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RESEARCH PAPER

Physiological and proteomics responses of nitrogen assimilation and glutamine/glutamine family of amino acids metabolism in mulberry (*Morus alba* L.) leaves to NaCl and $NaHCO_3$ stress

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ABSTRACT

In order to find out the response mechanism of nitrogen assimilation and glutamine/glutamine family of amino acids metabolism in mulberry (*Morus alba* L.) leaves under NaCl and $NaHCO_3$ stress, and to reveal its role in salt alkali adaptation. The effects of the nitrogen metabolism of mulberry leaves were studied under 100 mmol L^{-1} NaCl and $NaHCO_3$ stress.The results showed that the activity of NR and the content of TN and SP did not change significantly, the expression of NiR, Fd-NiR, *Fd-NiR* gene and theactivity of NiR increased significantly under NaCl stress, but nitrogen assimilation was inhibited under $NaHCO_3$ stress. NaCl stress had no significant effect on the expression and activity of GS and GOGAT in mulberry leaves. Under $NaHCO_3$ stress, the expression of Fd-GOGAT, Fd-GOGAT2, *Fd-GOGAT* gene, and the activity of GS and GOGAT were significantly decreased. NaCl stress can promote the accumulation of Pro, Put and Spd in mulberry leaves. The accumulation of Pro under $NaHCO_3$ stress is greater than that under NaCl stress. NaCl stress also induced the up-regulation of GAD, GAD1 and *GAD1* gene expression, so promoting the synthesis of GABA may be an adaptive mechanism for mulberry to cope with NaCl stress, but the expression of GAD did not change significantly and *GAD* gene expression lower than CK under $NaHCO_3$ stress. Although both NaCl and $NaHCO_3$ stress could promote the synthesis of GSH by up-regulation of GCLM expression, GSH under $NaHCO_3$ stress was significantly higher than that under NaCl stress, the content of H_2O_2 was still significantly higher than that of NaCl stress, that means GSH may not play a key role in alleviating the oxidative damage in mulberry leaves caused by salt and alkali.

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Nitrogen is one of the most important factors limiting the growth of plants, and the yield and quality of crops.[1–3] Nitrogen metabolism mainly consists of nitrogen uptake, transport, assimilation, etc. NO_3^-N and NH_4^+-N are the main forms of nitrogen used by plants. To be specific, NO_3^- can be assimilated as the glutamine/glutamine family and amino acid organic nitrogen by glutamine synthetase (GS) and glutamate synthase (GOGAT) only after being reduced into NH_4^+ under the actions of nitrate reductase (NR) and nitrite reductase (NiR).[4–6] Amino acids synthesize proteins in cells, which are then modified, transported, and stored before becoming components of plant organisms. Reasonable nitrogen metabolism constitutes a precondition of normal plant growth. However, nitrogen metabolism is also a process relatively sensitive to saline-alkali adversity stress. Saline-alkali stress leads to the disorder of nitrogen uptake and metabolism in plants.[7] The amino acids of the glutamine/glutamine familymetabolites mainly include glutamate (Glu), glutamine (Gln), arginine (Arg) and proline (Pro), as well as γ-aminobutyric acid (GABA) and polyamines (PAs).Glu and Gln play vital roles not only in the nitrogen metabolism of plants, but also in the regulation of their carbon metabolism.[8,9] Under abiotic stress, plants often accumulate Pro and PAs to regulate intra-cellular osmotic potential and promote water uptake. There is a positive correlation between the accumulation of Pro and the adaptability of plants to drought and salt stress ,[10] Pro also functions as signaling molecules in the growth and development of plants (such as inducing the expression of ABA) ,[11,12] serves as an inducer for osmotic stress-related genes and a scavenger for reactive oxygen species (ROS) ,[13–15] and plays a crucial part in regulating adversity stress in plants.[16] Under salt stress, the accumulation of Pro can also effectively reduce Na^+ uptake by plant roots and lower Na^+/K^+ ratio in plants.[17,18] The accumulation of PAs is a protective plant response to stress that enhances the resistance of plants to biotic and abiotic stresses.[19–21] Besides osmotic regulation ,[22,23] PAs are also capable of directly stabilizing cell membrane structures.[24,25] GABA is also considered as a signaling molecule that is capable of regulating the growth and development of plants, and plays an important role in terms of improving the water retention capacity of cells ,[26,27] regulating the transduction of cell signals ,[28] and adjusting the metabolism of ROS in plants, etc..[29,30] The products generated in the metabolic process of the glutamine/glutamine family, such as GABA and NO, can also function as signaling molecules in regulating the growth and development of plants and their responses to adversities.[31–33] In brief, the metabolic process of glutamine/

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glutamine family is not only affected by environmental factors, but also one of the most important mechanisms for plants to actively respond to adversities and improve their adaptability to adversities.

Mulberry(*Morus alba* L.) is a deciduous tree species of *Moraceae*. Besides being used as silkworm feed, its leaves also contain antioxidative active ingredients, which have important economical and medicinal values.[34,35] In addition, mulberry is also resistant to drought, cold, and low temperature, and is a fine tree species with high economic and ecological values.[36,37] More concretely, saline-alkali stress serves as an important factor that limits the growth of plants, and the yield and quality of crops (as it inhibits plant photosynthesis and photorespiration).[38] This results in metabolic disorders of ROS ,[39] and destroys proteins, nucleic acids, and other structures.[40] In the natural environment, soil contains both neutral salts (NaCl and Na_2SO_4), and alkaline salts ($NaHCO_3$ and Na_2CO_3) ,[41] the ion toxicity of alkaline salts is equivalent to that of neutral salts, but alkaline salts cause more serious harm to plants, mainly due to their higher pH values.[42,43] The Songnen Plain in Northeast China is extensively covered by $NaHCO_3$-based saline-alkali soil, where the highly alkaline environment restricts the promotion of mulberry to a great extent.[44,45] However, the current research in this field rarely involves the effects of saline-alkali stress on the metabolic process of glutamine/glutamine family in plants. Under this context, it is necessary to reveal the depth mechanism of nitrogen assimilation and the mechanism by which the metabolic process of the glutamine/glutamine family responds to saline-alkali stress; this is of vital significance for regulating the saline-alkali tolerance of plants. Our previous study showed that mulberry was tolerant of neutral salts, but relatively sensitive to alkaline salts based on NaHCO3.[38,46–48] To clarify the mechanism of this phenomenon and provide a theoretical basis for the reasonable planting of mulberry, this experiment investigated nitrogen assimilation and the metabolic process of the glutamine/glutamine family of amino acids, combined proteomic technology and plant physiology, explored the effects of NaCl and $NaHCO_3$ stresses at the same Na^+ concentration (100 mmol L^{-1}) on nitrogen assimilation, and the metabolic process of the glutamine/glutamine family of amino acids in mulberry leaves. The study mainly focused on the responses of GABA, Pro, PAs, and other metabolic processes to NaCl and $NaHCO_3$ stresses, aiming to identify the mechanism of the effects of NaCl and $NaHCO_3$ stresses on nitrogen metabolismin mulberry leaves, and to reveal the adaptation mechanism of the seedling leaves of mulberry under NaCl and $NaHCO_3$ stresses through nitrogen metabolism.

