Transcriptome Analysis of *Gossypium hirsutum* L. Reveals Different Mechanisms among NaCl, NaOH and Na$_2$CO$_3$ Stress Tolerance

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As an important source of fiber and edible oil, cotton has great economic value. In comparison to their individual studies, association and differentiation between salt and alkaline tolerance has not been focused yet by scientists. We have used next-generation RNA-Seq technique to analyze transcriptional changes under salt and alkaline stresses in cotton. Overall, 25,929 and 6,564 differentially expressed genes (DEGs) were identified in roots and leaves, respectively. Gene functional annotation showed that genes involving ionic homeostasis were significantly up-regulated under NaCl stress and Na$_2$CO$_3$ stress, and genes enriched in starch and sucrose metabolism were up-regulated under NaOH stress and Na$_2$CO$_3$ stress. Furthermore, a synergistic enhancing effect between NaCl and NaOH stress was also observed in this study. Likewise, our studies indicate further that genes related with starch and sucrose metabolism were regulated to respond to the high pH under Na$_2$CO$_3$ stress, inducing plant hormone signal transduction and key enzyme reactive oxygen species (ROS) activity to respond to ionic toxicity and intracellular ionic homeostasis. By analyzing the expression profiles of diverse tissues under different salt and alkaline stresses, this study provides valuable ideas for genetic improvements of cotton tolerance to salt-alkaline stress.

Plant growth and development is highly influenced by various biotic and abiotic factors, resulting in a destructive impact on their production. In the north east area of China, soil alkalinity is a major abiotic stress which is responsible for the decline of agricultural production and causes environmental hazard. Soils are considered as Saline-Alkaline soils, upon salinity in soils goes above 0.3%.

Soil affected by salt are basically classified in three categories: saline soils, alkaline soils and salt-alkaline soils. Saline soils comprises of excessive amount of neutral salts, which includes NaCl and Na$_2$SO$_4$, as a major part, resulting in salt stress. NaHCO$_3$ and Na$_2$CO$_3$ are responsible for the alkalization of soils by creating a high pH value, with a destructive effect on plants growth. Stress resulted from alkaline soils causes several issues of osmotic pressure stress, different types of ionic injuries and high pH stress. Plants under salt-alkaline conditions suffer from both salt stress caused by excessive salt ions and alkaline stress caused by high pH.

Salt-alkaline stress causes several damage to plants because of the presence of salt ions and high pH. Previous studies have shown that plants maintain intracellular homeostasis through osmotic adjustment, generating an active oxygen scavenging system and adjusting organic acid under NaCl stress. Genes associated with brassinosteroid biosynthesis were upregulated under alkaline stress (Na$_2$CO$_3$). Cao et al. found that the expression of Gshdz4 was induced by NaHCO$_3$, indicating that Gshdz4 is only responsible for resisting HCO$_3^-$, but not high pH. The response to NaHCO$_3$ of *T. hispidu* involves multiple physiological and metabolic pathways. The Aft2 gene plays a negative role, and Na$^+$-ATPase ENA1 is regulated during alkaline pH resistance in *Saccharomyces cerevisiae*. Three major mechanisms of plant resistance to salt stress have been found: maintenance of ionic and osmotic homeostasis, detoxification and growth regulation. High pH can also affect ion balance, root growth, organic acid accumulation and cellular processes. However, a little is known about the difference between salt and alkaline stress.

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As an important source of fiber, vegetable protein and edible oil, cotton has great economic importance. Soil salt-alkalinity has become an increasingly serious factor in cotton yield because of irrigation and secondary salinization. Much attention was paid to salt tolerance in cotton. However, the alkaline tolerance of cotton has not been well studied. Few research has been conducted to show the different responses to salt and alkaline stress in cotton. Recent studies have showed that genome-wide transcriptome analysis of cotton has become popular for studying stress tolerance. Because the genes involved in salt-alkaline response are numerous, salt-alkaline genes can be more easily identified via transcriptome and DEG analyses.

