Exogenous metabolites spray, which identified from metabolomics analysis and transcriptomic analysis, can improve salt tolerance of Chinese cabbages (*Brassica rapa* L. ssp pekinensis)*

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**ABSTRACT**

Soil salinization is one of the causes of the decline of the available cultivated land. By improving the salt tolerance of vegetables, the abandoned salinized land can be effectively utilized, saving the arable land for major crops. We investigated the salt tolerance of hundreds of Chinese cabbage cultivars, by which the two most tolerant and sensitive cultivars were selected and studied by metabololome and transcriptome analyses. The results showed that, under salt stress, metabolites’ response was not closely correlated with gene expressions in the tested Chinese cabbages. However, some KEGG pathways were significantly regulated at both metabolic and transcriptional levels. Furthermore, we identified several critical metabolites in the co-regulated pathways, including acetyl-CoA, pyruvic acid, ATP, nicotinic acid and Coenzyme Q10, which could alleviate the salt stress level in the tested Chinese cabbage. Thus, our findings provide candidate agents which can be used to improve salt tolerance in Chinese cabbage.

1. Introduction

During the past centuries, excessive agricultural activities have led to a rapid expansion of farmland salinization worldwide (Parida and Das 2005; Rozema and Flowers 2008). As a result, it is estimated that, by the year 2050, over half of the agricultural land will be vulnerable to salt stress (Wang et al. 2003), which poses a severe threat to the global food supply. Thus, cultivating salt-tolerant crops and uncovering the tolerance mechanism becomes more imperative and urgent.

Salt stress can cause sodium ions (Na+) toxicity, hypertonic stress, and oxidative damage in plants (Ashraf 2009; Zhu 2016; Shah et al. 2018). Excessive Na+ triggers several pathways in response, such as the SOS pathway, the MAP kinase pathway, and the ABA-signaling pathway (Zhu 2002; Bartels and Sunkar 2005; de Zelicourt et al. 2016). Studies have shown that engineering salt stress-related genes in these pathways can enhance salt tolerance in plants (Xu et al. 1996; Hu et al. 2006; Zhang et al. 2018; Ayadi et al. 2019). However, transgenic crops are limited in many countries and areas according to their agricultural practice regulations. Thus, other approaches, such as biochemistry methods, should also be developed to reduce soil salinization on crop production. Metabolites are the end products of many biological processes, which could directly regulate plant growth and development. Studies have shown that salt stress influences the presence and levels of many metabolites in plants (Forieri et al. 2016; Hossain et al. 2017; Lee et al. 2016; Tang et al. 2020), which can be considered as the final response of the plant to environmental changes and stresses (Fiehn 2002; Lee et al. 2016; Wi et al. 2020). Previous reports have indicated that adding some exogenous metabolites can alleviate the stress damage (Nounjan and Theerakulpisut 2012; Li et al. 2017; Irani and Todd 2018; Xiong et al. 2018). These metabolites’ critical functions have been discovered at the metabolic level through metabolomic profiling by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS).

*Brassica rapa* (Chinese cabbage – Pekinensis group) is the most popular vegetable in Northeast Asia, widely cultivated in China, Japan and Korea. However, all three countries’ cultivated land per capita is limited, and utilizing large-scale fertile land for vegetable production is not suitable for their...
current agricultural situation. Effective utilization of salinized land for Chinese cabbage can reduce the production cost and save more farmland for major crops. Hence, it is critical to understand Chinese cabbage’s functional mechanism in response to salt stress and identify the materials conveniently enhancing salt tolerance.

In this study, we planted 235 Chinese cabbage cultivars in salinized land. Ten and eight cultivars with the best and worst growth were selected for further tolerance studies in the laboratory. Finally, two cultivars from each category were selected and subjected to one-to-one comparison by metabolomics and transcriptome analyses. Differentially expressed metabolites (DEMs) and differentially expressed genes (DEGs) were identified and investigated in the four comparisons. Considering that the correlation between DEMs and DEGs was not consistent, we narrowed our study to the KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis and selected six co-regulated pathways. The critical metabolites from each pathway were selected, and their functions in the salt resistance of Chinese cabbage were investigated. Our results showed that acetyl-CoA, pyruvic acid, ATP, nicotinic acid and Coenzyme Q10 might be the candidate metabolites that could enhance the Chinese cabbage salt tolerance. Moreover, our study provides novel agents which can be utilized for reducing salt stress in Chinese cabbage.

