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RESEARCH PAPERS

GABA Enhances Muskmelon Chloroplast Antioxidants to Defense Salinity-Alkalinity Stress[1]

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Abstract—Salinity-alkalinity stress is a pivotal factor influencing plant growth, development, and yield. γ-Aminobutyric acid (GABA) protects plants against a variety of environmental stresses. However, it is remains largely unknown whether exogenous GABA increases the tolerance of *Cucumis melon* L. seedlings via effects on the chloroplast antioxidant system. In this study, the role of exogenous GABA application on the malondialdehyde content and antioxidant enzyme activities and the ascorbate-glutathione (AsA-GSH) cycle in seedlings of muskmelon was investigated. Plants were treated with foliar spraying of GABA (50 mM) under control or salinity-alkalinity stress conditions. Salinity-alkalinity stress induced cellular membrane damage. Treatment with GABA protected muskmelon seedlings from salinity-alkalinity stress by enhancing antioxidant enzyme activity and reducing malondialdehyde content. These effects of GABA resulted in maintenance of the membrane integrity of the muskmelon seedling. In addition, the status of both GSH and AsA redox played key roles in the regulation of the oxidative stress response in muskmelon seedlings under salinity-alkalinity stress.

Keywords: Cucumis melon, muskmelon, γ-aminobutyric acid (GABA), oxidative stress, salinity-alkalinity stress, ascorbate-glutathione (AsA-GSH) cycle

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INTRODUCTION

Salinity-alkalinity stress, one of the major limiting factors in global agricultural crop production [1], is caused by faulty irrigation practices combined with excessive nutrients, poor rainfall, inappropriate soil leaching, and strong evaporation [2]. This stress results in over-accumulation of reactive oxygen species (ROS), weakened photosynthesis, and cell death [3, 4]. All ROS are strong oxidizers, including hydroxyl radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2) [5, 6]. High levels of ROS have harmful effects that result in DNA damage, protein denaturation, enzyme activity impairment, lipid peroxidation, carbohydrate oxidation, pigment breakdown, and cell death [7]. Hence, maintaining moderate levels of ROS is essential to the ability of plants to withstand diverse abiotic and biotic stresses.

Maintaining a subtle balance between ROS generation and scavenging is important for plants and primarily controlled by the antioxidant defense system [8]. The central antioxidant defense system includes the ascorbate-glutathione (AsA-GSH) cycle, which consists of two dominating non-enzymatic antioxidants (GSH and AsA) and four enzymes (ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)). The two non-enzymatic antioxidants reduce ROS by spontaneous biochemical reactions with the assistance of the four enzymes [9, 10]. Superoxide dismutase (SOD) also plays an important role in the antioxidant system [11].

γ-Aminobutyric acid (GABA), a four-carbon non-protein amino acid, is an important component of the free amino acid pool in most prokaryotic and eukaryotic organisms [12]. This amino acid can also be produced by the catabolism of polyamines [13]. Under abiotic stress, GABA has been shown to stabilize intracellular pH and acts as a source of carbon and nitrogen for the tricarboxylic acid cycle [14]. In addition, GABA enhances the activity of some antioxidant enzymes and reduces malondialdehyde (MDA) content, which results in maintenance of membrane integrity [15]. It has been reported that exogenous

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Abbreviations: APX—ascorbate peroxidase; AsA—ascorbate; DHA—dehydroascorbate; DHAR—dehydroascorbate reductase; GABA—γ-aminobutyric acid; GR—glutathione reductase; GSH—glutathione reduced; GSSG—oxidized glutathione; MDA—malondialdehyde; MDHAR—monodehydroascorbate reductase; O_2^-—superoxide anions; SOD—superoxide dismutase.

GABA alleviates hypoxia damage by accelerating polyamine biosynthesis and conversion, and by preventing polyamine degradation in melon plants [16].

Muskmelon (*Cucumis melon* L.) is an economically important horticultural crop that is widely cultivated in China but is sensitive to salinity-alkalinity stress [14, 17]. Previous studies have illustrated that exogenous GABA improves the photosynthesis of muskmelon seedlings that are exposed to salinity-alkalinity stress [17]. However, there are few studies on the antioxidation effects of GABA in muskmelon under salinity-alkalinity stress conditions. Therefore, in this study, the salinity-alkalinity stress tolerance and antioxidant system changes in both GABA-treated and untreated leaves of muskmelon plants were investigated.

