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Comparative metabolomic profiling in the roots of salt-tolerant and salt-intolerant maize cultivars treated with NaCl stress

J.Y. YUE, L.H. WANG, X.T. DOU, Y.J. WANG, and H.Z. WANG*

Tianjin Key Laboratory of Animal and Plant Resistance, Tianjin Normal University,
Tianjin 300387, P.R. China

Abstract

Maize crops are sensitive to NaCl stress, which is one of the most harmful abiotic stresses affecting agricultural productivity. To gain further insights into the differential metabolic responses to NaCl stress, we employed metabolomics and physiological approaches to understand the response of salt-tolerant (PH6WC) and sensitive (PH4CV) cultivars of maize. Salt stress caused a significant reduction in root growth, lower root numbers, softened roots, leaf etiolation, inhibition of leaf formation, and decreased shoot height and stem width in both the tolerant and sensitive genotypes compared with the control. These morphological characteristics increased with the progression of the NaCl concentration, however, they were less prominent in the salt-tolerant genotype. Evans blue staining demonstrated that NaCl-induced root cell death, and the root cells of 'PH4CV' were almost completely dead following 9 d of exposure to 100 mM NaCl. Under treatment with 100 mM NaCl, 79 compounds in the roots of 'PH4CV' were identified as being significant metabolites, and 85 compounds were identified as being significant metabolites in the roots of 'PH6WC'. The NaCl-induced changes in the metabolomes of these two maize cultivars indicate that 80 root-based compounds were different between NaCl-treated plants and controls. Among these metabolites, 30 were found in both maize cultivars when responding to NaCl stress. These compounds were associated with the metabolism of some basic compounds such as *cis*-9-palmitoleic acid, L-pyrroglutamic acid, galactinol, deoxyadenosine, and adenine. The changing abundance of the 30 metabolites was not completely consistent in 'PH4CV' and 'PH6WC'. Glucose metabolism was exclusively induced by NaCl in the 'PH4CV' maize seedlings whereas nucleic acid metabolism was more significant in the 'PH6WC' maize seedlings in response to NaCl stress. Overall, 'PH6WC' and 'PH4CV' responded differently to NaCl stress, and this information is helpful in understanding how maize seedlings respond to this type of abiotic stress.

Additional key words: cell death, glucose metabolism, leaf elongation, nucleic acid metabolism, root morphology, *Zea mays*.

Introduction

Salt stress is a major environmental factor that severely endangers the growth and distribution of plants, especially in arid and semi-arid areas (Jung *et al.* 2017, El-Katony *et al.* 2019). Salt stress inhibits plant water uptake, and toxic concentrations of Na⁺ and Cl⁻ often accumulated in the cytosol (Jiang *et al.* 2019). Both the ion toxicity and osmotic stress induces subsequent oxidative stress (Flowers *et al.* 2015, Abd El-Gawad *et al.* 2016, Zhang *et al.* 2018, Carillo *et al.* 2019). Plant responses to salinity are not only dependent on plant species but also on the duration of salt exposure as well as the concentration and the type of salts (Carillo *et al.* 2019). At short-term salinity, plant growth inhibition can be explained by reduced leaf

elongation through decrease in pressure potential in cell of expanding tissues while long-term salinity affects many different aspects of growth, including those associated with water stress and those specific to NaCl (Shabala *et al.* 2016). Thus, when salinity tolerance and physiological responses are evaluated in species that have evolved under naturally occurring salinity stress, both the intensity and the time course over which the stress is imposed should be considered. According to previous reports, global warming and unreasonable irrigation of arable soil can further worsen salination in soil and massively decrease in production of salt-sensitive crops (Ceccarini *et al.* 2019, Issaoui *et al.* 2019).