2. Materials and methods

2.1. Experimental design

According to our proposal, the experiment was divided into three main steps (Figure 1). In the first step, we used 235 different Chinese cabbage varieties for the field salinity tolerance screening. In the second step, the two varieties with the most tolerance and the two varieties with the most susceptible were sent for the multi-omic (transcriptome and metabolome) analysis. After the correlation analysis between the DEMs and DEGs, the metabolites with potential salinity resistance were selected for the verification test. In the third step, the Chinese cabbage varieties with the most salinity tolerance were first put on the half-strength Murashige Skoog medium (½ MS) for germination for three days. Then the seedlings were transferred to the ½ MS medium with salinity stress and the corresponding metabolites for another ten days to verify the salinity resistance function.

2.2. Plant materials and treatment

Chinese cabbage seeds were germinated in the plug with normal soil in the climate room (20-25°C) for two weeks. Then, the seedlings were transferred to the identified saline (the salinity concentration equal to 31.3 mM NaCl) and alkali field in Lijin county, Dongying city, Shandong province, China (37°26′26.67″ N, 118°43′36.94″ E) for another 50 days.

For tolerance tests in media, the seeds were germinated on ½ MS for three days. Then, the same size seedlings were transferred to ½ MS with 31.3 mM NaCl for another seven days or added selected metabolites were for another ten days in a controlled environment at 22/20°C in a 16-h-light/8-h-dark photoperiod.

2.3. Metabolome with LC-MS/MS and data analysis

Samples (6 biological replicates) selected in salt tolerance phenotype in ½ MS with 31.3 mM NaCl were used to conduct metabolome analysis. At least 3 g tissues were frozen by liquid nitrogen in a mortar and homogenized manually using a pestle simultaneously, and the homogenate was resuspended with chilled 80% methanol and 0.1% formic acid. The samples were incubated on ice for 5 minutes and then centrifuged at 15000 g, 4°C for 5 minutes. The supernatant was diluted to a final concentration containing 60% methanol by LC-MS grade water. Subsequently, the samples were transferred to a fresh Eppendorf tube with a 0.22 μm filter and centrifuged at 15000 g, 4°C for 10 min. Finally, the 4 μl filtrate was injected into the LC-MS/MS system analysis. Liquid sample (100 μL) and chilled methanol (400 μL) were mixed totally.

LC-MS/MS analyses were conducted using Vanquish UHPLC system (Thermo Fisher) with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher). Using a linear gradient, samples were injected into a ThermoFisher Hyperil Gold column (ID 25002-102130, pore size 1.9 μm, length 100*2.1 mm). The eluent A (0.1% FA in Water) and eluent B (Methanol) were used for positive polarity mode. The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 16 min. Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and aux gas flow rate of 10 arb.

The raw data files generated by UHPLC-MS/MS were processed by the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) for each metabolite to conduct peak alignment, peak picking, and quantitation. The main parameters were set as follows: retention time tolerance: 0.2 minutes; actual mass tolerance: 5 ppm; signal intensity tolerance: 30%; signal/noise ratio: 3; and minimum intensity: 100000. Peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. Finally, peaks were matched with the mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) qualitative relative quantitative results. LC-MS/MS assay was performed by Novogene Co., Ltd.(Beijing, China).

2.4. Transcriptome sequencing and data analysis

Samples (3 biological replicates) selected in salt tolerance phenotype in ½ MS with 31.3 mM NaCl were used to perform RNA-seq analysis. The total RNA was isolated by Trizol, and mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, universal
PCR primers and index (X) Primer. Finally, PCR products were purified (AMPure XP system), and the library quality was evaluated on the Agilent Bioanalyzer 2100 system.

The clustering of the samples was conducted on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the cDNA libraries were sequenced on an Illumina Novaseq platform to generate 150 bp paired-end reads. For novel transcript prediction, each sample’s mapped reads were assembled by StringTie (v1.3.3b) (Pertea et al. 2015). StringTie used a novel network flow algorithm and an optional de novo assembly step to assemble and quantitate full-length transcripts, representing multiple splice variants for each gene locus. Novogene carried out total RNA isolation, library construction, sequencing, and raw data analysis.

