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INVESTIGATING THE POSSIBLE MECHANISMS INVOLVED IN ALUMINUM TOLERANCE THROUGH ANALYSIS OF TRANSCRIPTOME DATA FROM DIFFERENT GENOTYPES OF SOYBEAN

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Abstract

Aluminum is one of the main factors limiting crop growth in acidic soils, thus, revealing the mechanism involved in soybean aluminum resistance is an important measure in order to improve soybean yield in acidic soils. In this paper, we analyzed transcriptome data from different genotypes of soybean under aluminum stress and found that multiple genes may mediate differences in aluminum tolerance of different soybean genotypes. Through comparative analysis, we found that aluminum-tolerant soybeans had a higher organic acid metabolism compared to sensitive soybeans, while aluminum-tolerant soybeans suffered higher levels of oxidative stress compared to sensitive soybeans. The differences in root elongation of various genotypes of soybeans under aluminum stress is likely to be closely related to the changes in cell wall structural components, among which differential expression of genes encoding POD, PAL, PME, PGIP1, HRGPs and expansion and other enzyme genes plays a key role. These results provide a clear basis for further studies on the relationship between the cell wall and aluminum tolerance of soybeans and their regulation.

Key words: Aluminum tolerance, RNA seq, Soybean, Cell wall, Anti-oxidation.

Introduction

Crop yield limitation in acidic soils has global characteristics, and its main limiting factor is the inhibition of plant root growth by soluble trivalent aluminum ions, which show toxic effects even at very low concentrations. However, some species with natural resistance to aluminum can grow well in acidic soils. Over the past 30 years, researchers have gradually deepened their understanding of the physiological mechanism behind aluminum tolerance in important crops, benefiting from the isolation and identification of many genes involved in aluminum tolerance (Wu *et al*., 2014; Delhaize *et al.*, 1993). In some plants, only 1-2 alleles contribute significantly to aluminum tolerance (Riede & Anderson, 1996; Ma *et al*., 2004). Previous published studies have shown that the presence of two QTLs in soybean contributed to the variation in root elongation phenotype under aluminum stress, localized to chromosomes 8 and 16, respectively (Abdel-Haleem *et al.*, 2013). However, in addition to these major genes, cumulative effects of many minor genes have also been found to be crucial for plant aluminum tolerance (Jian *et al.*, 2013; Korir *et al.*, 2013). Numerous transcriptomic studies have shown that hundreds of genes exhibit differentially expressed characteristics under stress, suggesting that these genes actually respond to stress (Yong *et al.*, 2014; Sun *et al.*, 2010; Chandran *et al.*, 2008). In addition, one study found that European Aspen (*Populus tremula*), about 175 genes were up-regulated and 69 genes were down-regulated after 6 h of aluminum stress, while the number of differentially expressed genes decreased rapidly to 26 when the stress time increased to 2 d. This reduction in the number of aluminum-responsive gene populations was not thought to be due to irreversible aluminum toxicity, but rather that their aluminum-tolerant system was stimulated and formed (Grisel *et al.*, 2010). Researchers have screened more than 600 aluminum-responsive genes in soybean (*Glycine max*, culitvar Jiyu70) using cDNA chip technology. Except for some conserved MATE families and transcription factor genes of C_2H_2 type with clear annotations, more than 50% of the genes were of unknown function (You *et al.*, 2011). These previous studies provide us with evidence to suggest that there are still many aluminum-tolerant genes that are yet to be identified with unknown functions, and differences in the materials studied may also lead to new discoveries. Thus, further in-depth analysis of aluminum stress response genes is the only way to comprehensively reveal the mechanisms behind plant aluminum responses; and by comparing the similarities and differences of expression patterns of aluminum response genes in plants with different aluminum resistance genotypes, we hope to discover key candidate genes to pave the way for future research.

