Gamma-aminobutyric acid (GABA) alleviates salt damage in tomato by modulating Na+ uptake, the GAD gene, amino acid synthesis and reactive oxygen species metabolism

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Abstract

Background

Gamma-amino butyric acid (GABA), a four-carbon nonprotein ogenic amino acid, is involved in plant abiotic stress resistance. Previous studies have reported that GABA acts as a signal substance or metabolic product by regulating cytoplasmic pH, polyamine biosynthesis and degradation, NO_3^- reduction and assimilation, and antioxidant responses in a variety of crops under various environmental stresses. The main purpose of our study was to explore the regulatory mechanism by which exogenous GABA enhances salt tolerance in tomato (*Solanum lycopersicum* L.) and its effects on the functions of key enzymes.

Results

Exogenous application of 5 mM GABA significantly reduced the salt damage index and increased the plant height, chlorophyll content and dry and fresh weights of tomato plants treated with 175 mM NaCl. GABA significantly reduced Na^+ accumulation in leaves and roots by preventing Na^+ influx in roots and transportation to leaves. Cloning of the sequences of four *SlGAD* genes revealed that *SlGAD* genes played an important role in enhancing the resistance of tomato plants to NaCl stress with GABA application. Among the *SlGAD* genes, *SlGAD1* was the most sensitive and contributed the most to the increase in GAD activity under salt stress even if the *SlGAD2* transcriptional expression was the prominent under normal conditions. GABA increased the GAD activity and amino acid contents in tomato leaves compared with the levels under salt stress alone, especially the levels of GABA and proline. In addition, GABA treatment significantly alleviated the active oxygen-related injury of seedlings under salt stress by increasing the activities of antioxidant enzymes and decreasing the contents of active oxygen species (O_2• and H_2O_2) and malondialdehyde (MDA).

Conclusion

Our data revealed a positive effect of GABA on the resistance of tomato seedlings to salt stress, which was closely associated with GABA's effects on Na^+ flux and transportation, the expression and activity of *SlGADs*, amino acid contents and the metabolism of reactive oxygen species. Exogenous GABA influences NaCl-treated tomato plants by reducing Na^+ influx into root and inducing osmotic regulation and antioxidant reactions by increasing *SlGAD1* expression and GAD activity, the contents of endogenous GABA and proline and antioxidant enzyme activity.

Background

In recent years, soil salinization has become an alarmingly severe problem, affecting ~ 10% of the land surface in the world [1] as well as 42.9% of protected soil in China [2]. This has become the main obstacle for the sustainable production of protected agriculture. Tomato (*Solanum lycopersicum* L.), one of the most widely cultivated vegetable crops, is a moderately salt-sensitive crop among most crop species [3, 4]. However, soil salinization often severely affects tomato fruit yield and quality by decreasing photosynthetic efficiency and disturbing physiological metabolism due to ion toxicity, osmotic stress, nutrient deficiency, etc. Apparently, compared with the slow progress of molecular breeding [1], the regulation of salt stress tolerance by exogenous

substances is a fast and effective method to relieve salt damage in crops, especially by regulating various ion transport pathways and the related metabolism.

Gamma-aminobutyric acid (GABA), a four-carbon non-proteinogenic amino acid, connects the two major metabolic pathways of carbon and nitrogen in plants and its content is significantly higher than that of other non-protein amino acids [5]. It has an important effect on plant growth and abiotic stress resistance as a signal substance or metabolic product by regulating cytoplasmic pH, acting as a temporary nitrogen pool and inducing antioxidant responses [6, 7]. Previous studies have demonstrated that anabolic metabolism of GABA could be activated by salt stress induction and as a result, GABA accumulation has been observed to increase rapidly in a number of plant species, such as tomato, tea, tobacco, alfalfa, *Arabidopsis*, barley and soybean [8–12]. Among these plants, the GABA content was enhanced approximately 20-fold in *Arabidopsis* seedlings under 150 mM NaCl [11], and GABA levels increased significantly in seedlings of lentils treated with 25–100 mM NaCl [13]. Furthermore, it has been demonstrated that the synthesis-related accumulation of endogenous GABA is closely related to exogenous GABA supplementation, which increased by 29% in hulless barley and by 1-fold in *Caragana* treated with 0.5 mmol·L^{-1} and 10 mmol·L^{-1} GABA, respectively, under salt stress [14–15]. Therefore, it is believed that endogenous GABA, which is affected by salt stimulation and exogenous GABA induction, plays a vital role in improving plant resistance to salt stress via regulatory metabolic pathways [16–18].

The improved salt tolerance due to GABA in plants is related to many physiological metabolic pathways, including the control reactive oxygen species (ROS) accumulation in tomato [19], regulation of redox balance and chlorophyll biosynthesis [20], enabling of cytosolic K^+ retention and Na^+ exclusion in *Arabidopsis* [1] and alteration of cell wall composition [11]. We previously reported that GABA synthesis and supplementation are crucial for enhancing salt tolerance by decreasing ROS generation and photosynthesis in tomato [21] and accelerating NO_3^- reduction and assimilation in pakchoi [22]. Although there are many physiological metabolic pathways involved in the GABA-related plant salt tolerance, the mechanism by which GABA improves plant salt tolerance has not been elucidated clearly. However, these functions of GABA in plants are performed mainly via a short pathway known as the GABA shunt [7]. During the process, GABA accumulation plays a vital role due to irreversible synthesis catalysed by glutamic acid decarboxylase (GAD; EC 4.1.l.15) [10, 23] as well as uptake and transport of exogenous GABA [20].

It has been proven that *GAD* is the most sensitive gene for GABA metabolism in response to abiotic stress. GAD enzyme activity and gene expression levels are closely related to the GABA-mediated enhancement of plant stress resistance [10]. GAD simultaneously catalyses glutamic acid (Glu) degradation and GABA synthesis. To date, *GAD* genes have been cloned and identified in various plant species, including tomato [23], citrus [24], tea [9] and other plants. Moreover, GABA levels are regulated by *GAD* transcriptional expression and enzyme activity regulation, which contributes approximately 61% to the accumulation of endogenous GABA in NaCl-treated soybean [25] and the GABA levels decrease by approximately 50–81% in mature green fruits of *SlGAD2*-suppressed lines [23]. *GAD* gene expression significantly increases the accumulation of GABA in five wheat cultivars under salt and osmotic stress [26]. Os*GAD2* was the most important gene for GABA accumulation, exhibiting increased activity both in vitro and in vivo, showing that transgenic *OsGAD2* had over 40-fold higher activity than the WT [27]. *SlGAD2* and *SlGAD3* play key roles in regulating GABA levels in tomato fruit, showing that transgenic over-expression lines contained higher levels of GABA (2.7- to 5.2-fold) than the WT [23]. However, there were significant differences in expression sites in different plants under NaCl. *CiGAD1* was expressed in the stem, leaf and seed coat of *Caragana intermedia*, while *CiGAD2* was highly expressed in the bark [15]. The increase in expression of *CiGAD1*

and *CiGAD2* induced GABA accumulation within 24 h of salt treatment [15]. *GAD2* gene expression in all parts of *Arabidopsis* and tobacco was significantly enhanced, accompanied by increased GAD activity and increased GABA content [10, 11]. The *CsGAD* gene enhanced the salt and alkali tolerance of melon by increasing leaf GAD activity and GABA content [20]. However, only a limited number of studies have examined the relationship between GADs, GABA and tomato salt tolerance, and the metabolic processes and related metabolism have not been identified.

To experimentally elucidate the relationship between GABA supplementation and tomato plant salt tolerance, we investigated plant growth and changes in Na^+ flow, and accumulation in NaCl-treated plants. Furthermore, we cloned four *SlGAD* genes, determined the transcript expression levels of all the *SlGADs* and detected the amino acid synthesis (including GABA) and metabolism of ROS in leaves to explore the physiological functions of GABA in salt-damaged tomato seedlings. The objective of the paper was to elucidate the regulatory mechanism by which exogenous GABA enhances salt tolerance in tomato plants, which might provide new information about the molecular regulation of *GAD* genes and the subsequent effect of GABA on ion uptake or metabolic processes in tomato plants under high-NaCl conditions.

Results

Effects of exogenous GABA on the phenotype of tomato seedlings under salt stress

Extensive damage was apparent in the roots and leaves of seedlings cultivated under salt stress (Fig. 1). The roots of the NaCl treatment group exhibited discoloration and atrophy at 1 d after salt stress, and the new leaves wilted at 2 d. The seedling heights were significantly shortened, while the leaves and roots were severely shrunken, after salt stress for 4 d. Until 6 d of NaCl stress, the seedlings were relatively weak, which manifested as nearly half of the leaves turning yellow and wilting, and the roots lost viability. However, exogenous GABA significantly alleviated the plant phenotypic symptoms of salt injury. At the initial stage of salt stress, the seedlings subjected to GABA treatment did not show root discoloration and their leaves did not wilt, while a small number of aerial roots appeared at 2 d. At 4 d of NaCl and GABA treatment, the seedlings were obviously shorter than those of the control. The leaves remained stretched and did not exhibit obvious wilting and there were more aerial roots than those under NaCl stress. At the end of the experiment (6 d), most of the leaves and roots of seedlings treated with NaCl + GABA remained clearly healthier as compared to the seedlings under salt stress alone.

The salt damage index of the seedlings treated with NaCl increased with extension of treatment time, but GABA application significantly decreased the salt damage index of the seedlings (Fig. 2A). The salt damage index of the seedlings treated with NaCl + GABA decreased by 54.5%, 38.6%, 41.5% and 22.5% compared with that of the NaCl treatment group. The growth rate in terms of plant height was investigated during the same experimental period (Fig. 2B). The rate of increase in seedling height was approximately 3.2% under normal growth conditions, while NaCl significantly inhibited the height growth of the seedlings and the rate of increase in seedling height gradually decreased with prolonged treatment time. Although GABA treatment did not enhance plant height under control conditions, GABA could alleviate the inhibitory effect of salt stress on seedling height growth under NaCl treatment. The plant height growth rate of the NaCl + GABA treatment group was dramatically higher than that of the NaCl treatment group, showing 39.2%, 63.6%, 126.3% and 92.0% improvement at 1, 2, 4 and 6 d, respectively.

The fresh weight of tomato seedlings treated with NaCl and NaCl + GABA was significantly lower than that of the control and GABA treatment groups (Fig. 3). Compared with the fresh weight of the NaCl treatment group, than that of the NaCl + GABA treatment group was significantly increased by 23.7%, 28.8% and 37.9% at 2, 4 and 6 d, respectively, after treatment. The dry weight of the NaCl treatment group was decreased significantly compared with that of the control treatment group but was not significantly different from that of the NaCl + GABA treatment group. Chlorophyll content was greatly reduced in the leaves of seedlings under NaCl treatment compared with control treatment and decreased gradually with the prolonged salt stress (Fig. 4). Exogenous GABA could delay the decrease in chlorophyll a and chlorophyll b levels under NaCl treatment. During the whole salt stress period, the levels of chlorophyll a and b in the NaCl + GABA treatment group were significantly higher than those in the NaCl treatment group, with a range of promotion of 132.1% and 50.0%, respectively, at 6 d after salt stress.

Effects of exogenous GABA on Na^+ flux and Na^+ content in leaves and roots under salt stress

To further clarify the process of Na^+ transport from roots to shoots, non-invasive micro-test technology (NMT) was used to measure the Na^+ flux in leaves and roots after salt treatment for 2 d (Fig. 5A and 5B). Net Na^+ efflux and influx were measured in leaves and roots, respectively. Under normal conditions, net Na^+ efflux in leaves and net Na^+ influx in roots were very low (nearly close to 0) both with and without GABA treatment. NaCl stress significantly increased net Na^+ efflux in leaves and net Na^+ influx in roots, with averages of 2309 $pmol \cdot cm^{-2} \cdot s^{-1}$ and 305.08 $pmol \cdot cm^{-2} \cdot s^{-1}$ in leaves and roots, respectively. However, net Na^+ efflux in leaves and net Na^+ influx in roots were obviously reduced in GABA-treated seedlings under NaCl treatment, with an approximately 43.2% decline in leaves and a 50.2% decline in roots under NaCl treatment.