Maize is characterized by high yield and a wide geographical scope for growth (Gao *et al.* 2016). Its

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* Corresponding author; fax: (+86) 22 2376 6539, e-mail: 421052455@qq.com

kernels contain a diverse range of phenols and carotenoids, and one unique constituent (GNA-maize) which has the potential to inhibit the activity of the AIDS virus. However, a number of studies have indicated that salt-sensitive maize cultivars are harmed and produce lower yields when salt concentration in soil solution reach 150 mM (Rouf Shah *et al.* 2016, Du *et al.* 2017, Luo *et al.* 2017b). In order to increase maize yields, many researchers have set out to study its salt tolerance mechanism from the perspectives of physiology, biochemistry, and molecular biology and thus developed novel salt-resistant germplasms. However, because of the complicated relationship between maize yield and soil salinity, the current level of information about the salt tolerance mechanism of maize is far from comprehensive (Juhos *et al.* 2015).

The emergence of "omics" has become a research hotspot in different scientific fields including metabolomics, proteomics, genomics, and transcriptomics (Singh *et al.* 2015, You *et al.* 2019). In recent years, some omics-oriented reports concerning the salt tolerance mechanism of maize have been produced, but these focus more on regulatory changes in the proteome and transcriptome of maize under salt stress and rarely report the metabolome of salt tolerance or sensitive maize (Richter *et al.* 2015, Cai *et al.* 2017, Du *et al.* 2017, Luo *et al.* 2017a, Wang *et al.* 2017, Trevisan *et al.* 2019). The mechanisms of salt tolerance in plants are not fully understood. Metabolomics is the scientific study of internal changes in substances present in biological organisms to adapt to disturbances or stimuli in the external environment. Featuring "systematic-dynamic-integrated-analytical" principles, metabolomics accords with the dynamic process of plants in response to environmental change (You *et al.* 2019). Therefore, metabolomics-based approaches may be used to facilitate a systematic understanding of the salt stress-related mechanism in maize.

NaCl generally dissolves easily in soil. In non-saline conditions, NaCl can be beneficial for plants. When present in a range of 0.2 - 2 mg g⁻¹(f.m.), it can stabilize the oxygen evolving complex of photosystem II, maintain cell membranes electric potential, and regulate tonoplast H⁺-ATPase, pH-stat, amylases, and asparagine synthetase. However, when its concentration is more than 4 mg g⁻¹(f.m.), NaCl can be toxic impairing photosystem II quantum yield and photosynthetic electron transport rate and consequently causing other physiological dysfunctions in plants (Carillo *et al.* 2019 and references therein). Harmful changes in the external environment cause severe damage to plants in their early stages of development, and seedling growth determines the subsequent productivity of the crop. Therefore, investigation of the mechanism underlying seedling responses to NaCl stress is of critical significance (Farooq *et al.* 2015, Shelke *et al.* 2017, Zezulka *et al.* 2019). In the present study, the salt-sensitive (PH4CV) and salt tolerant (PH6WC) maize genotypes were used as experimental materials to compare the variation of phenotypic characteristics and root activity under NaCl stress. The metabolic profiling in two maize genotypes with contrasting ability to cope with NaCl stress was compared, the key metabolic pathways involved in

NaCl response in maize were identified by metabolomic analysis. The outcomes of this study can provide molecular evidence for elucidating the molecular mechanism of plant responses to salt stress.

Materials and methods

Plants, growth conditions, and treatments: The test species were salt-sensitive PH4CV and salt-tolerant PH6WC maize (*Zea mays* L.) genotypes. 'PH6WC' and 'PH4CV' were the female and male parents of maize hybrid Xianyu335 bred by *Tieling Pioneer Seed Research Co. Ltd.* 'PH6WC' was bred from the crossing PH01N × PH09B from Reid population. 'PH4CV' was bred from the crossing PH7V0 × PHBE2 from Lancaster population. These two maize PH4CV and PH6WC cultivars were preliminarily selected from 23 cultivars in the seedling stage where 'PH4CV' was found as the salt-sensitive cultivar and 'PH6WC' as the salt-tolerant cultivar. Seeds were firstly washed with tap water then rinsed with distilled water thrice before being soaked in distilled water for 12 h. The seeds were then transferred to a culture basin covered with a wet gauze. A small amount of distilled water was sprayed onto the seeds for culture at ambient temperature. The distilled water was changed every 24 h until the maize grew to the one-leaf-and-one-bud stage. The uniform seedlings were transplanted into a 1/10 Hoagland solution for further culture. The nutrient solution was changed every 2 d until the maize grew to the three-leaf-and-one-bud stage.