Materials and Methods

Material culture and treatment: In this experiment, aluminum-resistant genotype soybean BaXi 10 (BX10, Brazilian soybean variety) and aluminum-sensitive genotype soybean BeDi 2 (BD2, a widely cultivated variety in South China) were used as research materials (Zhen *et al.*, 2007; Chandran *et al.*, 2008). The soybean seeds were preserved and bred in our laboratory (Institute of Plant Molecular Biology, Nanjing University). We

selected several seeds with full granules and bright color, submerged the seeds with an appropriate amount of 0.1% $HgCl_2$ for 15 min, then immediately rinsed them with sterile distilled water for 3-5 times to completely remove the residual $HgCl_2$. The sterilized perlite was then spread in the germination box (plastic, 30×30cm), and the seeds were evenly spread on the perlite to avoid seed accumulation. Seeds were then covered with perlite about 2-3 cm thick; sufficient sterilized distilled water was poured into the germination box so that the seeds could fully absorb the water. The germination cassette was placed in a light-free incubator at 25°C to germinate. After 4 days, the seedlings were transferred to a 1/2 Hoagland nutrient solution in pots, placed in a light incubator and continued to culture for 2 days under the following conditions: 26 C/22 C (day/night), 16/8 h photoperiod, illumination of about 400 mmol m^{-2} s^{-1}, about 70% humidity. After 2 days, seedlings with the same growth potential were selected and transferred to the following solutions for treatment: the control group was 100 mol/L $CaCl_2$ solution, adjusting pH 4.5; the aluminum treatment group was 100 mol/L $CaCl_2$ added to $AlCl_3$, making Al_3+ 50 mol/L, adjusting pH 4.5.After 2 days of treatment, the root tips of the seedlings were cut about 2 cm, frozen immediately with liquid nitrogen, and finally wrapped with aluminum foil and stored in an ultra-low temperature refrigerator at -80°C for future use.

Hiseq 2000 sequencing and data analysis: Total RNA was isolated by using Trizol reagent (Takara, Dalian, China). The mRNAs were purified by using Dynabeads Oligo (dT)25 mRNA isolation beads (Thermo Fisher Scientific Inc., USA). The obtained mRNAs were immediately reverse transcribed into first-strand cDNA and then used for second-strand cDNA synthesis. The products were purified using AM PureXP beads (NEB, USA), and each library was normalized by adjusting the cDNA concentration to 10 nM before subjecting to high-throughput sequencing on an Illumina HiSeq 2000 sequencer at Personalbio Co., Ltd (Shanghai, China).The raw reads were filtered using FastQC package to discard contaminant sequences and low quality reads (phred quality score < 30 and read length < 50 bp). The obtained clean reads were mapped to reference genome (Glyma1.0) using Bowtie/Tophat program. BLASTp searches (E-value < 1e-5) were conducted for the following databases: Ensembl, JGI, Kyoto Encyclopedia of Genes and Genomes (KEGG) and egg NOG to find the corresponding transcripts in soybean genome and acquire the annotations of these transcripts. Expression abundance was normalized by using reads per kilo bases per million reads (RPKM).This part was consistent with our previously published work (Huang *et al.*, 2017).

Results and Discussion

Organic acid secretion-related genes: Aluminum-induced citric acid secretion is a key defense mechanism adopted by red adzuki bean (*Vigna umbellata*) roots against aluminum toxicity (Yang *et al*., 2006); in addition, aluminum-induced malic acid secretion is also an important way for soybean (*Glycine max*) to adapt to aluminum stress in acidic soils (Liang *et al*., 2013). MATE family proteins have the ability to promote citrate transporters induced by aluminum and thus play a major role in enhancing the aluminum tolerance of sorghum (Magalhaes *et al.*, 2007). In this study, GLYMA13G27300 encoded the Arabidopsis homologous FRD3 protein, which was up-regulated 62.15-fold and 1309.98-fold in BX10 and BD2, respectively, with citrate transporter activity; similarly, GLYMA02G31370 and GLYMA10G37710 also encoded the MATE protein, with the former up-regulated 3.71-fold and 12.04-fold in BX10 and BD 2, and the latter up-regulated 1.87-fold and 2.95-fold, respectively. The expression of these genes is not consistent with the secretion of citric acid in two genotypes of soybean under aluminum stress (Dong *et al*., 2004), which may be compensatory expression caused by insufficient citric acid synthesis in aluminum-sensitive soybean under aluminum stress (that is, compensating for the insufficiency of citric acid quantity by enhancing the ability of citric acid efflux).

