Characterization of metabolic responses to salt stress in soybean seedling using gas chromatography-mass spectrometry

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A B S T R A C T

Soil salinity is a critical obstacle in modern agriculture which devastates crop growth. Many plants have developed different strategies to sense, transduce, and develop tolerance to salinity. Plant adaptation to salinity stress includes complicated metabolic pathways, genes and molecular networks. Here, we used gas chromatography-mass spectrometry (GC-MS) to understand the metabolic responses of soybean seedlings upon various levels of salt stress treatments. To this end, one salt tolerant and one salt sensitive soybean cultivar, namely Dongnong 69 and Dongnong 63 were used in this study. A total of 10 metabolites, including sugars, amino acids and organic acid, were identified as differential biomarkers. Our results indicated that these biomarkers were closely related to salinity tolerance in soybean seedlings. In particular, three metabolites, namely isoleucine, serine and aspartic acid, were found respond significantly differently between the different soybean cultivars. These three metabolites can be therefore served as potential biomarkers to screen for salt tolerant soybean cultivars. Overall, results of this study help to improve our knowledge with respect to plant salt tolerance in general, and soybean in particular.

Keywords: GC-MS; soybean; salt stress; organic acids; amino acids

INTRODUCTION

Soil salinity remains one of the significant challenges in agriculture. Currently, over 6% of the world`s land and 20% of the arable land are salt-affected, which causes a great threat to agricultural production (Yang and Guo 2018a; El Moukhtari et al. 2020). It has been estimated that the salt-affected lands continue to increase at a rate of 1-2% annually due to reasons such as poor irrigation. High salinity is generally caused by concentrations of Na^+ and Cl^- in the soil. In general, a soil is regarded salt-affected when its electric conductivity is higher than 4 dS m^{-1}, resulting in the elevated osmotic pressure to about 0.2 MPa, and as a consequence impeding the water and nutrients absorption by plants from the soil (Acosta-Motos et al. 2017).

Plants has also evolved various molecular and biochemical strategies to deal with salt, including control of the ion in plants, selectively accumulating and eliminating ions, compartmentalizing ions at the subcellular and cellular levels, producing compatible solutes, alternating photosynthetic pathways, changing the structure of membranes, and biosynthesis of additional plant hormones (Parida and Das 2005). Therefore, understanding plant adaptation to salt stresses could provide us valuable insights into improving the growth and yield of crops grown in high-salt environments. Significant improvements towards plant-salt stress response have been achieved over the last two decades via molecular genetics and genomics analysis (Yang and Guo 2018b). For instance, various elements have been found to involve in salt stress signal transduction and pathway regulation by studying Arabidopsis, rice and barley (Yang et al. 2018; Hazzouri et al. 2018; Yang et al. 2019; Islam et al. 2019). The examinations of candidate salt tolerance genes has enhanced our understanding in regulatory pathways that influence salt-stress tolerance; and discovery of novel candidate genes could improve plants salt tolerance through genetic engineering (Jamil et al. 2011;

Mishra and Tanna 2017; Tang et al. 2019; Zhang et al. 2020). Some studies aimed to research the metabolites changes to salt stress, including *Oryza sativa* (Gupta and De 2017; Chang et al. 2019), *Glycine max* (Zhang et al. 2016; Jung et al. 2021), and *Zea mays* (Sun et al. 2015; Richter et al. 2015). These studies demonstrate that metabolomic analyses provide an important means to improve our understanding the mechanisms to salt stress.

Currently, the soybean growth is threatened by hostile factors including drought and salt stress, resulting in a significant yield reduction (Silvente et al. 2012; Li et al. 2017). Here, we have used GC-MS to characterize and compare the metabolome variations of different soybean cultivars, i.e. salt tolerant cultivar Dongnong 69 and salt sensitive cultivar Dongnong 63, in response to salt stresses at seedling stage. The objective of study is to understand and compare the metabolic responses and adjustments of the two different soybean cultivars upon different levels of salt stresses.