Each genotype was planted in plastic pots (50 cm in diameter and 40 cm in height; 10 plants per pot) containing the 1/10 Hoagland nutrient solution (pH 5.2) with 0 mM NaCl to form the control group and various concentrations of NaCl (10, 25, 50, and 100 mM) to form the salt stress treatment group. During the experiment, salinity gradually increased to avoid salt shock to the plants. NaCl concentrations increased in 25 mM increments every second day for salt adaption until the final concentration was reached. The nutrient solution was exchanged at 1 d intervals.

Determination of root activity: Evans blue staining can be used to perform fast identification of dead cells. Deeper staining of more sites indicates less cell activity in the root. Evans blue staining liquid can penetrate into the cells through damaged membrane and combine with the proteins to stain them blue (Gholami *et al.* 2018). The staining approach was modified based on the methods proposed by Baker (1994) and Chalivendra (2017).

Three seedlings were harvested from both non-NaCl-treated and NaCl-treated groups at 0, 1, 3, 6, and 9 d after the onset of NaCl stress. They were washed with tap water, distilled water, and deionized water, respectively, and then dried with filter paper. The root tips (1.5 cm) were sampled and placed in 0.25 % Evans blue staining liquid for 8 min in darkness. Then, the tissues were rinsed with deionized water until it became colorless. After 1 h, the tissues were photographed under a stereoscopic microscope (*Nikon*

C-fled2, Nikon, Tokyo, Japan) to record the staining of the roots.

The stained portions of the seedling roots were removed and cut into pieces before being added to Evans blue extracting liquid (at the ratio of 1 mg root tissue to 0.01 cm³ of extracting liquid prepared with 50 % (v/v) methyl alcohol, 1 % (m/v) sodium dodecyl sulfate (SDS), and water. Extraction was performed in a water bath at 60 °C for 1 - 2 h until the tissues became completely colorless. Then, the extracting liquid was washed with flowing water at ambient temperature. A microplate reader was used to measure the absorbance at 600 nm.

Metabolomic assay - sample collection and preparation:

Ammonium acetate, ammonium hydroxide, ammonium fluoride, and formic acid were purchased from *Sigma Aldrich* (Saint Louis, MO, USA). Acetonitrile was purchased from *Merck* (Shanghai, China).

Seven seedlings of salt-sensitive and salt-tolerant maize cultivars were randomly selected from three pots where they were grown under control conditions and 100 mM NaCl treatment for 6 d. After being washed with tap water, distilled water, and deionized water in turn, the seedlings were gently dried with filter paper and cut quickly into 2-cm pieces of root tips. From each sample, 0.6 g of tissue was weighed and placed into 15-mL Eppendorf tubes, frozen in liquid nitrogen and then kept in a freezer at -80 °C. Samples from each group were six times. Moreover, control samples were prepared for the purpose of stabilizing and calibrating experimental instruments and approaches. The samples from the salt-sensitive 'PH4CV' control group were labeled as C1 and those from the treatment group as T1, samples from the salt-tolerant 'PH6WC' control group were labeled as C2 and those from the treatment group as T2.

