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Analysis of metabolite pathway in saline-alkali flax leaves by nano-iron

Zhang Yanping[1], Zhao Wei[2*], Wang Limin[2], Jia Xiuping[2], Qi Yanni[2], Xie Yaping[2], Wu Zhiguo[3], Chai Zongwen[4], Allah Wadhayo Gandah[5], Abdul Ghaffar Shar[6]

[1] Institute of Biotechnology, Gansu Academy of Agricultural Sciences, 1# Xin village, Agricultural Science Academy, Lanzhou, 730070, China
[2] Crop Research Institute, Gansu Academy of Agricultural Sciences, 1# Xin village, Agricultural Science Academy, Lanzhou, 730070, China
[3] School of Physical Science and Technology, Lanzhou University, 222# South Tianshui Road, Lanzhou, 730000, China
[4] Gansu General Station of Agro-Technology Extension, 708# West Jiayuguan Road, Lanzhou, 730020, China
[5] Department of Soil Science, Sindh Agriculture University, Tandojam, 70060, Pakistan
[6] Xi'an Fanyi University, 123# Taiyigong street, Xi'an, 710105, China
* Corresponding author's e-mail: 308214921@qq.com

ABSTRACT

Iron is the most demanded trace element among the essential micronutrients for plants, participating in the electron transport chain processes such as cytochrome and ferredoxin in photosynthesis. Iron can activate enzymes like catalase and peroxidase, helping to eliminate free radicals and enhance the stress resistance of crops. In recent years, nano-iron powder has been widely applied in agricultural production, including in grains, oilseeds, and Chinese herbal medicines, with significant yield-increasing effects. However, there are no reports on the application of nano-iron in improving the salt and alkali tolerance of flax and its physiological mechanism. This study aimed to verify the effect of nano-iron on the growth traits of flax in saline-alkali soil through the analysis of growth traits and metabolic pathways, and to explore the mechanism of action of related metabolites in promoting salt tolerance in flax through statistical analysis. The results showed that, emergence rate, survival rate, root diameter and stem diameter of flax increased by 24.8%, 22.5%, 25.7% and 6.9%, respectively, in saline-alkali soil after nano-iron treatment. Through non-targeted metabolomics testing, 15 up-regulated metabolite types including amino acids and their derivatives, ketones, and lactones were screened out, and their information related to the salt tolerance of flax was obtained. By screening metabolic pathways and analyzing the salt tolerance mechanism, four pathways were selected from 20 metabolic pathways, and the metabolites related to salt tolerance were precisely screened out on each pathway. Among them, five metabolites, including arginine-threonine-lysine-arginine peptide, Histidine leucine, N-L-histidine L-leucine, Arginine-isoleucine-threonine-valine-lysine polypeptide and Na-ser-oh, were screened out on the amino acid metabolism pathway; two metabolites, PA and PC, were screened out on the lipid metabolism synthesis pathway; one metabolite, lupeol, was screened out on the sesquiterpene and triterpene biosynthesis pathway; one metabolite, 3,5-dihydroxydecanoate, was screened out on the alcohol biosynthesis pathway.

Keywords: flax, saline land, nano-iron, metabolic pathway.

INTRODUCTION

Nanomaterials (1–100 nm) are significantly different from conventional materials and widely gained attention in agricultural research during recent years (Muhammad et al., 2020; Tripathi et al., 2023; Zaman et al., 2025). Iron is the most demanded of essential trace elements for plants and is the "core driver" of chlorophyll synthesis. When iron is lacking, chloroplasts in plants cannot develop normally, which hinders photosynthesis (Therby-Vale et al., 2022). Iron is also involved in the process of electron transport chains, such as cytochrome and ferroredoxin

in photosynthesis convert energy into ATP (Rai et al., 2022; Alam et al., 2025). In addition, iron can activate catalase, peroxidase, etc., which help eliminate free radicals and enhance crop stress resistance (Azam et al., 2025). Metal nanomaterials are zero-dimensional and has exotic properties that are different from macroscopic objects, individual atoms and chemistry (Muhammad et al., 2020). In recent years, nano-iron powder has been widely used in agricultural production, involving grains, oils, and Chinese herbal medicines, with notable yield-increasing effects (Dewi et al., 2022; Ahmad et al., 2024; Pérez-Hernández et al., 2024). Relevant research on crops showed that nano iron powder significantly promoted the root development, leaf thickness and chlorophyll content, and improve the stress resistance of crops (El-Desouky et al., 2021; Rani et al., 2022; Rahman et al., 2023; Azam et al., 2025).