MATERIALS AND METHOD

Sample preparation

Two soybean cultivars, i.e. salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S), were first surface sterilized using 20% commercial bleach, extensively washed using sterile water, and germinated in darkness at 28 °C for 3 days. The soybean seedlings were then moved to 10-cm diameter plot containing washed sand. Salt stress experiments were conducted with 7-day-old seedlings. Seedlings of each cultivar were divided into 3 groups, including a control group, a 50mM NaCl treatment group (50) and a 100 mM NaCl treatment group (100), with each group contains at least 24 pots. Seedling were watered with Hoagland's solution, except that the two salt stress groups were also supplemented with 50 and 100 mM NaCl for 14 days. After treatment, the whole shoots were collected, weighted and kept at -80 °C for GC-MS analysis (Fig. 1a).

Metabolite extraction and GC-MS analysis

Six above-ground seedlings of each cultivar were collected, homogenized, and 100 mg sample was weighed from each seedling. They were then extracted as described earlier (Kim et al. 2017; Hyeon et al. 2020) (Fig. 1b). The Agilent 7890A GC system, coupled to a Pegasus TOF-MS (LECO, USA) was used for GC-MS analysis in this study. Detailed instrumental parameters are as described elsewhere (Kim et al. 2020) (Fig. 1c).

Mass spectral analysis

Raw data files were first converted into.cdf with ChromaTOF (version 4.50.8.0, LECO) software. Data preprocessing, including peak picking, grouping, alignment and deconvolution, was performed on the.cdf files using the R packages CAMERA (Kuhl et al. 2012) and XCMS (Smith et al. 2006). The peak list was then used for statistical analysis. Principal component analysis plot and bar plots were produced with R package ggplot2 (Wickham 2016); and heatmap was produced using R package Superheat (Barter and Yu 2018). A metabolite was defined differential when the p-value < 0.05 and log2 FC > 1. The differential metabolites were identified using ChromaTOF software (version 4.50.8.0, LECO) (Fig. 1d).

RESULTS

The profiling of polar plant metabolites was achieved for all treatments using GC-MS. Peak-picking with XCMS (Smith et al. 2006) using signal-to-noise ratio (S/N) threshold of 3 resulted in 4219 mass features. After fragment feature grouping and irrelevant data (e.g., noise and back ground peaks) filtering using R package CAMERA (Kuhl et al. 2012), 66 compounds were detected. The extracted compound list was first submitted to multivariate statistical analysis in order to evaluate sensitivity of our GC-MS approach to discriminate between control and salt-stress treated soybean groups. To this end, PCA analysis was used to evaluate the trends, outliers and quality of our GC-MS data. The result showed that the six groups from the two soybean cultivars were well separated by PCA score plot. The result could cover 65.22% and 21.23% of the total variance of the data (Fig. 2). The obvious discrimination of the 2 soybean cultivars, i.e. salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S), indicated that the metabolic responses between the cultivars were significantly different upon salt treatments. In addition, the clear separation of the three different salt treatment groups, i.e. control (0), 50 mM NaCl (50) and 100 mM NaCl (100), showed that the levels of salt treatments could also alter the metabolic profiles in both Dongnong 69 and Dongnong 63 soybean cultivars (Fig. 2).