The samples frozen at -80 °C were removed and ground under liquid nitrogen. Further, 60 mg was weighed out and added to 1 cm³ of methanol:acetonitrile:water solution (2:2:1, v/v) before being shaken for 60 s on a vortex shaker and underwent to a 30 min low-temperature ultrasound. Afterwards, the samples were left at -20 °C to settle the proteins, filtered with a filter pipe, and centrifuged at 14 000 g and 4 °C for 20 min. Finally, the supernatant was removed for freezing and drying, and the samples were kept at -80 °C.

Analysis by liquid chromatography - mass spectrometry / mass spectrometry analysis: Analyses were performed using an ultra high performance liquid chromatography system (*1290 Infinity LC*, *Agilent Technologies*, Santa Clara, CA, USA) coupled to a quadrupole time-of-flight system (*AB Sciex TripleTOF 6600*, *Shanghai Applied Protein Technology Co.*, Shanghai, China).

For hydrophilic interaction liquid chromatography separation, samples were analyzed using a 2.1 mm × 100 mm *ACQUITY UPLC BEH 1.7 μm* column (*Waters*, Wexford, Ireland). In both electron spray ionization positive and negative modes, the mobile phase contained A = 25 mM ammonium acetate and 25 mM ammonium

hydroxide in water and B = acetonitrile. The gradient was 95 % B for 1 min, linearly reduced to 65 % over 13 min, and then reduced to 40 % over 2 min, and maintained for 2 min, then increased to 95 % over 0.1 min, followed by a 4.9 min re-equilibration period.

The electrospray ionization source conditions were set as follows: ion source gas1 (Gas1) as 60, ion source gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature 600 °C, ion spray voltage floating ± 5500 V. In mass spectrometry only acquisition, the instrument was set to acquire data over the m/z range 60 - 1 000 Da and the accumulation time for a time-of-flight mass spectrometry scan was set at 0.20 s/spectra. In auto mass spectrometry/mass spectrometry acquisition, the instrument was set to acquire data over the m/z range 2 - 1 000 Da and the accumulation time for the product ion scan was set at 0.05 s/spectra. The product ion scan was acquired using information-dependent acquisition with the high sensitivity mode selected. The parameters were set as follows: the collision energy was fixed at 35 ± 15 eV, declustering potential 60 V (+) and -60 V (-), exclude isotopes within 4 Da, candidate ions to monitor per cycle: 6.

Data analysis: For root activity experiments, three replicates were performed. All data measured were subjected to analysis of variance. The mean differences were compared using the Duncan's multiple range test. Differences with $P \leq 0.05$ were considered significant.

For metabolomic data analysis, the raw mass spectrometry data (wiff.scan files) were converted to MzXML files using *ProteoWizard MSConvert* before importing into a freely available *XCMS* software. For peak picking, the following parameters were used: centWave m/z = 25 ppm, peakwidth = c (10, 60), and prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, and minfrac = 0.5 were used. *CAMERA* (collection of algorithms of metabolite profile annotation) was used to annotate isotopes and adducts. In the extracted ion features, only the variables with more than 50 % of the nonzero measurement values in at least one group were kept. Identification of metabolites was performed by comparing m/z values (< 25 ppm) and mass spectrometry / mass spectrometry spectra with an in-house database established with available authentic standards.

After normalizing to total peak intensity, the processed data were uploaded before being imported into *SIMCA-P* (v. 14.1, *Umetrics*, Umea, Sweden), where they were subjected to multivariate data analysis, including *Pareto*-scaled principal component analysis and orthogonal partial least squares discriminant analysis (OPLS-DA). Seven-fold cross-validation and response permutation tests were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. Metabolites with the VIP value >1 were further applied to the Student's *t*-test at the univariate level to measure the significance of each metabolite. Differences with $P \leq 0.05$ were considered as significant.

Results

During the experimental period, NaCl stress caused a significant reduction in the root growth, lowered root numbers, softened roots, caused leaf etiolation, inhibited leaf formation, and decreased shoot height and stem width in both the tolerant and sensitive genotypes compared with the control and the effects increased with the progression of NaCl stress (Fig. 1 Suppl.). The sensitive genotype showed significant changes in these morphological characteristics in response to NaCl stress compared with plants grown under control conditions. However, the morphological changes were less apparent in the tolerant genotype. The ‘PH4CV’ maize seedlings withered following 9 d of NaCl stress.