Flax is a major oil crop grown in arid and semi-arid regions in Gansu and Inner Mongolia, China, and has very important nutritional and economic value (Liu et al., 2011). Flax seeds are rich in omega-3 and alpha-linolenic acid, lignans and dietary fiber (Noreen et al., 2025). Flax metabolites can provide effective assistance in studying biological processes and mechanisms (Gao et al., 2024; Ehsan et al., 2023: Khan et al., 2024). The metabolic basis of macroscopic phenotypes in different crop individuals can be compared through qualitative and quantitative analysis of metabolites in different metabolic pathways or networks (Somalraju and Fofana, 2023; Dong et al., 2024; van Aubel et al., 2024). Non-targeted metabolome analysis can be used to find the differential metabolites between treatments and control group, and to clarify their links to biological processes or states (Yang et al., 2018; Wu et al., 2021). This study discussed the effect of nano-iron on the growth traits of flax from saline-alkali soils, and identified the mechanism of nano-iron on salt resistance of flax associated with the metabolic pathways.

MATERIALS AND METHODS

Study site and materials

The study site is located in Luyang Town, Jingtai County, Gansu Province and was a severely saline-alkali land with soil pH value of 8.63 and total salt content of 0.81%. Nano iron was provided by Gansu Gushuo Nano Agricultural Technology Co., Ltd., and the flax variety Longya No.14 was provided by Gansu Academy of Agricultural Sciences. 15 g nano-iron per hectare was applied, including 7.5 g for dressing with 75 kg flax seed, 3 g for spraying during the fir period (5–10 cm), and 4.5 g for spraying during the rapid growth period. The test materials are grouped as shown in Table 1.

Sample testing

One week after the treatments were completed, the leaves of the treated and control flax were taken respectively (three repetitions) and stored in an ultra-low temperature refrigerator for metabolic pathways analysis. Non-target metabolomic tests were conducted to identify the differential metabolites. The flax samples were first placed in a freezer dryer and vacuum freeze-dried for 63 hours. The dried samples were ground into a fine powder by a grinder set at 30 Hz for 1.5 minutes. 50 mg of the sample powder was extracted with 1200 μL of 70% methanol-water solution (precooled at -20 °C). The mixture was vortexed for 30 seconds, which repeated six times at 30-minute intervals, and then centrifuged for 3 minutes at 12,000 rpm. At last, the supernatant was collected and filtered through a 0.22 μm micropore membrane, and stored for UPLC-MS/MS analysis.

Data analysis

In this study, the principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed.

Table 1. The experimental materials

Species	Tissue	Sample	Group
Flax	leaf	NL-1	NL
Flax	leaf	NL-2	NL
Flax	leaf	NL-3	NL
Flax	leaf	CKL-1	CKL
Flax	leaf	CKL-2	CKL
Flax	leaf	CKL-3	CKL

Univariate analysis primarily involved fold change (FC) analysis. The metabolites were identified based on variable importance in projection (VIP) scores derived from the OPLS-DA model. Further screening of differential metabolites was conducted by integrating VIP values with P-values, false discovery rates (FDR), or fold change values obtained through univariate analysis. By combining both univariate and multivariate approaches, a total of 550 metabolites were ultimately identified, of which 253 were up-regulated and 297 were down-regulated.

RESULTS

The effects of nano-iron on growth traits of flax in saline-alkali land

The test results show, Nano-iron treatment had significant effects on the growth traits of flax. Compared with the control group, the emergence rate, seedling preservation rate of flax from saline-alkali land increased by 24.8%, 22.5%; The standard for root thickness is the most obvious. was that the experimental treatment was increased compared with the control 25.7%,The thickness of the stem only increased 6.9%, We believe that the thickening effect of nano-iron on the roots of flax might be the main reason for its strong stress resistance (Table 2).

Metabolomic analysis

Group principal component analysis

In this study, PCA was first performed on the grouped samples to observe the degree of variation between different groups of samples. The results showed that the NL three replicates were all distributed in the range of less than PC1 value -40, while the CKL three replicates were all greater than 40, indicating significant differences among the samples (Figure 1).

