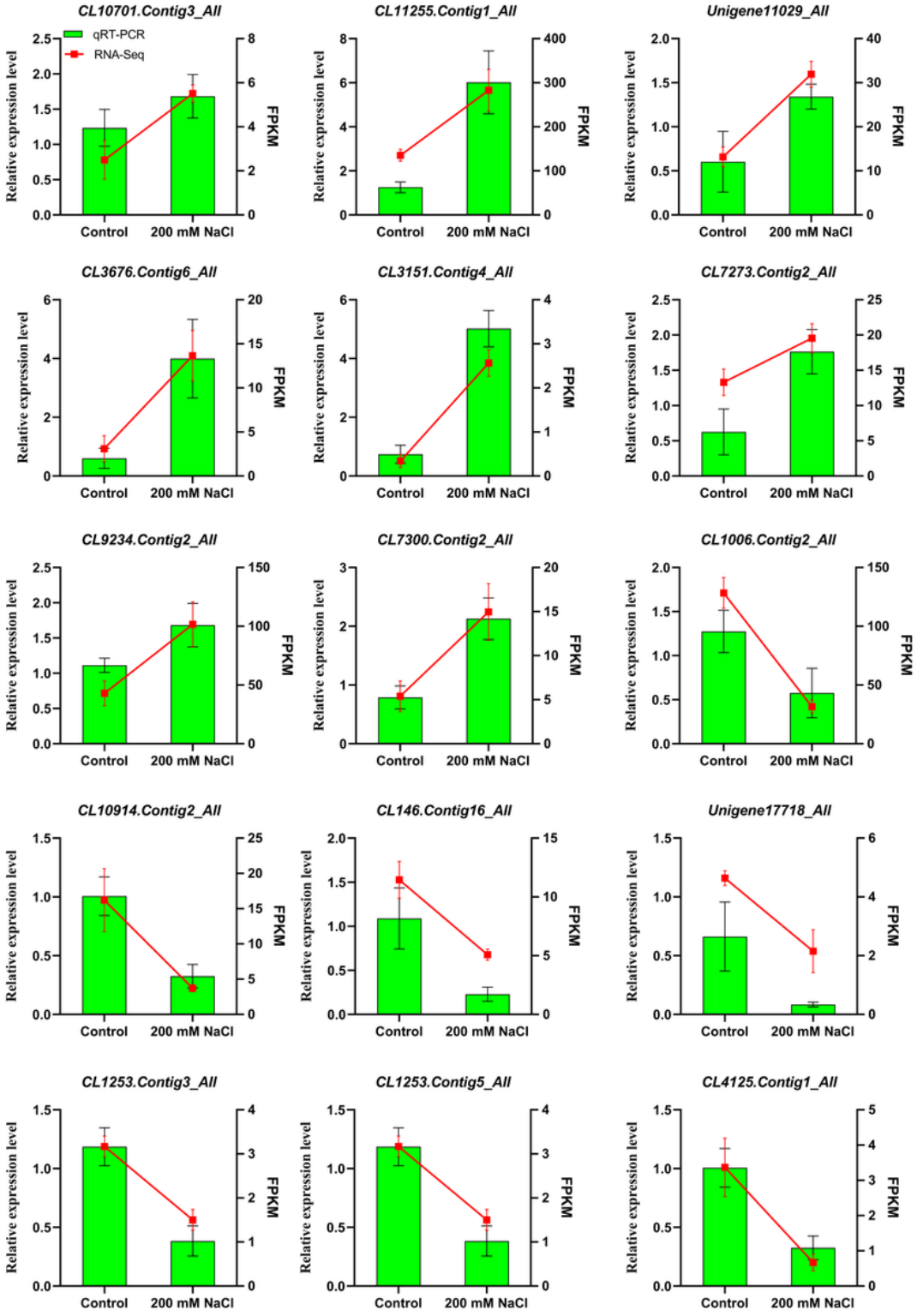


Figure 3

The qRT-PCR verification of the genes associated with lipid metabolism and ion transport in *S. europaea* with or without 200 mM NaCl