Na^+ accumulation in leaves and roots is the main symptom of salt stress and the results showed that the roots accumulated more Na^+ than the leaves under all treatments (Fig. 6). There was no significant difference in Na^+ content in leaves and roots between the control and GABA treatments. The Na^+ content in the leaves and roots of NaCl-treated seedlings was markedly higher than that of the control. However, exogenous GABA significantly inhibited Na^+ accumulation in leaves and roots under salt stress, yielding reductions of 28.6% and 32.4%, respectively, relative to the control levels at 4 d after treatment.

Effects of exogenous GABA on the expression levels of four GAD genes and GAD activity in leaves under salt stress

We cloned the four *GAD* genes, and the conserved region of the sequences showed high homology, with a sequence alignment consistency of 83.73% **(Fig. S1).** The initial relative expression pattern of all four GAD paralogues in the leaves of tomato seedlings was analysed under normal culture conditions, which showed that four *GAD* gene transcripts had significant differences (Fig. 7). Among these genes, *SlGAD2* was the most highly expressed, with approximately 10.7-, 47.8- and 69.6-fold higher expression than *SlGAD1, SlGAD3* and *SlGAD4*, respectively. Among the genes, *SlGAD4* exhibited the lowest expression.

Salt stress significantly increased the expression of *SlGAD1-3* compared with the control (Fig. 8). *SlGAD1-3* showed the same trend in levels across the treatments (NaCl + GABA > NaCl > C + G and the control). NaCl + GABA treatment induced a greater amount of *SlGAD1-3* expression than NaCl treatment alone. In contrast, *SlGAD4* showed levels in the order C + G > control > NaCl > NaCl + GABA, with the lowest expression level, only 0.039-fold that of the control, observed in the NaCl + GABA treatment at 12 h. Among the four genes, *SlGAD1* exhibited the

largest change in transcription level, reaching the highest level after 6 h of NaCl + GABA treatment, approximately 19.4-fold that of the control; in the NaCl treatment group at 6 h, it had reached 14.5-fold that of the control. The change range of *SlGAD2* and *SlGAD3* was far lower than that of *SlGAD1*. At 12 h after NaCl treatment, the maximum variation in *SlGAD2* and *SlGAD3* was only 2.45-fold and 3.64-fold higher than that of NaCl + GABA treatment. The expression of *SlGAD4* decreased significantly under salt treatment, so it showed the lowest expression among the four genes. But it had little effect on the change of the general up-regulation of *SlGADs* gene change trend.

The activity of GAD treated with NaCl + GABA or NaCl was significantly higher than that of the control (Fig. 9) and showed an increasing trend for 6–48 h followed by a decreasing trend for 96 h. The GAD activity of the NaCl + GABA treatment group was the highest during the entire processing period and markedly higher than that of the NaCl treatment group, with an increasing rate of 20.3–61.3%.

Effects of exogenous GABA on amino acid contents in leaves under salt stress

To analyse the changes in amino acid content, 16 amino acids in leaves from the different treatment groups were detected (Fig. 10A and 10B). Based on the general trend, the levels of most of the amino acids increased to varying degrees under salt stress compared to the control treatment. Among these amino acids, the levels of methionine (Met), GABA, alanine (Ala), proline (Pro) and glycine (Gly) were significantly higher than those in the control treatment after 2 d of salt stress, and the lysine (Lys), leucine (Leu), GABA, alanine, proline, threonine, glutamate and aspartic acid (Asp) levels were significantly higher than those in the control treatment after 4 d of salt stress. GABA, Glu and Pro showed prominent variations among all the amino acids induced by NaCl stress. The levels of GABA, which this article focuses on, significantly increased by 1.5- and 1.3-fold after 2 d and 4 d of salt stress, respectively. The levels of GABA showed the following trend: NaCl + GABA > C + G > NaCl > control, and the levels in the NaCl + GABA and C + G treatment groups were significantly higher than those after salt stress by 1.6- and 1.3-fold, respectively, 2 d after treatment. The proline levels, which are representative of stress characteristics, significantly increased 1.38-fold under salt stress. The addition of exogenous GABA led to an 18.9% increase in proline compared to the level under salt stress. However, the addition of exogenous GABA had no significant effect on the proline level under normal treatment. Glutamate exhibited a notable increase in the NaCl treatment group compared with the control treatment group. Exogenous GABA further improved glutamate levels under salt stress, with increases of 16.3% and 15.7% compared with the levels in the NaCl treatment group.

Effects of exogenous GABA on the activity of antioxidant enzymes under salt stress

Superoxide dismutase (SOD) activity in leaves treated with NaCl, C + G or NaCl + GABA gradually increased with increasing treatment time, and all the treatment groups showed significantly higher SOD activity than the control (Fig. 11A). The SOD activity in the NaCl + GABA treatment group was the highest and significantly higher than that in the NaCl treatment group during the entire treatment process, with increases of 25.1%, 22.1%, 23.4% and 18.6% at 1, 2, 4 and 6 d, respectively. The NaCl treatment ranked second, with a rate of increase of 19.0%-35.4% compared with the control.

With increasing treatment time, the peroxidase (POD) activity in leaves treated with NaCl + GABA, C + G or NaCl significantly increased and obviously higher than that in the control group (Fig. 11B). The NaCl + GABA treatment group showed the highest POD activity, followed by the GABA treatment group and the NaCl treatment group had the lowest POD activity. The POD activity of the NaCl + GABA treatment group significantly increased by 22.5%,

18.7%, 28.3% and 49.2% compared with the NaCl treatment group and that of the NaCl treatment group significantly increased by 11.0%-56.5% compared with the control.

During the entire treatment period, the catalase (CAT) activity in tomato leaves showed the following change trend: NaCl + GABA > GABA > NaCl > control (Fig. 11C). Exogenous GABA treatment significantly increased CAT activity under salt stress compared to that under salt stress alone and the increase was 33.9%, 25.9%, 50.1% and 30.2% higher than that under NaCl treatment alone.

Effects of exogenous GABA on reactive oxygen production in leaves under salt stress

Under salt stress, the production rate of $O_2^{\bullet-}$ in tomato leaves was markedly higher than that in the control group (Fig. 12A). When GABA was added under salt stress, the production rate of $O_2^{\bullet-}$ in leaves was significantly lower than that in the NaCl treatment group, with a reduction proportion of more than 11%. In Fig. 12B, blue spots indicates the amount of $O_2^{\bullet-}$. The number of blue spots under salt stress was significantly higher than that under the control and GABA treatments, but the number of blue spots under the NaCl + GABA treatment was markedly lower than that under NaCl treatment.

H_2O_2 content was determined by DAB staining method (Fig. 13A). The H_2O_2 content increased with prolongation of salt treatment. The levels of hydrogen peroxide increased significantly under salt treatment, while GABA application significantly inhibited H_2O_2 accumulation under NaCl treatment, with a reduction of 21.9%-23.5%. Under salt stress, the brown spots in tomato leaves indicated the amount of H_2O_2 (Fig. 13B). The amount of H_2O_2 in leaves under NaCl + GABA treatment was significantly lower than that under NaCl treatment. Exogenous GABA treatment significantly alleviated the active oxygen-related injury of seedlings under salt stress.

It can be seen from Fig. 14 that malondialdehyde (MDA) content in leaves treated with NaCl was markedly higher than that in the control treatment during the extension of treatment time. After adding GABA into the nutrient solution of seedlings treated with NaCl, the MDA content decreased significantly, with reductions of 15.8%, 15.1%, 22.8% and 14.0%. This result indicated that exogenous GABA could significantly reduce the MDA content in the leaves under salt stress to alleviate the damage caused by active oxygen in tomato seedlings under salt stress.

Relationships between phenotypic indexes and levels of reactive oxygen species under salt stress

Correlation analysis of phenotypic and physiological indexes revealed several significant correlations(Table 1). The salt damage index was negatively correlated with rate of increase in plant height, total chlorophyll content and fresh weight, and positively correlated with leaf sodium ion content. The content of sodium ions was significantly correlated with all phenotypic indexes and the levels of all active oxygen species. The levels of reactive active oxygen species were significantly correlated with the salt damage index, all phenotypic indicators and sodium ion content.

Table 1
Correlation analysis between phenotype and physiological index

Index	Salt damage index	Growth rate of height	Chla + b Content	Fresh weight	Na^+ content	$O_2^{\cdot-}$ productive rate	H_2O_2 content	MDA content
Salt damage index	1.000	-0.885**	-0.962**	-0.799**	0.979**	0.828**	0.918**	0.876**
Growth rateof height		1.000	0.913**	0.863**	-0.942**	-0.837**	-0.920**	-0.820**
Chl(a + b) content			1.000	0.871**	-0.974**	-0.861**	-0.979**	-0.897**
Fresh weight				1.000	-0.924**	-0.781**	-0.911**	-0.758**
Na^+content					1.000	0.824**	0.997**	0.921**
$O_2^{\cdot-}$productive rate						1.000	0.850**	0915**
H_2O_2content							1.000	0.886**
MDA content								1.000
Note:'*'shows significant correlation at the level of $p < 0.05$, '**'shows extremely significant correlation at the level of $p < 0.01$.								

Discussion

Exogenous GABA improved salt stress tolerance by alleviating phenotypic symptoms of salt damage

Biomass is a comprehensive indicator of the response to salt stress in many crop species. High salt concentrations reduce biomass, as reflected by reductions in crop growth and yield [28]. Previous studies have indicated that salinity typically reduces plant growth [29] by altering the water potential and inducing nutrient deficiency [30–31]. Exogenous application of GABA has been shown to alleviate the inhibitory effects of salt stress on plant growth by reducing chlorophyll degradation and maintaining high photosynthetic capacity [18]. For example, the fresh and dry shoot masses of GABA-treated lettuce plants were less negatively affected by saline water than non-GABA-treated plants [21, 32]. In the present study, the tomato seedlings gradually exhibited typical symptoms of salt damage, including reductions in growth rate, fresh and dry weights and chlorophyll content. Our results also indicated that treatment with exogenous GABA mitigated the damage due to salt stress by increasing the values of growth-related physiological indicators, including growth rate, fresh and dry weights and chlorophyll level. These results indicated that reductions in growth could be mitigated by the addition of exogenous GABA, consistent with our previous study of cucumber [33]. GABA may have alleviated the inhibitory effects of salt stress on plant growth by preventing stress-induced damage to chloroplast structure.

Exogenous GABA regulated Na^+ flux and Na^+ content in leaves and roots under salt stress

The damage to plant cells under salt stress and the resulting growth inhibition are mainly caused by the excessive absorption and accumulation of Na^+ [34]. The ability of a plant to minimize the accumulation of toxic Na^+ in sensitive shoots is a crucial feature of salinity tolerance [35]. Therefore, the accumulation of sodium ions in tissues is often considered one of the indicators of the degree of salt stress injury. A previous study indicated that under exogenous GABA treatment the absorption of Na^+ by sweet sorghum seeds was reduced [36]. Furthermore, our previous study showed that exogenous GABA application influenced the absorption and inhibition of mineral elements in cucumber seedlings under NaCl stress and the addition of 5 $mmol \cdot L^{-1}$ GABA significantly reduced the accumulation of sodium ions in cucumber roots under salt stress [33]. However, there was no strong evidence that GABA directly reduced Na^+ to relieve salt stress. In our study, Na^+ concentration was higher in roots than in leaves. Exogenous GABA significantly reduced the accumulation of sodium ions in leaves and roots with the reduction of net Na^+ influx in roots and Na^+ efflux from leaves. Alqarawi suggested that GABA could maintain the hormones and mineral nutrients and reduce lipid peroxidation under environmental stress [37]. Our results indicated that exogenous GABA was absorbed by plant roots, resulting in increases in the osmotic regulation ability of cells, inhibition of Na^+ absorption by roots and reductions in ion toxicity caused by excessive Na^+ accumulation in leaves.