2.5. Correlation analysis between DEMs and DEGs

Three independent biological replicates from the six were randomly selected in the DEMs and co-analyzed with DEGs from the independent biological replicates. Fifty DEMs and one hundred DEGs whose P-value range from the lowest value from all the DEMs and DEGs were analyzed by the Pearson correlation coefficient (Adler and Parmryd 2010).

2.6. Statistical analysis

All experiments were conducted in triplicates. Data were analyzed using variance (ANOVA) analysis with Duncan’s multiple range test (DMRT). Standard errors were calculated for all mean values, and differences were considered significant at the $p \leq 0.05$ level.

3. Results and discussion

3.1. Screening salt-tolerant cultivars from salinized farmland

For Chinese cabbage, different cultivars exhibit different shapes, growth cycles and stress tolerance. Therefore, it is of significant interest to us to directly select stress-tolerant cultivars in the agricultural land. Thus, in the past three years, we carried out salinity tolerant cultivar selection of Chinese cabbage in the salinized farmland of

![Figure 1. Workflow of the experiment.](image-url)
Shandong province, China. Before the selection, five soil samples from different locations widely and randomly spread in the salt-affected field were mixed and collected to measure exchangeable Na⁺. The average concentration of the Na⁺ was 720 mg/kg (31.3 mM). A total of 235 Chinese cabbage cultivars were transferred to the soil after a two-week germination period in the climate room. For each row, one cultivar was planted, which included twenty-one plantlets. As shown in Figure 2A and B, ten cultivars showing the highest survival rate (more than 16/21) and the most robust growth phenotype, and eight cultivars showing the lowest survival rate and salt-sensitive phenotype were selected for further analysis.

3.2. Selecting salt-tolerant cultivars under the laboratory condition

To exclude other factors in the field, such as irregular rain, changes of climate, and other abiotic stresses besides NaCl stress, and to narrow the target salt-tolerant cultivars, we carried out the tolerance test using eighteen selected cultivars in the ½ MS media with 31.3 mM NaCl in the controlled chamber. To avoid the difference in germination, the seeds germinated on ½ MS for three days and then transferred to the media with salt to grow for seven days. After three independent tolerance tests, two of the ten selected species, Lvyingxiaobaicai and Fengkang50, showed significant salt-tolerant phenotypes, which grew stronger with NaCl than the control (Figure 2C). These data are consistent with our tolerance tests in the field and can be used for further analysis.

3.3. Metabolite profiling and data analysis

The salt tolerance and sensitive phenotypes are likely due to the difference of specific metabolites in different cultivars. As metabolites can directly change the plants physiological and biochemical traits, a metabolome analysis was conducted on salt-treated plants compared with salt-tolerant species, Fengkang50 and Lvyingxiaobaicai against salt-sensitive cultivar Banzha819 for discovering the mechanisms of salt-tolerance. Two thousand one hundred eighty-three metabolites were identified following three days of germination and then exposed to 7 d of salt stress by LC-MS/MS. Two thousand one hundred eighty-three metabolites were identified using the positive ion mode, and 1266 were identified using the negative ion mode (Supplementary Table S1). Principal component analysis (PCA) of the total samples showed that the quality control samples were clustered together. Therefore, all six independent biological replicates from each group are in the surrounding areas of the quality control samples (Supplementary Fig. S1A and B). The partial least-squares discriminant analysis (OPLS-DA) was also carried out to detect the differences between each pair of the four varieties. As shown in Supplementary Fig. S1C-J, it is obvious that a non-negligible genotypic difference exists between each pair. These data suggested that the experimental data were well controlled.

3.4. Differentially expressed metabolites identified from metabolome analysis in the four comparison groups pairs

To determine the salt tolerance mechanism in Chinese cabbage at the metabolic level, we compared the metabolites identified from the two salt-tolerant cultivars with salt-sensitive cultivars. In the four comparison pairs, the metabolite profile of Fengkang50 compared to the Julongqiulv75 is hereafter referred to as comparison 1 or C1; likewise, Fengkang50 compared to Banzha819 is C2; Lvyingxiaobaicai compared to Julongqiulv75 is C3; Lvyingxiaobaicai compared to Banzha819 is C4. Differentially expressed metabolites (DEMs) with statistically significant change, variable importance in the projection (VIP) of PLS-DA > 1.0, fold change (FC) > 2.0 or < 0.5, and P-value < 0.05 (t-test), were selected. In C1, 188 DEMs are up-regulated and 237 down-regulated; In C2, 388 DEMs are up-regulated and 278 down-regulated; In C3, 332 DEMs are up-regulated and 295 down-regulated; and there are 440 up-regulated and 295 down-regulated DEMs in C4 (Figure 3A). As shown in Figure 3B, 1398 DEMs were involved in the four comparisons, and the intersection analysis showed that only 28 DEMs were included in all four comparisons (Figure 3A and C) (Supplementary Table. S2).