In our study, we used RNA-seq technology to analyze the expression profiles of diverse tissues under different salt and alkaline stresses. Firstly, the phenotypic changes in roots and leaves of cotton under Na$_2$CO$_3$, NaCl, and NaOH stress were observed, and the physiological indexes were measured to demonstrate phenotypic differences. Secondly, we studied the transcriptome changes between the stress groups and the control group. Finally, the difference between salt and alkaline stress was obtained. This study provides new ideas for genetic improvement of cotton tolerance to salt-alkaline stress.

### Results

**Phenotypic and physiological responses to different salt-alkaline stresses in *Gossypium hirsutum* L.**

Previous studies have reported that cotton is more sensitive to abiotic stresses at three-leaf stage. Different morphological has been observed in *G. hirsutum* Zhong 9807 during its three-leaf stage under various concentrations of Na$_2$CO$_3$ stress. We found that seedlings became obviously different between the treatment and control groups under 50 mM Na$_2$CO$_3$ stress after 12 h (Fig. S1). Two treatments with parallel concentrations of 100 mM NaCl and NaOH respectively were considered to study the comparative effects of CO$_3^{2-}$, Na$^+$ and high pH (Table 1). The salt-alkaline tolerance of Zhong 9807 was examined by comparing salt stress (NaCl stress) with two other alkaline stresses, NaOH stress and Na$_2$CO$_3$ stress. The phenotypic changes of the three treatments were shown (Fig. 1A,B). Leaves withered and lost lustre slightly under NaCl stress, while roots have no

| Component | Na$_2$CO$_3$ | NaCl | NaOH |
|-----------|-------------|------|------|
| Na$^+$ (mM) | 100 | 100 | 0.125 |
| pH | 11.32 | 7.00 | 11.32 |

Table 1. Stress comparison for 50 mM Na$_2$CO$_3$ with 100 mM NaCl and 0.125 mM NaOH.
evident changes in phenotype, suggesting roots may have stronger resistance than leaves. However, slight tarnish in leaves and nigers were found in roots under NaOH stress. When seedlings were subjected to Na2CO3 stress, roots became seriously withered and nigrescent, leaves tarnished heavily and veins almost darkened. The variance analysis results of the chlorophyll content and relative water content (RWC) under Na2CO3 stress (Fig. 1C,D) were significantly different from those of the control group except the RWC in roots.

Transcriptome sequencing and alignment. Using the allotetraploid species Gossypium hirsutum Zhong9807, RNA-Seq analyses were conducted on three biological repetitions of each sample. RNA samples of roots and leaves were collected at 12 h post-salt stress and alkaline stress. Seedlings transplanted to normal conditions were used as controls. 24 qualified libraries were established (Table S1). Raw reads were processed to remove adapter and low-quality reads initially. Clean reads were then mapped to the G. hirsutum reference genome using TopHat228. Approximately 213.36 Gb of clean reads was obtained. On average, 6.15 Gb of clean reads was obtained from each library. More than 87.02% of reads’ Q-score was Q30, and 78.23–83.44% of the total reads were aligned.

The aligned sequences were assembled with Cufflinks21, which was guided by a annotation genomes of tetraploid species G. hirsutum from CottonGen22. RNA-Seq assays revealed that there were 60,369 unigenes with 95.79% (57,825 unigenes) annotated genes and 9.18% (5,544 unigenes) novel genes (Table 2). For the evaluation of DEGs’ reliability and the filter of abnormal samples, Pearson correlation coefficient (PCC) analysis was conducted. The correlation analysis indicated that under all three salt-alkaline stresses, roots and leaves showed more than 85% similarities except for the genes between RCK-1 and RCK-2 and LSS-1 and LSS-3 (Fig. 2).

The gene expression profile under NaOH stress was almost the same as the situation of control group in roots. The gene expression profile under NaCl and Na2CO3 stresses, however, were different. This result demonstrated that the damage caused by NaOH stress was the slightest one. It also showed the damage caused by Na2CO3 stress was the most serious situation. The expression correlation analysis showed the similar results in leaves and roots (Fig. S2).

GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), NR (RefSeq non-redundant proteins), Swiss-Prot, COG (Cluster of Orthologous Groups), Pfam, and eggNOG (evolutionary genealogy of genes) annotation of the novel genes were conducted. Totally, 4,202 and 4,014 novel genes were annotated in roots and leaves, respectively. We found that genes in roots were enriched in “nucleic acid binding” and “RNA-dependent DNA binding” GO terms. The genes in leaves were found enriched in “nucleic acid binding” and “DNA binding” terms. KEGG pathway analysis indicated that genes were enriched in “carbon metabolism”, “amino acid biosynthesis” and “plant hormone signal transduction” pathways (Fig. S3).

Differentially expressed genes analysis in leaves and roots under various salt-alkaline stresses and control group. Gene expression levels were estimated by fragments per kilo base of transcript per million fragments mapped (FPKM). Differential expression analysis of treatments and control group was performed using the DESeq. A threshold of Fold Change ≥2 and FDR < 0.01 was used for identifying DEGs. In total, 25,929 DEGs (NaCl: 14,176; NaOH: 6,843; Na2CO3: 20,492) and 6,564 DEGs (NaCl: 452; NaOH: 50; Na2CO3: 6,458) were obtained in roots and leaves, respectively. DEGs numbers under different stresses in roots and leaves were presented as Na2CO3 > NaCl > NaOH stress, which illustrated that Na2CO3 stress induced a significant biological response in the plant as compare to NaCl stress and NaOH stress. In response to salt-alkaline stresses in roots and leaves, majority of genes got down-regulated in roots and up-regulated in leaves (Fig. 3B). These results indicated their tissue-specificity expression in response to stress29.

Approximately 8,800 (42.94%) of the total DEGs under Na2CO3 stress were found as root specific and 6,071 (94.00%) were leaf specific DEGs. Furthermore, 4,056 (28.59%) of the total DEGs under NaCl stress were root specific, while 85 (18.8%) DEGs were root specific. Moreover, 930 (13.59%) of the total DEGs under NaOH stress were root specific genes and 19 (38.00%) were leaf specific DEGs (Fig. 3C,D). Under Na2CO3 stress, 762 DEGs were commonly identified in roots and leaves (Fig. 3A). To study the expression profiles of these 762 genes under different salt-alkaline stresses, the FPKM of genes was normalized, and K-means cluster analysis with a normalized FPKM was used. These genes were divided into eight clusters. The genes in each cluster had the same expression profile (Fig. 4). Cluster 2 was the largest one in roots (164 genes, 21.52% of 762 genes), followed by cluster 8 (152 genes, 19.94%), cluster 4 (145 genes, 19.02%) and cluster 7 (101 genes, 13.25%). In leaves, cluster 4 was the largest profile (167 genes, 21.52% of 762 genes), followed by cluster 3 (162 genes, 21.26%), cluster 1 (121 genes, 15.88%) and cluster 7 (117 genes, 15.35%). The similar expression profile in roots and leaves was cluster 4, in which DEGs was up-regulated under Na2CO3 stress and unchanged under NaCl and NaOH stress. We mapped common cluster 4 genes within roots and leaves (Table S2) in the GO database. Results are presented in Fig. 5.

GO enrichment analysis of differentially expressed genes. To further investigate the function of DEGs, we mapped all of DEGs to the GO database24. 5,769 (87.89%) and 20,508 (79.09%) DEGs in leaves and

| Genes          | RSS | RAS | RSAS | LSS | AS | LSAS | Total |
|----------------|-----|-----|------|-----|----|------|-------|
| Annotation gene| 18436| 10122| 55903| 8129| 7672| 46078| 60369 |
| Novel gene     | 3318 | 3307 | 3240 | 3440 | 985 | 3179 | 5544  |

Table 2. Statistics of functional genes with Cufflinks. RSS: Root, 100 mM NaCl; RAS: Root, 0.125 mM NaOH; RSAS: Root, 50 mM Na2CO3; LSS: Leaf, 100 mM NaCl; LAS: Leaf, 0.125 mM NaOH; LSAS: Leaf, 50 mM Na2CO3.
roots were annotated, respectively. "Response to stimulus" (GO:0050896) was enriched in both leaves and roots after the results were classified. The enriched DEGs were different among the salt-alkaline stresses (Table 3), which were consistent with the phenotypic changes under different stresses. Under NaCl and NaOH stress, more genes related to "response to stimulus" were identified in roots than in leaves (Table 3), while more genes were recognized in roots as compared to those in leaves under Na2CO3 stress. The results implied that a more complicated physiological process occurred in roots than in leaves when the cotton plants were damaged by both NaCl and NaOH stresses. This was in contrast to the situation when seedlings were under Na2CO3 stress.