Exogenous GABA elevated GAD activity by upregulating SlGAD expression

Four *GAD* genes in the tomato genome have been reported [38–40]. Previous studies on the *GAD* genes in tomato mainly focused on the effect of *SlGAD1-3* on the accumulation of GABA during fruit development [23]. Our study is the first to detect the relative expression of *SlGADs* in tomato leaves under salt stress. The transcription level of the *SlGAD1-3* gene increased and that of *SlGAD4* decreased significantly under salt stress. Among these genes, the *SlGAD1* gene showed the most sensitive response to salt stress. Moreover, *SlGAD1* level and GAD activity were significantly positively correlated. Therefore, we speculated that *SlGAD1* plays the most important role among the four *SlGAD* genes in altering GAD activity under salt stress.

Previous studies have shown differences among *GADs* in the expression induced under stress. In poplar, only two of the six *GAD* genes were upregulated under NaCl stress, with levels significantly higher than those of the other four genes [41]. In current study, *SlGAD1-3* were upregulated, but *SlGAD4* was downregulated, mainly due to the fact that different isoforms have different functions under stress. In previous work, exogenous GABA improved *PSGAD2* and *PSGAD4* transcription levels compared with those under single hypoxia treatment [42]. Our results showed that the relative expression of *SlGAD1-3* in the NaCl + GABA treatment group was significantly higher than that in the NaCl treatment group. The possible reason is that GABA is absorbed by plant roots and transported to leaf cells. During stress, the accumulation of GABA in the cells induced the expression of *SlGAD*s, and the effect was stronger than that of salt stress alone. Previous reports have shown that GAD enzymatic activity is stimulated by either cytoplasm acidification or rising intracellular Ca^{2+} levels [43, 18]. The strongly induced expression of *SlGAD*s in the presence of exogenous GABA under salt stress should also be related to the changes in intracellular calcium and hydrogen ions.

GABA accumulation in plant cells is generally believed to occur mainly due to the activation of glutamate decarboxylase [44]. GAD activity in hulled barley and poplar leaves has been found to be significantly higher under salt stress [14, 41]. Furthermore, exogenous GABA treatment has been shown to enhance GAD activity under $Ca(NO3)_2$ stress [20]. Our results showed that GAD activity in leaves of plants within the NaCl treatment group was significantly increased compared with that in plants leaves in control group and exogenous GABA

improved GAD activity over that observed in plants under NaCl treatment. The change in GAD activity was consistent with the expression patterns of *SlGAD1-3.* These results indicated that the accumulation of GABA in tomato leaves was mainly due to the change in activity of the GAD enzyme under salt stress treatment.

Exogenous GABA increased the amino acid contents in leaves under salt stress

GABA is an important component of the free amino acid pool, which plays an important role in regulating the response of plant cells to stress and enhancing the adaptability of plants to stress [18, 14]. Shelp et al. found that GABA may act as an osmotic molecule to alleviate water stress, which is often the result of cell water shortages caused by drought, salinity or freezing injury [17]. An increase in GABA content can rapidly increase the contents of the components of cell solutes, thus reducing dehydration damage to the cell membrane and alleviating cell death [45–46]. GABA accumulation is generally considered highly dependent on GAD activity and glutamate content [17]. Previous studies have shown that the level of endogenous GABA increases under salt stress [23, 47]. In our study, we observed that leaves treated with NaCl exhibited increased accumulation of GABA and increased Glu levels. We also observed a rapid increase in the level of GAD enzyme under salt stress. The increase in Glu content provides a large amount of raw material for the synthesis of GABA and improves the activity of GAD for the synthesis of a large amount of GABA. Considering these results together, we speculate that GABA accumulation in leaves is induced by activation of the decarboxylation pathway of glutamate under salt stress. This process can consume H^+ and thus reduce the degree of acidification of cells. This is consistent with our previous study of hypoxia stress [48]. In a previous study, the GABA content of hulless barley was increased by more than 50% under salt stress and the accumulation of GABA was induced by exogenous GABA [14]. In our study, exogenous GABA was found to induce an increase in endogenous GABA content in plants under either control or stress conditions. This finding was due to the fact that exogenous GABA can be absorbed directly by plants [49–50] and then engage in downstream metabolism and be converted into other substances [19]. The glutamate content of plants in the C + G treatment was lower than that of control plants owing to feedback regulation of GAD activity. In plants receiving Na + G treatment, the accumulated GABA in leaves was derived from exogenous GABA as well as from endogenous production as part of the stress response induced by salt stress. The accumulation of GABA in leaves was associated with the accumulation of glutamate and the enhanced mobilization of GAD activity upon exogenous GABA treatment under salt stress. Glu could maintain the flow of the GABA shunt and TCA metabolism under stress [51]. The lack of apparent feedback regulation of Glu may be due to plant consumption of large amounts of GABA, thereby inducing Glu decarboxylation, to alleviate the cell acidification caused by salt stress. The accumulation of Pro is an adaptation of plants subjected to NaCl stress [52]. Exogenous application of GABA has been shown to increase leaf Pro accumulation under stress conditions [53–55]. Our study found that the Pro content increased with exogenous GABA application under salt stress. This finding is consistent with the report that exogenous GABA can improve salt tolerance by influencing osmotic regulation in leaf cells [56].

Exogenous GABA improved salinity stress tolerance by regulating the antioxidant system

ROS accumulation in plant cells under stress is considered to cause reductions in photosynthetic system efficiency [57]. Induction of the antioxidant defence system can protect plants from ROS accumulation [58]. Salt stress induces osmotic and oxidative stresses that perturb plant metabolism [14], lead to membrane damage and the accumulation of lipid peroxides [59]. Our results indicated that salt stress significantly affected the activities of SOD, POD and CAT accompanied by a rapid increase in active oxygen metabolites, consistent with previous

studies [60–61, 21]. Liu et al. found that GABA exhibits ROS scavenging ability and can contribute to alleviating stress [62], although studies are required to determine whether GABA can directly scavenge ROS to relieve stress. In the current study, exogenous GABA enhanced the activities of the three enzymes mentioned above, thereby promoting the conversion of $O_2^{\bullet-}$ into H_2O_2 and the subsequent decomposition into water and oxygen. The results also showed that both $O_2^{\bullet-}$ and H_2O_2 decreased significantly following exogenous GABA application. MDA content is regarded as an important indicator of oxidative damage in plants [63]. We observed a significant decrease in MDA content following exogenous GABA treatment under salt stress. Some studies have proposed that exogenously applied GABA acts as a signalling molecule in the scavenging of ROS and in modulating antioxidant enzyme activities [11, 64].Our results support the view that exogenous GABA plays an important role in maintaining the redox state and eliminating the ROS generated in leaves.

Conclusions

Our results suggested that exogenous GABA alleviates NaCl stress by modulating Na^+ uptake, *GAD* expression, amino acid synthesis and reactive oxygen species metabolism. Exogenous GABA improved the phenotype of tomato seedlings under salt stress by reducing the salt damage index and increasing plant height, chlorophyll content and dry and fresh weights. GABA produced these phenotypic effects by delaying the release and absorption of Na^+ from leaves and roots, thus reducing the accumulation of sodium ions. Among the studied *SlGAD* genes, *SlGAD1* was most sensitive to salt stress and contributed the most to the increase of GAD activity under salt stress. GABA accumulation in leaves was induced by activation of the decarboxylation pathway of glutamate under salt stress, mainly due to the change in activity of the GAD enzyme. GABA influenced osmotic regulation by increasing GAD activity and the amino acid contents of leaves. Furthermore, exogenous GABA was important for maintaining the redox state and eliminating the ROS generated in leaves.

Methods

Plant materials and cultivation

The experiment was carried out in a plastic greenhouse at the Experimental Farm of Hebei Agricultural University in Baoding, Hebei Province, during a spring-summer growing cycle (March-July 2019). Seedlings of the tomato cultivar *S. lycopersicum* L. 'Zhongza9', a salt-sensitive intermediate-type variety [65] with large fruits (70-80 mm) purchased from China Vegetable Seed Technology Co., Ltd (Beijing, China), were transplanted at the four true-leaf stage into 20-L grey plastic pots (ten plants per pot) without bottom holes and filled with 16 L of Hoagland's nutrient solution (pH 6.5, EC 2.0-2.2) for hydroponics. An air pump was used to maintain normal ventilation. The treatments described below were carried out after three days of pre-culture.

Treatments and experimental design

(1) Control: normal cultivation with Hoagland's nutrient solution.

(2) Salt treatment (Na): 175 mmol·L^{-1} NaCl was added to the nutrient solution.

(3) GABA treatment (C+G): 5 mmol·L^{-1} GABA was added to the nutrient solution.

(4) Salt + GABA treatment (Na+G): 175 mmol·L^{-1} NaCl and 5 mmol·L^{-1} GABA were added to the nutrient solution.

All plants used for an experiment were germinated on the same day and were kept in the same growth environment. In the pre-test, 175 mmol · L^{-1} NaCl was the concentration for significant phenotypic difference in the plants screened. Unless otherwise specified, all chemicals were of analytical grade and were obtained from Sigma-Aldrich (USA). For each treatment, leaves of the second leaf from the top (four replications) were harvested after transplantation, immediately frozen in liquid nitrogen and stored at -80°C until further molecular analysis. Leaves and roots (four replications) were harvested after transplantation and stored at -20°C until further biochemical analysis.

Determination of the salt damage index and plant height growth rate

At 0, 1, 2, 4 and 6 days after treatment, 30 seedlings were selected for each treatment for determination of the statistical salt damage index [66], and the rate of increase in plant height was calculated according to the formula (determined plant height - previously determined plant height) / previously determined plant height×100%

Determination of fresh weight, dry weight and chlorophyll content

At 2, 4 and 6 days after treatment, the fresh weight of the plants was determined. Then, the plants were killed at 105°C in the oven for 30 min and dried at 80°C to constant weight, and the dry weight of the plants was measured. The chlorophyll content of the seedlings was extracted with acetone-ethanol (1:1) [67].

Quantification of Na^+ flux and Na^+ content in leaves and roots

Quantification of the activity of the Na^+ efflux system was performed according to the 'recovery protocol' [68]. For this, the net Na^+ flux was measured using NMT (YoungerUSA LLC, Amherst, MA, USA), ASET 2.0 (Sciencewares, Falmouth, MA, USA) and iFluxes 1.0 (YoungerUSA) software [69]. After 2 days of treatment, the leaves and roots of the control and the other three treatment groups were rinsed with distilled water and transferred to the measurement solution containing very little salt (0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.1 mM NaCl, 0.3 mM MES, pH 6.0). Plant specimens were immobilized in the middle of poly-lysine-coated coverslips (2×2 cm) in the measuring chamber. The net flux was measured after 15 min (for leaves) and 30 min (for roots) of equilibration in low-Na^+ solution. The measurement sites in the leaves were mesophyll cells. The measurement site in the root was 100 μm from the root tip, and vigorous Na^+ flux was observed in our experiment. The magnitude of the steady-state ion flux was calculated from data recorded over a 300-s period. The glass micropipettes and measuring solutions were prepared as described by Lei et al. (2014) [70].

The Na^+ content was determined as described in Chen et al. 2010 [71]. After 2 and 4 days of treatment, 100 mg of the leaves and roots of tomato seedlings was dried, and 5 ml of HNO_3 (65-68%) was added to the microwave digestion system for digestion. The digested sample was diluted to the designated volume with deionized water and filtered with a 0.25-μm pore filtration membrane. The Na^+ content was determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer Inc., ELAN DRC-e).

RNA extraction, cDNA synthesis, cloning of *SlGADs* and qRT-PCR for leaves

Leaf samples (100 mg) from tomato plants were obtained after 0, 6, 12, 24, 48 and 96 h of treatment. RNA extraction was carried out as described previously with minor modifications [72]. Total RNA was extracted with the Eastep® Super Total RNA Extraction Kit (Shanghai Biotechnology Co., Ltd., Shanghai). The concentration of

RNA was quantified using a NanoDrop (NanoDrop 2000) and the integrity of the RNA was verified on a 1% agarose gel. cDNA was synthesized with the Transcript® One Step gDNA Removal and cDNA Synthesis Supermix Reverse Transcription Kit (TransGen Biotech, Beijing).