Next, ANOVA was used to analyze the significant difference of each metabolite responsible for the group discrimination. In total, 10 out of 66 mass features were found differential. Subsequently, these 10 differential biomarkers were used to plot a heatmap plot (Fig. 3). The heatmap plot clearly displays two major blocks of metabolites: the first block (the first 6 columns) contains a group of metabolites that show highest contents in salt sensitive cultivar (S). The 6 metabolites were putatively identified as mannitol, fumaric acid, aspartic acid, asparagine, glycine, and isoleucine, respectively; The first block (the last 4 columns) includes a list of metabolites that show highest contents in salt tolerant cultivar (T). These
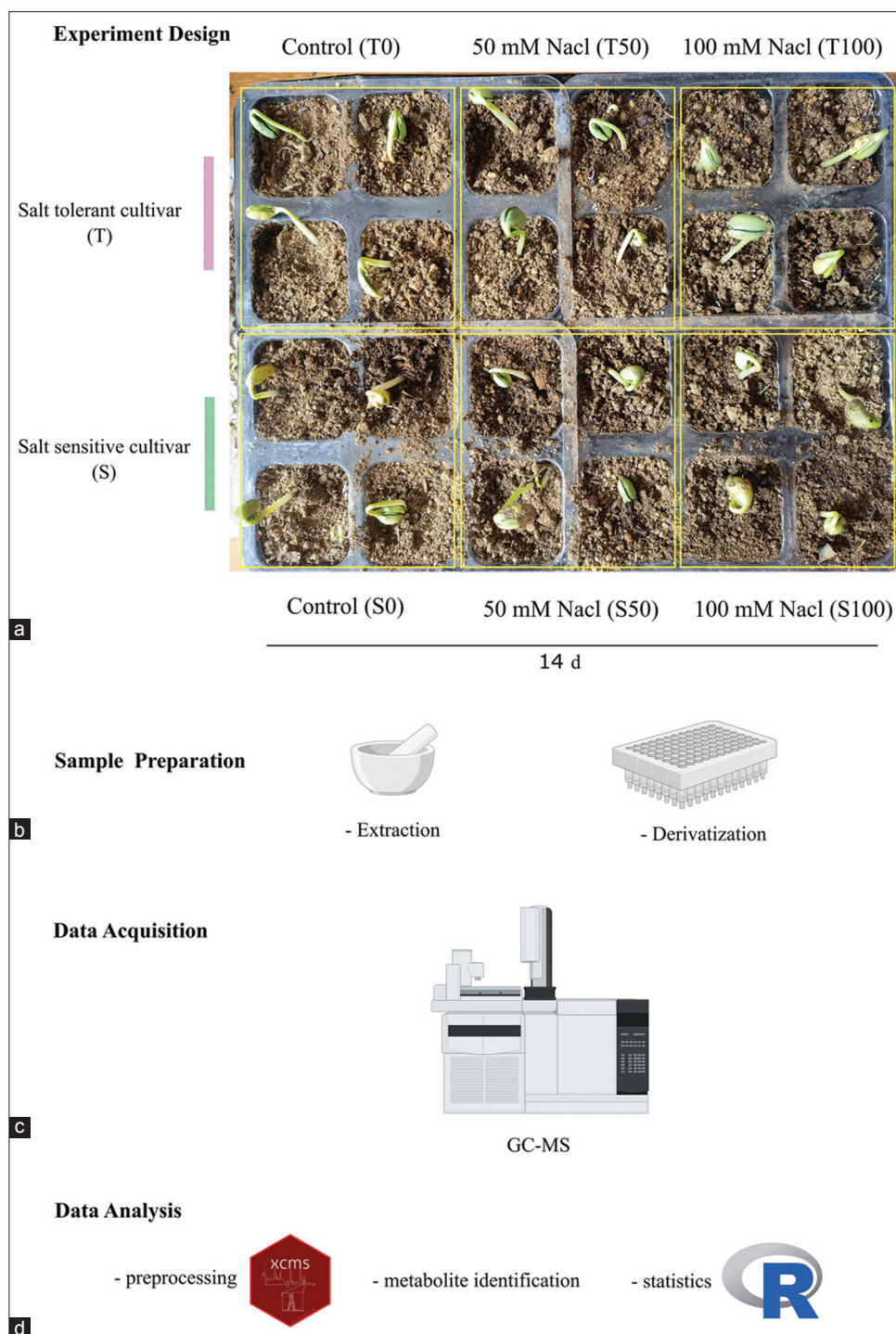
Fig 1. The workflow employed in this study. (a) Representative images of seven-day-old seedings of two bean cultivars, i.e. salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S). They were subjected to different salt treatment. The 14-day salt treatment groups include a control group (0), a 50mM NaCl treatment group (50) and a 100 mM NaCl treatment group (100), with each group contains at least 24 pots. (b-c) The samples were extracted and derivatized for GC-MS analysis. (d) The raw data were first converted into .cdf and preprocessed with R package XCMS. The resulting peak list was then used for statistical analysis.

metabolites were putatively annotated as proline, serine, inositol and glutamic acid. (Fig. 3).