Functional enrichment of the annotated genes was conducted using topGO25, and the results of the enrichment were sorted by p-value numbers. Then the first 20 GO terms with the smallest p-values were chosen. GO enrichment analysis revealed an enrichment of genes involved in plant responses to salt stress, ionic homeostasis, organic substance, hormone signal pathways and osmotic stress in leaves and roots under salt-alkaline stresses (Fig. S4). The results revealed that "divalent metal ion transport", "hyperosmotic salinity response" and "cellular cation homeostasis" genes had higher expression levels under Na2CO3 stress. "Hyperosmotic salinity response", "response to osmotic stress" and "protein serine/threonine phosphatase activity" genes reached a higher expression level in NaCl stress, while these genes were non-significantly enriched under Na2CO3 stress. These genes are related to osmotic adjustment and maintenance of intracellular ionic homeostasis, playing vital roles in plant tolerance to salt stress26. According to statistics, DEGs under SS, AS and SAS appeared as follows: Na2CO3 > NaCl > NaOH stress (Table S3). Genes related to ion absorption and compartmentalization, such as SOS2, SOS3-Like, CCX, CDPK and ABC transporters, were detected from these genes27–31. These were up-regulated under Na2CO3 and NaCl stress, and were down-regulated under NaOH stress (Fig. 6).

**KEGG metabolic pathways annotation of sugar metabolism in responses to different alkaline stresses.** Annotation analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways32 of DEGs
contributed to the decoding of gene functions. A total of 1,392 (21.21%) and 5,510 (21.25%) DEGs were annotated in roots and leaves, respectively. The KEGG enrichment pathways (Fig. S5) indicated that the specific DEGs, found under three salt-alkaline stresses in roots and leaves, were widely enriched in the pathways of plant hormone signal transduction, photosynthesis, peroxidase and glutathione metabolism. According to previous studies, these pathways are related to abiotic stresses tolerance.

DEGs were significantly enriched in the pathway “starch and sucrose metabolism” (ko00500) under NaOH and Na2CO3 stresses, but not significantly enriched under NaCl stress. The sucrose content of leaves increased significantly when *lupin* was under NaCl stress. It has reported that exogenous sugar affected the hormone signal transduction, key enzyme metabolism and sucrose metabolism of grape seedlings. Among DEGs enriched in starch and sucrose metabolism (Figs 7A and S6), we found some genes that had up-regulated under NaOH and Na2CO3 stress and down-regulated under NaCl stress in roots (Fig. 7B). The TPS1-TPS2 (trehalose-6-phosphate synthase) lines displayed a significant increase in drought, freezing, salt and heat tolerance.

Transcription factors analysis and annotation. Many studies have reported that transcription factors (TFs) play an important role in stress tolerance. Transcription factor annotation was performed among 762 specific DEGs (Fig. 3A). These TFs were classified into 18 families and three protein kinases families. Except for bZIP (basic region/leucine zipper), NAC (NAM/no apical meristem, ATAF/Arabidopsis transcription activation factor, and CUC/cup-shaped cotyledon), MYB (v-myb avian myeloblastosis viral oncogene homolog) and ERF (ethylene response elements) families were related to salt-alkaline tolerance, C2H2 (Cys2/His2) transcription factor family was also enriched (Fig. 8). C2H2, a zinc finger protein, is related to the osmotic stress tolerance of *Arabidopsis thaliana*.

Validation of RNA-Seq data by quantitative real-time PCR. To verify the reliability of sequencing, quantitative real-time PCR (qRT-PCR) was performed using the same RNA samples that were previously used for RNA-seq. A total of 20 genes were randomly selected for qRT-PCR, including 10 up-regulated genes and 10 down-regulated genes. Linear correlation analysis of the data of these two groups was performed. The fold change...
(FC) of genes between salt-alkaline-stressed tissues and controlled treatments using qRT-PCR were compared to those ones using RNA-Seq. The correlation coefficients between qRT-PCR and RNA-seq were significant in the roots and leaves under different salt-alkaline stresses (Fig. S7).