The full-length sequences of the *SlGAD1*, *SlGAD2*, *SlGAD3* and *SlGAD4* genes in tomato were published in GenBank as AB359913.1, NM_001246893.1, NM_001246898.2 and XM_004237202.4, respectively. The primers used in this experiment are shown in Table 2. The cDNA of the tomato leaves was used as template for PCR amplification. The reaction system was as follows: cDNA template, 2 μL; forward and reverse primers, 0.5 μL; 2× Flash Hot Start Mastermix (dye), 12.5 μL; ddH_2O, 9.5 μL. The reaction conditions were as follows: 94°C for 3 min; 35 cycles of 98°C for 5 s, annealing for 10 s, 72°C for 15 s; 72°C for 10 min. The annealing temperatures for *SlGAD1*, *SlGAD2*, *SlGAD3* and *SlGAD4* were 55°C, 52°C, 47°C and 50°C, respectively. Agarose gel electrophoresis (1.2%) was used to detect the amplified products. The target fragment was recovered by a DNA Gel Recovery Kit (TIANGEN, Beijing). The amplified product was ligated to the pMD19-T cloning vector (TaKaRa, Beijing) at 16°C overnight and then transformed into *Escherichia coli* DH5α (TransGen Biotech, Beijing). The cells were plated on LB solid medium coated with ampicillin (Amp) and the plate was inverted and incubated for 12-16 h at 37°C. The positive spots were picked out and sent to Shanghai Biotechnology Company for sequencing, and finally, the full-length sequence of the gene cDNA was obtained. The conserved regions of the *SlGAD1*, *SlGAD2*, *SlGAD3* and *SlGAD4* genes were compared with DNAman software.

Table 2 List of primers used for Cloning of *SlGADs*

Gene name	Primer name	Primer sequence (5'-3')
SlGAD1	*SlGAD1*-F	CGCTCCCGCATTTATACC
	SlGAD1-R	TACAGGATGGCGATGGAA
SlGAD2	*SlGAD2*-F	CTCTTTTGCTTTACTCTTTGAT
	SlGAD2-R	GGTCCAATTTTACATTGTAGAT
SlGAD3	*SlGAD3*-F	ATGGTTCTCTCAAAAA
	SlGAD3-R	CTTCCCTAACAAATAGATGC
SlGAD4	*SlGAD4*-F	TTCCTCACTTTACGCCAAA
	SlGAD4-R	CCTTCAACAAGTAATCCTTCC

To determine the expression levels of *SlGADs* in tomato, qRT-PCR was performed. The primers were synthesized by Shanghai Biotechnology Company. The reaction mixture contained the following: 2 × Super Evagreen Master Mix, 10 μL; upstream and downstream primers (10 $\mu mol \cdot L^{-1}$), 1 μL each; cDNA, 2 μL; water added to a final volume of 20 μL. The reaction procedure was as follows: predenaturation at 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 5 s and elongation at 72°C for 25 s. The experimental results were analysed by the $2^{-\triangle\triangle CT}$ method. The relative expression level of each *SlGAD* gene was normalized to the expression level of the *Actin-7* gene (GenBank accession number X58253), which was used as an internal control. The primer sequences used in this experiment are shown in Table 3.

Table 3 List of primers used for qRT-PCR of *SlGADs*

Gene name	Primer name	Primer sequence (5'-3')
SlGAD1	*qSlGAD1*-F	GGGGCGGTTCGATATTGTCT
	qSlGAD1-R	GCAGCACAGCAATGTGTTCA
SlGAD2	*qSlGAD2*-F	TGTGATGAGCCCTGAGAAAG
	qSlGAD2-R	ATTGGAGTGTCCCACCCTGT
SlGAD3	*qSlGAD3*-F	TGACATCGTCAAGGTCCTCC
	qSlGAD3-R	CAAAACTCAGCAATTGCCCT
SlGAD4	*qSlGAD4*-F	CTCCACCTTTGCTTCTCGCT
	qSlGAD4-R	TCTGGCTCCATCCATGTTGT
SlACT7	*qSlACT7*-F	ACCACCACTGCTGAACGG
	qSlACT7-R	ACCTCTGGGCAACGGAAC

Determination of GAD enzyme activity and amino acid content in leaves

Leaf samples (100 mg) of tomato plants were taken after 0, 6, 12, 24, 48 and 96 h of treatment. The GAD activity in tomato leaves was determined by using precolumn derivatization with 2,4-dinitrofluorobenzene (DNFB) and reversed-phase high-performance liquid chromatography (RP-HPLC). One unit of GAD activity was defined as the amount of enzyme required to release 1 mmol of GABA every 30 min (40°C) in every gram of plant tissue [73]. After 2 and 4 days of treatment, the amino acid content was determined by precolumn derivatization with DNFB and RP-HPLC [74].

Determination of antioxidant enzymes and ROS

SOD activity was determined as described by Giannopolitis et al. (1977) [75]. Inhibition of photochemical reduction of NBT by 50% was used as an activity unit (U). POD activity was determined as described by Zeng et al. (1997) [76]. CAT activity was determined as described by Dhindsa et al. (1982) [77]. The activity unit (U) of the enzyme was defined as 0.1 OD per minute. The production rate of O_2^- was determined as described by Wang Aiguo et al. (1990) [78]; histochemical staining of O_2 and H_2O_2 was performed as described by Christensen et al. (1997) [79]; and the MDA content was determined by using thiobarbituric acid [80]. Each process was repeated three times.

Statistical analysis

The effects of the various treatments were analysed by the SAS 8.1 statistical program (SAS Institute, Cary, NC) using Fisher's least significant difference (LSD) test in conjunction with one-way ANOVA, with differences evaluated at a 0.05 level of significance.

Abbreviations

CAT: Catalase; GABA:Gamma-aminobutyric acid; GAD:Glutamate decarboxylase; H_2O_2:Hydrogen peroxide; MDA:Malondialdehyde; O_2 Superoxide anion; POD:Peroxidase; ROS:Reactive oxygen species; SOD:Superoxide

dismutase

Declarations

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Authors' contributions

XW and HG conceived and designed the research. XW, QJ and SJ conducted the experiments. BG, JL and GL contributed new reagents and analytical tools. All authors read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

