Identification of alkaline salt tolerance genes in Brassica napus L. by transcriptome analysis

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Research Article

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**Abstract**

**Background**

Soil salt alkalinization is one major abiotic factor reducing the productivity of crops worldwide, including rapeseed (*Brassica napus* L.), an indispensable oil crop and vegetable. The mechanism studies of alkali-salt-tolerance are helpful for the breeding of high resistant varieties and the expansion of the planting area. However, the study of the alkaline salt tolerance mechanism in rapeseed is limited. This study aimed to identify candidate genes related to the regulation of alkali salt tolerance in *B. napus*.

**Results**

In the current study, *B. napus* line 2205 exhibited stronger tolerance to alkaline salt than *B. napus* line 1423. In line 2205, the lesser plasma membrane damage index, the accumulated osmotic adjustment substances, and higher antioxidant enzyme activities were contributed to alkaline tolerance. Ultrastructure observation found that the mesophyll cells of line 2205 had higher integrity compared with line 1423, further confirming that line 2205 suffered a lesser injury under alkali-salt stress. Transcriptome analysis showed that more genes responded to alkaline salt in line 2205, and the expressions of respective specific response genes were different in these two cultivars. The expression level of the line-1423-specific-response genes was lower in line 2205, which were primarily annotated to the cytosol, spliceosome, and ubiquitin-mediated proteolysis, whereas most of the specific response genes in line 2205 had a higher expression, which were mainly enriched in carbohydrate metabolism, photosynthetic processes, ROS regulating, and response to salt stress. It can be seen that the tolerance to alkaline salt is attributed to the high expression of some genes in these pathways. Based on these results, twelve cross-differentially expressed genes, including *BnMDH2*, *BnGAPCs*, *BnACO1*, *BnGOXs*, and *BnGGTAs*, were proposed as candidate genes for regulating alkali-salt tolerance in rapeseed.

**Conclusions**

The present study demonstrates that tolerant rapeseed exhibits superior physiological indicators and actively mobilizes genes involved in energy metabolism and ROS-related pathways to resist undesirable adverse signals from alkaline salts. The candidate genes provide reliable clues for further analysis of the resistance mechanism of rapeseed.

**Background**

Salinity is among the most detrimental stresses in plants, as salt hampers plant growth, development, yield, and quality [1]. Soil salinization is intensifying because of unsuitable irrigation practices and other causes by climate changes and environmental pollution [2]. The Global Map of Salt-affected Soils displays that 85% topsoils (0-30 cm) and 62% subsoils (30-100 cm) are saline, 34% and 19% of which are sodic and saline, respectively. Salt-affected soils are distributed in over 100 countries, especially in the arid and semi-arid regions, including China. However, the basic national conditions of China’s large population make it difficult to grow crops in non-saline land. Therefore, rational exploitation and improvement of the salinized land can relieve the pressure for cultivated land resources. According to reports, the total area of available saline-alkali soil is 3.67 × 107 ha in China, 30% of which shows great agricultural potential [3, 4].

Salt-affected soils are mainly categorized into two groups: saline and sodic (alkaline). Generally, there is the presence of excess sodium on exchange sites in both soils, while the anions are different. The dominant anions are chloride and sulfate in saline soils. However, the high concentration of carbonate/bicarbonate anions in sodic soils causes high pH, high sodium absorption ratio, and low electrical conductivities compared with saline soils [5]. In salt-stress plants, the imbalance of osmotic potential induces water stress, the high concentration of Na+ causes severe ion toxicity, and the interaction between salts and mineral nutrients results in nutrient imbalance and membrane injury [1, 6]. In addition, plants growing in alkaline salt often suffer more severe damage due to high pH [7]. The disruption of pH homeostasis affects absorption of inorganic ions and causes the precipitate of metal ions, resulting in a decrease in the availability of plant nutrients [8]. Thus, plants subjected to alkali-salt stress not only have physiological drought and ion toxicity, but also have to maintain intracellular pH balance.

Many studies have revealed the responding mechanisms to salt stress (NaCl) in temporal and spatial in the model plant Arabidopsis thaliana. Briefly, a series of early responses (including K+ transport, Ca2+ signaling, H+ transport, phospholipid modifications, reactive oxygen species (ROS) production, and protein kinase activity) are induced after the initial perception of the sodium signal [1, 9]; downstream of these signaling, biosynthesis and transport of phytohormone are altered, and some gene expressions are changed in phytohormones-dependent and -independent manners [10]; finally, these cascade signals lead to adaptive responses to salt stress, such as the production and transport of ion and adjustment of growth and development[11, 12]. Under salt stress, the excessive ROS disrupt redox homeostasis and cause oxidative damage to plant cells [13]. To alleviate the negative effect of oxidative stress, non-enzymatic (like glutathione, ascorbic acid, α-tocopherol, and flavonoids) and enzymatic defense systems (e.g. superoxide dismutase (SOD), peroxidase (POD), and catalase) perform the functions in scavenging or detoxifying the over-generation ROS. In addition to these reactions, osmotic unbalance caused by salt stress leads to stoma close, which reduces the amount of CO₂ available for photosynthesis and the CO₂-fixing-enzyme activity decreases. Moreover, Na+ disrupts the proton-motive force and chloroplast function, which decreases the photosynthetic rate [14]. Hence, plants change the distribution of their photosynthesis-fixed carbon to cope with the cut photosynthetic and sustain growth. In Arabidopsis, salt stress induces carbon partitioning to sugars, instead of starch [15]. van Zelm et al. revealed that spatial carbon influx might be related to the tissue-specific sodium content [1].