**Discussion**

**Synergistic enhancing effect of salt stress and alkaline stress.** Salt-alkaline situation often results in osmotic stress to plants firstly, following by ionic toxicity and consequently oxidative stress, which leads to an increase in intracellular active oxygen. Numerous studies on molecular mechanisms of cotton tolerance to salt stress have been conducted by mainly focusing on salt tolerance, but few have investigated the difference between salt tolerance and alkaline tolerance. In comparison with the situation under NaCl and NaOH stresses, maize seedlings suffered more changes under Na$_2$CO$_3$ stress. In this study, significant phenotypic differences were observed in roots and leaves under Na$_2$CO$_3$ stress.

RNA-seq analysis of roots and leaves under NaCl, NaOH and Na$_2$CO$_3$ stresses was performed to investigate different molecular mechanisms under salt and alkaline stress. The expression profiles induced by Na$_2$CO$_3$ and NaCl in *Puccinellia tenuiflora* and maize were relatively pronounced, while those induced by NaOH were relatively unchanged. Results indicated that NaOH stress have triggered few biological responses, probably related to the neutralization of pH by organic acid, such as ascorbic acid accumulation, alleviating serious damage. Significant biological responses occur under NaCl stress, because plants cannot eliminate ions immediately and may constantly suffer from osmotic stress and ionic toxicity. More biological responses may occur in plants if there is both high pH and salt stress under Na$_2$CO$_3$ stress. In our study, the DEGs numbers are presented as: Na$_2$CO$_3$ > NaCl > NaOH.
stress which showed in Fig. 3. It indicates that there is a complicated and synergistic effect between NaCl and NaOH stresses. It also explains that NaCl and NaOH stresses together exhibit significant damages to plants.

Ion homeostasis in response to Na\(^+\) stress. Plant under salt-alkaline stress may first generate a series of substances for osmotic adjustment and then regulate relevant genes expression to maintain ionic homeostasis and balance the cellular osmotic potential\(^{6,49}\). According to GO function enrichment of DEGs, we found that the GO enrichment terms “hyperosmotic salinity response”, “response to osmotic stress”, “protein serine/threonine phosphatase activity”, “divalent metal ion transport”, and “cellular cation homeostasis” genes, which are related to osmotic adjustment and maintenance of intracellular ion homeostasis, were enriched under NaCl and Na\(_2\)CO\(_3\) stresses, but not under NaOH stress\(^{27,30}\). Hence, we paid close attention to these genes related to osmotic adjustment and ion homeostasis. As shown in Fig. S4, there may be a special mechanism of Na\(^+\) stress tolerance.
The ABC (ATP-binding cassette) transporters use the energy produced by hydrolysis of ATP for the transport of micromolecules and play a vital role in ion homeostasis\(^5\). In our study, several ABC transporter genes were up-regulated under NaCl and Na\(_2\)CO\(_3\) stresses and down-regulated under NaOH stress. We found that the\(\text{ABCB}21\) (Gh\(_\text{A12G1090}\)), \(\text{ABCG}36\) (Gh\(_\text{A10G0583}, \text{A05G1089}, \text{Sca006272G01}\)), and \(\text{ABCA}2\) (Gh\(_\text{A09G1286}\)) genes in the roots of \(\text{Gossypium hirsutum}\) were up-regulated under Na\(^+\) stress. Kamimoto \textit{et al.} demonstrated that \(\text{Arabidopsis}\) \(\text{ABCB}21\) imported/exported auxin\(^5\). Auxin (IAA) regulated NAC transcription factors of \(\text{NTM}2\) expression under salt stress\(^5\). In our study, we found that the NAC family was enriched under Na\(_2\)CO\(_3\) stress. Kim \textit{et al.} found that \(\text{AtPDR}8\) (\(\text{ABCG}36\)) was an efflux pump of Cd\(^{2+}\)\(^5\). \(\text{AtPDR}12\) mediates the uptake of ABA (abscisic acid) in \(\text{Arabidopsis}\)\(^\text{54}\). ABA stimulates calcium-dependent protein kinases (CDPKs) and serine/threonine phosphatase activity (SOS2). The level of SOS2 and SOS3-Like (also known as CBLs) proteins were both up-regulated in roots and leaves under Na\(^+\) stress. The protein SOS2 can regulate Na\(^+\)/K\(^+\) transport proteins and CBLs worked as a calcium sensor in plant salt tolerance\(^5\). \textit{cpk} mutation is salt-insensitive\(^5\). CBLs and CDPKs can alter the