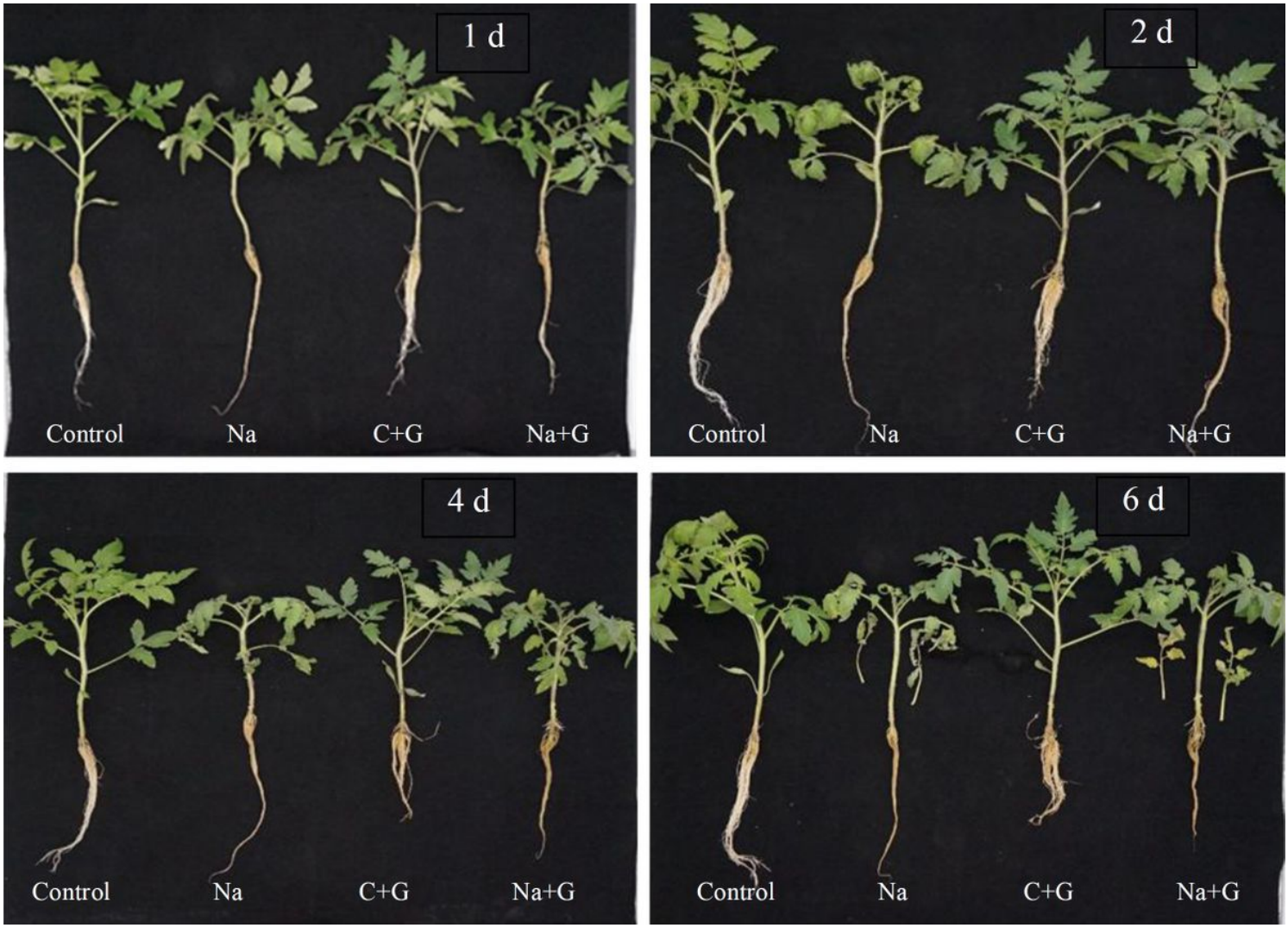

Figure 1

Growth of tomato seedlings in control and NaCl treatment with or without GABA

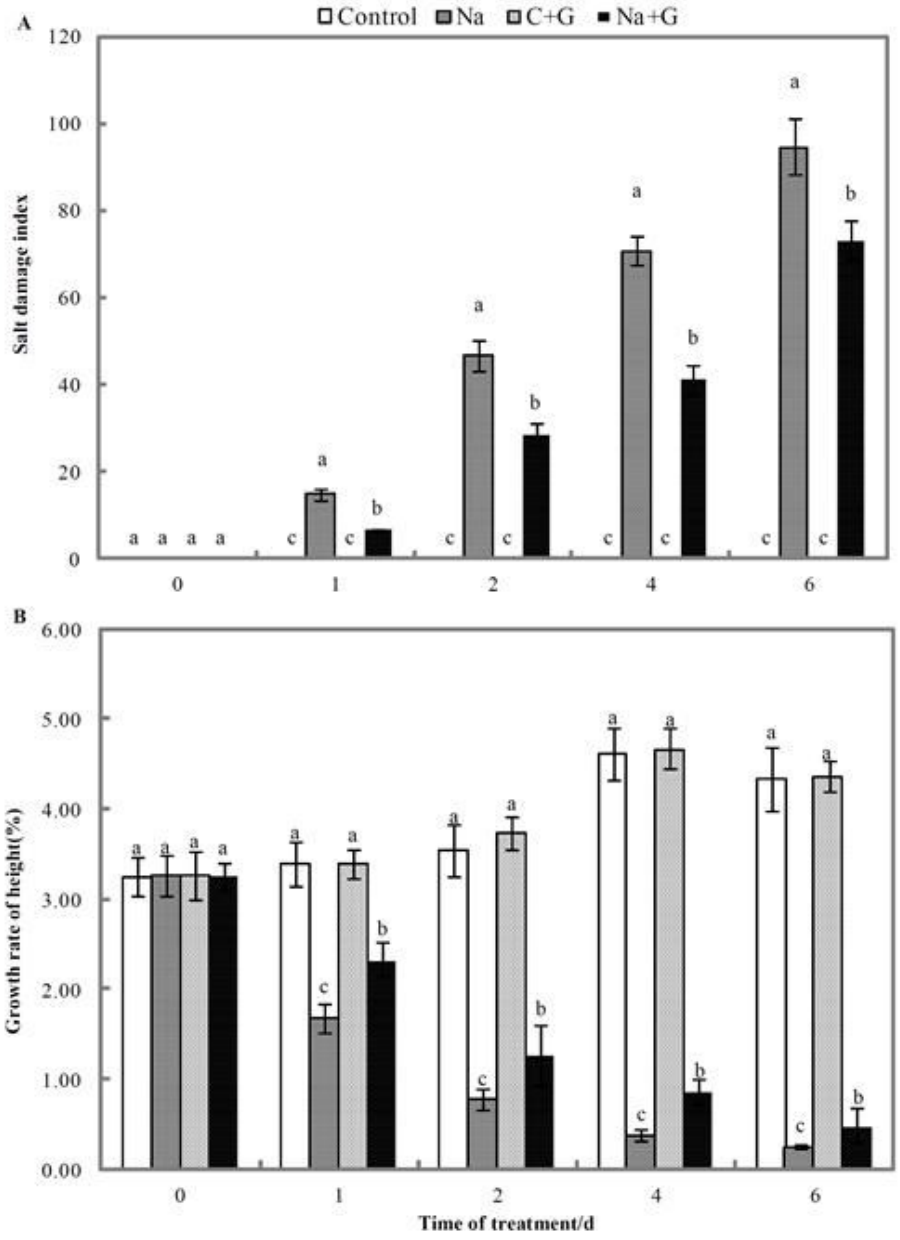

Figure 2

Salt injury index and plant height growth rate of tomato seedlings under NaCl stress with or without GABA. (A) salt injury index, (B) plant height growth rate. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

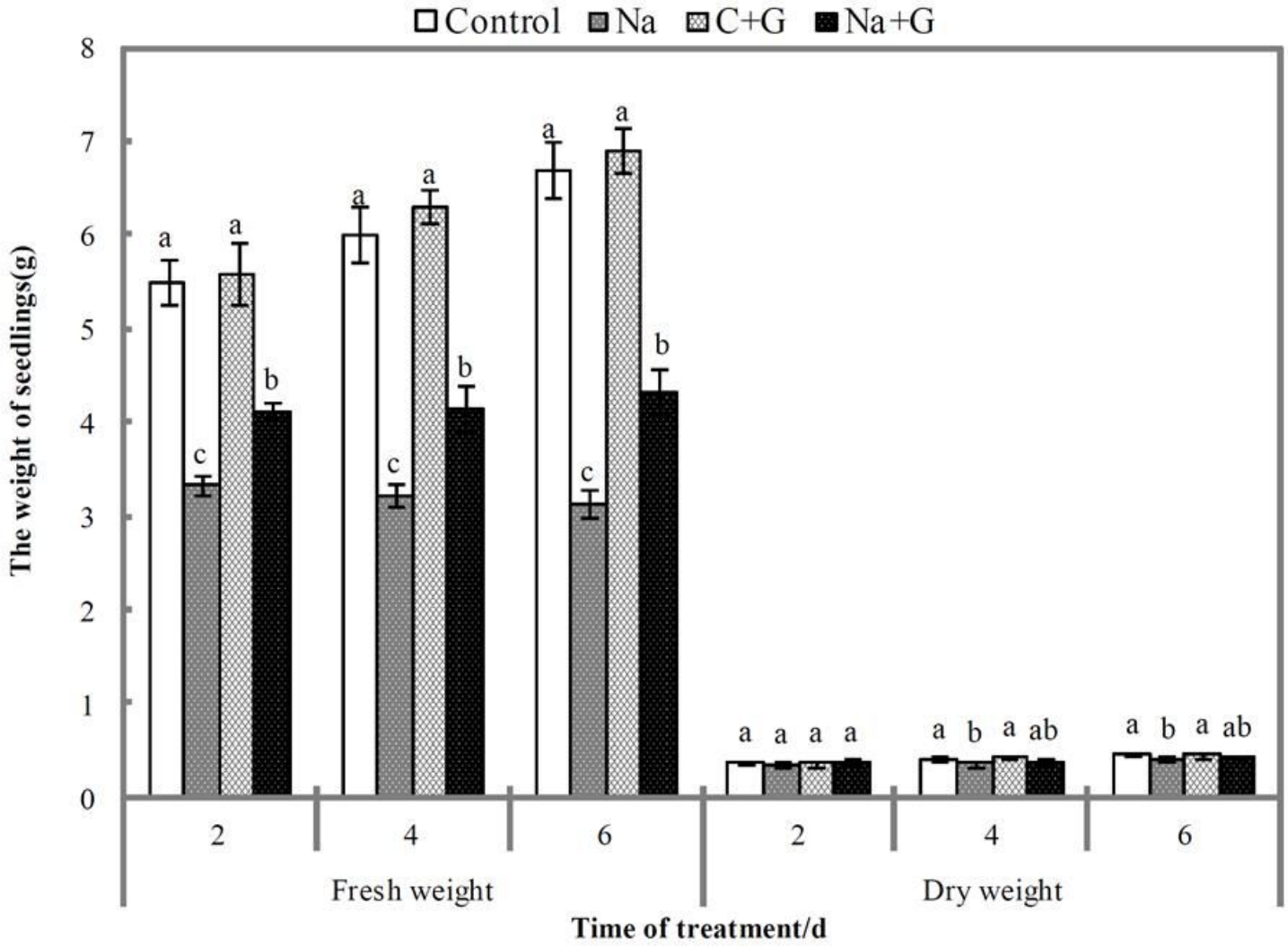

Figure 3

Fresh weight and dry weight of tomato seedlings under NaCl stress with or without GABA. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

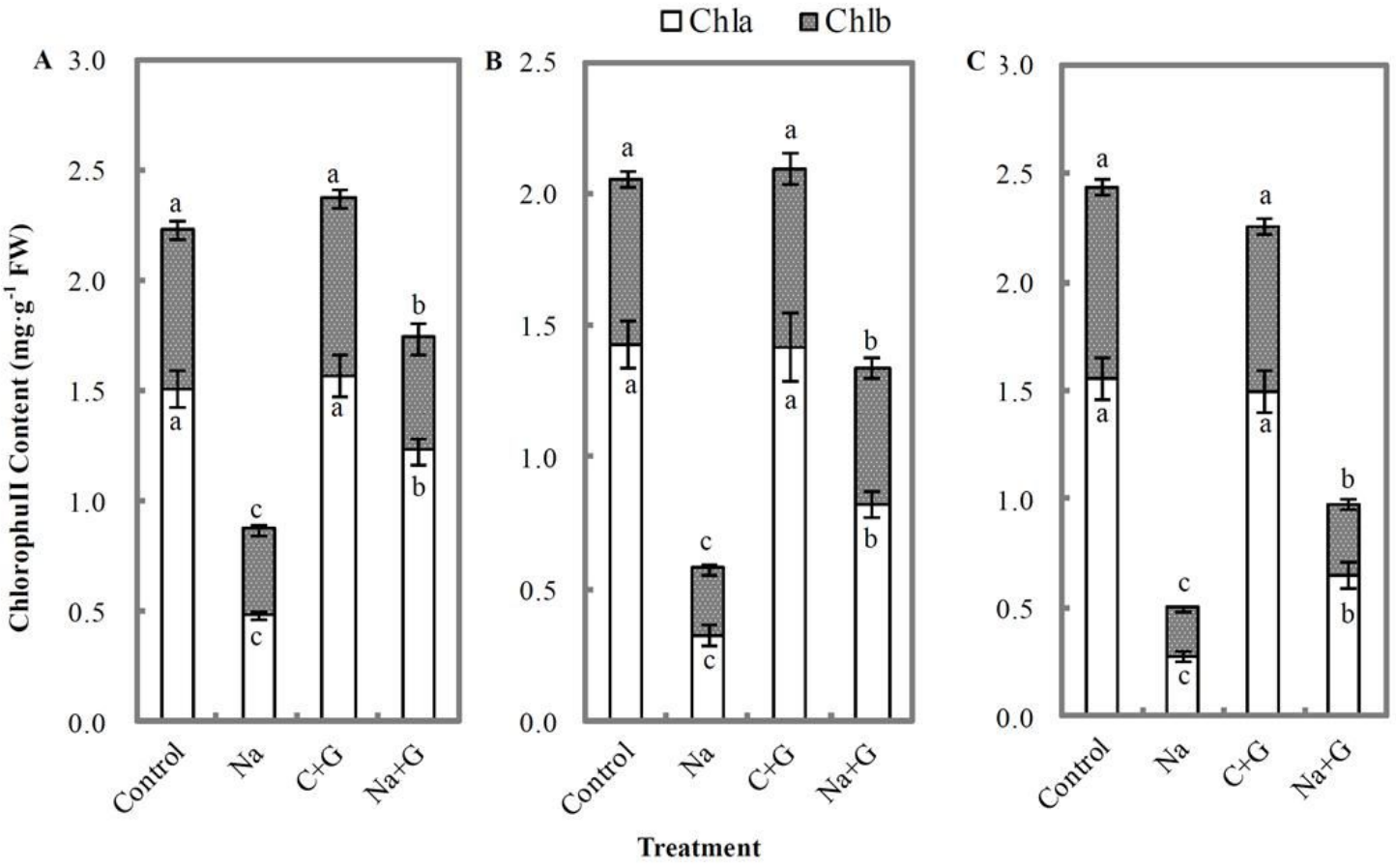

Figure 4

Chlorophyll contents of tomato seedlings under NaCl stress with or without GABA for 2(A) , 4 (B) and 6 (C)d. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at P<0.05 by Duncan's test.