1 Materials and methods

1.1 Experimental materials and treatments

This experiment was carried out in the Soil Science Laboratory of Northeast Agricultural University (Harbin, China) in 2018. One-year-old mulberry seedlings were selected and planted in plastic pots with a diameter of 30 cm and a height of 28 cm. Two plants were planted in each pot, with a seedling height of about 30 cm. The culture medium was fully mixed with peat soil and perlite at a volume ratio of 1:1. NaCl or $NaHCO_3$ treatment were irrigated with 1 L NaCl or $NaHCO_3$ solutions at a concentration of 100 mM (Na^+ content is 2.3 gkg^{-1}). The salt concentration and irrigation volume were simulated according to the types and contents of the main salt in saline-alkali soil of Songnen Plain in China. A plastic tray was attached under each pot to prevent any loss of salt/alkali solution. Any solution found in the tray was poured back into the culture medium. The remaining five pots were irrigated with the same volume of distilled water (1 L for each pot) and used as the control (recorded as CK). On the 7th day after irrigation, the parameters were measured after differences in growth phenotypes were apparent.[38]

1.2 Parameters and measuring methods

Determination of total nitrogen (TN): plant leaves are dried at 80 °C, crushed and screened at 40 mesh, digested with concentrated H_2SO_4-H_2O_2, and determined by Micro-Kjeldahl method.[49] Determination of soluble protein (SP) and proline [Pro) content using fresh samples by 50. H_2O_2 content was determined as described in 51. Nitrate reductase (NR], nitrite reductase (NiR), glutaminesynthetase (GS), glutamate synthase (GOGAT) activity and reduced glutathione (GSH) content were determined using the kits produced by Suzhou Comin Biotechnology Co., Ltd (Suzhou, Jiangsu, China). Determination of putrescine (Put), spermidine (Spd) and spermine (Spm) content: Add 4 ml of precooled 5% perchloric acid to the leaves of fresh mulberry seedlings, grind in ice bath and centrifugate at 4 °C for 30 min (15000 × g). Take 500 µl supernatant and add it into 10 ml centrifuge tube, then add 7 µl benzoyl chloride and 1 ml 2 M NaOH, swirl for 20 s, then react in water bath at 37 °C for 20 min.Add 2 ml of saturated NaC1 solution and mix well, add 2 ml of ether for extraction, centrifugate 1500 × g for 5 min, take 1 ml of ether phase for vacuum drying, dissolve with 100 µl methanol, and then pass through 0.45 µM filter membrane. The content of putrescine (Put), spermidine (Spd) and spermine (Spm) were determined by high-performance liquid chromatography (HPLC, Agilent),in which the chromatographic column was Spherisorb C18 (Waters 3.9 × 150 mm, 10 µm),the UV detection wavelength was 230 nm, the mobile phase was methanol: water = 60:40 (V/V), the flow rate was 0.7 ml min^{-1}, and the injection volume was 20 µl. All the above indexes were measured three times of biological repetition.

Determination and analysis of proteomic:The leaves of mulberry seedlings in different treatments were collected and precooled with liquid nitrogen, and then sent to PTM Biolabs in Hangzhou Eco & Tech Developmental Area [Hangzhou, Zhejiang, China) in an incubator with dry ice for proteomics determination. Proteins related to nitrogen assimilation and glutamine/glutamine family of amino acids metabolism that showed differential expression by 1.2-fold were subjected to further analysis according to 38.

qRT-PCR analysis: Based on the findings of proteomics analysis, we chose nitrogen assimilation and glutamine/glutamine family of amino acids metabolism proteins for RT-PCR analysis for the verification of proteomics data according to 46. Gene primers sequences (5′-3′] are as follows:

NRT1.3: F: TCGTCTTCACTGAATGTATCGTCA; R: TACGCCCAAGTGAAAGCTCA.

Fd-NiR: F: TGATCCCAGTTTGCAGAGCC; R: TGCACACTCGAGTTCTTTTTAGGT.

Fd-GOGAT: F: CCGCACGCGTGTGTATATAAAAT; R: GTTTTCATGGGTCTCGCATGT.

GS: F: GCGGATCTGGTTTGGACGTA; R: TGCAGCAGCATGTCTCTTGT.

P5 CS: F: GGAGATTGGCGTTGGGAAGA; R: GGTGACATCGAGCTGGCTAA.

GAD1: F: CATGTGGATGCAGCAAGTGG; R: TTCTCCAAATGACCCACCCG.

OAT: F: CTGGAGAGCACGGAAGTACC; R: GGCTTGGCAAGAACTCCTCT.

SPDS: F: CCGACATATCCAAGTGGTGTCA; R: TCAGAAAGGTTGGCAAGGCA.

1.3 Data processing

Excel and SPSS (22.0) were used to analyze the measured data. All data were the mean ± standard error (SE) of three repetitions, and the differences among different treatments were compared by one-way ANOVA and LSD. Taking $P < .05$ as significant according to Duncan's multiple range test, $P < .01$ as very significant according to Duncan's multiple range test.