So as to deeper understand the metabolic changes of the 10 differential biomarkers, we have semi-quantitatively

compared their abundance among the three different salt treatment groups in both soybean cultivars Dongnong 69 (T) and Dongnong 63 (S), respectively. In salt tolerant cultivar Dongnong 69 (T), the contents of 6 metabolites, including mannitol, inositol, aspartic acid, glutamic acid,
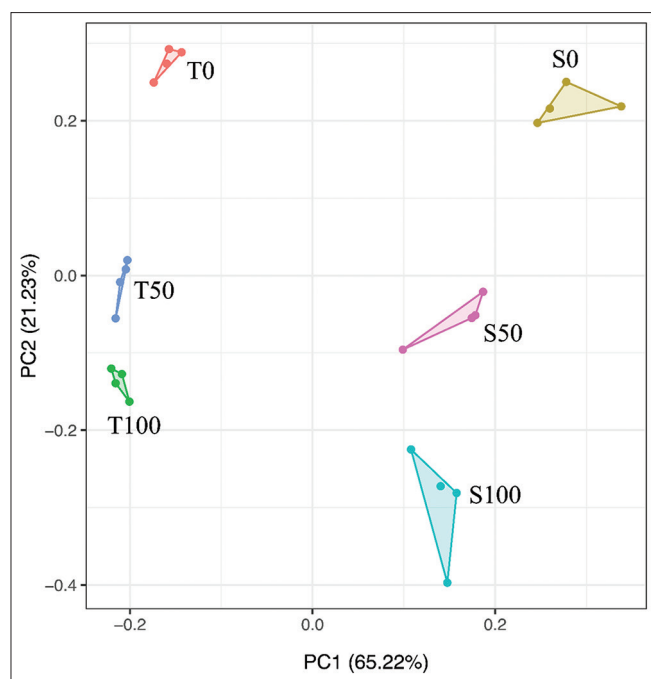
Fig 2. Principal component analysis (PCA) for soybean seedlings analyzed by GC-MS. PCA score plot (n = 4) of normalized ion intensities of 66 metabolites indicates the differences of metabolic profiles among the salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S). In addition, the different salt treatment groups from the two cultivars, including control (0), 50 mM (50) and 100 mM (100) NaCl treated groups, were also found to have distinct metabolic profiles.

Fig 3. Hierarchical clustering heatmap of statistically significant metabolites affected by salt stress treatments. Ten metabolites were found statistically changed in both salt tolerant Dongnong 69 (T) and salt sensitive Dongnong 63 (S) soybeans. Each colored cell (red higher, blue lower) on the heatmap corresponds to a z-score normalized metabolite. Each row represents a sample, and each column represents a metabolite. Values are calculated using Euclidean distance with a Ward clustering algorithm (n = 4).

serine and fumaric acid, were statistically significantly reduced (p-value < 0.05) after salt treatments There is a generally trend that contents of all the 6 metabolites tend to reduce with the increase of salt concentration (Fig. 4a). By contrast, the content of another 4 metabolites, i.e. asparagine, isoleucine, glycine and proline, were statistically elevated after salt treatment. A similar trend was observed for the 4 metabolites that their abundance increased with the increase of salt concentration (Fig. 4b). The 10 metabolites were also found to respond differently upon different levels of salt stresses in salt sensitive cultivar Dongnong 63 (S). Among them, the contents of glutamic acid, inositol, isoleucine, mannitol, glycine and fumaric acid were reduced (Fig. 5a). In contrast, the levels of serine, asparagine, proline and aspartic acid were elevated (Fig. 5b).