Relative to the mechanisms responding to salt, limited information is known on the impact of alkaline salt. Most studies about alkali stress are conducted on the genome, physiological and biochemical levels. For example, alkali stress causes the loss of wheat seedling biomass and accumulated inorganic ions with different trends compared with salt stress [16]. The effects on the growth and photosynthesis of wheat under alkali than salt stress have also been
investigated by Guo et al. [17]. The investigation of two Chinese cabbage cultivars found photosynthesis was affected under alkaline stress and the varying photosynthetic capacity was associated with the synthesis of organic acids and carbohydrates [7]. The physiological responses to NaCl and NaHCO₃ were also studied in tomatoes, which showed organic acids and regulation of ROS might be related to the alkalai stress tolerance of tomatoes [18]. Many quantitative trait loci and genome-wide association studies related to alkali-salt have been reported in rice [19, 20], and some alkali salt-regulation genes have also been cloned in soybean [21-23], rice [24, 25], and alfalfa [26, 27].

Rapeseed (*Brassica napus* L.) is the second oil crop, an important edible vegetable, and a crucial source of biofuel [28]. Oilseed in China mainly relies on imports because of the low production from lower plantings [29]. Expanding the cultivated area of rapeseed by planting the salt- and alkali-tolerant rapeseed in salt–alkali soils, especially in the idle lands in the north and coastal areas of China, can remit the over-reliance on the international market. The expound about the alkaline-salt tolerance mechanism is beneficial to the breeding of new resistant varieties. Although many rapeseeds cultivate with high diversity in salt-resistance have been reported [30], the details about genes and mechanisms of tolerance to alkalinity are largely unknown in rapeseed. Transcriptomic analysis can provide mRNA expression of genes at high throughput and is widely applied to screen candidate genes involved in stress responses [30, 31]. In the present study, an RNA-seq was performed on alkaline-tolerance and -sensitive rapeseed cultivars to screen alkaline salt-tolerance-related genes, which will lay a solid foundation for the breeding and mechanism of alkaline salt tolerance in *B. napus*.

**Results**

**Differences of alkaline-salt tolerance and physiological indicators between lines 2205 and 1423 under alkaline salt stress**

The growth of line 2205 and 1423 showed no difference under normal conditions (Fig. 1A-B). However, they showed different phenotypes after 7 days under alkaline salt stress. At this stage, the leaves of line 1423 almost withered, while the leaves of line 2205 just began to yellow and wilt (Fig. 1C-D), indicating line 2205 is more tolerant to alkaline salts than line 1423.

To further evaluate the difference in alkaline-salt tolerance between these two cultivars, the physiological indexes related to stress response were measured. The results showed NaHCO₃ stress increased the relative electric conductivity (REC), the content of soluble sugars (SS), soluble protein (SP), proline (Pro), and the activity of POD and SOD in both cultivars, and the tendency increased over the stress time (Fig. 1). The content of SP was higher in line 2205 than line 1423, and the gap was increased after 24-h stress (Fig. 1E). Under the normal and alkaline-salt conditions, the concentration of SS and REC was significantly lower in line 2205 compared with line 1423, which was opposite to the SOD activity (Fig. 1F-H). There was no significant difference in the Pro content and the POD activity between line 2205 and line 1423 under the normal growth condition and 12-h stress, which were significantly elevated in line 2205 after 24-h stress compared with line 1423 (Fig. 1I-J). These results implied that the physiological responses to alkaline salt were different between line 2205 and line 1423.

**Ultrastructural-feature differences between line 2205 and line 1423 under alkaline salt stress**

Observation on microstructures showed that the mesophyll cells of lines 2205 and 1423 exhibited typical features under normal conditions. The chloroplasts were spindle-shaped structures, the basal particles were arranged in an orderly manner, the thylakoids were arranged compactly, and starch grains were present in the chloroplast of both lines (Fig. 2A, B, G, H). However, the cells of line 2205 were approximatively round, and more and tighter chloroplasts were found, which showed a long oval shape in line 1423 (Fig. 2C, I). After alkaline salt stress for 7 days, line 1423 suffered a greater disruption, obvious plasmolysis occurred and the organelles in the cells decreased or dissolved. However, many intact organelle structures can still be observed in line 2205 (Fig. 2F, L). In contrast to the shrunken manifestation occurring on the thylakoid and starch grains of chloroplasts in the sensitive-salt line 1423, the thylakoid of 2205 swelled and the starch grains in the chloroplasts became larger and more transparent. Moreover, the basal particles of line 2205 still maintained the regular arrangement to some extent, while those of line 1423 were arranged loosely and distorted. Besides, line 2205 had fewer osmiophilic granules compared with line 1423 (Fig. 2D, E, J, and K). From these results, we speculated the stronger resistance of line 2205 was related to the more complete chloroplast structure under NaHCO₃ stress.

**Illumina sequencing and differentially expressed genes between line 2205 and line 1423**

In order to explore the discrepant genes responding to alkaline salt between line 2205 and line 1423, RNA-seq was performed after 0-h (control), 12-h, and 24-h NaHCO₃ stresses on three independent biological replicates. In total, 116.40 GB clean data from 18 RNA libraries were obtained after filtering. The clean data of each sample reached 4.90 GB, the GC content was between 45.85%-46.88%, and the percentage of Q30 base was more than 91.71%. The efficiency of sequence comparison between the clean reads of each sample and the reference genome ranged from 83.09% to 88.39% (Additional file 1: Table S1).