2 Results

2.1 Nitrogen assimilation

In Figure 1, TN and SP contents and NR activity of mulberry leaves under NaCl stress did not change significantly, but NiR activity increased by 17.79% ($P < .05$) compared with CK, while TN, SP content and NR, NiR activity under $NaHCO_3$ stressdecreased very significantly under $NaHCO_3$ stress compared with CK.

2.2 Glu-Gln cycle

In Figure 2, the activity of GOGAT and GS in mulberry leaves under NaCl stress did not change significantly, but the activity of GOGAT and GS under $NaHCO_3$ stress decreased by 60.18% ($P < .01$) and 29.42% ($P < .01$) respectively compared with CK.

2.3 Pro and PAs metabolism and other metabolic processes of Glu

Under NaCl and $NaHCO_3$ stress, the Pro content in mulberry leaves increased by 28.57% ($P < .01$) and 43.13% ($P < .01$) respectively compared with CK (Figure 3a).The content of Put and Spd under NaCl stress increased by 26.72% ($P < .01$) and 18.85% ($P < .01$) compared with CK, respectively, and the content of Spm did not change significantly. The content of Put and Spd increased

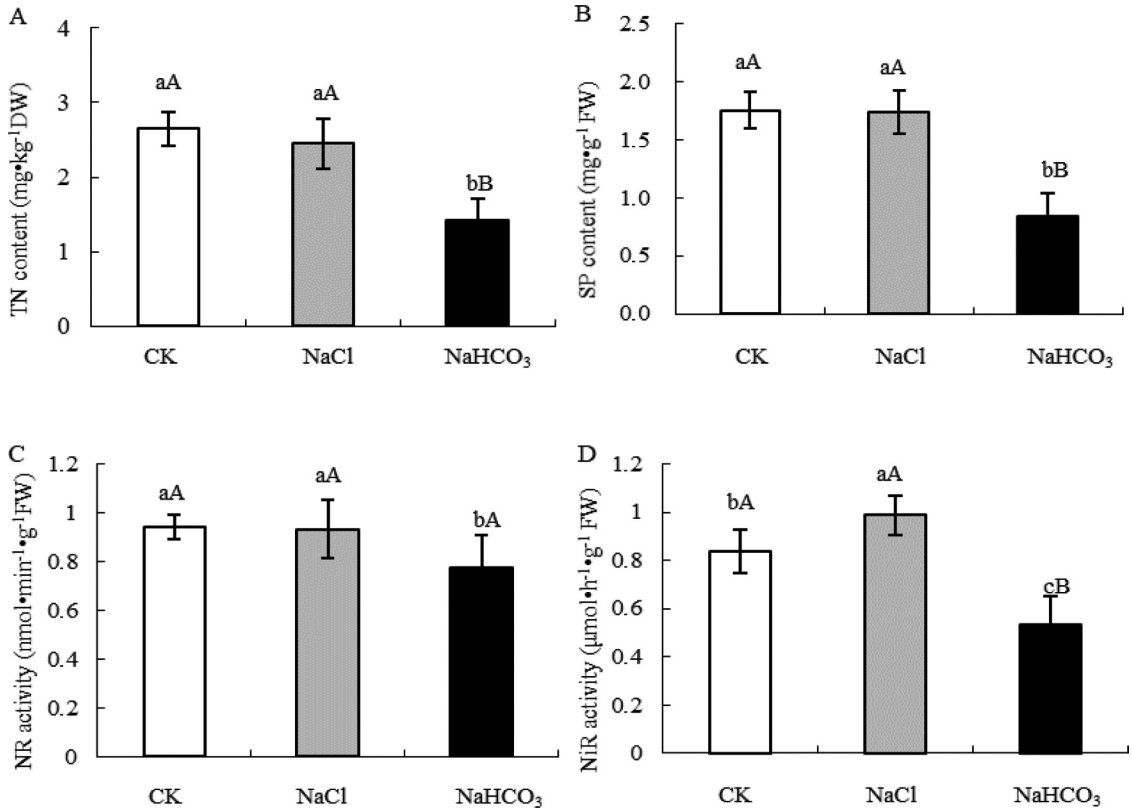


Figure 1. TN content (a), SP content (b), NR activity (c) and NiR activity (d) of mulberry leaves under NaCl and $NaHCO_3$ stress. Note: The data are from three replicated experiments (n = 3), and represent means ± SE. Significant differences were expressed by different small letters ($P < .05$), and very significant differences were expressed by different capital letters ($P < .01$).

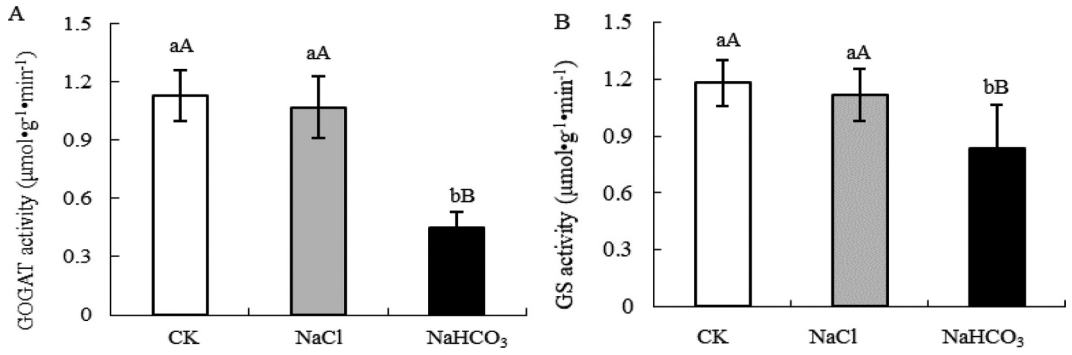


Figure 2. GOGAT activity (a) and GS activity (b) of mulberry leaves under NaCl and $NaHCO_3$ stress. Note: The data are from three replicated experiments (n = 3), and represent means ± SE. Significant differences were expressed by different small letters ($P < .05$), and very significant differences were expressed by different capital letters ($P < .01$).