As shown in Fig. 1, there were differences in organic acid metabolism between two genotypes of soybeans under aluminum stress. The activity of pyruvate kinase (PK) in aluminum-resistant soybeans increased significantly, which promoted the conversion of phosphoenolpyruvate (PEP) to pyruvate, thereby promoting the increase of acetyl CoA content. On the other hand, the activity of phosphoenolpyruvate carboxylase (PEPC) increased in aluminum-resistant soybeans, catalyzing the direct conversion of PEP to pyruvate. Oxaloacetic acid; elevated malate dehydrogenase (MDH) activity also promotes the conversion of malic acid to oxaloacetic acid. The increase of acetyl CoA and oxaloacetic acid content is beneficial to the synthesis of citric acid. However, we did not detect any significant changes in citrate synthase activity in our study. It was previously found that the citrate synthase activity of BX10 and BD2 did not significantly change after 6 h of 50 mol/L aluminum stress, but the secretion of citric acid did significantly increase (Pereira *et al.*, 2006). This suggested that citric acid synthesis and secretion were not similar in the two genotypes of Soybean studied under aluminum stress.

In addition, citrate transport-related channel protein activity was significantly increased under aluminum stress, while malate transport-related channel protein was not found to change, suggesting that citrate secretion from BX10 and BD2 genotypes of soybean under aluminum stress is the main organic acid resistant to aluminum stress.

Cell wall-related genes: The cell wall is the first barrier faced by aluminium to enter cells, and contains a large number of negatively charged substances that fix aluminium. Therefore, most of the aluminum absorbed by roots is concentrated in the cell wall (Clarkson *et al.*, 1967; 1967). It is the accumulation of aluminum in the cell wall that limits the relaxation of the cell wall and changes the composition of cell wall polysaccharides, thus inhibiting root elongation (Van *et al.*, 1994; Ma *et al.*, 1999). Pectin in the cell wall is considered to be the main binding site for aluminum (Blamey *et al.*, 1993), with the content of pectin and its methylation level having an important correlation with plant aluminum resistance (Eticha *et al*., 2005). Pectinesterase (PME) catalyzes the demethoxylation of cell

wall pectin to produce pectic acid. Studies have shown that PME in rice roots is induced by aluminum, and its activity is higher in aluminum-sensitive varieties than in aluminum-sensitive varieties; demethylation of pectin exposes the negatively charged carboxyl groups, thus increasing aluminum binding and preventing aluminum ions from entering the cell interior (Yang *et al.*, 2008). Previous study also obtained similar results in the study of PME activity under wheat aluminum stress (Tang *et al.*, 2006). In our study, we found that GLYMA03G37391 and GLYMA19G39990 encoded soybean PME, the former was up-regulated 1.48-fold and 3.24-fold in BX10 and BD2, and the latter was up-regulated 4.67-fold and 6.38-fold, respectively. These two genes were up-regulated more in BD2, indicating that the root system of BD2 soybean binds more aluminum ions, and thus the root elongation is inhibited more.