Orthogonal partial least squares discriminant analysis

PLS-DA method was used to solve insensitivity of variables with low correlation. Compared with PCA, PLS-DA maximizes the distinction between groups, which is beneficial for finding differential metabolites. Where X is the sample quantitative information matrix and Y is the sample grouping information matrix. OPLS-DA performs $\log^2$ conversion on the original data and then centralizes it. The results showed that the

Table 2. Effects of nano-iron on flax growth in saline-alkali land

Serial number	Emergence rate (%)	Seedling preservation rate (%)	Root thickness (mm)	Stem thickness (mm)
NL	75.5	66.9	2.66	2.02
CKL	60.5	54.6	2.12	1.89
Increase (%)	24.8	22.5	25.7	6.9

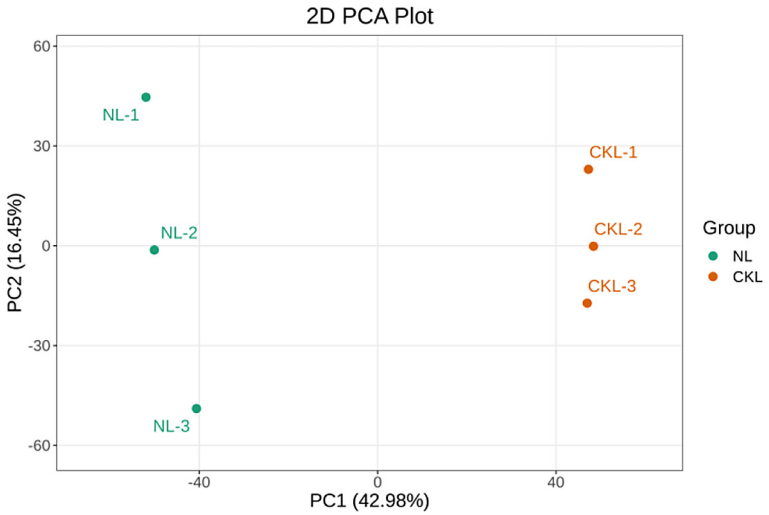

Figure 1. Principal component analysis of the groups

grouped samples were mostly distributed at the two ends of the plus - minus 50 intervals on the X-axis, with significant differences (Figure 2).

Volcano plot of Differential metabolites

A Volcano Plot is primarily used to show the difference in the relative content of metabolites between two groups of samples and the significance of the difference statistically. In this study, the metabolites were screened by the condition "metabolites with VIP > 1; metabolites with fold change ≥ 2 and fold change ≤0.5". If the differences of metabolites between the control and treatments were more than 2 times or less than 0.5 times, the result was considered significantly. The experimental results show that the number of up-regulated metabolites is 491, the number of down-regulated metabolites is 503, and there is no significant change in 5.351 metabolites (Figure 3).

Bar chart of differential metabolites

By analyzing 491 upregulated metabolites, we ultimately screened out 15 metabolites with significant differences.The 15 upregulated metabolites were amino acids and their derivatives, ketones, lactones, phosphatidylic acids, phosphatidylcholine, benzene and its derivatives, triterpenoids, organic acids, alcohols, etc (Table 3).

Metabolic pathway screening

The screening of metabolic pathways in this study was mainly based on the differential abundance Score (DA Score), which captures the overall changes of all metabolites in a certain pathway. The DA score is an analytical method based on metabolic changes in pathways, and is the difference between up-regulated number of differential metabolites and down-regulated number of differential metabolites annotated to the total number of metabolites of the pathway. The top 20 pathways were shown in Figure 4).

In this study, four of the 20 metabolic pathways had corresponding upregulated metabolites. Among them, 5 metabolites were identified for the D-Amino acid metabolism pathway, 2 metabolites were screened on the Ether lipid metabolism synthesis pathway; 1 metabolites was screened for Sesquiterpenoid and triterpenoid biosynthesis metabolic pathway; 1 metabolite screened for the Steroid biosynthesis metabolic pathway (Table 4).