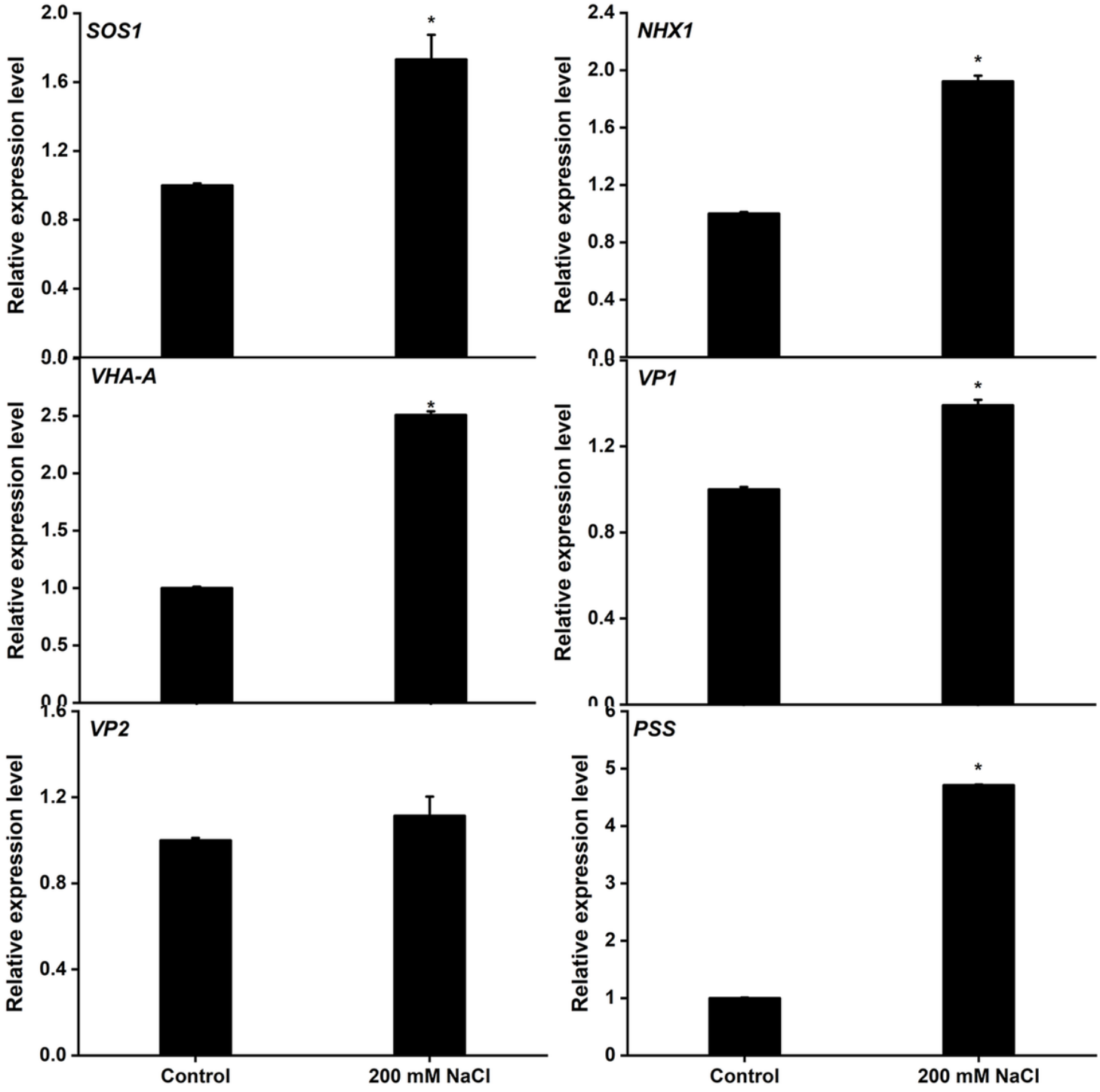


Figure 4

The qPCR analysis of *SeSOS1*, *SeNHX1*, *SeVHA-A*, *SeVP1*,*SeVP2* and *SePSS* expression with or without 200mM NaCl treatment in *S. europaea*. The transcript levels all were normalized to *Tubulin* gene expression. One-way Duncan's test was performed, and statistically significant differences are indicated by different letters ($P<0.05$). The experiments were repeated at least three times with similar results, and data from one representative experiment are presented