Interestingly, we have found that the metabolic responses in salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S) were different. In particular, isoleucine, serine and aspartic acid showed opposite responses towards salt stress in the two soybean cultivars. For instance, the contents of serine and aspartic acid were decreased in Dongnong 69 (T) while their levels were significantly increased in Dongnong 63 (S) after salt stress; and the content of isoleucine was elevated in Dongnong
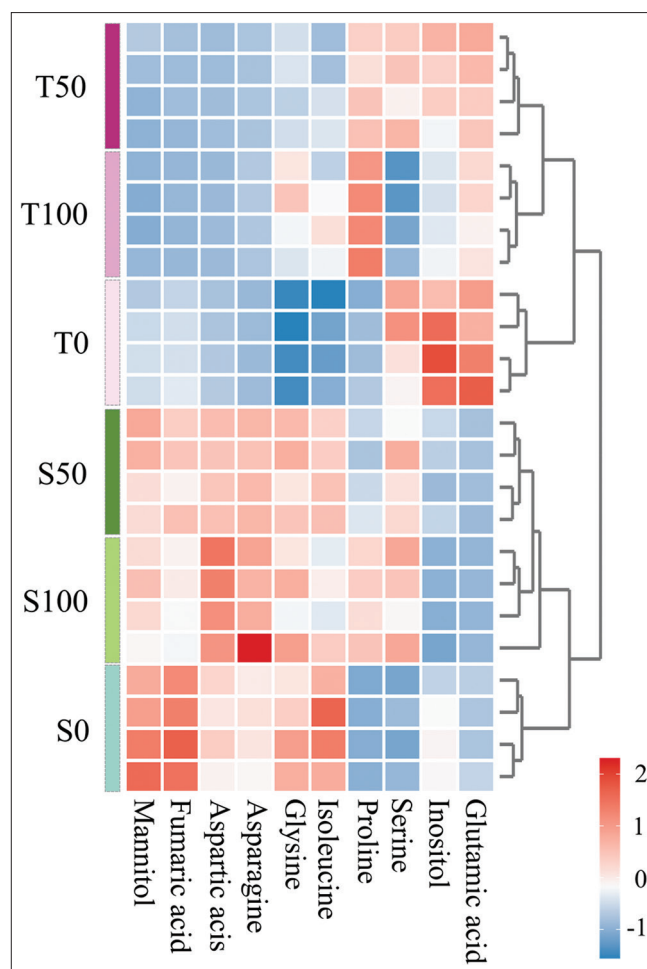
69 (T) and decreased in Dongnong 63 (S) (Fig. 4 and 5). Statistical analysis of the 3 metabolites between the two different soybean cultivars revealed that their contents in the soybean seedlings were all statistically different before salt stress (Control group, p < 0.05) (Fig. 6a). They can be therefore used as potential biomarkers for rapid screening of salt tolerant soybean cultivars without any salt treatments using GC-MS. The trend of fold-change of the 3 metabolites between Dongnong 63 (S) and Dongnong 69 (T) further confirmed that their responses are opposite in the two soybean cultivars (Fig. 6b).

DISCUSSION

Salinity has been demonstrated to have different harmful effects on plants, including interference of photosynthesis and respiration, water stress, ion imbalance and nutritional
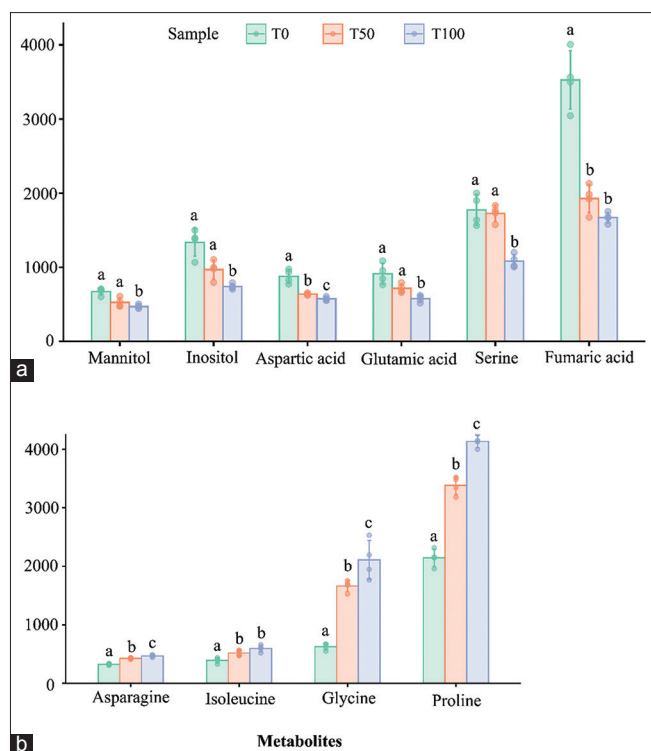
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Fig 4. Semi-quantitative comparison of different differential metabolites among control, 50 mM and 100 mM salt stress treated groups in salt tolerant cultivar Dongnong 69 (T). (a) Bar plot of differential metabolites that are more abundant in the control group. (b) Bar plot of differential metabolites that are highly induced in salt stress treated groups. Bar plot represents mean + SD (n = 4) of square root transformed ion intensity. Different letters indicate statistical differences (p-value < 0.05) among the control (0), 50 mM (50) and 100 mM (100) salt stress treated groups.