DISCUSSION

Effects of nano-iron application on flax growth in saline-alkali land

Results of this study showed that nano-iron treatments had a significant effect on the growth traits of flax from saline-alkali land. Compared with the control group, the emergence rate, seedling preservation rate, root and stem thickness all increased. As a nanomaterial, nano-iron has high reactivity and can reduce the accumulation of excessive sodium ions in plants by adsorbing or binding them, thereby reducing salt stress damage to flax (Shahzad et al., 2024). In addition, nano-iron

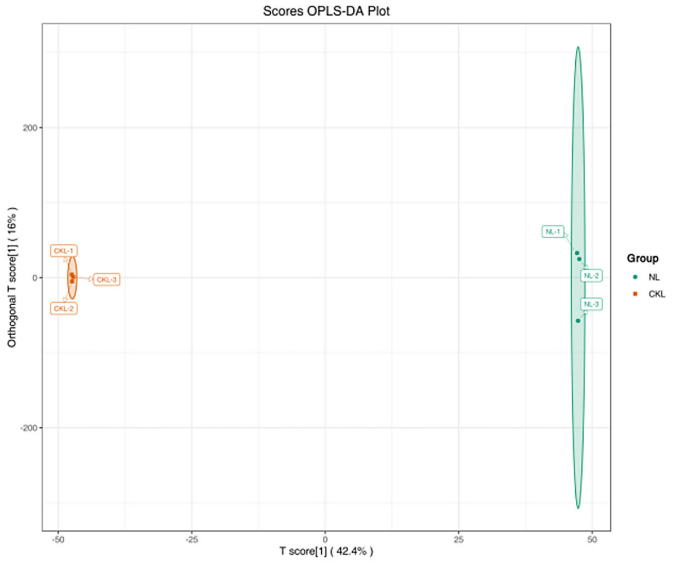

Figure 2. Path of OPLS-DA score graph

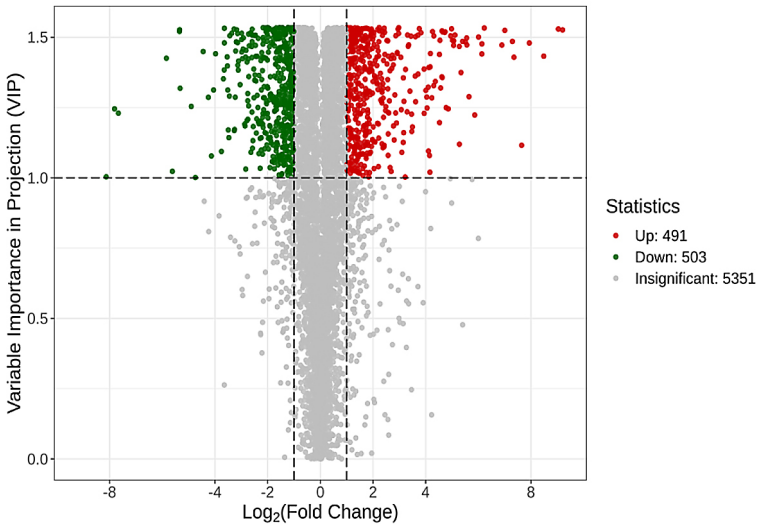

Figure 3. Volcano map of differential metabolites

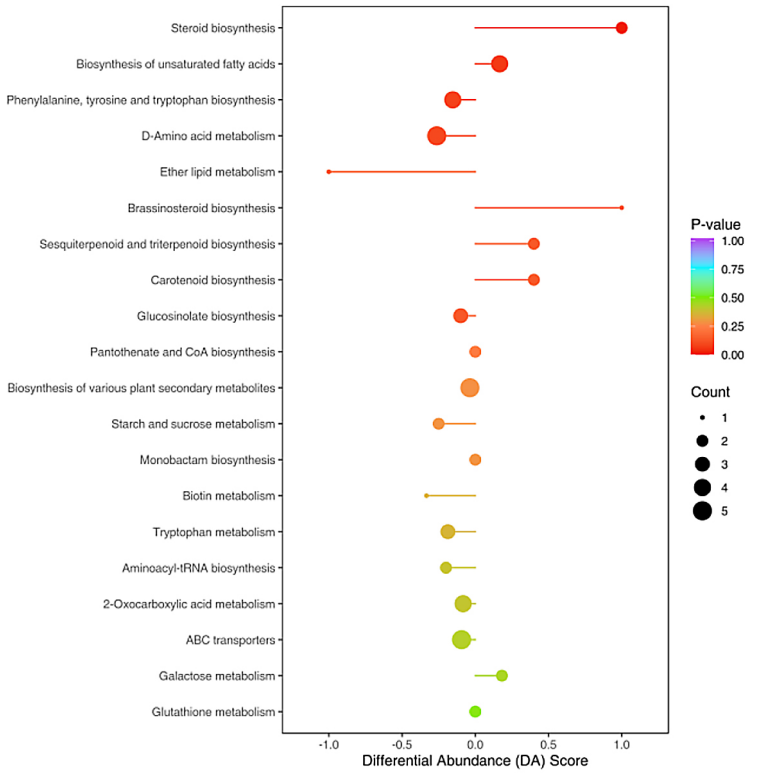

Figure 4. Variance abundance score graph

can reduce oxidative stress caused by salt and regulate the activity of antioxidant enzymes in plants, which protect the integrity of cell membrane structure and function (Alqudah et al., 2025). Secondly, the effect of nano-iron on salt resistance of flax is also reflected in promoting plant nutrient absorption. Salt stress not only causes ionic poisoning, but also prevent plants absorbing essential nutrients, such as iron whose deficiency will aggravate the growth inhibition of plants (Verma et al., 2018; Sarkar and Kalita, 2023). Nano-iron, as an iron source, can be effectively absorbed by flax roots, and can continuously provide iron to plants for the slow-release properties of nanoparticles, and then to promote chlorophyll synthesis and photosynthesis (Ullah et al., 2024). In addition, by improving the rhizosphere microenvironment, nano-iron can help flax absorb other nutrients such as phosphorus

Table 3. Differential metabolite screening

Serial number	Names of metabolites	Metabolite type	Molecular formula
1	Arg-Thr-Lys-Arg	Amino acids and their derivatives	C22H45N11O6
2	Histidylleucine	Amino acids and their derivatives	C12H20N4O3