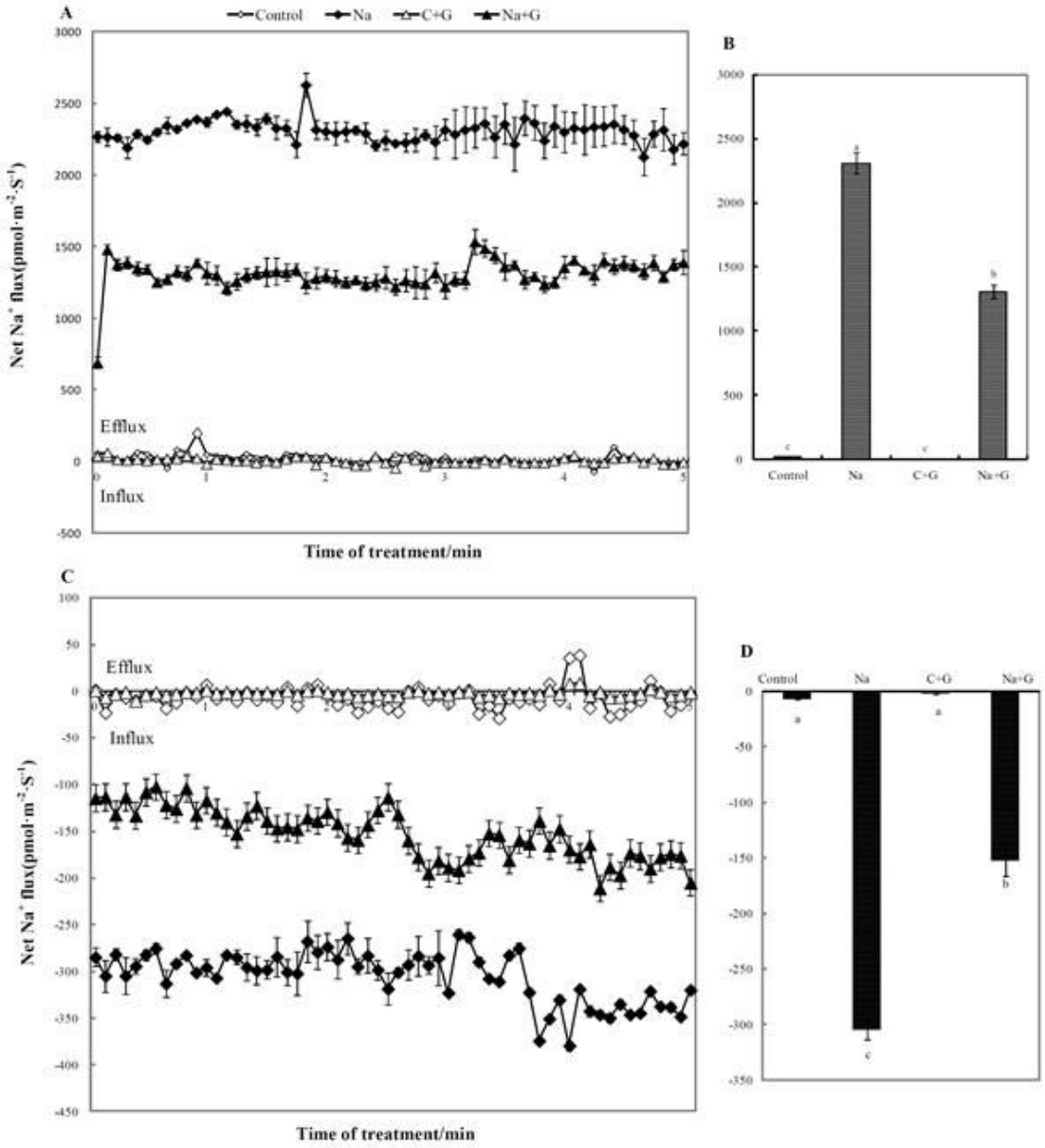

Figure 5

Net Na+ flux in leaves and root under NaCl stress with or without GABA 2d after treatment. (A) and (B) leaves, (C) and (D) root. Note: The positive value of the ordinate indicates that the ions are discharged (Efflux) and the negative value indicates that the ions are absorbed (Influx).Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

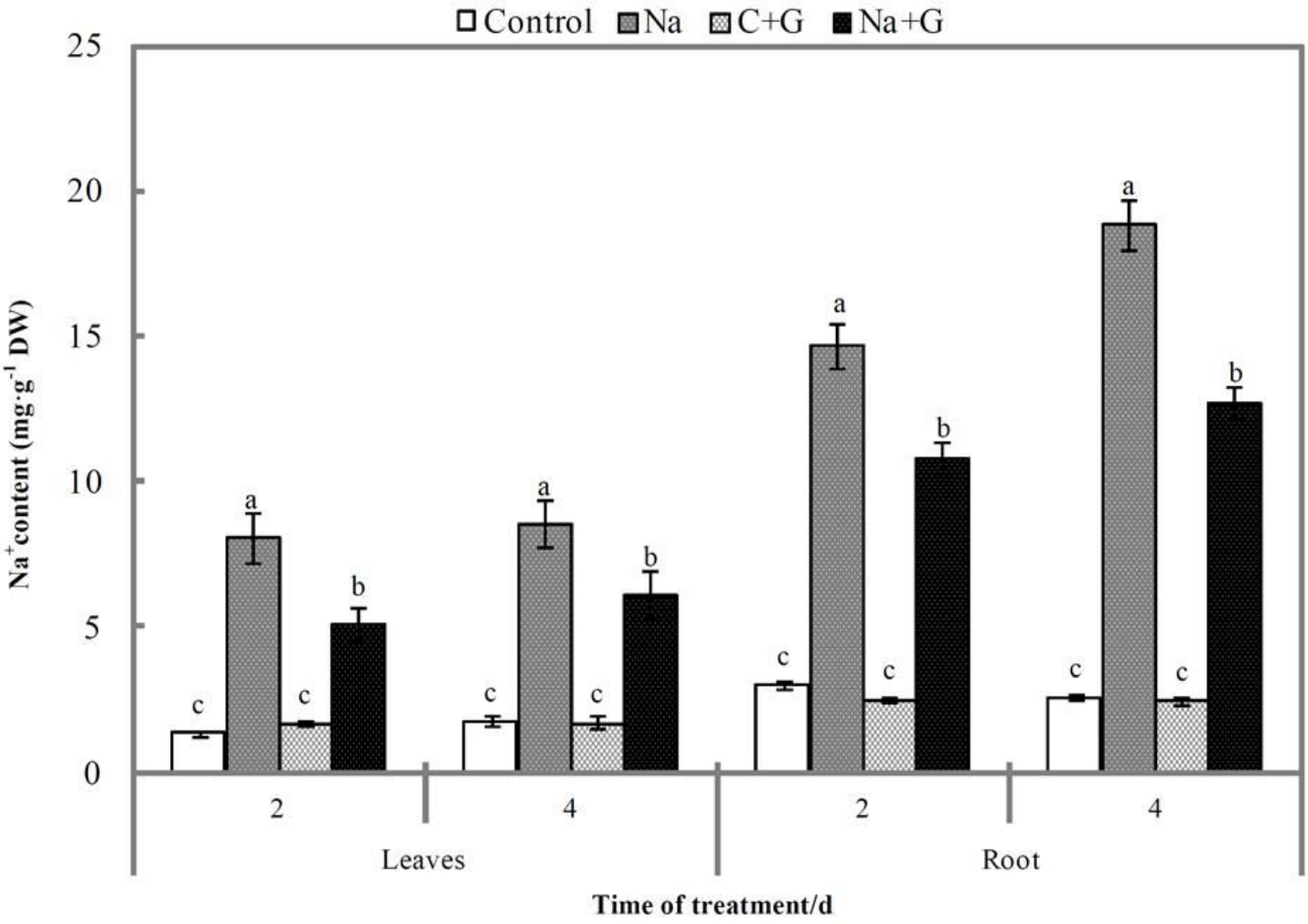

Figure 6

Na+ accumulation in leaves and root under NaCl stress with or without GABA 2 d and 4 d after treatment. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at P<0.05 by Duncan's test.

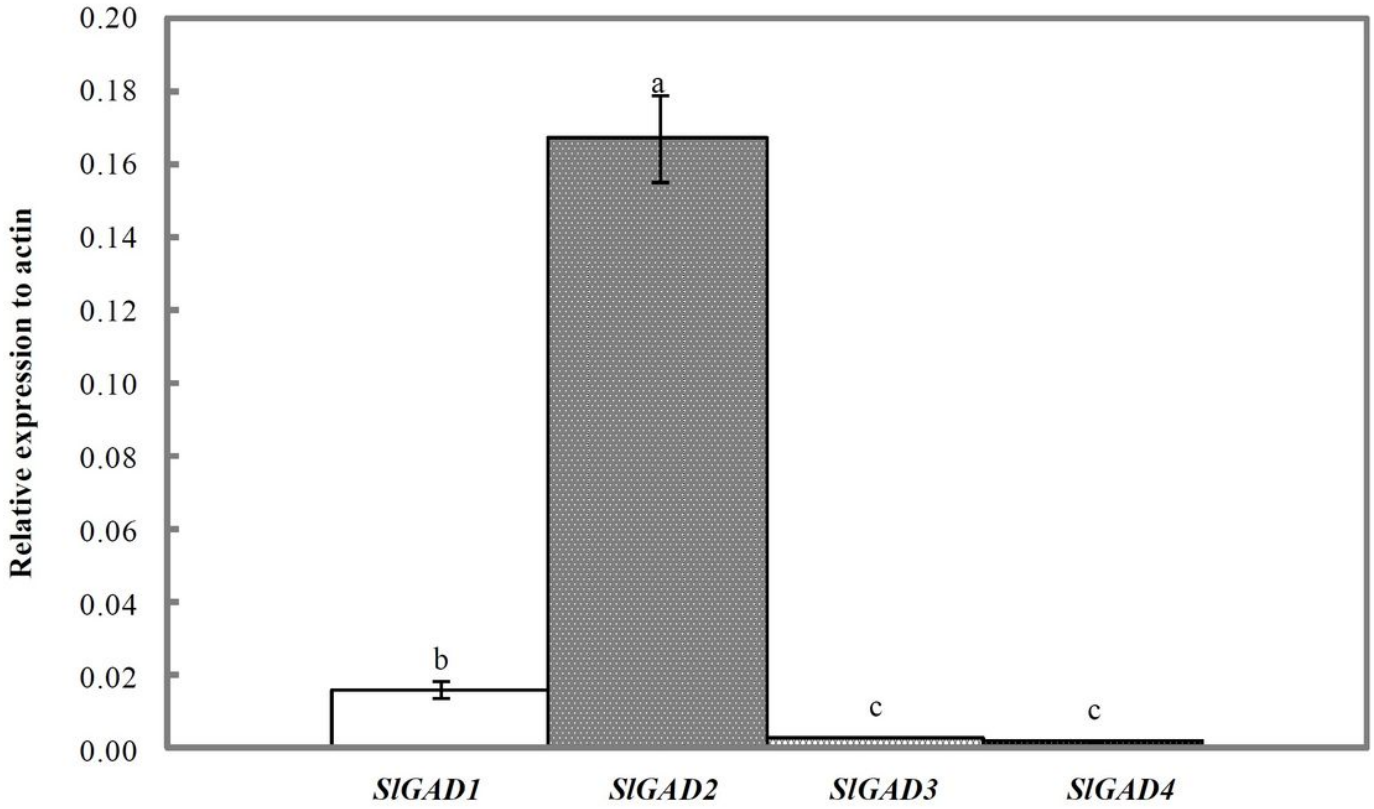

Figure 7

Expression analysis of GAD genes in leaves. Plants of 4-week-old were used. Note: Values are means of three biological replicates. Error bars represent the standard error of means; different lower-case letters in each gene indicate significant difference at $P<0.05$ by Duncan's test.