MATERIALS AND METHODS

Plant materials. Muskmelon seeds (*Cucumis melon* L., cv. Yipintianxia no. 208) were surface sterilized in 10% Na_3PO_4 for 20 min, immersed in distilled water for 6 h, and germinated at 27°C in the dark. After two days, germinated seeds were sown in washed commix medium (Xintiandi Co., Yangling, Shaanxi, China), and placed in a seedling greenhouse with an average day/night temperature of 26–30°C/16–18°C, a 12 h light and 12 h dark photoperiod, and 50–90% relative humidity. A total of 25 plants for each treatment and three independent experiments were performed.

Salt-alkaline and GABA treatment. Seedlings fully expanded with four true leaves were grown in the nutrient medium with or without 50 mM salinity-alkalinity solution (NaCl : Na_2SO_4 : $NaHCO_3$: Na_2CO_3 at a 1 : 9 : 9 : 1 molar ratio) which final concentration of 50 mM Na^+ and pH of the final nutrient solution is 8.6. The muskmelon seedlings were subjected to the following four experiment groups: (1) control (CK), normal nutrient medium plus leaf spraying of 0 mM GABA; (2) control + GABA (CG), normal nutrient medium plus leaf spraying of 50 mM GABA; (3) stress (S), nutrient medium containing 50 mM salinity-alkalinity solution plus leaf spraying of 0 mM GABA; and (4) stress + GABA (GS), nutrient medium containing. Then, a 50 mM solution of GABA in water was applied by spraying all leaves per plant on a daily basis. This concentration of GABA was chosen based on previous results [14]. Seedlings were treated with the same amount of GABA or distilled water at 9:00 a.m. for seven consecutive days. After 0, 1, 3, 5, and 7 days of stress treatment, the third fully expended leaves of four experimental groups, numbered basipetally starting at the uppermost fully expended leaf, was collected to analyse malondialdehyde content, and antioxidant enzymes activities and the content of antioxidant in the chloroplasts, respectively. Isolated chloroplasts from the leaves using the method described by Shu et al. [18] with a slight modification were analyzed. Fresh leaves (15 g) were homogenized for 3 s in a Polytron blender with 30 mL of 330 mM sorbitol, 30 mM MES, 2 mM ascorbate, and 0.1% BSA, adjusted to pH 6.5 with Tris. The homogenate was filtered through four layers of cheesecloth, and the dark green filtrate was centrifuged at 1800 *g* for 2 min. The pellets were resuspended in 2 mL of buffer (330 mM sorbitol, 30 mM HEPES and 0.2% BSA, adjusted to pH 7.6 with Tris), put into a tube containing 8 mL of resuspension medium plus 40–80% (v/v) Percoll and centrifuged for 3 min at 2000 *g*. The interlayer between 40 and 80% Percoll contained intact chloroplasts. All procedures were carried out at 4°C, and the percentage of intactness of chloroplasts was about 85%.

Extraction of chloroplast antioxidant enzymes and antioxidants. 3 mL aliquot of chloroplast-containing supernatant was mixed with 3 mL ice-cold HEPES buffer (25 mM, pH 7.8) containing 0.2 mM ethylenediaminetetraacetic acid and 2% (w/v) polyvinylpyrrolidone. The mixture was then centrifuged at 12000 *g* and 4°C for 20 min. The resulting supernatant was used to assay the antioxidant enzyme activity and determine the content of antioxidants (AsA and GSH) as described below [19].

Malondialdehyde measurements. Malondialdehyde (MDA) was measured according to the method of Xu et al. [20]. The leaf samples (0.15 g) were homogenized in 4 mL of 10% TCA and centrifuged at 10000 *g* for 15 min. The supernatant (1 mL) was mixed with 1 mL of 0.6% thiobarbituric acid, heated at 95°C for 30 min and then was quickly cooled down on ice. After centrifugation at 10000 *g* for 10 min, absorbance of the supernatant was measured with an UV-visible spectrophotometer at 450, 532 and 600 nm, respectively.

Measurement of SOD, APX, GR, MDHAR, and DHAR activities. The SOD activity was assayed by monitoring SOD-mediated inhibition of the photochemical reduction of nitro blue tetrazolium [21]. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of the reduction of nitro blue tetrazolium as monitored by spectrophotometer at 560 nm. The APX activity was assayed by monitoring the ascorbate oxidation rate using spectrophotometer at 290 nm according to the method of Nakano and Asada [22]. The GR activity was measured by tracking nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, which was determined by monitoring the decrease in absorbance at 340 nm over 3 min [23].