3	Ar (4) - 4, 8 aalpha - Dimethyl - 6 alpha - [1 - methyl - 1 - (beta - D - glucopyranosyloxy) baton rouge] 1, 4-trichlorobenzene abeta, 5,6,7,8,8 octahydronaphthalene - a 2-one	Ketone compounds	C21H34O7
4	(3,6, 9-trimethylidene-2-OXO-3A,4,5, 6A,7,8, 9A,9b-octahydroazuleno[4,5-b]furan-8-yl) acetate	Lactone compounds	C17H20O4
5	Arg-Ile-Thr-Val-Lys	Amino acids and their derivatives	C27H53N9O7
6	PA(14:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Phosphatidic acid	C39H63O8P
7	1-(9Z,12Z-octadecadienoyl)-2-tetradecanoyl-glycero-3-phosphocholine	Phosphatidylcholine	C40H76NO8P
8	L-Tyrosyl-L-Threonine	Amino acids and their derivatives	C13H18N2O5
9	thio-Miltefosine	Phosphatidylcholine	C21H46NO3PS
10	Nap-Ser-OH	Amino acids and their derivatives	C21H18N2O7
11	(e) 20-7 - butyl - 11,16,23,24,25 pentahydroxy - 10,19,20 - trimethyl - 15 - methylidene - 8,13,28,29 - tetraoxatetracyclo [22.3.1.13, 6.01 2, 14] nonacos - 20 - ene - 9, 18 - dione	Benzene and its derivatives	C33H52O11
12	Lupeol	Triterpenoids	C30H50O
13	Ganoderic acid Ma	Organic acids	C34H52O7
14	[3-Hydroxy-1-[3-hydroxy-1-oxO-1 -(2,3,4,5, 6-pentahydroxyHexoxy)decan-5-yl]oxy-1-oxodecan-5-yl] 3,5-dihydroxydecanoate	Alcohols	C36H68O15
15	5 - N - (6 - aminohexyl) - 7 - N - benzyl - 3 - propan - 2 - ylpyrazolo [1, 5 - a] pyrimidine - 5, 7 - diamine	Benzene and its derivatives	C22H32N6

Table 4. Metabolic pathways and screening of metabolites

Metabolic pathways	Related metabolites on the pathway
D-Amino acid metabolism pathway	Arginine-threonine-lysine-arginine polypeptide; Histidine leucine; N-L-histidine L-leucine; Arginine-isoleucine-threonine-valine-lysine polypeptide; Na-ser-oh;
Ether lipid metabolism pathway	Polyamines (PA), phosphatidylcholine (PC)
Sesquiterpene and triterpene biosynthetic pathways	Lupine: lupeol
Steroid biosynthesis pathway	3, 5-dihydroxydecanoate

and potassium, and then improve the salt tolerance of plants (Verma et al., 2018; Sarkar and Kalita, 2023). This study also preliminarily verified the effect of nano-iron on the improvement of growth traits of flax in saline-alkali land, particularly on emergence rate, seedling preservation rate, root and stem thickness.