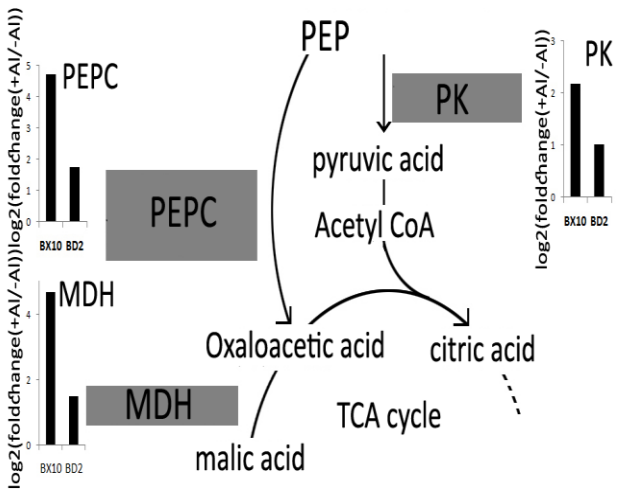

Fig. 1. The scheme of organic acid metabolism in soybean BX10 and BD2 under Al stress. The activity of pyruvate kinase (PK) in BX10 increased significantly, which promoted the conversion of phosphoenolpyruvate (PEP) to pyruvate, thereby promoting the increase of acetyl CoA content. The activity of phosphoenolpyruvate carboxylase (PEPC) increased in BX10, catalyzing the direct conversion of PEP to pyruvate. Oxaloacetic acid; elevated malate dehydrogenase (MDH) activity also promotes the conversion of malic acid to oxaloacetic acid. The increase of acetyl CoA and oxaloacetic acid content is beneficial to the synthesis of citric acid.

The binding of aluminum to cell wall pectin affects cell wall stability and reduces the ion exchange capacity (CEC) of the cell wall. The binding of aluminum causes cell wall stiffness, reduces its ductility and affects cell elongation (Kochian *et al.*, 2005). Studies have shown that PGIP1 (polygalacturonase inhibiting protein 1), a protein component of the plant cell wall, can interact with fungal polygalacturonase to inhibit fungal invasion, and is an important pathogen defense protein (Lidon *et al.*, 1999). It was found that PGIP1 was regulated by the transcription factor STOP1, and its expression level was increased under aluminum stress, which participated in the aluminum tolerance response in Arabidopsis. The authors speculated that PGIP1 could maintain the stability of pectin in an acidic environment (Sawaki *et al.*, 2009). In our study, we found that GLYMA08G08381 encoded PGIP1, which was increased 10.84-fold and 17.38-fold in BX10 and BD2, respectively, and thus played an important role in maintaining cell wall stability of the two genotypes of soybean roots under acid-aluminum stress.

Hydroxyproline-rich glycoproteins (HRGPs) are important components of plant cell walls, which participate in plant defense responses under adverse conditions by increasing their content and also play an intracellular signaling role in fungal infection defense (Showalter *et al.*, 1985). It was showed that the content of HRGP in aluminum-tolerant genotype rice was significantly higher than that in sensitive genotype rice under aluminum stress, and the accumulation of HRGP promoted cell wall thickening, thus alleviating aluminum toxicity (Pan *et al.*, 2010). In this study, two genes encoding HRGP protein, GLYMA14G1020 0 and GLYMA08G37180, were found to be up-regulated 36-fold and 20-fold in BX10 and BD2, respectively, while the latter was up-regulated 121-fold and 4-fold, respectively. This indicates that HRGP plays an important role in the resistance of soybean to aluminum stress and may also be one of the reasons for the differences in aluminum resistance among different genotypes of soybean.

Expansins are key regulators of cell wall elongation during plant growth. Expansin activity in wheat was promoted by K^+ and Mg^{2+} and inhibited by Cu^{2+} and Al^{3+}, and the elongation of hypocotyls was closely related to the expression of expansins, particularly beta-expansins (Gao *et al.*, 2008). In our study, the GmEXPB2 gene was up-regulated 74-fold in BX10 under aluminum stress, while there was no significant change in BD2, suggesting that the expression of this gene has a significant effect on maintaining the root elongation of Soybean under aluminum stress, which also explains to some extent why the relative elongation of BX10 under aluminum stress is higher than that of BD2.

Lignin plays a role in maintaining the mechanical properties of plant cell walls, and lignin content increases under stress, thereby enhancing stress resistance (Ying *et al.*, 2003). Under aluminum stress, wheat root tips rapidly accumulated lignin, and the lignin content of aluminum-sensitive cultivars was higher than that of aluminum-resistant cultivars, which was positively correlated with the degree of inhibition of root growth. Resistant cultivars had less inhibition of root elongation due to the low lignin content (Taylor *et al.*, 1995). Lignin is synthesized through the phenylpropane metabolic pathway, in which POD (EC 1.11.1.7) catalyzes monomer lignin polymerization and is the last key enzyme for lignin formation. In this study, we found four transcripts encoding POD, which catalyze the last step of lignin synthesis reaction. These genes were up-regulated more in BD2, indicating that the lignin content in the cell wall of BD2 root system was higher than that of BX10, and thus its root elongation was inhibited to a greater extent.