The activities of MDHAR and DHAR were assayed according to the method described by Zhang et al., with a slight modification [24]. MDHAR activity was assayed using spectrophotometer at 340 nm in a 1 mL sample containing 50 mM HEPES-KOH (pH 7.6), 25 mM AsA, 1 mM nicotinamide adenine dinucleotide hydride (NADH), 0.5 units of ascorbate oxidase, and 50 μL of enzyme extract. DHAR activity was assayed

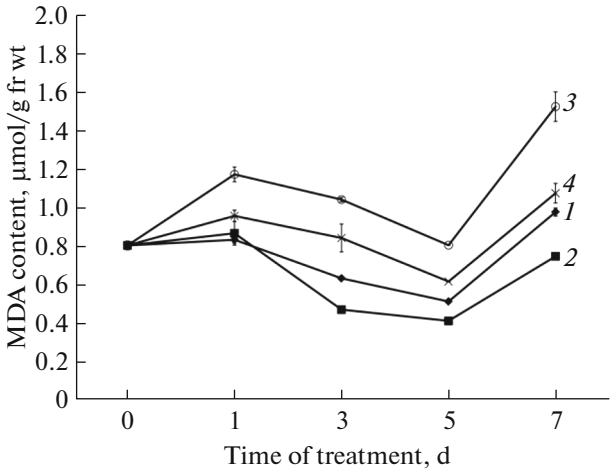

Fig. 1. GABA decreased the MDA content of muskmelon chloroplasts under salinity-alkalinity stress. *1*—The muskmelon leaves were treated with distilled water under normal condition; *2*—the muskmelon leaves were treated with 50 mM GABA under normal condition; *3*—the muskmelon leaves were treated with distilled water under salinity-alkalinity stress; *4*—the muskmelon leaves were treated with 50 mM GABA under salinity-alkalinity stress. Each data point is the mean ± *SE* ($n = 3$).

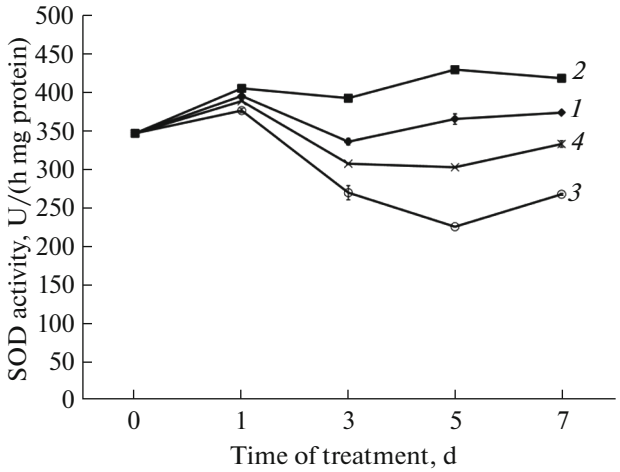

Fig. 2. GABA improved the SOD activity of muskmelon chloroplast under salinity-alkalinity stress. *1*—The muskmelon leaves were treated with distilled water under normal condition; *2*—the muskmelon leaves were treated with 50 mM GABA under normal condition; *3*—the muskmelon leaves were treated with distilled water under salinity-alkalinity stress; *4*—the muskmelon leaves were treated with 50 mM GABA under salinity-alkalinity stress. Each data point is the mean ± *SE* ($n = 3$).

using spectrophotometer at 265 nm in a 2.9 mL sample containing 100 mM HEPES-KOH (pH 7.6), 25 mM reduced GSH, 2 mM dehydroascorbate (DHA), and 50 μL enzyme extract. Protein was determined according to the method of Bradford, using bovine serum albumin as a standard [25].

Determination of AsA and GSH content. The AsA content was determined according to the method of Shu et al. [18] with a minor modification. The reaction mixture contained 200 μL 5% trichloroacetic acid, 100 μL 0.4% H_3PO_4-ethanol, 100 μL 0.03% $FeCl_3$-ethanol, 200 μL 0.5% BP-ethanol, and 300 μL extract. The sample was incubated at 40°C for 1 h, after which the absorbance was measured using spectrophotometer at 534 nm. The AsA content was calculated based on an ascorbic acid standard curve.

The GSH content was assayed as described by Li and Cheng [26] and determined by subtraction of oxidized glutathione from total glutathione.

Statistical analysis. All data were statistically analyzed with SAS 9.0 software (SAS Institute, Inc., Cary, NC, USA) using Duncan's multiple range test at the $P < 0.05$ level of significance.