3.5. Transcriptome analysis in the four comparison pairs

The salt-tolerant and sensitive phenotypes of the four cultivars could also be associated with gene expression. RNA-seq analysis was conducted on the four comparisons (C1, C2, C3 and C4) to verify the DEGs' expression pattern. Differentially expressed genes (DEGs) with statistically significant change (a fold change > or < 1, DESeq adjusted P-value < 0.8) (Love et al. 2014) in C1, C2, C3 and C4 were selected. In the four comparison pairs, the largest number of DEGs (3680 up-regulated and 3399 downregulated) was identified in C2 (Fengkang50 vs Banzha819), and the least number of DEGs (1901 up-regulated and 1802 downregulated) was identified in C3 (Lvyingxiaobaicai vs Julongqiulv75) (Figure 4A). As shown in Figure 4B, 524 DEGs are common to the four comparisons. However, more DEGs specifically exist in different comparisons. These results suggested that different Chinese cabbage cultivars could share specific salt tolerance mechanisms at the transcriptional level.

3.6. Co-analysis of metabolomics and transcriptome

Correlation analysis was performed between metabolomics and transcriptomics to reveal the consistency between DEMs and DEGs. One hundred DEGs and fifty DEMs were randomly selected according to the P-value (from lowest to highest scores) and compared. As shown in Supplementary Fig. S2, the correlation between DEMs and DEGs is not well consistent. In C1, over half of the selected DEMs and DEGs were negatively correlated. It is suggested
that the DEMs at the metabolic level did not directly associate with the DEGs at the transcriptional level. According to the KEGG database, the DEMs and DEGs were compared and assigned to further explore the fundamental functional salt-tolerant pathways. Tables 1 and 2 show that eleven KEGG pathways were identified in the four comparison groups at the metabolic level, and twenty-six KEGG pathways were identified at the transcriptional level. We also conducted a correlation analysis of KEGG pathways at metabolic and transcriptional levels (Supplementary Fig. S3). The results indicated that DEMs and DEGs' enrichments from the KEGG database were inconsistent with the Chinese cabbage with salt stress.

3.7. Identification of metabolite candidates in salt tolerance of Chinese cabbage

However, among the pathways, Citrate cycle (TCA cycle), Pyruvate metabolism, Oxidative phosphorylation, Nicotinate and nicotinamide metabolism, alpha-Linolenic acid metabolism, and Ubiquinone and other terpenoid-quinone biosynthesis were co-enriched in the four comparison groups during the metabolic and transcript KEGG analysis. Therefore, we speculated that these functional pathways could play an important role in salt tolerance in Chinese cabbage. Then, we selected the critical metabolites or ultimate products in these six metabolic pathways, including

![Figure 2. Salt tolerance identification from 235 different Chinese cabbage cultivars in the saline field (37°26′26.67″N, 118°43′36.94″E). (A) The ten most salt tolerance species were selected from the 235 independent lines in the saline farmland. (B) The eight most salt-sensitive species were selected from the 235 independent lines in the saline farmland. White numbers in the parenthesis are the survival ratio. (C) Chinese cabbage plants germinated on ½ MS plates vertically for three days were transferred to plates with (or without) 31.3 mM NaCl for another seven days. Representative results from three independent experiments are shown.](image-url)
Acetyl-CoA from the TCA cycle; Pyruvic acid from the Pyruvate metabolism; ATP from the Oxidative phosphorylation; Nicotinic acid from the Nicotinate and nicotinamide metabolism, Linolenic acid from the alpha-Linolenic acid metabolism; and the Coenzyme Q10 from the Ubiquinone and other terpenoid-quinone biosynthesis for the salt resistant functional test in the Chinese cabbage. As shown in Figure 5 A and B, five of the six selected metabolites (acetyl-CoA, pyruvic acid, ATP, Nicotinic acid and Coenzyme Q10) can enhance the salt tolerance of salt-sensitive Chinese cabbage cultivars: Banzha819 and Julongqiulv75.
5.8. Potential function of the metabolites in salinity stress adaptation