Pectin methylesterase (PME) and expansin activities are regulated by plant endogenous ethylene (Kunert 2006; Baxter *et al.*, 2013), while PME activity is also regulated by reactive oxygen species (Hawes *et al.*, 1998). The synthesis of plant cell wall lignin is promoted by both ethylene and reactive oxygen species (Hawes *et al.*, 1998).

Therefore, it is thought that reactive oxygen species and ethylene are involved in the regulation of root cell wall structure modification under aluminum stress.

Soybean resists aluminum toxicity by increasing cell wall lignin content, improving cell wall stability, increasing cell wall aluminum adsorption capacity, cell wall thickness and maintaining cell wall extensibility (Fig. 2). PME activity of aluminum-sensitive soybeans was higher than that of resistant soybeans, but expansin activity was significantly lower than that of resistant soybeans. These results suggest that on the one hand, the root pectin hydrolysis degree was higher, more aluminum ions were adsorbed, but on the other hand, the cell wall relaxation was lower, coupled with a large amount of lignin deposition, so the root elongation was greatly inhibited. A large amount of synthesis of HRGP in aluminum-tolerant soybean promotes cell wall thickening of the root system and has strong cell wall elongation ability, so the root elongation is less inhibited and aluminum resistance is stronger under aluminum stress.

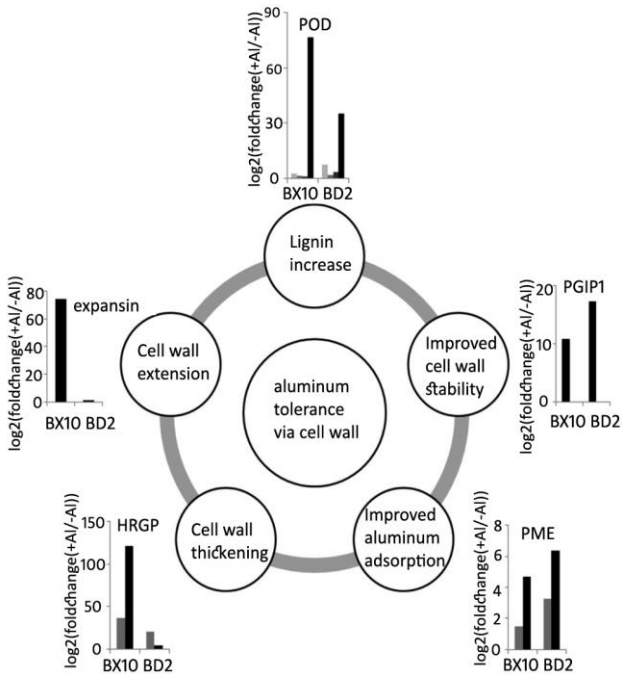

Fig. 2. Relationship between the alteration of cell wall and Al tolernance in soybean BX10 and BD2 under Al stress. POD is involved in lignin synthesis, thus help to increase the content of lignin. PGIP1can maintain the stability of pectin, which is very important in protecting the cell wall. PME catalyzes the demethoxylation of cell wall pectin to produce pectic acid, which is a good combining site of Al^{3+}. The accumulation of HRGP promoted cell wall thickening, their high expression in BX10 can facilitate the aluminum resistant. Gene encoding expansin is highly expressed in BX10, suggesting that the relative elongation of BX10 under aluminum stress is higher than that of BD2.