Fig 5. Semi-quantitative comparison of different differential metabolites among control, 50 mM and 100 mM salt stress treated groups in salt sensitive cultivar Dongnong 63 (S). (a) Bar plot of differential metabolites that are more abundant in the control group. (b) Bar plot of differential metabolites that are highly induced in salt stress treated groups. Bar plot represents mean + SD (n = 4) of square root transformed ion intensity. Different letters indicate statistical differences (p-value < 0.05) among the control (0), 50 mM (50) and 100 mM (100) salt stress treated groups.

deficiency (Acosta-Motos et al. 2017). Here, the amounts of 6 metabolites were found significantly reduced upon salt stress in salt tolerant cultivar Dongnong 69 (Fig. 4a). Our result was contrary to several previous studies on different plant species that sugar such as inositol and mannitol and organic acids (OAs) tend to accumulate under salt stress (Xu et al. 2013; Gupta and De 2017). Indeed, Sugars and OAs have been reported to serve as metabolically-active solutes in balancing cation excess and adjusting osmotic pressure (Chen et al. 2009). By contrast, some other studies reported a strong and exclusive decrease of OAs during salt stress, similar as what we have found in this study (Sanchez et al. 2007; Zhao et al. 2014; Chang et al. 2019). Especially, it has been shown that salt tolerant wild-type soybean tend to accumulate OAs whereas the OA contents are reduced in semi-wild and cultivated soybeans during salt stress treatment (Li et al. 2017). These intra- and inter-species variations reported from different studies suggest that the change of OAs levels upon salt stress treatment is an important criterion to differentiate between wild and cultivated crop against salinity.
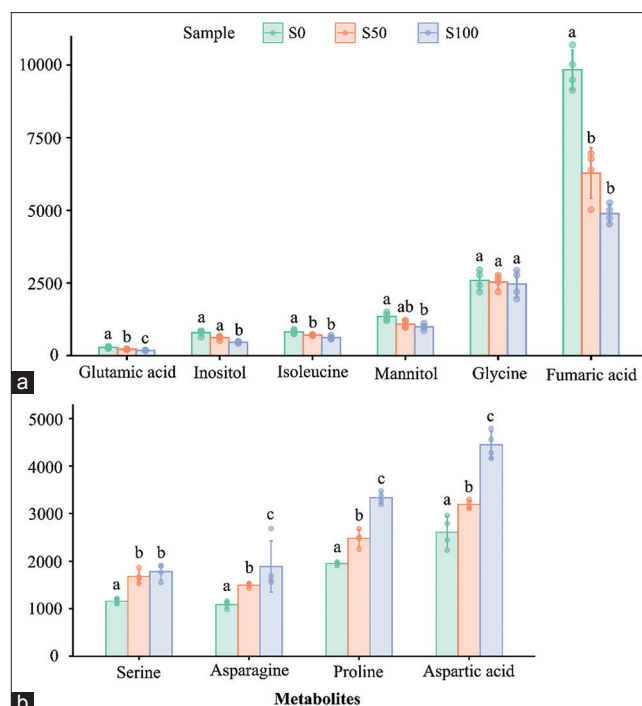