The exposure to NaCl stress increased the Evans blue uptake more significantly in ‘PH4CV’ than in ‘PH6WC’, as compared with respective controls. Staining intensified

with an increase in the duration of exposure to NaCl. Control roots not treated with NaCl showed almost no staining. The ‘PH4CV’ maize seedlings turned blue after 9 d of NaCl stress, indicating that the root cells were no longer viable. The microscopic observation indicated similar results (Fig. 1, Fig. 2 Suppl.).

In the present study, the metabolic changes in two maize cultivars displayed contrasting responses to NaCl stress. Based on the principal component analysis, a clear separation between the NaCl-treated group and controls could be observed. Furthermore, the two cultivars were distinguished distinctly (Fig. 3 Suppl.). These results suggest that distinct metabolic programs are engaged at the different stages of NaCl stress and differential metabolic responses between ‘PH4CV’ and ‘PH6WC’ could be the basis of their contrasting tolerance to NaCl stress.

Single variable analysis was used to assess the difference in metabolites between the two groups of samples. After

Fig. 1. The absorbance of the Evans blue solution extracted from roots of two maize cultivars under NaCl stress at different time points. Means $\pm$ SEs, $n = 3$, different letters indicate statistically significant differences at $P < 0.05$.

Fig. 2. Changes in the percentage of the total number of metabolites in the roots of two maize cultivars PH4CV and PH6WC under NaCl stress. T1 - NaCl-treated ‘PH4CV’, C1 - ‘PH4CV’, T2 - NaCl-treated ‘PH6WC’, C2 - ‘PH6WC’ (right part: * indicates significance ($P < 0.05$), ** indicates high significance differences ($p < 0.01$); left part - different letters indicate statistically significant difference at $P < 0.05$).

6 d of 100 mM NaCl treatment, the metabolites in salt-treated maize roots showed significant changes compared with the control group. As shown in Fig. 4 Suppl., the red dot represents the different metabolites with $FC > 1.5$ and $P < 0.05$. These results suggested that potential marker metabolites could be further screened.

We profiled the metabolic changes in two maize cultivars displaying contrasting responses to NaCl stress. Changes associated with response to NaCl stress were calculated as a ratio of relative metabolite concentration of the treated plants to that of the controls. The patterns varied widely, with some metabolites upregulated and some downregulated. Certain metabolites were present in mixed patterns between 'PH4CV' and 'PH6WC' following NaCl treatment. Dramatic variations in the significant root

metabolites among the four groups, *i.e.*, C1, C2, T1, and T2 (cutoff of two-fold log 2, $P < 0.05$), were compared.

'PH4CV' is sensitive to NaCl. Under 100 mM NaCl treatment; 79 compounds in its roots were identified as significant in comparisons between NaCl-treated plants and controls with five replicates. A total of 51.9 % of the metabolites were accumulated whereas 48.1 % of the metabolites decreased. At least 33 metabolites showed a more than two-fold increase compared with controls. Amino acids (17), organic acids (15), polyols and sugars (6), fatty acids (7), and alkaloids (4) were the major groups of metabolites that were altered because of NaCl stress (Fig. 2).

'PH6WC' is tolerant of NaCl stress. Under 100 mM NaCl treatment conditions, 85 compounds in the roots

Fig. 3. The *KEGG* pathway enrichment analysis of the differentially accumulated metabolites between maize cultivars PH4CV (A) and PH6WC (B) at 6 d under NaCl stress.

were identified as significant compared with the control plants. A total of 48.75 % of the metabolites were positively accumulated whereas 51.25 % of the metabolites decreased. At least 47 metabolites exhibited a more than two-fold increase compared with control. Amino acids (23), delspray (10), polyols and sugars (8), organic acids (6), and alkaloids (5) are major groups of metabolites altered in 'PH4CV' and 'PH6WC' because of NaCl stress (Fig. 2).