**Figure 6.** Heatmap for the co-expression of genes clusters related to ionic homeostasis in roots and leaves under different stresses. RCK: Root, Control group; RSS: Root, 100 mM NaCl; RAS: Root, 0.125 mM NaOH; RSAS: Root, 50 mM Na\(_2\)CO\(_3\); LCK: Leaf, Control group; LSS: Leaf, 100 mM NaCl; LAS: Leaf, 0.125 mM NaOH; LSAS: Leaf, 50 mM Na\(_2\)CO\(_3\). (A) Heat map log\(_2\) FC (Fold Change) value of the expression level cluster of roots under different stresses. (B) Heat map log\(_2\) FC (Fold Change) value of the expression level cluster of leaves under different stresses. Red = high expression level of genes, and Green = low expression level of genes.
transcriptional profile, such as the transcription factors (MYB, WRKY, and bZIP), which are regulated and expression of the downstream genes is changed. MYBs regulated the genes of the anthocyanin pathway in Rosaceae. Kim et al. introduced a double knockout mutant of bZIP17 and bZIP28 to analyze the function of bZIP17. They found that mutant plants exhibited multiple developmental defects including scarce root elongation. These TFs were significantly up-regulated under Na\(^+\) stress in roots and leaves. We also found that CCX-related genes were up-regulated.

Chen et al. speculated that AtCCX1 is vital for Na\(^+\) resistance and serves as a Na\(^+\)/K\(^+\) exchanger in vacuoles. These results indicate that ionic homeostasis may not be affected by the high pH induced by NaOH stress. However, high pH may increase osmotic stress together with Na\(^+\) and produce a synergistic enhancing effect of NaCl and NaOH stresses. The reason is that Na\(^+\) easily affects the dynamic equilibrium of the cytomembrane under environment with high pH. The thorough mechanism of the synergistic enhancing effect needs further study.

**Figure 7.** Analysis of differential genes in pathways of starch and sucrose Metabolism. RCK: Root, Control group; RSS: Root, 100 mM NaCl; RAS: Root, 0.125 mM NaOH; RSAS: Root, 50 mM Na\(_2\)CO\(_3\). (A) A schematic diagram of sugar metabolism. Digits represents regulatory enzyme for specific process. (B) Up-regulated DEGs under Na\(_2\)CO\(_3\) and NaOH treatments and down-regulated DEGs under NaCl treatment.

**Figure 8.** Annotation of transcription factors of specific DEGs tolerance to Na\(_2\)CO\(_3\) stress.
Oxygen deficiency in response to high pH. For further detection of the differences between salt and alkaline stresses, KEGG pathways enrichment analysis of DEGs was performed. The results demonstrate that under NaOH and Na$_2$CO$_3$ stresses, DEGs were found significantly enriched in “starch and sucrose metabolism” pathways, but non-significantly under NaCl stress. It has been noted in results that genes enriched with this pathway may have some relationship with high pH. It has been reported in previous studies that the cytosolic pH of maize roots decreases from 7.5 to 6.5 under oxygen deprivation. Anna belle et al. found that the transcription level of Sus2 (sucrose synthase) was ABA-dependent and specifically induced by O$_2$ deficiency. We found that genes encoding Sus were up-regulated under high pH stress (under both NaOH and Na$_2$CO$_3$ stresses, but not NaCl stress). Hence, we speculate that high pH results insufficient oxygen level in plant cells, followed by induction of Sus genes and increased transformation of glucose into sucrose β-glucosidase (BGLU) was accumulated in the ER (endoplasmic reticulum) body, which plays a vital role in the defense system of plants. β-glucosidase is a substrate of glucose production, and BGLU genes were up-regulated. Plants use HKX (hexokinase) as a glucose sensor in response to a changing environment. Mu et al. showed that trehalose-6-phosphate synthase (TPS) genes are stress-related in cotton. Nelson et al. found that AtTPS1 is probably a member of the HKX1-dependent Glc-signaling pathway, and that O$_2$ deficiency is regulated by the expression of Ugp1. Studies have reported that the use of exogenous sugars may affect hormone signal transduction, key enzyme metabolism and sucrose metabolism of grape seedlings. Genes enriched in “starch and sucrose metabolism” pathways are up-regulated under NaOH and Na$_2$CO$_3$ stress and down-regulated under NaCl stress. These genes then participate in plant hormone signal transduction and affect the activities of some key enzymes (such as ROS, ATPase and RuBisCO). Galacturonosyltransferases (GalATs/GAUT) are required for the synthesis of pectin. Pectin methyl esterase (PME) catalyzes pectin deesterification, releasing acid pectin and methanol, which cause cell wall changes. The Subcellular locations of GAUT, PME and UDP-D-glucuronate 4-epimerase (GAE) are Golgi, suggesting that PME, GAUT and GAE were related to synthesis of cell wall.