Stress response-related genes: Plants suffer various stresses during their growth and development, and a change in environmental conditions will stimulate the plant's own defense system to participate in the stress response. Under the same experimental conditions as our study, Zhen *et al.*, (2009) found that the POD activity of BD2 was significantly higher than that of BX10 (Zhen *et al.*, 2009). Other studies have found that aluminum inhibition of barley root elongation is significantly associated with aluminum-induced potentiation of POD activity (Tamás *et al.*, 2003) as well as with cell death caused by POD triggering H2O2 production (šimonovičová *et al.*, 2004). These studies indicate that the enhancement of antioxidant enzyme activity under aluminum stress is a resistance measure when plants are injured, and it is more significant in aluminum-sensitive varieties. In this study, 14 peroxidase-encoding genes were found, which were divided into three categories according to their expression pattern: type I, which was up-regulated in both BX10 and BD2, but with a greater amplitude in BX10; type II, which was significantly up-regulated in BD2, but not significantly different to BX10; type III, which was down-regulated in both BX10 and BD2, but with a greater down regulation in BX10. This different response characteristic may be related to the diversity of POD functions.

Plant NADPH oxidase (NADPH oxidase), a homologous protein of respiratory burst oxidase homologues (RBOHs), is an extracellular enzyme that can catalyze the formation of superoxide anion radicals ($O_2^{\cdot-}$) from O_2. It is involved in the regulation of plant growth and development and their response to environmental stress via the reactive oxygen signaling pathway (Marino *et al*., 2012). Previously published studies have shown that in Maize, Ca^{2+} can bind to the EF chiral domain of NADPH oxidase to activate the enzyme, regulate the production of reactive oxygen species and participate in the ABA signal transduction pathway (Marino *et al*., 2012). Further studies have also revealed that calcineurin B-like calcium sensors CBL1 and CBL9, together with their corresponding protein kinases (CIPKs), interact with the N-terminal domain of Arabidopsis NADPH oxidase RBOHF to regulate its activity and promote the production of reactive oxygen species (Drerup *et al*., 2013). However, in our study, we found two NADPH oxidase genes whose expression levels decreased significantly under aluminum stress, indicating that aluminum inhibited the expression of the NADPH oxidase gene. Since the presence of aluminum decreases Ca^{2+} uptake by the roots (Huang *et al.*, 1992), the decrease in NADPH oxidase gene expression activity is likely related to the decrease in Ca^{2+} concentration. Therefore, the extracellular ROS signaling pathway triggered by NADPH oxidase in Soybean under aluminum stress may be inhibited, with the sensitive cultivars even more inhibited.

The production of reactive oxygen species in plants is mainly coupled with the electron transport chain, in which the NADH dehydrogenase complex (I) and the coenzyme Q-cytochrome BC1 reductase (III) are the main sites for the production of reactive oxygen species (Hawes, 1991; Cai *et al.*, 2011). In scavenging reactive oxygen species, the contribution of alternative oxidase (AOX) in vitro, and uncoupling protein (UCP) in the inner mitochondrial membrane, is positive. Adverse stress results in the blockage of the main electron transport pathway of cytochrome c, shunting of electrons to AOX to maintain a certain level of electron transport, and maintaining the ubiquinone pool in a higher oxidation state. UCP belongs to the mitochondrial anion transporter, which can reduce

the proton gradient across the membrane and maintain normal electron flow at a low rate of ATP synthesis, thus maintaining the normal metabolism of plants (Tamás *et al.*, 2005). In our study, GLYMA04G14800 and GLYMA01G02950 encoded soybean AOX and UCP, respectively, and their expression levels were up-regulated in BX10 and BD2, with the larger in BD2, so they could be regarded as the common protective mechanism against oxidative stress in these two genotypes of soybean. GLYMA17G07210 encodes NADH dehydrogenase, which is up-regulated in BX10 and down-regulated in BD2; the increase of DADH dehydrogenase activity is beneficial to the transport of electrons and reduces the leakage of electrons; while the decrease of its activity leads to the leakage of electrons from the inner membrane, increasing the contact opportunity with O2 and leading to the production of reactive oxygen species.