Screening of different metabolites and correlation with salt resistance

To explore the mechanism of nano-iron on germination rate, seedling preservation rate and root thickening of flax in saline-alkali land, this study screened out differential metabolites and information related to salt resistance traits of flax through non-target metabolomics tests. Combined with univariate and multivariate statistical analysis methods and multi-angle analysis results, a total of 550 metabolites were screened, including 253 upregulated and 297 down-regulated metabolites. Among them, 15 metabolites such as amino acids and their derivatives, ketones and lactones were screened for upregulation, which played an important role in enhancing plant resistance. Amino acids and their derivatives help maintain the integrity of the cell membrane structure, prevent membrane lipid

peroxidation under adverse conditions, and protect the cell from damage (Cai and Aharoni, 2022; Romanenko et al., 2024). As precursors to many secondary metabolites, such as phenolic compounds, alkaloids, etc., Amino acids not only have defensive functions but also enhance the plant's resilience to adverse conditions (Romanenko et al., 2024). Ketones also perform well in plant resistance, for example, flavonoids show significant enrichment under salt stress (Feng et al., 2023), and Lactone compounds extend plant quality and increase plant resistance to adverse conditions (Mostofa et al., 2018; Gao et al., 2025). Organic acids can not only reduce water evaporation from crops by regulating stomatal opening to enhance their drought resistance, but also reduce the toxic effects of saline-alkali on crops(Panchal et al., 2021).

Screening of metabolic pathways and analysis of salt resistance mechanisms

In this study, four out of 20 metabolic pathways were selected, and metabolites related to salt resistance were precisely screened on each pathway, mainly amino acid polypeptide compounds, including Arginine-threonine-lysine-arginine polypeptide, histidine leucine polypeptide, N-L-histidine L-leucine polypeptide, arginine-isoleucine-threonine-valine-lysine polypeptide, NA-serine derivatives, etc. Amino acid peptides, as an important biostimulant, play a significant role in enhancing plant resistance, eliminating reactive oxygen species (ROS) antioxidant effects, activating plant defense responses through signal transduction. Amino acid peptides can also serve as carriers to help plants better absorb mineral elements, especially trace elements, which is crucial for enhancing plant resistance (Cao et al., 2025). The application of serine is mainly reflected in plant growth regulation. Serine derivatives can not only promote the growth and development of plants, but also enhance the drought resistance and cold resistance of plants (Clemente et al., 2019). In addition, polyamines (PA), a class of small nitrogen-containing compounds, are widely present in plants, mainly including humidines, spermidines and spermidines, enhance plant salt tolerance by regulating ion balance, antioxidant defense and other pathways (Amiri et al., 2024). Research has found that with increasing in phosphatidylcholine (PC) content, the absorption of Na^+ and excretion of K^+ in wheat decreased, which means that ionic toxicity reduced and thus could better resist to salt stress (Farooq et al., 2024; Wang et al., 2024). Few reports considered that lupanol might indirectly enhance the salt resistance of plants by eliminating reactive oxygen species or increasing the activity of antioxidant enzymes to protect cell membranes from oxidative damage (Makhubu et al., 2024).

CONCLUSION

This study preliminarily verified the effect of nano-iron on improving the growth traits of flax from saline-alkali land. After nano-iron treatment, the emergence rate, seedling preservation rate and root thickness of flax grown on saline-alkali land increased by more than 20%, and the stem thickness also increased by 6.9%. 15 upregulated metabolite related to flax salt resistance were screened, including amino acids and their derivatives, ketones, lactones, etc, which played an important role in enhancing plant stress resistance. Four out of 20 metabolic pathways were screened and then precisely screened metabolites related to salt tolerance on each pathway. Five metabolites were screened on the amino acid metabolic pathway; Two metabolites such as PA and PC screened for lipid metabolism synthesis pathway; One lupanol screened for sesquiterpene and triterpene bioanabolic pathways; One metabolite of dihydroxydecanoate screened for alcohol bioanabolic pathways.

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