Heilongjiang is the largest soybean producing area in China, with a large area of arable land (about 118,000 square kilometers) and complex soil conditions. Therefore, the demand for soybean varieties is also very diverse. Although there are many soybean varieties in Heilongjiang, there is a big gap between the yield per unit and the main soybean producing countries such as Brazil, the United States and Argentina. The main reasons include the decline of soil organic matter content, thinning of soil layers, soil compaction, and destruction of soil biodiversity, as well as blindly applying too much nitrogen fertilizer. The application of nitrogen, phosphorus and potassium fertilizers is too much, while the application of medium and trace element fertilizers is less, resulting in obvious shortage of soil nutrients. Breeding techniques and methods are not advanced enough, biological breeding and molecular breeding techniques have not been widely used in soybean breeding. As a new soybean variety with high oil, high yield and disease resistance, Dongnong 69 (Taking Hefeng 50 as the female parent and Beijiao 922 as the male parent) has been promoted on a large scale since 2017. Dongnong 63（Take Huajiang 2 as the female parent and Hefeng 55 as the male parent）, is the main cultivar in the third and fourth accumulated temperate zone, with
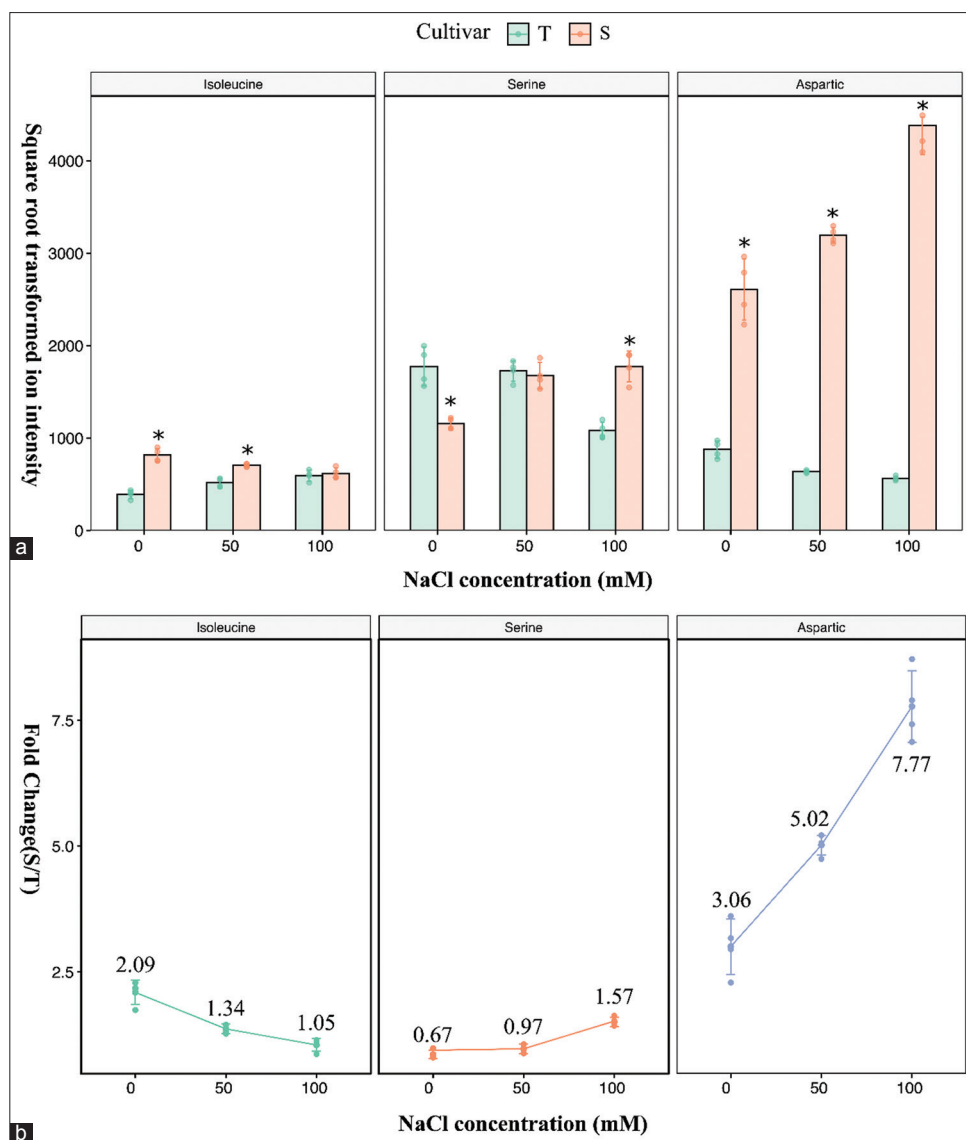
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Fig 6. The metabolic response of three metabolites were found different between salt tolerant cultivar Dongnong 69 (T) and salt sensitive cultivar Dongnong 63 (S) subjected to different salt treatments. (a) Bar plots of isoleucine, serine and aspartic acid that are highly induced in salt stress treated groups in Dongnong 69 (T) and Dongnong 63 (S). Bar plot represents mean + SD (n = 4) of square root transformed ion intensity. * denote significant differences (p < 0.05). (b) Line plots of fold change between Dongnong 63 (S) and Dongnong 69 (T) of the three metabolites after different salt treatments.