The survival of plants under abiotic stress conditions relies on integrating stress adaptive metabolic and structural changes into endogenous developmental programs. For example, it is reported that cucumber increases the metabolic efficiency of the glycolytic (EMP) pathway–TCA cycle, which produce a large amount of energy and intermediates to support plant growth, thereby enhancing their salt tolerance (Li et al. 2020). In this research, the Acetyl-CoA might also participate in the TCA cycle to help Chinese cabbage adapted to the salinity stress. Since Pyruvic acid was the final product of the EMP pathway, and it not only plays an essential role in improving salt tolerance (Wu et al. 2013), but it also links the EMP pathway to the TCA cycle. Pyruvic acid produced in the cytoplasm is transported to the mitochondrial matrix by a specific transporter on the mitochondrial inner membrane, a substrate for TCA cycle metabolism. This proof could also explain the co-expression pattern of the TCA cycle and Pyruvate metabolism in this research. Excessive salinity in soil can also induce reactive oxygen species (ROS) accumulation in plants (Achard et al. 2008), which helps enhance the resistance of plants to salt stress (Miller et al. 2007). The Oxidative phosphorylation was believed to participate in the last phase of the aerobic respiration pathway in mitochondria to generate ROS, enhancing plants’ tolerance to salinity stress (Huang et al. 2016; Mignolet-Spruyt et al. 2016). Thus, ATP in this study might be an energy material in ATP binding cassette (ABC) transporter and a signal molecule to promote downstream biosynthesis machinery activity and reinforce defence response to salinity stress.

Table 1. KEGG pathways of DEMs identified in C1, C2, C3 and C4. Pathway terms whose P-value ≤ 0.05 were selected as the significantly enriched terms. X: the number of DEMs involved in the KEGG pathway items; Y: the number of metabolites in the KEGG pathway items.

| Comparison | Pathway | P-value | x | y | MEDs | Ion detection mode |
|------------|---------|---------|---|---|------|-------------------|
| C1         | * Citrate cycle (TCA cycle) | 0.00186176 | 3 | 5 | Fumaric acid, Citric acid, Succinate | ESI- |
|            | Alanine, aspartate and glutamate metabolism | 0.00612541 | 3 | 7 | Fumaric acid, Citric acid, Succinate | ESI- |
|            | * Pyruvate metabolism | 0.0159733 | 2 | 3 | Fumaric acid, Succinate | ESI- |
|            | * Oxidative phosphorylation | 0.05636181 | 5 | 5 | Fumaric acid, Succinate | ESI- |
|            | Butanoate metabolism | 0.03636181 | 2 | 5 | Fumaric acid, Succinate | ESI- |
|            | * Nicotinate and nicotinamide metabolism | 0.03636181 | 2 | 5 | Fumaric acid, Succinate | ESI- |
| C2         | * alpha-Linolenic acid metabolism | 0.03248404 | 2 | 2 | Traumatic Acid, 9-Oxononanoic acid | ESI- |
|            | Flavonoid biosynthesis | 0.02284404 | 2 | 2 | Isoliquiritigenin, Prunin | ESI- |
| C3         | Flavone and flavonol biosynthesis | 0.00433756 | 4 | 9 | Kaeppherol, Rhoifolin, Quercetin 3-O-sophoroside, Quercetin | ESI+ |
| C4         | Plant hormone signal transduction | 0.03112967 | 2 | 2 | Jasmonic acid, (+)-Abscisic acid | ESI+ |
|            | Flavone and flavonol biosynthesis | 0.04019061 | 3 | 5 | Kaeppherol, Apiin, Quercetin | ESI+ |
|            | * alpha-Linolenic acid metabolism | 0.01998842 | 2 | 2 | Traumatic Acid, 9-Oxononanoic acid | ESI- |

Table 2. KEGG pathways of DEGs identified in C1, C2, C3 and C4. Pathway terms whose P-value ≤ 0.05 were selected as the significantly enriched terms.