To identify changes in transcriptional expression under alkalai stress, the differentially expressed genes (DEGs) were identified by comparing the expression of genes between 12 and 24 h and those at 0 h in both cultivars. In the sensitive line 1423, 5220 (12/0 h) and 7049 (24/0 h) genes were altered at the transcriptional level, whereas 8395 (12/0 h) and 12960 (24/0 h) were differentially expressed in the tolerant line 2205 (Fig. 3A and Additional file 1: Table S2). It can be found that line 2205 has nearly twice as many DEGs as line 1423. Among these DEGs, 9401 and 2802 genes were specifically expressed in line 2205 (T. spe.) and line 1423 (S. spe.), respectively (Fig. 2A). However, 4161 of 9401 and 1136 of 2802 genes were not differentially expressed between these two lines neither normal condition nor alkali-salt treatment. These genes may not be the key genes for alkali-salt resistance, which was not considered in this
study. We focused on the remaining 5239 DEGs in T. spe (as subset ∆), and 1666 DEGs in S. spe (as subset □ Fig. 3B). Besides, DEGs were further analyzed between line 1423 and line 2205 at the same time points. A total of 10753 (S0 vs T0), 11139 (S12 vs T12), and 11309 (S24 vs T24) DEGs were confirmed (Additional file 1: Table S2). Among these DEGs, 10284 responded simultaneously to alkaline salt in these two lines, but their expression exhibited significant differences. Thus these genes were defined as subset ∆ (Fig. 3B).

Co-expression clustering of filtered DEGs

To excavate the potential alkali-salt-regulating genes, the co-expression clustering analyses of filtered DEGs (subsets ∆, □, and ○) were performed, and the values of Log_{10}(FPKM + 1) were used for normalization classification. Each subset could be divided into three classes (Fig. 4). On the subset ∆, 72.25% of DEGs (2195 + 1590/5239) had higher expression in T0, T12, and T24 (Fig. 4A, and B). However, the higher expressed DEGs just accounted for 28.36% in subset □, 73.37% of which (538 + 651/1666) was expressed at a lower level in the tolerant line 2205 (Fig. 4C-D). On the subset ○, the top two catalogs were down (4553 DEGs) and up (3508 DEGs) expressed in S0,12,24 vs. T0,12,24 (Fig. 4E-F). Taken together, the 3785 DEGs in subset □ and the 3508 DEGs in subset ○ with an up-regulated expression in line 2205 might be responsible for the salt tolerance, and the other 1189 (in subset □) and 4553 (in subset ○) genes with an up-expressed expression in line 1423 might have negative effects on the salt resistance.

To analyze the functional differences of the DEGs in these two groups, the synchronously up- and down-regulated genes (FDR ≤ 0.01, |log_{2} (fold change)| ≥ 1) in groups S0 vs T0, S12 vs T12, and S24 vs T24 were further investigated. As a result, 2236 of 7293 (up-R. in T., higher expression in line 2205) and 2240 of 5742 DEGs (down-R. in T, lower expression in line 2205) were selected as the important DEGs (Additional file 1: Table S3).

Gene ontology enrichment of the important DEGs

Functional cluster analyses of 2236 (up-R. in T.) and 2240 (down-R. in T.) DEGs were implemented using BMKCloud. For the 2236 DEGs, Gene Ontology (GO) analysis showed 78 significant GO terms were enriched (q-value < 0.05), including 51 terms in biological process (BP), 23 terms in cellular component (CC), and 4 terms in molecular function (MF). Among these, the terms “response to salt stress” (6.91%), “chloroplast” (16.35%), “pyridoxal phosphate binding” (1.87%) accounted for the highest gene ratio in BP, CC, and MF, respectively. Besides, these terms referred to photosynthetic processes and carbohydrate metabolism were also highly enriched in BP, such as “photorespiration”, “photosystem II assembly”, “photosynthesis, light reaction”, “chlorophyll biosynthetic process”, “chloroplast relocation”, “photosynthetic electron transport in photosystem I”, “photosynthesis”, “PSII associated light-harvesting complex II catabolic process”, “photosystem II oxygen evolving complex assembly”; “glycolytic process”, “starch biosynthetic process”, “glucogenesis”, and “glucose catabolic process”. In addition to the cellular component related to the chloroplast, apoplast, mitochondria, and peroxisome were also significantly enriched. However, only three GO terms, “cytosol” in CC, “snRNA binding” and “sucrose-semialdehyde dehydrogenase (NAD+) activity” in MF, were significantly enriched among the down-regulated 2240 DEGs in line 2205 (Fig. 5A).

Several studies have shown that the photosynthetic mechanisms of different salt-tolerant germplasms have different trends under salt stress [7, 32]. Therefore, we hypothesized the higher expression of multiple genes participating in photosynthetic systems delayed the attenuation of photosynthesis in the tolerant line 2205. Chloroplasts are not only the site of photosynthesis, but also ROS-production [33]. Besides, mitochondria and peroxisome significantly enriched in CC are also important sites for ROS releasing. Many researchers have indicated that ROS plays a dual role under abiotic stresses [13]. Thereby, we speculated that ROS homeostasis was stronger in line 2205 than line 1423, which increased the tolerance to alkaline salt.