RESULTS

Effect of GABA on MDA Content in Salinity-Alkalinity Stressed Muskmelon Seedling Chloroplasts

Our results show that after three days under normal conditions, there was a dramatically decrease in the content of MDA in the CG (GABA alone)-treated plants compared with CK (no treatment) plants. Compared with the CK-treated plants, the MDA content in the S-treated plants was remarkably improved and peaked on the seventh day at a level of 55.8%. Treatment with salts and GABA (SG treatment) dramatically reduced the MDA content to 29.4% lower than S-treated plants (Fig. 1).

Effect of GABA on the Chloroplast Antioxidant System of Salinity-Alkalinity Stressed Muskmelon Seedling Chloroplasts

Treatment with GABA alone or with salts (CG or SG-treated plants, respectively) under both normal and salinity-alkalinity stress conditions dramatically increased the SOD activity compared with the CK. In contrast, under the same conditions, treatment with only salts (S-treated plants) remarkably decreased the SOD activity compared to the CK-treated plants (Fig. 2). Five days after treatment, SOD activity in S-treated plants was 38.1% lower than that in CK-treated plants, whereas it was 34% higher in SG-treated plants than in S-treated plants (Fig. 2).

Compared to CK treatment, CG treatment enhanced the MDHAR and GR activities after three days (Figs. 3b and 3d). Compared with CK, the S treatment improved the activities of APX, MDHAR, DHAR, and GR early, and then decreased later, the APX, MDHAR, and DHAR activities in the S-treated plants peaked on day 3, whereas the GR activity peaked on day 5 (Fig. 3). The SG treatment also improved the activities of APX, MDHAR, DHAR,