The metabolite induced by NaCl stress in two different maize cultivars showed that 80 compounds in roots were significantly different between NaCl treatment and control. And 44.71 % of metabolites were accumulated, 55.29 metabolites decreased. At least 23 metabolites exhibited a more than two-fold increase compared with controls. Amino acids (20), organic acids (14), delspray (10), polyols and alkaloids (6), and sugars (4) were the major groups of metabolites that were altered because of NaCl stress. Among these different metabolites, 30 were found in both maize varieties responding to the NaCl stress. These metabolites were involved in the metabolism of some basic compounds such as *cis*-9-palmitoleic acid, L-pyroglutamic acid, galactinol, deoxyadenosine, and adenine, suggesting that primary metabolism was relatively highly impacted by NaCl stress in 'PH4CV' and 'PH6WC' (Table 1 Suppl.). The trends in accumulation of the 30 metabolites were not completely consistent between 'PH4CV' and 'PH6WC'. Of the 30 metabolites, 14 were upregulated and the remaining 16 were downregulated in 'PH4CV' whereas 15 were upregulated and the remaining 15 were downregulated in 'PH6WC'. However, glucose metabolism was exclusively induced by NaCl in the 'PH4CV' maize seedlings whereas nucleic acid metabolism was more significant in the 'PH6WC' maize seedlings in response to NaCl stress, demonstrating different metabolic responses to NaCl stress in the different maize cultivars.

Hierarchical cluster analysis was performed to reveal the accumulation patterns of the significantly altered metabolites after the exposure of maize seedlings to 100 mM NaCl for 6 d. All metabolite data from the roots of both cultivars under control and stress conditions were analyzed by hierarchical cluster analysis to provide a global view of the metabolite changes in 'PH4CV' and 'PH6WC' (Fig. 6 Suppl.). Clustering analysis of the metabolic profiles from the two cultivars showed that all the biological replicates were grouped together, indicating a good correlation between replicates and high reliability of our data. No clear separation between 'PH4CV' (C1) and 'PH6WC' (C2) could be observed in the control group but T1 and T2 were grouped into different clusters under NaCl stress. The results of *ANOVA* showed that, 79 metabolites were differentially accumulated between C1 and T1, 85 metabolites between C2 and T2, and 80 metabolites between T1 and T2. In addition, NaCl treatment significantly affected metabolites mainly in the classes of nucleotides and their derivatives, organic acids, amino acid derivatives, fatty acid, and alkaloids.

Correlation analysis was used to reveal relationships among metabolites. Both positive and negative correlations occurred among metabolites in both 'PH4CV' and

'PH6WC' after NaCl stress. Generally, high correlations observed in both 'PH4CV' and 'PH6WC' were either among amino acids or between amino acids and nucleotides or organic acids (Fig. 5 Suppl.).

Discussion

Salt stress restricts the entire growth process of plants, but seedlings are most sensitive to salt in the early stages of growth (Tounsi *et al.* 2017, Zezulka *et al.* 2019). Maize is a salt-sensitive C4 monocot crop and excessive salt concentrations in the soil lead to the abortion of maize kernels (Zhang *et al.* 2019b). This study probed the salt tolerance mechanism of maize by combining observations of seedling phenotype, root activity, and root metabolomics. The results indicate that treatment of maize seedlings with NaCl significantly inhibited their height, leaf size, stem diameter, and root growth. Furthermore, as the treatment is prolonged and NaCl concentration is raised, the inhibitory effects were more evident. This is consistent with the findings reported by Turk (2019).