The expression of these genes indicates that high pH firstly causes oxygen deprivation stress to cotton plants. Lacking of oxygen leads to anaerobic respiration of plants and produce ethylene which causes roots rotten. In addition, there were nigrities in roots under NaOH and Na$_2$CO$_3$ stresses and in leaves under Na$_2$CO$_3$ stress. Hence, we speculated the phenotypic changes under high pH were related to the oxygen deprivation signal pathway. Consequently, glucose synthesis and decomposition, TPS synthesis and other sugar metabolisms are involved in defending against oxygen deprivation. What is more, genes related to the synthesis of pectin were up-related. Pectin is an important cell wall polysaccharide that allows primary cell wall extension and plant growth. We inferred that high pH induce genes that recoded proteins of cell wall synthesis (PME, GAUT and GAE) were up-regulated to strengthen cell wall and defend high pH damage.

Conclusion
This paper described the possible mechanism response to Na$_2$CO$_3$ tolerance in cotton. In general, most of the genes and proteins related to Na$^+$ stress (treated with NaCl) and high pH (treated with NaOH) are also involved in the pathways against Na$_2$CO$_3$ stress (Fig. 9). We speculate that cotton may regulate the metabolism of starch and sucrose due to the toxicity of high pH, which results in the specific expression of some genes under Na$_2$CO$_3$ and NaOH stresses. High pH leads to oxygen deprivation stress, which causes cotton organs nigrities and rotteness. In addition, high pH also increases the synthesis of pectin-related enzymes, which strengthens cell wall to defense damage of high pH. In the process of the hydrolysis of ATPase, extra H$^+$ produced help to neutralize OH$^-$ within the cytoplasm.

Besides, genes and proteins related with ion homeostasis under Na$^+$ stress were also found in our study, such as protein kinases, transcription and transporters (Fig. 9). And these genes and proteins have been reported in previous studies. MYBs that regulate genes of the anthocyanin pathway were up-regulated under Na$_2$CO$_3$ stress, which always cause leaves to turn red or orange in apple. bZIPs play an important role in roots development under salt stress.

The mechanism of salt and alkaline tolerance in cotton are complicated. Further study is still needed, even though several genes have been transported into cotton and other plants. Here our study provides some candidate genes particularly responding to high pH and Na$^+$ stresses. For instance, Hexokinase (HKX) acted as a sugar sensor in eukaryotic cells, being found to be up-regulated under high pH stress, which indicates that genes encoding HKX may be related with high pH stress.