| Comparison | Pathway | P-value | x | y | MEDs | Ion detection mode |
|------------|---------|---------|---|---|------|-------------------|
| C1         | Phagosome | 0.00052383 | 37 | 6 | 31 | ESI- |
|            | Protein export | 1.73E-02 | 21 | 2 | 19 | ESI- |
|            | Homologous recombination | 4.40E-02 | 30 | 14 | 16 | ESI- |
|            | * Nicotinate and nicotinamide metabolism | 4.81E-02 | 9 | 4 | 5 | ESI- |
|            | Photosynthesis | 6.30E-18 | 55 | 1 | 54 | ESI- |
|            | Photosynthesis - antenna proteins | 3.84E-10 | 20 | 0 | 20 | ESI- |
|            | Ribosome | 8.04E-05 | 130 | 112 | 18 | ESI- |
| C2         | Carbon fixation in photosynthetic organisms | 0.00119843 | 38 | 17 | 21 | ESI- |
|            | Glycolylate and dicarboxylate metabolism | 0.00132315 | 40 | 15 | 25 | ESI- |
|            | Fatty acid elongation | 0.00296042 | 24 | 1 | 23 | ESI- |
|            | Carbon metabolism | 0.00300383 | 114 | 60 | 54 | ESI- |
|            | Glycine, serine and threonine metabolism | 0.00313479 | 36 | 12 | 24 | ESI- |
|            | Thiamine metabolism | 0.00946009 | 13 | 4 | 9 | ESI- |
|            | Peroxisome | 0.01059394 | 41 | 21 | 20 | ESI- |
|            | Aminoacyl-tRNA biosynthesis | 0.02804002 | 28 | 24 | 4 | ESI- |
| C3         | Cutin, suberine and wax biosynthesis | 0.04001972 | 16 | 2 | 14 | ESI- |
|            | * Pyruvate metabolism | 0.00134096 | 23 | 8 | 15 | ESI- |
|            | * Citrate cycle (TCA cycle) | 0.00136011 | 19 | 10 | 15 | ESI- |
|            | Homologous recombination | 0.00264832 | 24 | 7 | 17 | ESI- |
|            | Carbon metabolism | 0.00464589 | 57 | 29 | 28 | ESI- |
|            | Ribosome | 0.02158922 | 58 | 37 | 21 | ESI- |
|            | * Oxidative phosphorylation | 0.02387215 | 29 | 18 | 11 | ESI- |
|            | Steroid biosynthesis | 0.03601239 | 10 | 7 | 5 | ESI- |
| C4         | Photosynthesis | 2.16E-11 | 39 | 3 | 36 | ESI- |
|            | Photosynthesis - antenna proteins | 3.75E-08 | 16 | 1 | 15 | ESI- |
|            | Ribosome | 0.00050936 | 94 | 82 | 12 | ESI- |
|            | Oxidative phosphorylation | 0.00096966 | 47 | 43 | 4 | ESI- |
|            | Aminoacyl-tRNA biosynthesis | 0.00096966 | 47 | 43 | 4 | ESI- |
|            | Aminoacyl-tRNA biosynthesis | 0.00218484 | 25 | 22 | 3 | ESI- |
|            | Glycolipid metabolism | 0.00661065 | 30 | 10 | 20 | ESI- |
|            | Fatty acid degradation | 0.01059394 | 24 | 1 | 23 | ESI- |
|            | Homologous recombination | 0.01683085 | 18 | 14 | 4 | ESI- |
|            | * alpha-Linolenic acid metabolism | 0.00702685 | 17 | 11 | 6 | ESI- |
|            | Steroid biosynthesis | 0.03601239 | 10 | 7 | 5 | ESI- |