Salt stress often induces oxidative damage, impairs membrane integrity, and causes apoptosis (Che *et al.* 2019, Zhang *et al.* 2019b). NaCl stress causes dehydration of the root cells as well as it damage membranes which may lead to cell death, as it was determined by Evans staining in this study. The results of Evans staining presented here concur with those of Tian (2019), who studied the effects of the herbicide quinolinic acid on maize. When confronted with this stress, root cells became less active (Sunohara and Matsumoto 2008, Yang *et al.* 2018, Tian *et al.* 2019). This confirmed that this approach was a suitable means of determining root cell viability.

The metabolomics is used to elucidate the differential metabolic reactions that result from geographical, climatic, and species-associated factors by analyzing changes in the content of small-molecule substances. Recently, many researchers have employed metabolomic approaches to study the resistance mechanisms of plants (Umair *et al.* 2019). The aforementioned substances are mainly organic acids, fatty acids, amino acids, polyhydric alcohols, sugars, alkaloids, and mineral elements. In present study, the content of sucrose, betanin, most amino acids, and some fatty acids tended to rise in 'PH6WC' and 'PH4CV' under NaCl stress. And the amount of amino and fatty acids in the roots of maize 'PH6WC' rose sharply. We may thus assume that 'PH6WC' had better osmotic adjustment under NaCl stress. This result is similar to the findings of Wang (2018) studying of metabolite changes in response to low-temperature stress.

The content of organic acids generally increases when plants are under stress (Yang *et al.* 2012). In the present study, both 1-aminocyclopropanecarboxylic acid and 16-hydroxypalmitic acid decreased in both 'PH6WC' and 'PH4CV' under NaCl stress, however, 'PH6WC' significantly accumulated citric acid, citraconic acid, *cis*-aconitate, aminoadipic acid, acetyl phosphate, cinnamic acid, *ect.*, in its roots. Organic acids not only act as the intermediates in energy cycle, but also play a role in plant

adaptation to nutrient shortage and other abiotic stresses. As a component of the TCA cycle, citric acid can help 'PH6WC' to maintain a healthy root system and uptake nutrients and water. This is consistent with the result of Kang (2019).

The accumulation of amino acids can enhance plant stress tolerance by detoxification of ROS in photosynthetic organs, adjustment to osmotic stress, and maintenance of the intracellular pH (Yancey 1994, Hare *et al.* 1998, Gill and Tuteja 2010, Kang *et al.* 2019). Declines in glutamic acid, purines, and pyrimidines can lead to reduced protein synthesis (Zhang *et al.* 2019a,b). In the present study, enhanced accumulation of amino acids in both 'PH6WC' and 'PH4CV' was noted due to the salt stress, but their relative intensity was higher in 'PH6WC' than in 'PH4CV'. L-pyrroglutamic acid, cytosine, adenine, and thymine were downregulated in both maize cultivars, suggesting a negative effect of NaCl stress on protein synthesis in maize. Some amino acids play an adaptive role in mediating osmotic adjustment and protecting subcellular structure (He *et al.* 2015). Proline can remove ROS and alleviate their effects on cell proteins and membranes (Tang *et al.* 2019). Comparing the two maize genotypes revealed higher tendencies for the upregulation of proline in the roots of 'PH6WC', which testifies that NaCl stress can induce salt-tolerant maize to accumulate more proline to reduce membrane damage. This finding is similar to that of Saeedipour (2013), who indicated that higher proline content was found in maize seedlings under a low water potential. This result also supports the evidence that proline alleviates the deleterious effect of stress (Saeedipour 2013). The content of γ -aminobutyric acid (GABA) also increased under NaCl stress. GABA-regulated developmental processes, pH, and stress tolerance (Umair *et al.* 2019).

Fatty acids are constituents of plant membranes. Under NaCl stress, the content of fatty acids decrease because of membrane damage (Yang *et al.* 2012, Hu *et al.* 2015). In the present study, when compared with the control group, the content of fatty acids in the roots decreased in 'PH4CV' and increased in 'PH6WC' under NaCl treatment. This may suggest that 'PH6WC' can respond quickly to NaCl stress by accumulating oleic acid, citrate, myristic acid, 21-hydroxypregnenolone, and GABA, *ect.*, to alleviate the damage of membranes caused by NaCl exposure.