Materials and Methods

Plant materials and salt-alkaline stresses conditions. G. hirsutum cultivars Zhong9807 was used for this study. Seeds were sown in sand soil pots. The sand was washed clean and sterilized at 121 °C for 8 h. Four seedlings in each pot were cultivated in a 28°C/14 h light and 25°C/10 h dark cycle with a light intensity of 150 μmol m$^{-2}$ s$^{-1}$ and 75% relative humidity for approximately 30 days. Seedlings containing three true leaves and one heart-shaped leaf were washed out carefully and transplanted into conical flasks containing 0, 25, 50, 75, and 100 mM Na$_2$CO$_3$ solution for 0, 6, 12, and 24 h to observe phenotypic changes. Then, 50 mM Na$_2$CO$_3$ over 12 h was chosen as the applicable stress concentration and time. In addition, for salinity stress treatment, seedlings were transferred into ddH$_2$O supplemented with 100 mM NaCl (pH = 7.0). For alkaline stress treatments, seedlings were transplanted into ddH$_2$O supplemented with 0.125 mM NaOH (pH = 11.32) and 50 mM Na$_2$CO$_3$ (pH = 11.32). Seedlings transplanted into normal ddH$_2$O were used as controls. After exposure for 12 h, antepenultimate leaf and whole root samples were collected. Each sample was tested three time. Samples were frozen in liquid nitrogen and stored at −80°C for physiological measurement and transcriptome analysis.
Measurement of relative water content and chlorophyll content. The relative water content (RWC) of roots and leaves was calculated following the method described previously73. The total chlorophyll content of leaves was measured spectrophotometrically using the method of Arnon 74, and variance analysis proceeded as described by Anders 19.

cDNA libraries preparation and RNA-seq. Total RNA of roots and leaves was extracted. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Qualified RNA samples were randomly digested with fragmentation buffer. Library preparation for RNA-Seq was performed using the Employed NEB Next Ultra TM RNA Library Prep Kit (NEB, USA). Library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq. 2500 platform and paired-end reads were generated.

Quality control, alignment and differential expression genes analysis. Raw data in the fastq format was first processed through in-house perl scripts. The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were mapped to the G.hirsutum genome using TopHat2 tool20. These mapped reads were spliced using Cufflinks software21 based on the reference genome sequence. Quantification of the gene expression levels were estimated as fragments per kilobase of transcript per million fragments mapped (FPKM)75. Differential expression analysis of two groups was performed using the DESeq R package (1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 were found using DESeq76 and were assigned as differentially expressed. Consequently, DEGs were obtained of three biological conditions. Fold Change ≥ 2 and FDR < 0.01 were taken as the thresholds for determining whether a gene had differential expression.

Gene ontology and gene pathway enrichment analysis. Gene Ontology (GO) enrichment analysis of the DEGs was implemented using the GOseq R packages based on the Wallenius non-central hyper-geometric distribution, which can adjust for gene-length bias in DEGs24. KEGG32 is a database resource for understanding high-level functions and utilities of the biological systems, such as the cell, organism and ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS77 software to test the statistical enrichment of differential expression of genes in KEGG pathways.

Figure 9. Model of the regulatory networks in response to Na⁺ stress and high pH. The left blue part of the networks is related to Na⁺ stress, the right red part of the networks is related to high pH. The crossed green part is related to both Na⁺ stress and high pH.
**qRT-PCR verification of RNA-seq data.** qRT-PCR was carried out using the same samples. 20 genes were randomly chosen (Table S4), including 10 up-regulated and 10 down-regulated genes from the roots and leaves according to the FPKM. qRT-PCR was performed using the Applied Biosystems® 7500 Fast instrument and Top Green qPCR SuperMix. Reactions were performed with three technological and biological repetitions: 0.4 μL of each primer (10 μM/μL), 0.4 μL of passive reference Dye and 10 μL of Top Green qPCR SuperMix at a final volume of 20 μL. The profile for amplification was as follows: 5 min at 95°C, followed by 40 cycles amplification of 95°C for 15 s, then 20 s at 58°C, and 30 s at 72°C. The ΔΔCt method was used to calculate the relative fold change for each sample. The GhHsH3 house-keeping gene was used as a control. The correlation coefficients between qRT-PCR and RNA-seq was performed.

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Author Contributions
Wuwei Ye conceived and designed the experiments. Binglei Zhang, Xiugui Chen and Xuke Lu performed the experiments, prepared the materials and conducted the manuscript. Na Shu, Xiaohe Wang and Xiaomin Yang participated in data analyses. Thanks Shuai Wang, Junjuan Wang, Delong Wang, Lixue Guo for providing some significant advices. All authors read and approved the final manuscript.

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