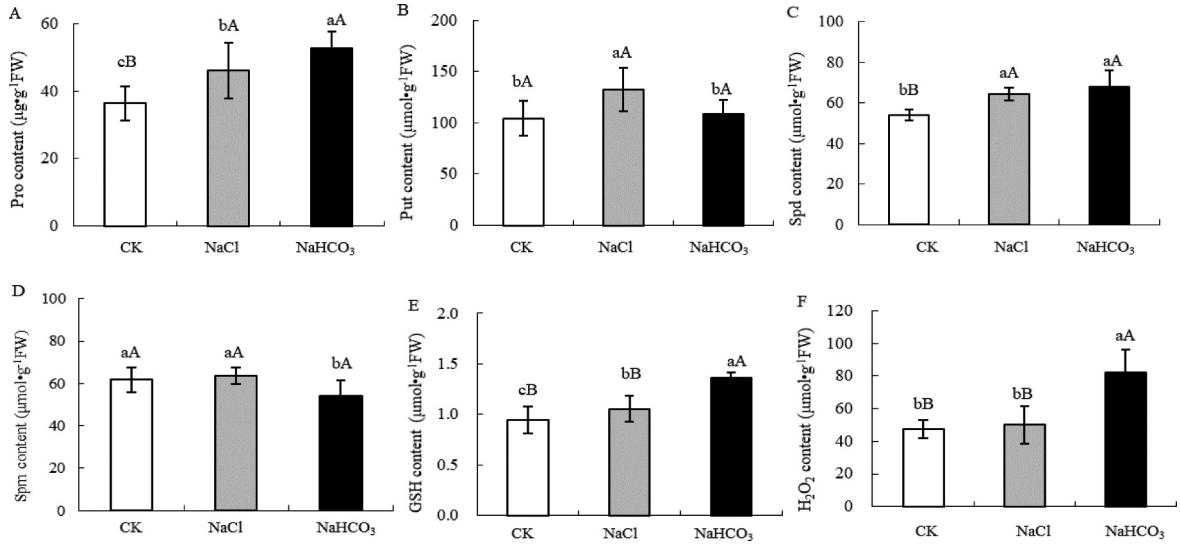


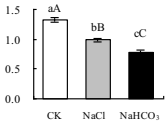
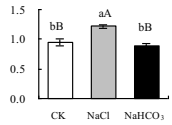
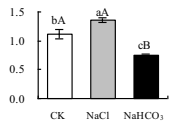
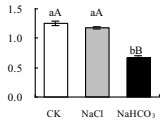
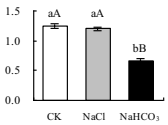
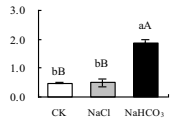
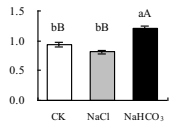
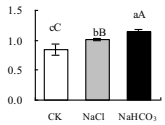
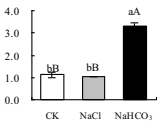
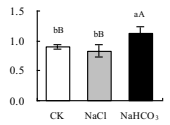
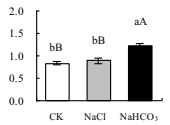
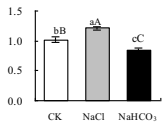
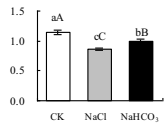
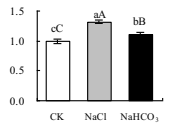
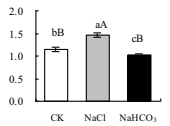
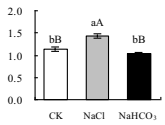
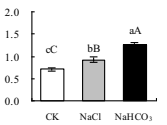
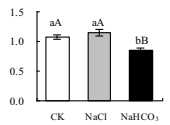
Figure 3. Pro content (a), Put content (b), Spd content (c), Spm content (d),GSH content (e) and H_2O_2 content (f) of mulberry leaves under NaCl and $NaHCO_3$ stress. Note: The data are from three replicated experiments (n = 3), and represent means ± SE. Significant differences were expressed by different small letters ($P < .05$), and very significant differences were expressed by different capital letters ($P < .01$).

slightly compared with CK under $NaHCO_3$ stress, the difference was not significant, but the content of Spm decreased by 11.99% ($P < .05$) compared with CK (Figure 3b–d). Under NaCl stress, GSH content increased by 11.66% ($P < .05$) compared with CK, and H_2O_2 content did not change significantly. However, under $NaHCO_3$ stress, GSH and H_2O_2 content were significantly higher than CK and NaCl treatment (Figure 3e,f).

2.4 Nitrogen assimilation and glutamine/glutamine family of amino acids metabolism related enzyme expression

In Table 1, under NaCl stress, NRT1.3 expression in mulberry leaves decreased by 25.59% ($P < .01$), and NRT1.3 expression under $NaHCO_3$ stress further decreased compared with CK and NaCl stress. Under NaCl stress, the expression of NiR and Fd-NiR increased by 27.97% ($P < .01$) and 21.92% ($P < .01$) respectively. There was no significant difference between NiR expression compared with CK under $NaHCO_3$ stress, but the expression of Fd-NiR was significantly down-regulated. The expression of Fd-GOGAT, Fd-GOGAT2 and GS (W9SA98 and W9SNP7) under NaCl stress did not change significantly. Under $NaHCO_3$ stress, Fd-GOGAT, Fd-GOGAT2 expression decreased by 47.35% ($P < .01$) and 47.19% ($P < .01$), but GS (W9SA98 and W9SNP7) were significantly higher than CK. Under NaCl stress, the expression of P5 CR was higher than CK, there was no significant change in P5 CS expression. The expression of P5 CR and P5 CS increased significantly compared with CK under $NaHCO_3$ stress. During the synthesis of Orn, the expression of ACOAT increased by 18.78% ($P < .01$) under NaCl stress, but decreased by 18.10% ($P < .01$) under $NaHCO_3$ stress compared with CK. Under NaCl stress, the change of OAT (W9RH73 and W9QV49) slightly different from those of CK, while under $NaHCO_3$ stress, OAT (W9RH73 and W9QV49) increased significantly. The OTC expression were significantly lower than that of CK under NaCl and $NaHCO_3$ stress. Under NaCl stress, the expression of SPDS did not change significantly, but under $NaHCO_3$ stress, it increased by 35.59% ($P < .01$) compared with CK. Under NaCl and $NaHCO_3$ stress, the expression of GAD and GAD1 in the leaves of mulberry showed similar trend, which were significantly lower than CK, but the decrease range under NaCl stress was larger than that under $NaHCO_3$ stress. The expression of GCLM

Table 1. Nitrogen assimilation and glutamine/glutamine family of amino acids metabolism related enzymes expression of mulberry leaves under NaCl and NaHCO3 stress.