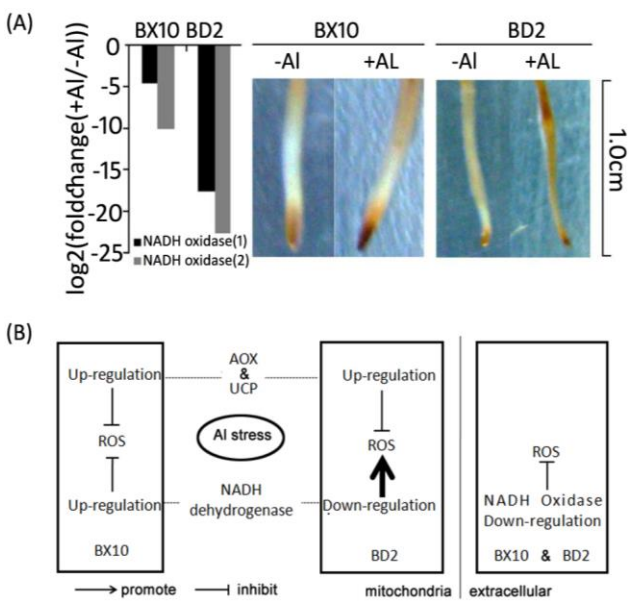

Fig. 3. (A) NADH oxidase expression patterns and H_2O_2 in situ observation in the roots of different soybean genotypes, (B) Differential pathway of mitochondrial and extracellular ROS production in different soybean genotypes under Al stress.

NADH oxidase is an enzyme that produces reactive oxygen species in mitochondria in vitro, and its expression level in both genotypes is inhibited by aluminum stress. As shown in the figure below, two NADH oxidase genes are down regulated in BX10 and BD2, with the down regulation in BD2 being greater. It has previously been shown that the Arabidopsis NADH oxidase RBOHD plays a key role in regulating the systemic ROS signaling pathway under various stress conditions (Aftab *et al.*, 2010). Thus, the ROS signaling pathway triggered by NADH oxidase was inhibited in both genotypes of soybean under aluminum stress, which did not result in a decrease in total ROS content (Fig. 3A); in fact, the content of H_2O_2 and $O_2^{\cdot-}$ in BD2 roots was found to be higher than that in BX10 roots after 48 h of 50 micromol/L aluminum stress. In addition, the permeability of the plasma membrane and the degree of membrane lipid peroxidation in BD2 roots was also seen to be higher than that in BX10 roots (Martha *et al.*, 2000). Since mitochondria are the main sites of reactive oxygen species production under stress conditions, the activity of electron transporters on the inner mitochondrial membrane plays an important regulatory role in the production of reactive oxygen species. As shown in Fig. 3B, the up-regulation of AOX and UCP activity in BX10 and BD2 has a positive effect on the scavenging of reactive oxygen species, but the balance of reactive oxygen species is inevitably broken under stress, and the decrease of NADH dehydrogenase activity in BD2 should be one of the factors leading to the higher content of reactive oxygen species in BX10.

Conclusion

The aluminum-tolerant soybean BX10 can maintain low levels of oxidative stress, and has strong citric acid metabolism ability. BX10 increases citric acid synthesis by inducing the up-regulated expression of genes related to the citric acid anabolic pathway under aluminum stress; changes in cell wall structural components are important characteristics of aluminum tolerance in soybean. Overall, soybean resists aluminum toxicity by increasing cell wall lignin content, improving cell wall stability, increasing cell wall aluminum adsorption capacity, increasing cell wall thickness, and maintaining cell wall extensibility. However, there are differences in the expression activities of genes involved in the regulation of cell wall components between BX10 and BD2, resulting in their different aluminum response abilities. Reactive oxygen species signaling pathways may differ among different genotypes of soybean. Extracellular reactive oxygen species (ROS) production was blocked by NADH oxidase in different genotypes of soybean under aluminum stress, but to a greater extent in BD2. Blocked ROS production may indicate that the signaling pathway mediated by ROS was inhibited. Mitochondrial-derived reactive oxygen species production, triggered by changes in NADH dehydrogenase activity, was found to be decreased in BX10 and in BD2, which may be one of the reasons why BD2 suffers from greater oxidative stress.

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