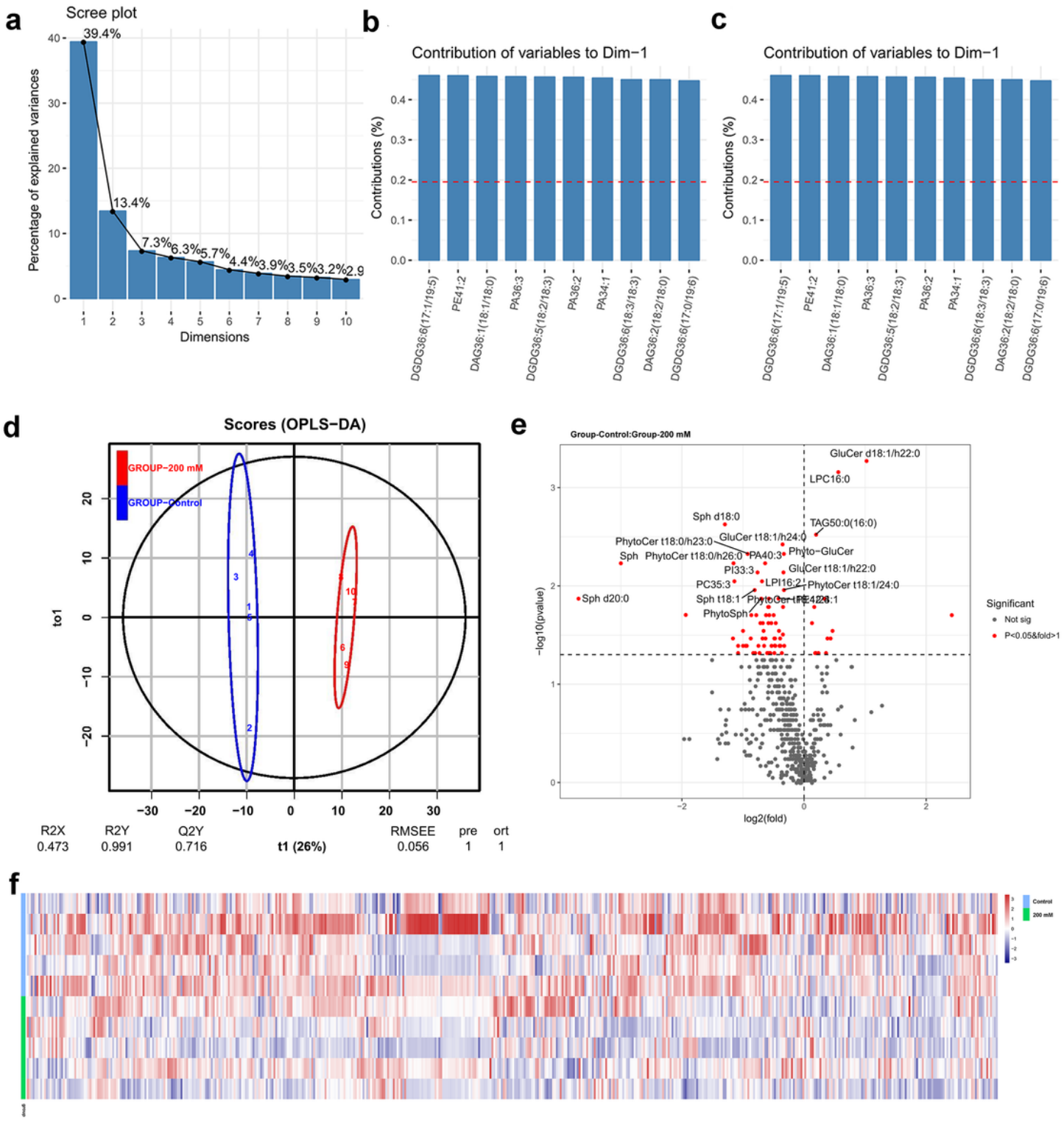


Figure 5

UPLC-QTRAP/MS-based lipidomics analysis with or without 200mM NaCl treatment in *S. europaea*. (**a**-**c**) Principal component analysis of lipid changes. (**d**) OPLS-DA score plots. (**e**) Volcano map of *S. europaea* with or without 200mM NaCl treatment. (**f**) Heat map of *S. europaea* with or without 200mM NaCl treatment