Protein name	NRT1.3	NiR	Fd-NiR	Fd-GOGAT2
Protein accession	W9SDQ8	S5FS06	W9RY59	W9RZU3
Protein description	Nitrate transporter 1.3	Nitrite reductase	Ferredoxin-nitrite reductase	Ferredoxin-dependent glutamate synthase 2
Species	*Morus notabilis*	*Morus alba*	*Morus notabilis*	*Morus notabilis*
Gene name	L484_004834	-	L484_027526	L484_019389
MW [kDa]	66.45	65.398	65.212	61.555
Score	13.736	191.3	5.8264	323.31
mean±SE				
Protein name	Fd-GOGAT	GS	GS	P5CR
Protein accession	W9SAH2	W9SA98	W9SNP7	W9QS99
Protein description	Ferredoxin-dependent glutamate synthase	Glutamine synthetase	Glutamine synthetase	Pyrroline-5-carboxylate reductase
Species	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*
Gene name	L484_019388	L484_004957	L484_001109	L484_024301
MW [kDa]	116.28	38.945	35.791	30.105
Score	323.31	296.69	81.024	7.5118
mean±SE				
Protein name	P5CS	OAT	OAT	ACOAT
Protein accession	W9QFW3	W9RH73	W9QV49	W9R2Z0
Protein description	Delta-1-pyrroline-5-carboxylate synthase	Ornithine aminotransferase	Ornithine aminotransferase	Acetylornithine aminotransferase
Species	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*
Gene name	L484_016970	L484_005329	L484_005328	L484_016256
MW [kDa]	76.762	26.935	32.311	48.054
Score	172.92	17.538	50.419	163.1
mean±SE				
Protein name	OTC	SPDS	GAD	GAD1
Protein accession	W9RD90	W9RK87	W9REF8	W9RQ01
Protein description	Ornithine carbamoyltransferase	Spermine synthase	Glutamate decarboxylase	Glutamate decarboxylase 1
Species	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*	*Morus notabilis*
Gene name	L484_000975	L484_002704	L484_007798	L484_007797
MW [kDa]	41.843	56.28	41.649	18.767
Score	121.09	121.31	32.679	146.32
mean±SE				
Protein name	GCLM	GGAT		
Protein accession	W9QH55	W9RCI8		
Protein description	Glutamate-cysteine ligase	Glutamate-glyoxylate aminotransferase		
Species	*Morus notabilis*	*Morus notabilis*		
Gene name	L484_024282	L484_027698		
MW [kDa]	65.45	53.503		
Score	122.73	323.31		
mean±SE				

Note: The data are from three replicated experiments (n=3), and represent means ± SE. Significant differences were expressed by different small letters ($P<0.05$), and very significant differences were expressed by different capital letters ($P<0.01$).

increased significantly compared with CK under NaCl and $NaHCO_3$ stress, and the increase of GCLM was more significant under $NaHCO_3$ stress. The expression of GGAT under NaCl stress had no significant change compared with CK, but the expression of GGAT decreased by 21.26% (P < .01) under $NaHCO_3$ stress.

2.5 *qRT-PCR* analysis of the key genes expression

NRT1.3, Fd-GOGAT and *GS* genes expression did not change under NaCl stress compared with CK, NaCl stress increased *Fd-NiR* gene expression by 19.61% (P < .05) compared with CK, but they were significantly lower than CK under $NaHCO_3$

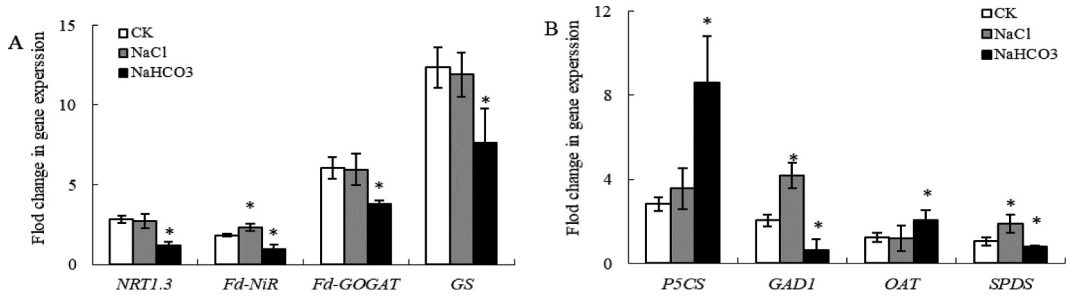


Figure 4. Effects of NaCl and $NaHCO_3$ stress on gene expression of nitrogen assimilation (a) and glutamine/glutamine family of amino acids metabolism (b) in leaves of mulberry seedlings. Note: * indicates significant difference with CK.

stress (Figure 4a). *Fd-NiR, Fd-GOGAT* and *GS* genes expression were consistent with that of proteins expression under NaCl and $NaHCO_3$stress (Table 1). There were no significant difference *P5CS* and *OAT* genes expression compared with CK under NaCl stress, *GAD1* and *SPDS* gene expression increased by 103.90% ($P < .01$) and 79.05% ($P < .05$), respectively. *P5CS* and *OAT* genes expression increased significantly under $NaHCO_3$ stress, but the genes expression of *GAD1* and *SPDS* were significantly lower than CK (Figure 4b). Except for the *SPDS* gene expression under $NaHCO_3$ stress, the change trend of *P5CS, GAD1* and *OAT* genes expression and protein expression was similar (Table 1).