Kyoto Encyclopedia of Genes and Genomes enrichment of the important DEGs

To further discern the functions of DEGs in line 2205 and line 1423, we conducted a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. The top 20 enriched pathways for the 2236 up-R. in T.) and 2240 (down-R. in T.) are shown in Fig. 4. Similar to GO enrichment, the two-group DEGs were enriched in different pathways. The four most significantly enriched pathways were “glycolate and dicarboxylate metabolism”, “carbon metabolism”, “peroxisome”, and “photosynthesis” for the 2236 up-regulated genes in line 2205 (Fig. 5B). The other four pathways, “biosynthesis of unsaturated fatty acids”, ‘N-Glycan biosynthesis’, ‘caffeine metabolism’, and ‘SNARE interactions in vesicular transport’, were the top pathways significantly enriched for these 2240 up-regulated genes in line 1423 (Fig. 5C). To sum up, GO and KEGG analyses both showed that carbohydrate metabolism, photosynthetic processes, and ROS regulating were enriched for these 2236 DEGs, which were not enriched for those 2240 DEGs. It is surmised that the higher expression of the genes in these pathways enhanced the tolerance to alkali salt stress of line 2205.

Candidate gene analysis

Based on the functional-enrichment results of the higher expression of DEGs in 2205, these DEGs in the top enriched pathways were further analyzed, including carbohydrate metabolism (210 DEGs, hereinafter to be referred as ‘Car-Met’ for simplicity), photosynthetic processes (585 DEGs, ‘Pho-Pro’ for simplicity), ROS regulating (47 DEGs), and response to salt stress (110 DEGs, ‘Res-Sal’ for simplicity) (Additional file 1: Table S4). Salt response is a complex trait, which is affected by the coordinated gene networks in several metabolic pathways [1]. Hence, we focused on the intersectant genes among target pathways, including 13 overlapped DEGs in “Car-Met, Pho-Pro, and ROS” pathways and 42 superimposed DEGs among “Car-Met, Pho-Pro, and Res-Sal” pathways (Fig. 6A). The expressions of the 55 genes were higher in line 2205 than those in line 1423 (Fig. 6B). The 55 genes were mainly annotated to carbon metabolism (ko01200), oxidative phosphorylation (ko00190), glycolate and dicarboxylate metabolism (ko00630), biosynthesis of amino acids (ko01230), carbon fixation in photosynthetic organisms (ko00710), 2-Oxocarboxylic acid metabolism (ko01210), glutathione metabolism (ko00480), and peroxisome...
Validation of the RNA-Seq data by qRT-PCR

To verify the differential expression detected by the Illumina RNA-Seq data, quantitative real-time PCR (qRT-PCR) was performed for six random genes using 18 RNA libraries. Similar expression trends were shown for the selected genes, and a high positive correlation (0.83) was obtained between RNA-seq and qRT-PCR data at different time points (Fig. 7), suggesting that the RNA-Seq data are reliable.

Discussion

Salt stress is a severe hazard that adversely affects all stages of plant growth, such as inhibiting seed germination, slowing plant development, decreasing biomass yield, and decreasing crop yield [1, 30]. Therefore, it is a great advantage to breed salt-tolerant rapeseed cultivars. Excavating the physiological and biochemical response mechanisms would be helpful to understand the molecular tolerance traits and develop salt-tolerant genotypes. However, our present knowledge on the mechanisms regulating salt tolerance (especially alkaline salt stress) in rapeseed is still in its infancy. Hence, the selection and identification of high alkaline-salt tolerance cultivars are crucial to expanding rapeseed production in saline-alkali soil.

Alkali-salt tolerance of line 2205 is reflected at multiple levels

Compared with line 1423, the 2205 cultivar exhibits strong tolerance not only to the neutral salt NaCl [34], but also to the alkaline salt NaHCO₃. The discrepancy between lines 2205 and 1423 is not only reflected in the visual phenotype, but also in physiological responses and ultrastructures of mesophyll cells. Relative electrical conductivity (REC) is an indicator used to quantify the cell membrane stability during adversity stress [35]. Reports have shown stress (such as heat, drought, and salt) can damage the plasma membrane, inducing an increase in REC [35]. In the current study, REC was significantly higher in line 1423, leading to severe damage and weak selectivity of the membrane compared with line 2205. Ultrastructural observation also discovered cell structures and organelles of line 1423 appeared to dissolve when subjected to 7-day alkali salt stress (Fig. 2), which may be correlated with the serious injury of the plasma membrane. Besides, as important osmotic regulators, salinity-induced accumulation of soluble sugar (SS) and soluble protein (SP) have been reported [36, 37]. In the current study, the increasing trend in SS and SP was detected after NaHCO₃ stress, which is consistent with previous reports. However, the tolerant line 2205 presented a lower SS content but an opposite pattern of SP (Fig. 1). Whether SP and SS play different roles in osmotic regulation in response to alkali salt needs further investigation. Many studies have reported that proline (Pro) plays an important and positive role in plant growth as an osmoprotectant and antioxidative defense molecule under abiotic stress [38]. We also found it was accompanied by a higher proline content in the more tolerant line 2205 under alkali salt (Fig. 1). Salt stress triggers the excess generation of ROS, which causes oxidative damage to cells and even results in plant death. The antioxidant defense system constituted of non-enzymatic and enzymatic components saves plants from oxidative damage by detoxifying the excessive ROS and maintaining the balance of ROS [39]. Wherein, superoxide dismutase (SOD) and peroxidase (POD) are two important constituents of the antioxidant defense system [6]. In this study, the higher POD and SOD activities in line 2205 endowed it with a higher scavenging ability for ROS (Fig. 1). We speculated this might partially contribute to the tolerance to alkali salt stress.