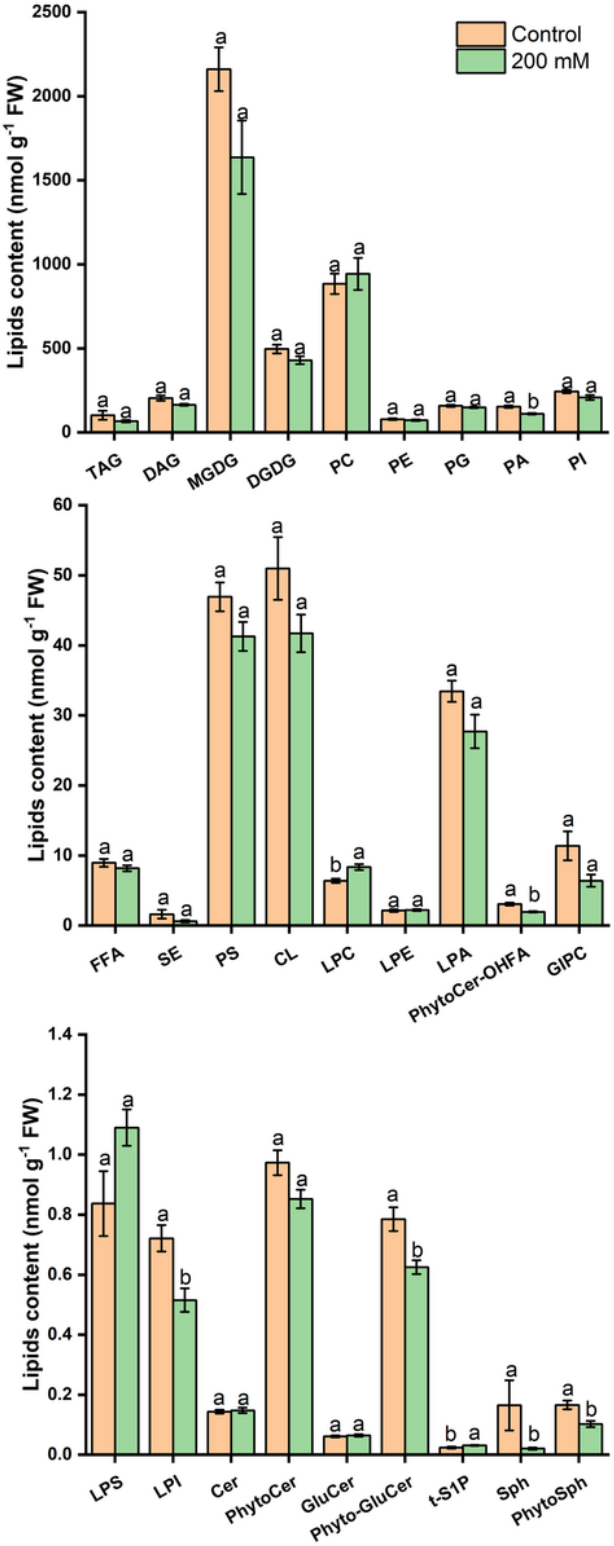


Figure 6

Significance analysis of total lipids with or without 200mM NaCl treatment in *S. europaea*. Data are presented as mean values ± SD of three independent experiments. One-way Duncan's test was performed, and statistically significant differences are indicated by different letters ($P<0.05$)