3. Discuss

NR is a key enzyme in the nitrogen assimilation process of plants. It is capable of catalyzing the reduction of NO_3^- to eventually generate NH_4^+ and nitrogenous compounds, oxidizing NADH to generate NAD^+ for electron transport, and supplementing NAD^+ in glycolysis to provide energy for plant growth.[52,53] In this experiment, under NaCl stress, NRT1.3 expression in mulberry leaves dropped significantly relative to that in the control leaves (CK), but NR activity did not experience any significant change. In contrast, under $NaHCO_3$ stress, NRT1.3, *NRT1.3* gene expression and NR activity both saw a substantial reduction. According to existing studies, NR is an induced enzyme, NO_3^- is the main signal inducing NR activity, and NR activity declines with the decrease of NO_3^- in nutrient media.[54] By means of lowering the transpiration rate of plants, saline-alkali stress can lower the uptake ratio of NO_3^-N, and consequently reduce the flow of NO_3^-N to leaves.[55] In this experiment, the decline of NR activity in mulberry leaves under $NaHCO_3$ stress was potentially caused by the inhibition of nitrogen uptake and transport by $NaHCO_3$ in the seedling roots of mulberry (TN and SP contents both dropped significantly). In addition, under NaCl stress, NiR and Fd-NiR and *Fd-NiR* gene expression in mulberry leaves both increased significantly relative to those in CK, accompanied by a significant increase in NiR activity. However, under $NaHCO_3$ stress, the above nitrogen assimilation process was uniformly inhibited to a very large extent. This partially explains one of our findings in preliminary research, that is, $NaHCO_3$ stress inhibits the growth and photosynthesis of mulberry.[38]

The Glu-Gln cycle of plants mainly relies on the NH_4^+ generated by GS and GOGAT through assimilation and photorespiration, and the NH_4^+ generated by NO_3^- through reduction in leaves. GS has a very strong affinity for NH_4^+, which keeps the concentration of NH_4^+ in plant tissues at an extremely low level.[56] In this experiment, NaCl stress did not impose any substantial effect on either GS activity or the expression of GS-associated proteins in mulberry leaves, which suggested that NaCl stress had no substantial effect on the assimilation process of NH_4^+ in the leaves of mulberry. This guaranteed that the NH_4^+ accumulated in leaves of mulberry would not cause any toxic effect. However, under $NaHCO_3$ stress, GS expression in the leaves of mulberry increased significantly relative to that in CK, but GS activity dropped significantly. Studies have shown that GS activity is induced by NH_4^+ ,[57,58] so this phenomenon is probably caused by the inhibition of nitrogen uptake and assimilation in the leaves of mulberry under $NaHCO_3$ stress. GOGAT has two forms, NADH-GOGAT using NADH as the electron donor and Fd-GOGAT using Fd as the donor. Fd-GOGAT plays a dominant role in plant leaves and accounts for 95% of GOGAT activity. In this experiment, Fd was used as the electron donor for two GOGAT differential proteins. NaCl stress did not exert any significant effect on either GOGAT activity or the expression of associated proteins in mulberry leaves. In contrast, under $NaHCO_3$ stress, Fd-GOGAT, Fd-GOGAT2 and *Fd-GOGAT* gene expression in mulberry leavesall decreased significantly, and GOGAT activity also saw a substantial reduction. Fd-GOGAT exists in chloroplasts, and its biosynthesis is photoinduced. It is related to photosynthesis and photorespiration, meaning that factors facilitating photosynthesis also contribute to the expression and synthesis of Fd-GOGAT.[59] For this reason, the decline of GOGAT activity and expression in mulberry leaves under $NaHCO_3$ stress might have something to do with the inhibition of its photosynthesis.[38,46]

Pro has a relatively strong affinity for water and can be used as an osmotic protective agent for plants. The increase in Pro content plays an important role in promoting water uptake by plants.[60,61] Pro is synthesized by Glu through catalyzation by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR).[62–64] Besides, pyrroline-5-carboxylate (P5C), the precursor of Pro can be synthesized by either Glu, or by Orn and Arg. Under the action of ornithine aminotransferase (OAT), Orn engages in a transamination reaction, generates glutamic-γ- semialdehyde (GSA), and forms P5C.[33,65]Salt stress and other adversities induce an increase in the expression of plant *P5CS* and *P5CR* genes, and promote the synthesis of Pro.[66] In contrast, in

Arabidopsis after knockout of the *P5CS1* gene, the synthesis of Pro is inhibited, and salt tolerance declines.[67] A similar conclusion was drawn by 68,in their study on rice after knockout of the *OSP5CS2* gene. In tobacco, with overexpression of the *OAT* gene, the accumulation of Pro is tripled, and transgenic tobacco can grow normally in 200 mmol L^{-1} NaCl.[10] In this study, under NaCl stress, P5CR expression in mulberry leaves increased significantly relative to that in CK, while OAT expression did not experience any significant change. In contrast, under $NaHCO_3$ stress, a significant increase was observed in P5CR and P5CS expression, as well as OAT and *OAT* gene expression. The amplitudes of their increases under $NaHCO_3$ stress were significantly higher than those under NaCl stress. The determination results of Pro content also showed that Pro content in seedling leaves of mulberry under $NaHCO_3$ stress was also significantly higher than under NaCl stress. That is to say, the mulberry leaves could adapt to saline-alkali stress through the accumulation of Pro under both NaCl and $NaHCO_3$ stresses. However, under NaCl stress, the P5C directly synthesized by Glu played the dominant part; under $NaHCO_3$ stress, Orn and Arg played extremely important roles through the P5C synthesized by OAT.