outstanding yield characteristics and an annual promotion area of more than 1 million mu, which has produced good returns. Therefore, with the promotion and application of molecular breeding technology, as long as the varieties are improved, local soybean cultivation still has great potential for development.

Many studies have shown that amino acids (AA) are essential to the development of salt tolerance (Guo et al. 2017). The results revealed that AA metabolism was largely involved in salt tolerant soybean cultivar to cope with salt stress, resulting in the substantial accumulation of asparagine, isoleucine, glycine and proline (Fig. 4b). The accumulation of these 4 AAs in Dongnong 69 may

partially explain the decrease of OA as OAs are precursors of AA biosynthesis and the salt stress results in the shift of biosynthetic pathway from OA to AA (Song et al. 2020). The elevated levels of glycine may suggest that it is important in keeping the osmotic potential and thus protecting cell injury from high sodium and chloride ions (Mishra et al. 2019). In addition, glycine is the precursors of glycine betaine, one of the key osmolytes; thus the accumulation of glycine therefore could keep high levels of glycine betaine and the soybean salt tolerance (Waditee et al. 2005). Frequent accumulation of proline has been repeatedly reported in plants, and it is a well-known osmoprotectant to conserve osmotic stability and prevent salt damage (Gharsallah et al. 2016). Our study also

showed that the free proline concentration of Dongnong 69 seedlings was significantly increased upon salt stress. Because of the nitrogen/carbon (N/C), asparagine is a key metabolites involved in nitrogen storage and transport from source to sink organs (Maaroufi-Dguimi et al. 2011). Our study here also supports the evidence that the content of asparagine increased to substantial levels when crops are subjected to salt stress (Gálvez-Valdivieso et al. 2005; Lea et al. 2007).

CONCLUSION

GC-MS technique employed in this study provides an efficient tool to characterize the physiological and biochemical changes in soybean seeding that are subjected to salt stress. Our results demonstrate that contents of a series of metabolites were substantially altered in salt stress treated soybean seedlings. A total of 10 metabolites were detected as potential differential biomarkers. Our results indicated that these biomarkers are vital in salinity tolerance in soybean. In addition, three metabolites, namely isoleucine, serine and aspartic acid, were found respond significantly differently between different soybeans. These three metabolites can be therefore served as potential biomarkers to screen for salt tolerant soybean cultivars. On the basis of the above observation, we can conclude that by maintaining metabolic homeostasis through biochemical changes, soybean seedlings are able to adapt to salt stress conditions. Therefore, this study helps to improve our knowledge with respect to plant salt tolerance in general, and soybean in particular.

ACKNOWLEDGMENT

Compliance with ethical standards
Author declares no conflict of interest.

Author contributions
XD performed the experiment; TP and YP carried out the experiment; XH and XL carried out the data and figures; MG designed the research.

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