It was found that the integrity of mesophyll cells of line 2205 was better than that of line 1423 under NaHCO₃ stress, and no plasmolysis was observed by microstructure observation (Fig. 2). Previous studies found the cell structure can be changed to minimize the damage when plants are subjected to saline-alkali stress [40]. The intact cell wall can be used as a barrier between the cell and the external environment to restrict ions from entering the mesophyll cells, thus protecting the cells from toxicity. The responses of mesophyll cells to saline-alkali stress in salt-tolerant and salt-sensitive materials are significantly different [41]. The mesophyll cells are important sites for photosynthesis; damage to mesophyll cells disturbs photosynthesis under stress [1]. In this study, the mesophyll-cell structure of line 2205 showed less damage compared with line 1423, indicating there may be more effective photosynthesis.

Synergistic up-regulation of genes in carbohydrates metabolism, photosynthetic process, and ROS equilibrium under NaHCO₃ stress

In this study, RNA-seq analysis identified more DEGs in the groups of T12 vs T0 and T24 vs T0 compared with the groups of S12 vs S0 and S24 vs S0, implying that more regulatory pathways had altered in line 2205 under 75 mM NaHCO₃ stress (Fig. 3A). This is consistent with the study of Mohamed et al. [30], which shows that the tolerant rapeseed YY9 has higher DEG regulation than the salt-sensitive one ZS11 on germination under salt stress. The higher number of DEGs in the tolerant line 2205 indicates that more pathways are involved in accommodating the alkali salt stress.

Further analysis discovered that 72.25% of the alkali-salt-specific response DEGs in line 2205 had higher expression than those in line 1423. However, 73.37% of line 1423-specific response DEGs showed lower expression in line 2205. Among these DEGs simultaneous responding to alkali salt in both cultivars, 44.27% and 34.11% DEGs possessed higher expression in line 1423 and line 2205, respectively (Figs. 3 and 4). From the differentially up- and down-regulated DEGs in these subsets, we speculated that their differences in alkali-salt resistance were due to the diverse coordinative capabilities of positive and negative regulation of alkaline salt tolerance.

In this paper, in order to excavate the functions and pathways regulating alkaline salt in rapeseed, the functional enrichments were performed for the target-group DEGs in line 2205. A number of significant terms and pathways were annotated by GO and KEGG analysis. The genes with lower expression in line 2205...
were mainly enriched in cytosol, spliceosome, and ubiquitin-mediated proteolysis (Fig. 5). Numerous studies have revealed that the splicing of stress-responsive genes and spliceosome components can be altered by salt stress, which influences the salt tolerance of plants by adjusting the homeostasis of ROS and osmosis. For example, serine/arginine-rich (SR)-like protein, as a component of spliceosome, have been reported to negatively regulate salt stress [42, 43]. Among the twelve serine/arginine-rich splicing factors among the selected DEGs in this study, nine genes (BnaA03g00590D, BnaA06g21030D, BnaA07g06170D, BnaC02g27300D, BnaC07g07660D, BnaC08g16960D, BnaC08g28300D, BnaC09g19170D, Brassica_napus_newGene_614) were from the set ‘down-R. in T.’ (Additional file 1: Table S3). It is speculated that the weak alkali-salt tolerance of line 1423 is correlated with the high expression of these genes.

However, the screened DEGs from ‘up-R. in T.’ were mainly enriched in carbohydrate metabolism, photosynthetic processes, ROS regulating, and response to salt stress (Fig. 5). A number of studies confirmed that there are complicated mechanisms in regulating salt tolerance [1, 34]. However, these extensive studies mainly focus on neutral salt NaCl, but not alkali salt, especially for the oil crop, B. napus. Our analysis showed that the higher expression of some genes in carbohydrate metabolism, photosynthetic processes, and ROS adjustment improved the alkali tolerance of rapeseed. The results were different from the annotated pathways (fatty acid and amino acids metabolisms) in response to Na₂CO₃ in canola [44].