Saccharides participate in the metabolism of galactose. They can also promote water absorption in seedling roots, regulate the osmotic potential of plant cells, and their content reflect the regulation of photosynthesis. Saccharide content indicates that photosynthesis has been affected under certain conditions (Umair *et al.* 2019). In this study, both maize species upregulated content of sucrose and betanin in response to NaCl treatment, indicating that the roots of maize seedling can adjust the water potential through saccharide accumulation. Hu (2019) studied the effect of stress on saccharide-related genes and discovered that saccharides could respond to salt stress. The comparison of maize cultivars indicated that salt-sensitive 'PH4CV' accumulated more saccharides whereas salt-resistant

'PH6WC' gathered more amino acids, organic acids, and fatty acids for osmotic adjustment. It can be assumed, that in the roots of salt-sensitive 'PH4CV' galactose metabolism is inhibited and photosynthesis is reduced. Compared with the control groups, the NaCl-treated groups of both cultivars exhibited lower concentrations of Mg^{2+} but 'PH6WC' exhibited less decline in Mg^{2+} . This suggests that 'PH6WC' maize can more effectively absorb Mg^{2+} and decelerate chlorophyll degradation. This is consistent with the findings of Jin (2017), who revealed that NaCl stress can affect absorption of Mg^{2+} and thus their photosynthesis. He also verified that low Mg^{2+} stress can affect chlorophyll content and organic acid accumulation in potato plantlets.

On the basis of the analysis of differential metabolites, this paper studied differential metabolic pathways and determined that maize adapts to the NaCl stress by initiating the metabolism of purines, pyrimidines, alanine, aspartic acid, and glutamic acid, synthesis of aminoacyl t-RNA, protein digestion and absorption, and adipocyte esterolysis and subsequently regulating the synthesis and decomposition of purines, pyrimidines, amino acids, and fatty acids (Fig. 3). Wang (2019) investigated the temporal metabolic responses to salinity in hulls of barley. They performed a widely targeted metabolomic analysis of 72 leaf samples from two contrasting cultivars and identified 642 compounds, 57 % of which were affected by salt stress in the two cultivars, these were principally amino acids and their derivatives, organic acids, nucleotides, and flavonoids. This study reveals that under NaCl stress, salt-sensitive and salt-tolerant maize cultivars are not metabolically consistent with each other. As 'PH4CV' underwent a significant change in the citrate cycle and metabolism of glyoxylic acid and dicarboxylic acid, 'PH6WC' changed mainly galactose metabolism, mineral absorption, biosynthesis and metabolism of fatty acids, and glyceryl phosphatide metabolism. By studying the response maize to Cd stress, Lian (2019) found that galactose metabolism, the citrate cycle, and the metabolism of related amino acids could relieve Cd poisoning of maize, which is similar to the findings of this paper. Changes in the content of saccharides, fatty acids, unsaturated fatty acids, and Mg^{2+} are all closely related to the salt tolerance of maize.

Conclusions

The results of the experiments conducted in this study revealed that the salt-tolerant and salt-intolerant maize cultivars displayed significant differences in response to treatment with 100 mM NaCl for 6 d. When compared with the control groups, content of organic acids, Mg^{2+} , L-pyrroglutamic acid, purines, and pyrimidines decreased while content of sucrose, betanin, most amino acids, and some fatty acids increased in treated groups. The salt stress reduced the chlorophyll content and photosynthetic rate. However, maize seedlings accumulate osmotic adjustment substances to lower the water potential and to eliminate

complete dehydration. The salt sensitive cultivar PH4CV was not able to regulate metabolic pathways as effectively as salt-resistant cultivar PH6WC.

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