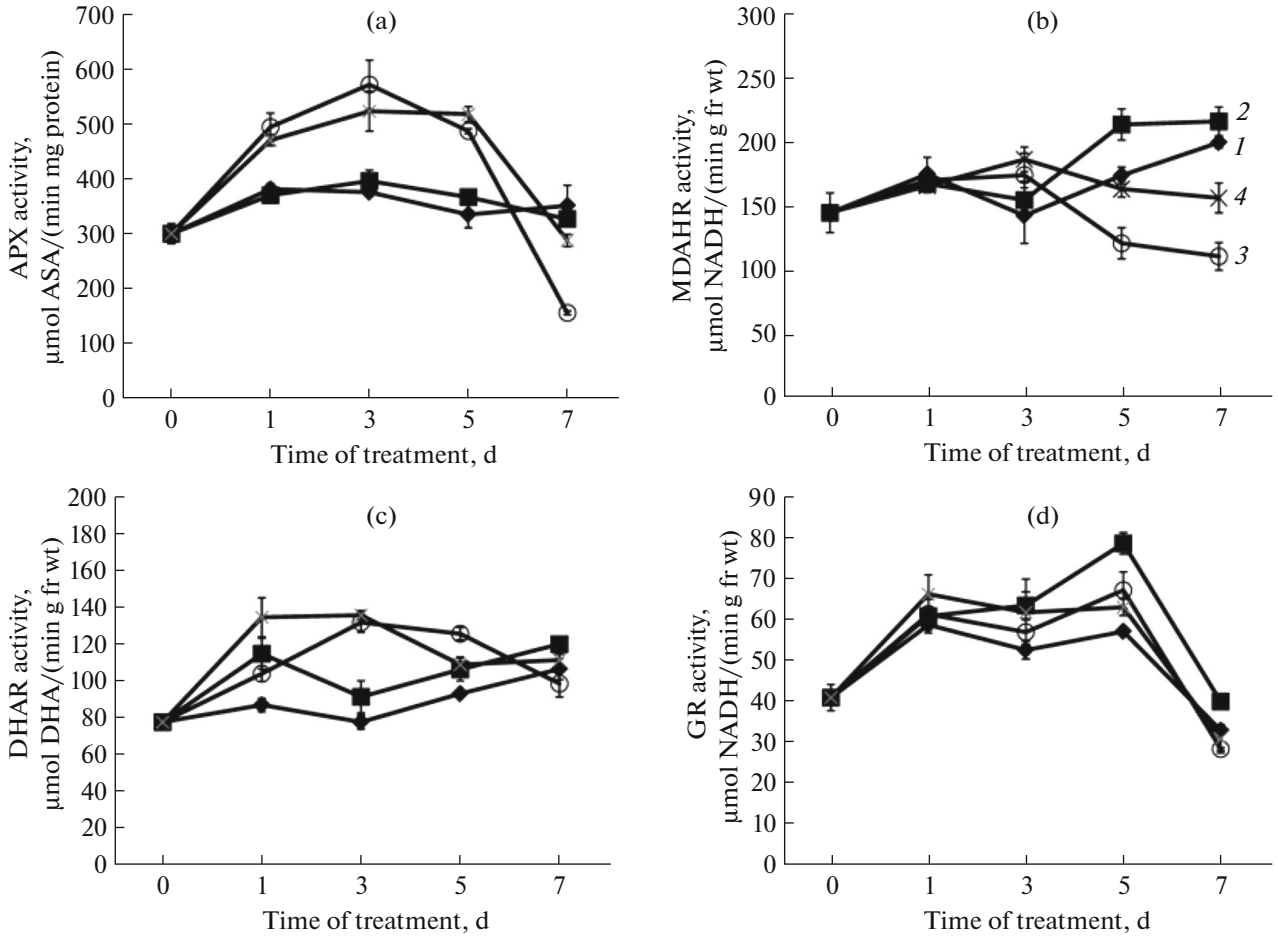

Fig. 3. GABA improved the antioxidase activities in AsA-GSH cycle of muskmelon chloroplast under salinity-alkalinity stress. *1*—The muskmelon leaves were treated with distilled water under normal condition; *2*—the muskmelon leaves were treated with 50 mM GABA under normal condition; *3*—the muskmelon leaves were treated with distilled water under salinity-alkalinity stress; *4*—the muskmelon leaves were treated with 50 mM GABA under salinity-alkalinity stress. Each data point is the mean ± *SE* ($n = 3$).

and GR compared to CK. In particular, inhibition of enzyme activities was alleviated by S treatment on day 7 (Fig. 3).

The CG treatment significantly increased the content of ASA + DHA, AsA, GSH + GSSG, and GSH compared with CK most of time (Fig. 4). The S treatment dramatically improved the content of ASA + DHA, AsA, GSH + GSSG, and GSH compared with CK after five days of treatment, with the exception of AsA content on day 5 (Fig. 4). The SG treatments exhibited similar remarkable improvements in the GSH + GSSG and GSH content compared with S treatment on day 7 (Figs. 4b, 4d).

DISCUSSION

Chloroplasts are the most important organelle in plants for the photosynthesis, growth, and yield formation, but they are sensitive to salt stress and the major source of ROS. Free tetrapyrroles that generate highly reactive singlet oxygen or oxygen reduction through the Mehler reaction in the chloroplasts may generate ROS, which in turn lead to membrane lipid peroxidation [27]. Environmental stresses exacerbate photoinhibition and, over a long period, may induce photooxidative damage, resulting in accumulation of ROS in chloroplasts. So, it is very important to scavenge the excessive ROS and maintain cellular homeostasis. Superoxide anions (O_2^-) may be quickly converted into H_2O_2 by SOD [5], which is then converted to H_2O or O_2 by an AsA and/or a GSH regenerating cycle [28]. In the present study, seedlings of muskmelon may increase chloroplast ROS accumulation which was resulting in an increased membrane damage under salinity-alkalinity stress (Fig. 1). In an efficiently functioning antioxidant system, a high level of antioxidant enzyme activity and high levels of nonenzymatic components are maintained [28]. Reduced GSH and AsA levels and reduced activities of some key enzymes (APX, GR, MDHAR, and DHAR) involved in the AsA-GSH cycle and SOD were improved dramatically with SG treatment compared