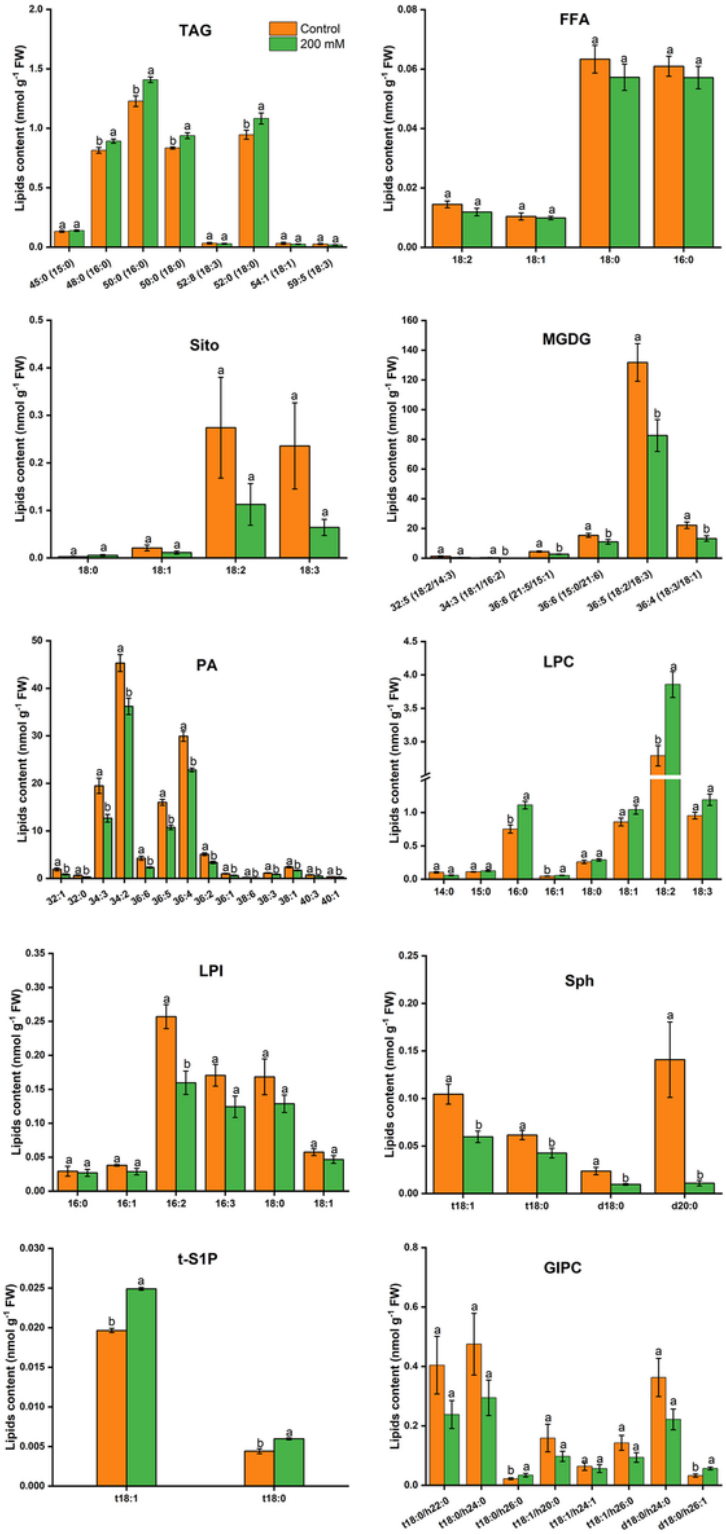


Figure 7

The content of different lipid compounds with or without 200mM NaCl treatment in *S. europaea*. The letters on the bar indicate the significant difference with a P-value < 0.05 using one-way ANOVA

Supplementary Files

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- SupplementaryMaterial.doc