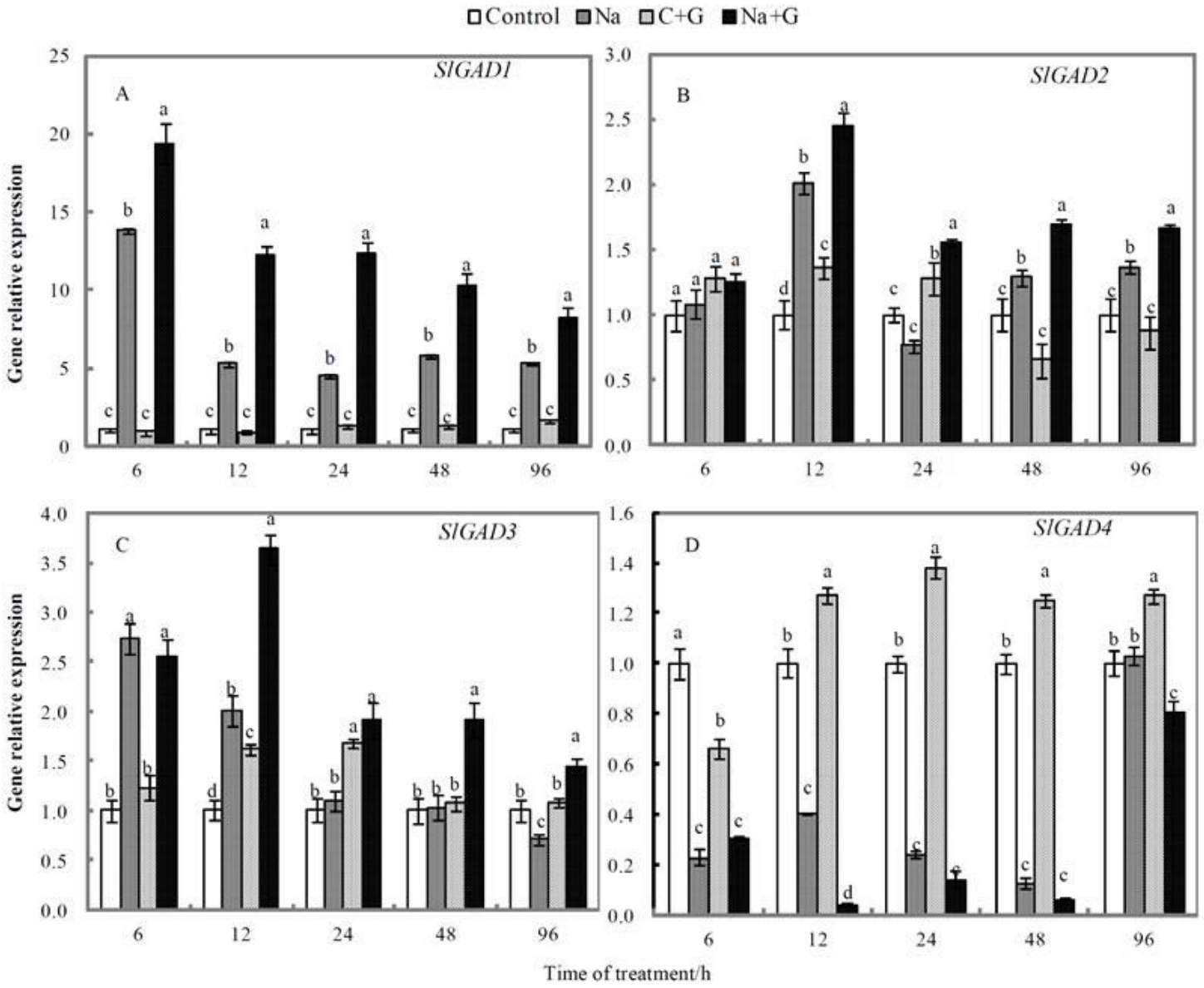

Figure 8

Dynamic changes in the relative expression of four tomato GAD genes in the leaves of tomato seedlings subjected to normal culture and NaCl treatment with or without GABA. Note: Gene expression of each treatment of 0 h was taken as 1, and the ordinate was taken as the ratio of gene expression of other time to that of 0h.The seedlings were shown to one of the following four treatments: Control (white squares), Na (salinity, grey squares), C+G(Control+GABA, grid squares), and Na+G (NaCl+GABA, black squares). Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

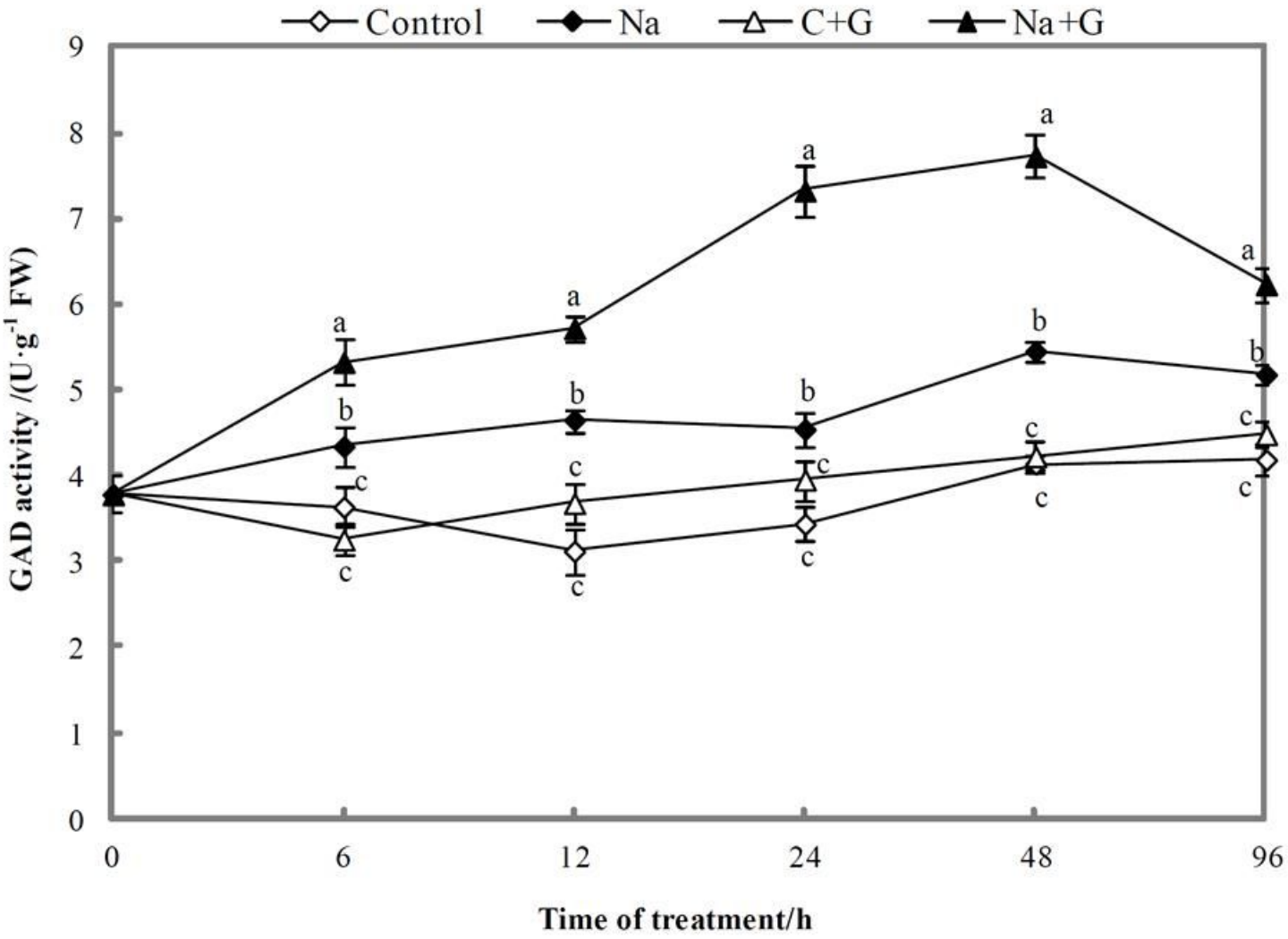

Figure 9

Dynamic changes of GAD activity in the leaves of tomato seedlings subjected to control and NaCl treatment with or without GABA . Note: The seedlings were shown to one of the following four treatments: Control (white diamond), Na (salinity, black diamond), C+G(Control+GABA, white triangle), and Na+G (salinity+GABA, black triangle). Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

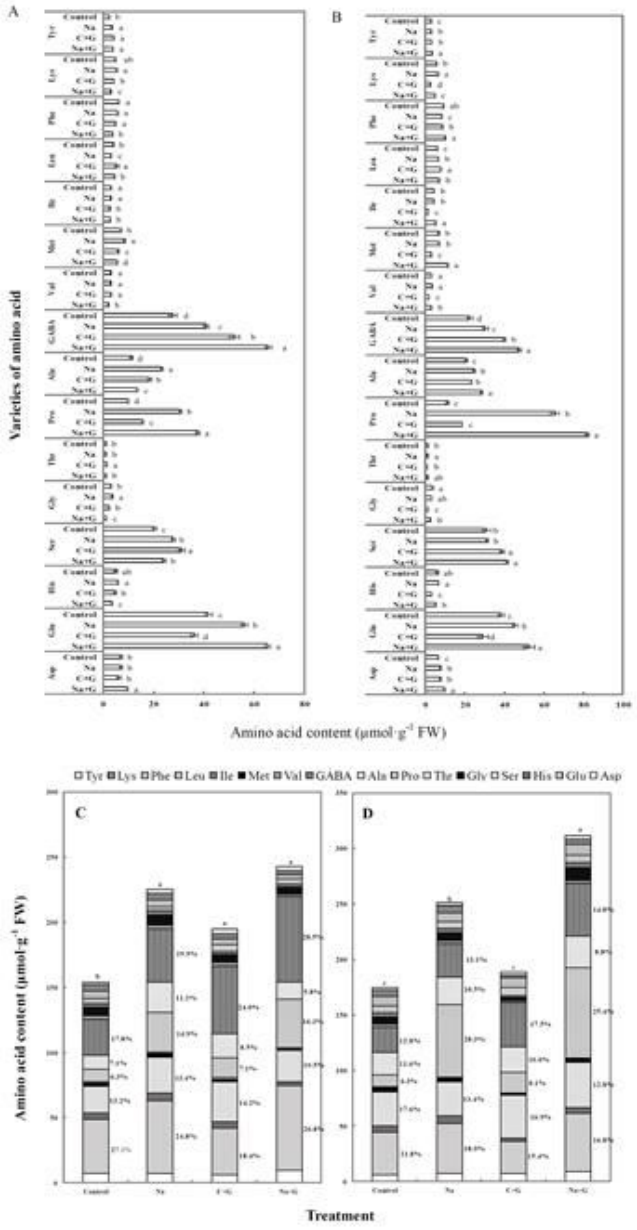

Figure 10

Different amino acids contents in leaves of control and NaCl treatment with or without GABA for 2and 4 d. (A) and (C) :2 days after treatment, (B) and (D) :4 days after treatment. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

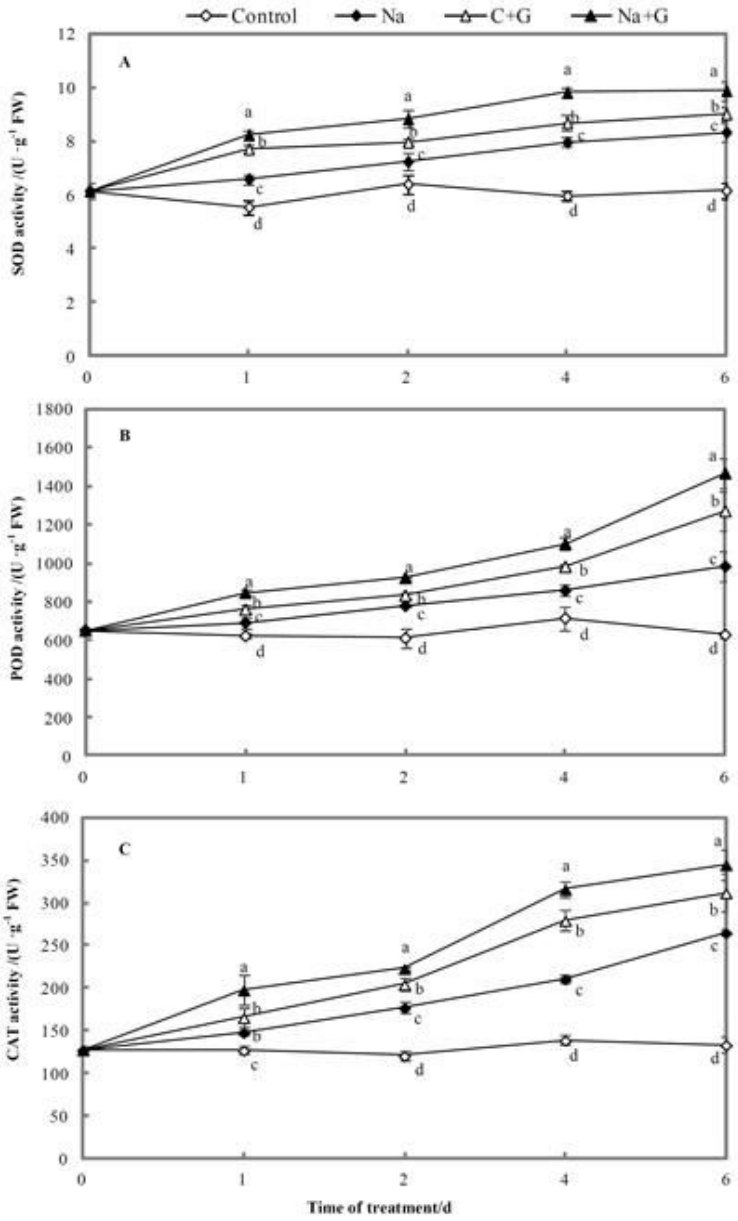

Figure 11

Antioxidant enzymes activities in leaves of tomato seedlings under control and NaCl treatment with or without GABA. (A): Superoxide dismutase.(B) :Peroxidase. (C) :Catalase. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