Considering the intricacy of quantitative traits, the intersection genes in target pathways were identified as candidate genes in regulating alkaline salt tolerance (Additional file 1: Table S5). Malate dehydrogenase 2 (MDH2), a mitochondrial tricarboxylic acid cycle gene, has been proven the mRNA level of MDH2 is higher in the tolerant rye than the sensitive one in response to aluminum stress [45], consistent with the candidate gene BnMDH2 (BnaA06g08600D) in this study. Various studies have testified cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) is participated in plant response to stresses, including heat [46], cold [47], oxidative stress [48], and cadmium [49], but no to alkali-salt stress. Two homologous genes, BnGAPC1 (BnaC05g47450D) and BnGAPC2 (BnaC01g40210D), were screened out in this study (Table S4). Both genes maintained high levels of expression in line 2205 even under the NaHCO₃ treatment, but not in line 1423 (Fig. 6). 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO), which participates in the final step of ethylene synthesis, is induced by heat, cold stresses, wounding of leaves, soil-flooding, etc. in potato [50]. In our study, two BnACO1 (BnaA01g01700D and BnaC01g02790D) showed high expressions in line 2205 (Fig. 6). Plants will burst ROS under stress. Excessive production of ROS will lead to damage for plants. However, when these molecules are at the right levels, they play an active role in signal transduction [13]. Glycolate oxidase (GOX), a typical enzyme of peroxisomes, can trigger. In plants, pathogen induced expression of GOX activates the expression of several defense genes and improves the resistance to pathogens by increasing H₂O₂ [51, 52]. In our study, three BnGOXs (BnaC01g36940D, BnaA05g25050D, and BnaA01g29410D) with significantly higher expression in line 2205 were identified, which may result in more glucose digestion and less SS, and simultaneously boost the POD and SOD activities in line 2205 (Fig. 1 and 6). Glutamate: glyoxylate aminotransferase (GGAT) plays an important role in photorespiratory carbon cycles and amino acid metabolism. The growth is strongly affected in aoat1-1 seedlings with a weak GGAT activity [53]. In our results, two BnGGTA1 (BnaA07g10020D and BnaC07g13140D) and BnGGTA2 (BnaC01g40210D) showed no expression in line 1423, but not in line 2205 (Fig. 6). Plants will burst ROS under stress. Excessive production of ROS will lead to damage for plants. However, when these molecules are at the right levels, they play an active role in signal transduction [13]. Glycolate oxidase (GOX), a typical enzyme of peroxisomes, can trigger. In plants, pathogen induced expression of GOX activates the expression of several defense genes and improves the resistance to pathogens by increasing H₂O₂ [51, 52]. In our study, three BnGOXs (BnaC01g36940D, BnaA05g25050D, and BnaA01g29410D) with significantly higher expression in line 2205 were identified, which may result in more glucose digestion and less SS, and simultaneously boost the POD and SOD activities in line 2205 (Fig. 1 and 6). Glutamate: glyoxylate aminotransferase (GGAT) plays an important role in photorespiratory carbon cycles and amino acid metabolism. The growth is strongly affected in aoat1-1 seedlings with a weak GGAT activity [53]. In our results, two BnGGTA1 (BnaA07g10020D and BnaC07g13140D) and BnGGTA2 (BnaC01g40210D) showed no expression in line 1423, but not in line 2205 (Fig. 6).

At present, there are few studies on the salt-tolerance mechanism of rapeseed. Only a handful of neutral salt regulated genes such as alternative oxidases (BnaAOXs [54]), calcineurin B-like proteins (BnaCBL), and CBL-interacting protein kinases (BnaCIPK [55]) were cloned, and even fewer alkali-salt-tolerance genes have been reported in B. napus. Our study proposed firstly candidate genes for alkaline salt tolerance in Brassica napus by RNA-seq, including twelve target genes in metabolic pathways (such as carbohydrate metabolism, photosynthetic processes, and ROS regulating), which positively responded to alkaline stress in rapeseed. Our study provides new insight into the mechanism of alkali-salt tolerance in B. napus. Cloning and functional analyses of these candidate genes have been arranged in follow-up projects to more accurately interpret these results.

Conclusion

In summary, alkaline salts can affect membrane permeability by stimulating many strong physiological responses to alkaline salts, and the damage to mesophyll cells in tolerant canola is attenuated and delayed. The responses include enhanced osmoregulation through the addition of soluble protein and proline, and more favorable ROS scavenging through higher SOD and POD activities. RNA-seq analysis demonstrated that high expression genes in lines 2205 and 1423 participated in different metabolic pathways. In line 2205, the high-expression genes participated in positive pathways such as carbohydrate metabolism, photosynthetic processes, and ROS regulating, which enhanced its tolerance. Twelve candidate genes were identified in multiple target pathways which might be the key genes responsible for alkaline salt responses in rapeseed.

Materials And Methods

Plant materials and growth conditions

The salt-tolerant line 2205 (T) and salt-sensitive line 1423 (S) were grown in a greenhouse at 25 °C and 60% relative humidity under a 16 h light/8 h dark photoperiod. Firstly, the seeds were germinated on filter papers with distilled water for three days until the cotyledons unfolded. Secondly, the buds were transplanted into the matrix [Mineral: vermiculite: Perlite = 3:1:1]. When the seedlings reached the two-leaf stage after ten days, the uniform seedlings were placed to the Hoagland nutrient solution (pH 6.56) [56] for hydroponics. After growth for 6 days, alkaline-salt stress was performed in the Hoagland nutrient solution containing 75 mmol/L NaHCO₃ (pH 8.12), while the control group contained only the nutrient solution. After treated with 75 mmol/L NaHCO₃ for 0, 12 and 24 hours, the young leaves of line 2205 (T0, T12, and T24) and 1423 (S0, S12, and S24) were collected for RNA-seq. The leaves from 10 plants were pooled to form one biological replicate and three replicates were generated for each sample. In total, 18 samples were collected and stored at -80°C as independent biological samples.

Measurement of physiological indexes
To further evaluate and analyze resistance differences to alkali stress between lines 2205 and 1423, six conventional physiological indicators related to stress response, including relative electric conductivity (REC), soluble sugar (SS), soluble protein (SP), superoxide dismutase (SOD), and peroxidase (POD), were examined using the young leaves after treated with 0, 12, and 24 hours. REC was detected using the method described by Lutts et al. [57], SS and Pro were measured by the method of Zhao et al. [58], the examination of SP was performed by the description of Woolhouse et al. [59], SOD was determined by the method of Beauchamp and Fridovich [60], and POD was detected based on the procedure provided by Zhao et al. [61].