2-Oxocarboxylic acid metabolism | 0.00132315 | 40 | 15 | 25 | ESI- |

3.8. Potential function of the metabolites in salinity stress adaptation

The survival of plants under abiotic stress conditions relies on integrating stress adaptive metabolic and structural changes into endogenous developmental programs. For example, it is reported that cucumber increases the metabolic efficiency of the glycolytic (EMP) pathway–TCA cycle, which produce a large amount of energy and intermediates to support plant growth, thereby enhancing their salt tolerance (Li et al. 2020). In this research, the Acetyl-CoA might also participate in the TCA cycle to help Chinese cabbage adapted to the salinity stress. Since Pyruvic acid was the final product of the EMP pathway, and it not only plays an essential role in improving salt tolerance (Wu et al. 2013), but it also links the EMP pathway to the TCA cycle. Pyruvic acid produced in the cytoplasm is transported to the mitochondrial matrix by a specific transporter on the mitochondrial inner membrane, a substrate for TCA cycle metabolism. This proof could also explain the co-expression pattern of the TCA cycle and Pyruvate metabolism in this research. Excessive salinity in soil can also induce reactive oxygen species (ROS) accumulation in plants (Achard et al. 2008), which helps enhance the resistance of plants to salt stress (Miller et al. 2007). The Oxidative phosphorylation was believed to participate in the last phase of the aerobic respiration pathway in mitochondria to generate ROS, enhancing plants’ tolerance to salinity stress (Huang et al. 2016; Mignolet-Spruyt et al. 2016). Thus, ATP in this study might be an energy material in ATP binding cassette (ABC) transporter and a signal molecule to promote downstream biosynthesis machinery activity and reinforce defense response to salinity stress.
Stress acclimating plants respond to abiotic and biotic stress by remodeling membrane fluidity and releasing ω-linolenic acid from membrane lipids (Chen, et al. 2021). Fatty acid desaturases, like Nicotinic acid, play an essential role in fatty acid metabolism and maintenance of the biological function of membranes in plant cells (Sui, et al. 2017). Under salinity stress, fatty acid desaturase activity and fatty acid composition were markedly changed in peanut plants (Sui, et al. 2018). In this research, the Nicotinic acid also increased the Chinese cabbage tolerance to salinity stress, implied it is a potential candidate metabolite for salinity adaptation. Coenzyme Q10 is primarily identified with its role in energy coupling, where it is involved in the generation of a proton gradient across membranes to drive ATP formation (Barros, et al. 2016). It is reported that Ubiquinone and other terpenoid-quinone biosynthesis DEGs were enriched in salinity tolerance variety RNA-seq data (Dugasa, et al. 2021). Thus, we believe Coenzyme Q10 might enhance the Chinese cabbage salinity tolerance by an unknown mechanism.

4. Conclusions

Salinity is one of the major stresses which limit the production of crops and vegetables. Different crops share specific mechanisms of stress resistance. Therefore, it is a realistic strategy to compare hundreds of different Chinese cabbage cultivars better to understand the specific salt tolerance mechanisms in Chinese cabbage. We analyzed two extremely salt-tolerant and sensitive cultivars of Chinese cabbage from hundreds of cultivars at the metabolic and transcriptional levels through modern omics technologies. Five of six critical metabolites or ultimate products, including acetyl-CoA, pyruvic acid, ATP, Nicotinic acid and Coenzyme Q10, could enhance the salt tolerance in the two cultivars. It is indicated that exogenous application of the metabolites could improve salt resistance for Chinese cabbage.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and material

The data that support the findings of this study are available from the corresponding author, Jian-Wei Gao, upon reasonable request.

Authors’ contributions

Lilong He, Shu Zhang, Jingjuan Li and Lin Shi, performed the experiments, including field experiments during the period of two or three years with assistance from Qianyu Zhao; Lilong He, Shu Zhang, Jingjuan Li and Lin Shi carried out most of the analyses with assistance from Yongqing Li, Shaohua Zeng and Zhigeng Wu; Lilong He, Shu Zhang and Jianwei Gao designed the project and experiments; Shu Zhang and Lilong He wrote and edited the manuscript, and Jianwei Gao, Feng-De Wang and Wei Zhang reviewed the draft. All authors read and approved the final manuscript.

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Figure 5. Salt tolerance bioassay in Chinese cabbage with the selected metabolites. (A) Chinese cabbage plants germinated on 0.5X MS plates vertically for three days were transferred to plates with 31.3 mM NaCl alone or adding acetyl-CoA (100 µM), pyruvic acid (100 µM), ATP (10 µM), Nicotinic acid (100 µM) and Coenzyme Q10 (100 µM) for another ten days. Representative results from three reproducible experiments are shown. Scale bar: 1cm. (B) The root length of five seedlings of each class was measured as the mean value (removing the top and lowest values). Error bars indicate ± SD from three independent experiments. P values from Student’s t-test were determined for the salt- and metabolite-treated seedlings compared with the same only salt-treated cultivar seedlings: *P < 0.05; **P < 0.01; ***P < 0.001.
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