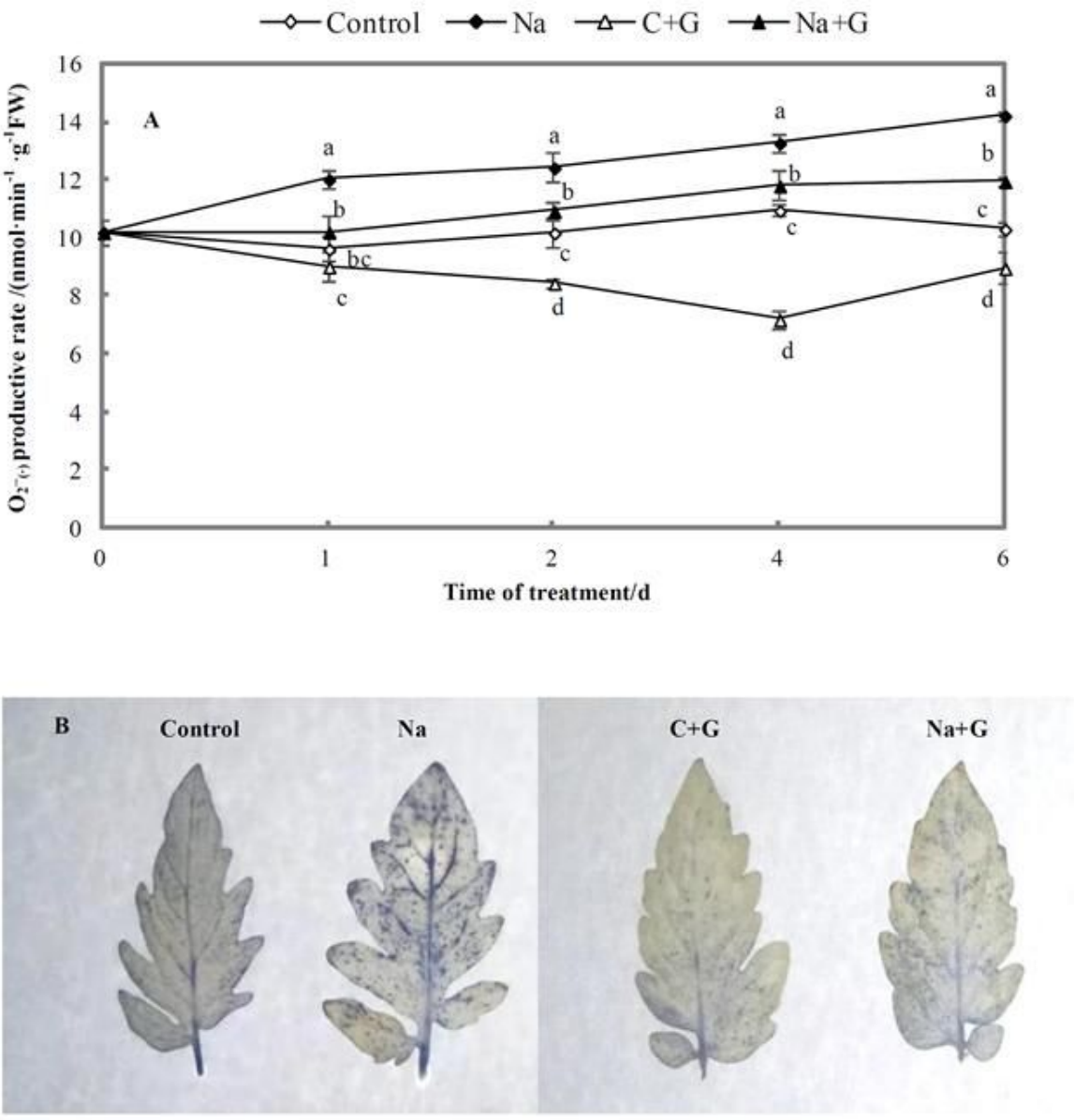

Figure 12

O2•- productive rate and tissue staining results in leaves of tomato seedlings under NaCl stress with or without GABA. (A): O2•- productive rate.(B) : Analysis of ROS production by NBT staining. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at $P<0.05$ by Duncan's test.

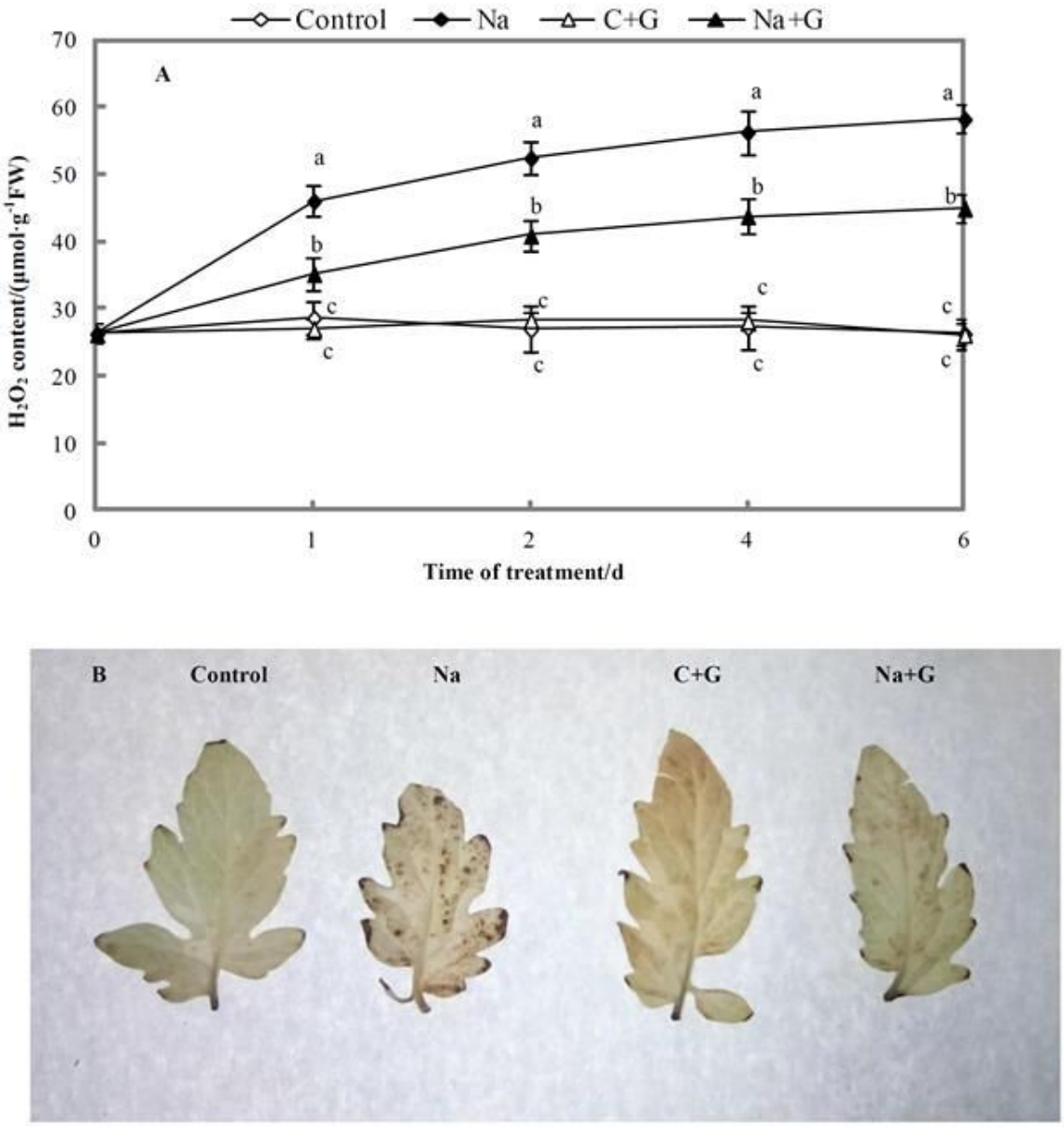

Figure 13

Content of H2O2 and tissue staining results in leaves of tomato seedlings under NaCl stress with or without GABA. (A): H2O2 content. (B) : Analysis of ROS production by DAB staining. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at P<0.05 by Duncan's test.

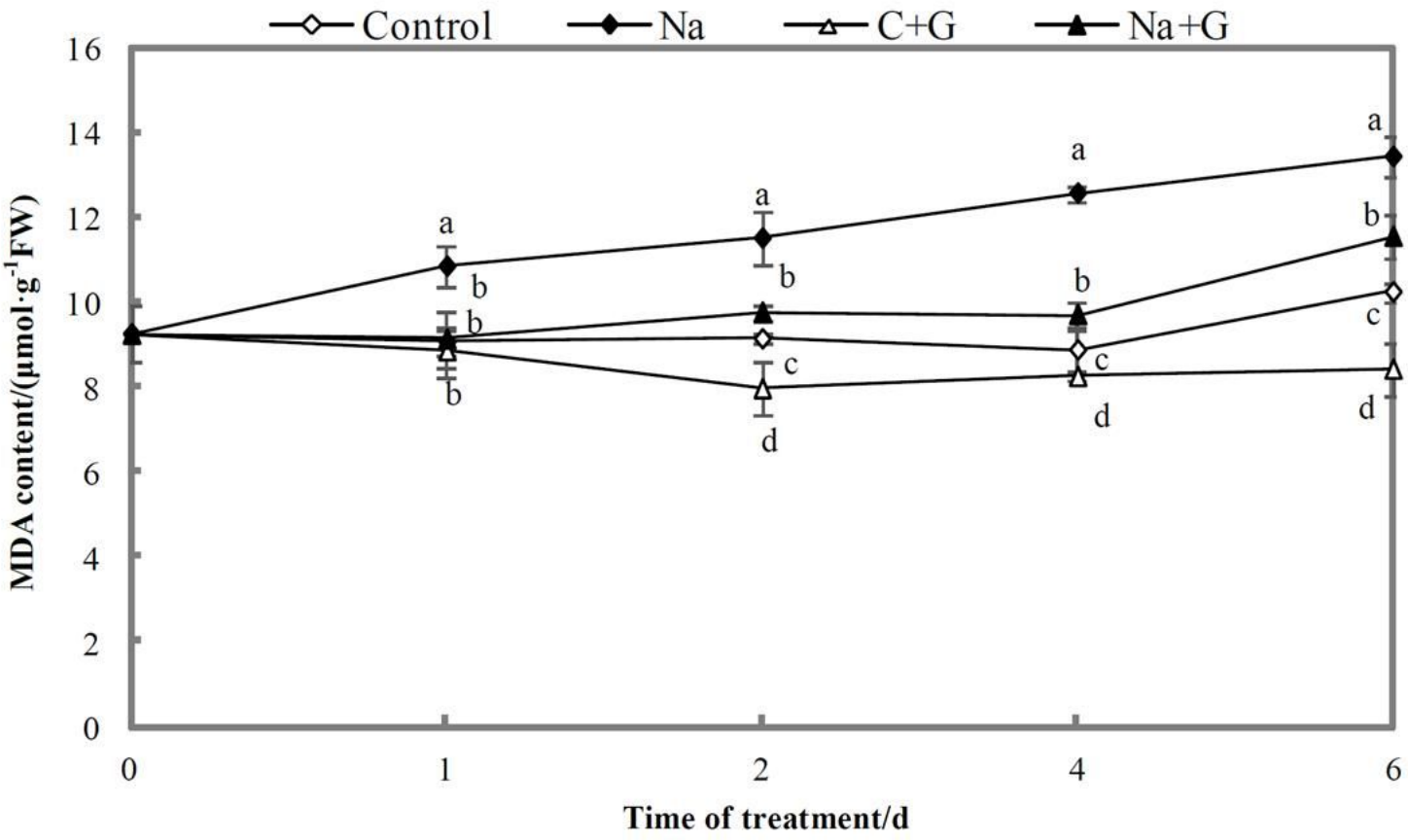

Figure 14

Content of Malondialdehyde in leaves of tomato seedlings under NaCl stress with or without GABA. Note: Each value is the mean ± SD of three independent experiments. Different lower-case letters in each column shape indicate significant difference at P<0.05 by Duncan's test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.jpg