Electron microscopic observation of seedling cells under NaHCO$_3$ stress

At the three-leaf stage, the third true leaf was collected at 0 h (CK) and 7 d after alkaline salt stress and washed with distilled water. Then, the tissue block with a width of 1 mm and a length of 5 mm along the middle of the main leaf vein was cut. The same part of each leaf was taken once, and the process was repeated using three plants. The cut tissue was quickly placed into the 4% glutaraldehyde solution (pH = 6.8) for 12 h after vacuum. Then, the tissue was washed with 0.1 mM phosphate buffer saline (PBS) buffer (pH = 6.8) for 10 min for four times. The leaves were transferred to 0.1 mM osmium acid for 1.5-2.0 h, washed with 0.1 mM PBS buffer (pH = 6.8) for 10 min for five times, and then dehydrated in ethanol solutions with gradient concentrations (30%, 50%, 70%, 80%, and 90%) for 15 min. The samples were then treated with 100% ethanol for 30 min, and 100% acetone for 30 min twice, infiltrated with acetone, permeated with pure glue for 24 h, and then embedded with resin. The half-thin section was sliced by microtome, stained by uranyl acetate and lead citrate, and observed by a transmission electron microscope (HT7700, Hitachi).

RNA-seq analysis

The total RNA of these samples was extracted with the RNAprep Pure Plant Kit (TIANGEN, DP441) and subjected to quality control with a Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA concentration was measured by NanoDrop 2000 (Thermo). A total amount of 1 μg RNA per sample was used as the input material for RNA sample preparations. The libraries were sequenced on an Illumina HiSeq X Ten platform at Beijing BMK Biotechnology Co., Ltd. (Beijing, China).

Quality control was performed by removing reads containing adapter, ploy-N, and low quality from raw data. The clean paired-end reads were mapped to the designated reference genome of B. napus (http://www.genoscope.cns.fr/brassicaceae/data/) by the Hisat2 tools soft [62]. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the genome. Gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped (FPKM) [63]. Pairwise differential expression analyses were performed using the DEseq [64]. Comparison schemes for these two lines, the salt-tolerant line 2205 (T) and salt-sensitive line 1423 (S), at three-time points (0, 12, and 24 h) were set up to analyze the gene expression, including S0 vs T0, S12 vs T12, S24 vs T24, S0 vs S12, S0 vs S24, T0 vs T12, and T0 vs T24. The resulting p values were adjusted using the method by Benjamini and Hochberg for controlling the false discovery rate (FDR). Genes with an FDR < 0.01 and |log$_2$ fold change| ≥ 1 were considered to be differentially expressed.

Further analyses of DEGs were performed using the tools on the platform BMKCloud (www.biocloud.net). Venn and clustering heatmap plots were generated by R package ‘VennDiagram’ and ‘heatmap’. The box plots of DEG sets were drawn using the GraphPad Prism v9.0 software (San Diego, CA, USA).

Functional enrichment analysis of DEGs

Gene Ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R packages with the significant threshold of $P < 0.05$ [65], and the significantly enriched terms were graphed using the GraphPad Prism v9.0 software. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched using the KOBASE online analysis database [66], and the top 20 pathways were displayed.

Verified DEGs by qRT-PCR

To verify the differential expression detected by the RNA-seq, the remaining RNA$_3$s of the 18 samples were reverse transcribed in a 20-μl reaction mixture with a FastKing RT Kit (With gDNase, TIANGEN, KR116). The transcriptional expression of six randomly selected DEGs was explored by quantitative real-time PCR (qRT-PCR). The Actin gene was used as the internal control for qRT–PCR data analysis. The primers for these genes were listed in Table S6 (Additional file 1). The reaction was performed in 8-tube strips on a QuantStudio™ 7 Flex (Thermo Fisher Scientific, USA) using SYBR Green Master ROX (TaKaRa, Japan). Three technical replicates for each biological-replicate sample were analyzed. Relative fold differences of the selected DEGs among two lines or experiments were calculated using the 2$^{ΔΔCt}$ method [67]. Pearson's correlation coefficient was used to express the correlation of log$_2$(fold change) between samples. The results were shown by Graphpad Prism 9.0.

Statistical analysis

Data processing was conducted using Microsoft Excel 2019 and figures were generated using the Graphpad Prism 9. All triplicate data were subjected to one-way analysis of variance (ANOVA) using IBM SPSS Statistics 21. The data are presented as the mean (± SD) of three replications. Means were separated by the least significant difference test at 5% level of significance.

Abbreviations

ACO: 1-Aminocyclopropane-1-carboxylate oxidase; AOX: Alternative oxidases; BP: Biological process; Car-Met: Carbohydrate metabolism; CBL: Calcineurin B-like proteins; CC: Cellular component; CIPK: CBL-interacting protein kinases; DEGs: Differently expressed genes; Down-R. in T.: Down expressed in 2205
comparing with 1423; FDR: False discovery rate; FPKM: Fragments per kilobase of transcript per million fragments mapped; GAPC: Glyceraldehyde-3-phosphate dehydrogenase; GGAT: Glutamate:glyoxylate aminotransferase; GO: Gene ontology; GOX: Glycolate oxidase; KEGG: Kyoto Encyclopedia of Genes and Genomes; MDH2: Malate dehydrogenase 2; MF: Molecular function; PBS: Phosphate buffer saline; PCR: Polymerase chain reaction; Pho-Pro: Photosynthetic processes; POD: Peroxidase; PRO: Proline; qRT-PCR: Quantitative real-time PCR; REC: Relative electric conductivity; Res-Sal: Response to salt stress; RNA-seq: RNA-sequencing; ROS: Reactive oxygen species; S: Salt-sensitive line 1423; S0: Sensitive line 1423 without alkaline salt stress; S12: Sensitive line 1423 under 12h-alkali-salt stress; S24: Sensitive line 1423 under 24h-alkali-salt stress; SOD: Superoxide dismutase; SP: Soluble protein; SS: Soluble sugars; T: The salt-tolerant line 2205; T. spe.: Genes specifically different expressed in tolerant line 2205; T0: Tolerant line 2205 without alkaline salt stress; T12: Tolerant line 2205 under 12h-alkali-salt stress; T24: Tolerant line 2205 under 24h-alkali-salt stress; Up-R. in T.: Up expressed in 2205 comparing with 1423.