GABA is synthesized mainly by Glu through catalyzation by glutamic acid decarboxylase (GAD). This is also an indirect way of synthesizing GABA: First, pyrroline is formed through the oxygenolysis of PAs by amine oxidase; then pyrroline is further disassimilated into GABA.[69] When the synthesis of Gln is blocked, the synthesis of proteins is reduced, and the rate of degradation is lowered in plants. Glu is converted into GABA in a larger amount.-[70] GABA is also regarded as a signaling molecule that can regulate the growth and development of plants (Lancien and Roberts, 71). Some studies have revealed that when a plant is exposed to multiple adversities (both biotic and abiotic), GABA content in the plant will increase rapidly.[72,73]GABA also induces the accumulation of Pro and PAs. Under salt stress, plants promote Pro synthesis and improve salt tolerance through regulating GABA pathways.[74] Exogenous GABA inhibits the generation of ethylene synthesis precursor SAMand thusinduce the accumulation of endogenous PAs [Shi et al., 75; 76]. According to the results of this experiment, the expression of GAD, GAD1 and *GAD1* gene in mulberry leaves were up-regulated under NaCl stress. However, $NaHCO_3$ stress did not experience any significant change. In other words, under NaCl stress, the seedling leaves of mulberry may have increased GABA content and improved salt tolerance by boosting the activity of GAD, but the synthesis of GABA did not exert any protective role under $NaHCO_3$ stress.

The increase of PAs content is a protective response of plants to stress, which enhances the resistance of plants to biotic and abiotic stressors.[21,77] PAs also have the function of osmotic regulation, which can help crops to deal with many different adversities and maintain normal physiological activities. Common PAs in plants include Put, Spd, and Spm, etc..[78,79] Put has two synthesis pathways in plants: In the first case, it is directly synthesized by Orn through ornithine carbamoyltransferase (ODC); in the second, first putrescine is formed by Arg through catalyzation by arginine carbamoyltransferase (ADC), and then putrescine is hydrolyzed into N-methyl putrescine ammonia (NCP), resulting in the formation of Put. Under adversity stress, the transcription level of the *ODC* gene in plants is raised,[80,81] and overexpression of the *ODC* gene can improve the salt tolerance of transgenic tobacco.[82] Saline-alkali stress also induces increases in *ADC* expression and PAs content in *Brassica juncea*.[83] Under salt stress, ADC activity and Put contents in rice plants with overexpression of the *Avena sativa* L. *ADC* gene were both significantly higher than those in non-GMO plants.[84] In this experiment, changes were not directly identified in ODC or ADC expression in mulberry leaves under NaCl and $NaHCO_3$stresses. However, under NaCl stress, the expression of acetylornithine aminotransferase (ACOAT) in mulberry leaves increased. In the process of Arg synthesis by Orn, the expression of key enzyme ornithine carbamoyltransferase (OTC) dropped significantly. Thus, NaCl stress facilitated the synthesis and accumulation of Orn in mulberry leaves, and promoted the direct synthesis of Put. The determination results of Put content also showed that Put content in mulberry leaves under NaCl stress increased significantly relative to that in CK. However, under $NaHCO_3$ stress, Put content did not manifest any significant difference between the seedling leaves of mulberry and CK. This probably could be explained by two aspects: First, the expression of ACOAT declined partially under $NaHCO_3$ stress in the synthesis process of Put; second, the expression of spermidine synthase (SPDS) increased significantly under $NaHCO_3$ stress, causing Put to decompose into Spd. Spd and Spm are formed by Put and decarboxylated S-Adenosylmethionine (SAM) through catalyzation by SPDS and spermine synthase (SPMS).[85,86] Plants with overexpression of the *SPDS* gene usually have an obviously enhanced resistance to multiple abiotic stresses.[87,88] In adversities such as saline-alkali stress and drought, SPMS can also raise Spd level, and enhance stress resistance.[89,90] In this experiment, the content of Put and Spd in mulberry leaves under NaCl stress increased significantly relative to those in CK. However, under $NaHCO_3$ stress, an increase was observed only in Spd content in mulberry leaves, and Spm content in mulberry leaves dropped significantly relative to that in CK. In other words, under NaCl stress, the seedling leaves of mulberry mostly relied on the Orn pathway for the promotion of Put and Spd synthesis, and the improvement of stress resistance. Under $NaHCO_3$ stress, the leaves adapted to the stress through the accumulation of Spd only.

Saline-alkali stress induces the burst of ROS in plants.[91,92] GSH is an important water-soluble antioxidative substance. In plant cells, it can directly reduce some ROS, and eliminate H_2O_2 through the AsA-GSH cycle.[93,94]The GSH in plants is synthesized by GCLM through linking up glutamate and glyoxylate.[95] This experiment showed that GCLM expression in mulberry leaves increased significantly under both NaCl and $NaHCO_3$stresses. In particular, the amplitude of increase was higher under $NaHCO_3$ stress, and the change in GSH content and GCLM expression presented similar trends. Namely, under NaCl and $NaHCO_3$stressors, the seedling leaves of mulberry could improve their saline-alkali tolerance through promoting GSH synthesis. In this experiment, $NaHCO_3$ stress promoted GSH synthesis in mulberry seedling leaves, and GSH content under $NaHCO_3$ stress was also significantly higher than under NaCl stress. However, H_2O_2 content in mulberry seedling leaves under $NaHCO_3$ stress was still