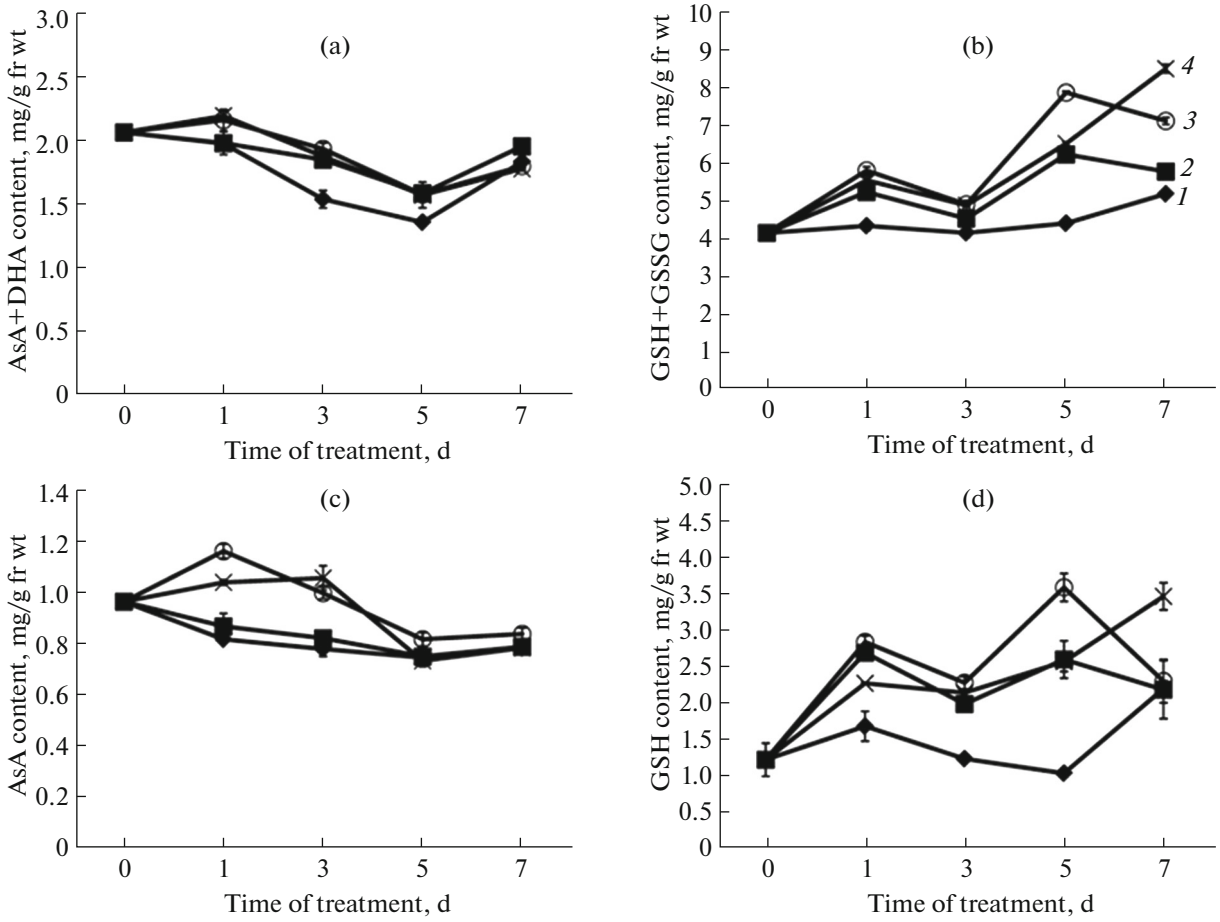

Fig. 4. GABA improved the AsA and GSH redox status of muskmelon chloroplasts to defend against salinity-alkalinity stress. *1*—The muskmelon leaves were treated with distilled water under normal condition; *2*—the muskmelon leaves were treated with 50 mM GABA under normal condition; *3*—the muskmelon leaves were treated with distilled water under salinity-alkalinity stress; *4*—the muskmelon leaves were treated with 50 mM GABA under salinity-alkalinity stress. Each data point is the mean ± *SE* ($n = 3$).

with S treatment (Figs. 2, 3, 4). These results suggest that O_2^- may be reduced to H_2O_2 by SOD, which was then scavenged by the AsA-GSH cycle induced by GABA. An increase in MDHAR activity might provide reducing equivalents for APX, which maintained the AsA-GSH cycle (Figs. 3, 4). In the present study, AsA regeneration under salinity-alkalinity stress was primarily driven by MDHAR and DHAR. GSH and AsA acted as substrates for DHAR and APX, respectively, and are considered the critical component of the AsA-GSH cycle for maintaining intracellular defenses against ROS-induced oxidative damage [9]. The increase in GR activity directly promoted conversion of oxidized glutathione to GSH, which eliminated H_2O_2 and reduced the accumulation of ROS in chloroplasts [29], resulting in the stabilization of the membrane.

In conclusion, salinity-alkalinity stress increased the membrane damage in muskmelon, and exogenous GABA alleviated the damage via SOD and the AsA-GSH cycle, which scavenged the excessive accumulation of ROS. Furthermore, both the GSH and AsA redox status was crucial in the regulation of the oxidative stress response. These results indicated that GABA played an important role in the relief of the harmful effects caused by oxidative stress.

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