Declarations

Ethics approval and consent to participate

The two rapeseed 2205 and 1423 were bred by the State Key Laboratory of Crop Stress Biology for Arid Areas/College of Agronomy. We have gained permission to grow the two rapeseed cultivars. In this paper, all methods were carried out in accordance with national, and international relevant guidelines and regulations.

Consent for publication

Not applicable

Data availability

The Global Map of Salt-affected Soils (GSASmap) V1.0.0 was released by the Status of the World's Soil Resources, Food and Agriculture Organization of the United Nations (https://www.fao.org/soils-portal/data-hub/soil-maps-and-databases/global-map-of-salt-affected-soils/en/). RNA-seq analyses were performed using BMKCloud (www.biocloud.net). The reference genome of B. napus was downloaded from http://www.genoscope.cns.fr/brassicanapus/data/. The RNA-seq data have been submitted to NCBI BioProject with BioProject ID: PRJNA682529. The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

ZH and AXX were responsible for designing this study. YX and SXT carried out RNA-seq analysis and drafted the manuscript. QZ, HW, PL carried out gene expression; YZ, AB, HMC measured indexes; MFQ, KW carried out seedling cultivation; YJS XL and LZ collected important background information and provided assistance for data acquisition, data analysis, and statistical analysis. All authors have read and approved the content of the manuscript.

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Contributions

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References


Figures
Figure 1

Phenotypical photographs and physiological responses of lines 2205 and 1423 under control and alkaline salt stress. Line 2205 (A) and line 1423 (B) seedlings in the Hoagland nutrient solution, 7-day-stress 2205 (C) and 1423 (D) seedlings using 75 mmol/L NaHCO$_3$. Bar graph represents the content of soluble protein (E), soluble sugar (F), relative electric conductivity (G), the activity of superoxide dismutase (SOD, H), and peroxidase (POD, I), and proline content (J). The different letters indicate significant differences detected by the least significant difference test ($P < 0.05$).

Figure 2
Ultrastructural observation of the tolerant line 2205 and sensitive line 1423 under control and NaHCO₃ stress. The ultrastructure of leaves of line 2205 (A-C) and line 1423 (G-I) without alkaline salt stress, 7-d NaHCO₃ treated 2205 (D-F) and 1423 (J-L). Ch, chloroplast; GL, basal grains; OG, osmophilic granules; SG, starch granules.

Figure 3

Venn diagram of DEGs among different groups. (A) Numbers of DEGs among the comparisons of control and 12-h or 24-h NaHCO₃ stress. The colored parts represent the genes that respond specifically to salt in line 2205 (T. spe.) and line 1423 (S. spe.). (B) The intersection of DEGs among T. spe. (the red filled part in A), S. spe. (the blue filled part in A), S0 versus T0, S12 versus T12, S24 versus T24. The purple, blue, and black DEGs with underlines mark the concrete genes in subset \( \beta \), \( \beta \), and \( \beta \) for further analysis, respectively.
Expression clustering of DEGs selected in Figure 2. Clustering heatmaps of $\log_{10}(\text{FPKM} + 1)$ values for these DEGs in subset (A), (C), and (E), respectively. (B, D, F) Box plots display the distributions of expression for the associated cluster in A, C, and E. According to the heatmap, each subset can be clustered into three groups. The numbers were shown on the top. In the box plots, the line marks the median, the top and bottom of the box represent the interquartile range, and the whiskers extend to 1.5 times the interquartile range.
Significantly enriched terms and pathways for the selected DEGs. (A) Significant Gene Ontology (GO) terms enriched by the up (full line) and down (broken line) expressed genes in line 2205. GO terms were classified into three groups (biological process, cellular component, and molecular function). The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were shown for the genes from ‘up-R. in T’ (B) and ‘down-R. in T’ (C).
**Figure 6**

Candidate genes in the target pathways. (A) The Venn diagram shows the DEGs in the carbohydrate metabolism, photosynthetic processes, and ROS regulating under salt stress. (B) The heatmap displays the Log$_2$FPKM of 13 DEGs (overlapped DEGs in photosynthetic processes and ROS regulating under salt stress) and 42 DEGs (overlapped DEGs in carbohydrate metabolism and photosynthetic processes under salt stress) for lines 2205 and 1423 under control, 12- and 24-hour stress.
Figure 7

Validation of RNA-Seq transcriptional data using qRT-PCR. (A-F) Comparisons of RNA-seq data (By FPKM; green color bars) with qRT-PCR (By the relative expression of reference gene; purple lines) of randomly selected six genes. (G) The scatter plot shows the Pearson's correlation of expression change by qRT-PCR (Y-axis) and RNA-Seq (X-axis).

Supplementary Files

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- Additionalfile1TableS1S6.xls