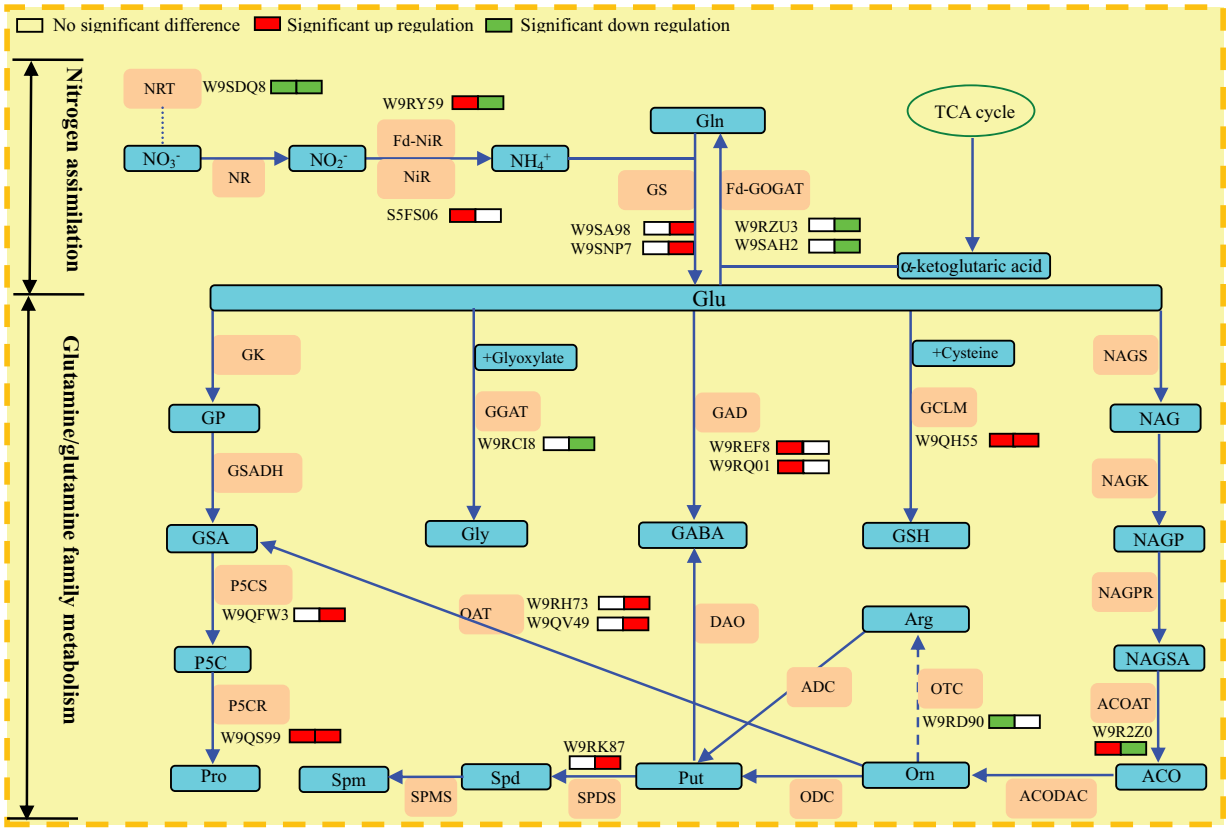


Figure 5. Schematic presentation of nitrogen assimilation and glutamine/glutamine family of amino acids metabolism of mulberry leaves under NaCl and $NaHCO_3$ stress. TCA cycle: Tricarboxylic acid cycle; Glu:Glutamate; Gly:Glycine; GSH: Glutathione; Gln: Glutamine; GP: Glutamine phosphate; GSA: Glutamic-γ-semialdehyde; P5C: Pyrroline-5-carboxylate; Pro:Proline; GABA: γ-aminobutyric acid; NAG: *N*-acetylgluamate; NAGP: *N*-acetylgluamamy phosphate; NAGS: *N*-acetylgluamic-γ-semialdehyde; ACO: α-*N*-acetylornithine; Orn: Ornithine; Arg:Arginine; Put: Putrescine; Spd: Spermidine; Spm:Spermine; NRT:Nitrate transporter; NR:Nitrate reductase; NiR: Nitrite reductase; Fd-NiR:Ferredoxin-nitrite reductase; GGAT:Glutamate-glyoxylate aminotransferase; GCLM:Glutamate-Cysteine ligase; GS: Glutamine synthetase; Fd-GOGAT: Ferredoxin-dependent glutamate synthase; GAD:Glutamate decarboxylase; GK:Glutamate kinase; GSADH: Glutamic-γ-semialdehyde dehydrogenase; P5CR:Pyrroline-5-carboxylate reductase; P5CS: Delta-1-pyrroline-5-carboxylate synthase; NAGS: *N*-acetylglutamate synthase; NAGK: *N*-acetylglutamate kinase; NAGPR: *N*-acetyl-γ-glatumy phosphate reductase; ACOAT:Acetylornithine aminotransferase; ACODAC:Acetylornithine deacetylase; OTC:Ornithine carbamoyltransferase; OAT: Arnithine aminotransferase; ODC: Ornithine decarboxylase; ADC: Arginine decarboxylase; SPDS: Spermidine synthase; SPMS: Spermine synthase

significantly higher than under NaCl stress, largely due to the increased sources of H_2O_2 in mulberry seedling leaves under $NaHCO_3$ stress, and also possibly due to the destruction of antioxidative pathways other than GSH. To confirm this, follow-up research will be necessary. The changes of proteins related to nitrogen assimilation and glutamine/glutamine family of amino acids metabolism of mulberry seedlings under NaCl and $NaHCO_3$ stress are shown in Figure 5.

The expression of related proteins are under NaCl and $NaHCO_3$ treatments from left to right. Red represents up-regulated expression,and green indicates for down-regulated expression.

4. Conclusion

NaCl stress did not significant effects on Glu-Gln cycleof mulberry leaves, but the activity and protein expression of NiR were up-regulated. NaCl promotes Pro, Put, GABA and GSH synthesis related enzymes and their gene expression to improve the resistance to NaCl stress. However, under the same Na^+ concentration of $NaHCO_3$, the TN and SP contents in mulberry leaves decreased, nitrogen assimilation and Glu-Gln cycle were inhibited, but $NaHCO_3$ stress could induce the increase of Pro and Spd content. Under the stress of $NaHCO_3$, the process of GSH synthesis by Glu was promoted, but the excess H_2O_2 could not be scavenged effectively. In short, nitrogen assimilation and glutamine/glutamine family of amino acids metabolism in mulberry leaves are sensitive process to NaCl and $NaHCO_3$ stress, the changes of these metabolic processes are important mechanisms for mulberry to adapt to salt and alkali stress.

Abbreviations

NR	nitrate reduce
TN	total nitrogen
SP	soluble protein
NiR	nitrite reduce
Fd-NiR	ferredoxin-nitrite reductase
GS	glutamine synthetase
GOGAT	glutamate synthase
Fd-GOGAT	ferredoxin-glutamate synthase
Pro	proline
Put	putrescine
Spd	spermidine
GAD	glutamate decarboxylase
GABA	γ-aminobutyric acid
GCLM	glutamate-cysteine ligase
GSH	glutathione

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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