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Research article

Application of γ-aminobutyric acid demonstrates a protective role of polyamine and GABA metabolism in muskmelon seedlings under $Ca(NO_3)_2$ stress

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ABSTRACT

The effects of exogenous γ-aminobutyric acid (GABA) application on growth, polyamine and endogenous GABA metabolism in muskmelon leaves and roots were measured. Plants were treated under control or 80 mM $Ca(NO_3)_2$ stress conditions with or without foliar spraying 50 mM GABA. $Ca(NO_3)_2$ stress significantly suppressed seedling growth and GABA transaminase activity, and enhanced glutamate decarboxylase (GAD) activity and endogenous GABA levels. Polyamine (PA) biosynthesis and degradation capacity increased in parallel with increasing GAD activity. Exogenous GABA application effectively alleviated the growth inhibition caused by $Ca(NO_3)_2$ stress, and significantly enhanced the activities of arginine decarboxylase (ADC), ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (SAMDC), polyamine oxidase (PAO), and diamine oxidase (DAO). Exogenous GABA also significantly reduced the accumulation of free putrescine (Put) and increased the levels of free spermidine (Spd) and spermine (Spm) in leaves, which improved the capacity for polyamine biosynthesis. Application of exogenous GABA under $Ca(NO_3)_2$ stress enables the plants to maintain a higher ratio of free Spd and free Spm with respect to free Put. Our data suggest that exogenous GABA has an important role in improving muskmelon seedling tolerance to $Ca(NO_3)_2$ stress by improving biosynthesis of PAs and GABA, and by preventing PA degradation. There is a potential positive feedback mechanism that results from higher endogenous GABA content and the combined effects of $Ca(NO_3)_2$ stress and exogenous GABA, which coordinately alleviate $Ca(NO_3)_2$ stress injury by enhancing PA biosynthesis and converting free Put to an insoluble bound PA form, and reduce PA degradation in muskmelon seedlings.

1. Introduction

Muskmelon (*Cucumis melon* L.) is an important horticultural fruit that is sensitive to salt stress. Muskmelon is widely cultivated in China. Salt stress is considered as one of the most severe environmental stresses, and salinization is a global threat to food security. In Chinese greenhouse, secondary salinization is caused by improper irrigation combined with excessive nitrogen fertilization, poor rainfall, inadequate soil leaching, and strong evaporation which severely reduces the productivity of greenhouse vegetables (Yuan et al., 2014). Previous studies report that Ca^{2+} accounts for >60% of total cations and NO_3^- accounts for 67–76% of total anions in secondary salinized soil (Li et al., 2004). Therefore, a high level of $Ca(NO_3)_2$ accumulation is one of the main characteristics of secondary salinization in Chinese agricultural greenhouses.

Plants have evolved an array of physiological, biochemical, and molecular regulatory mechanisms to survive salt stress, such as accumulating compatible solutes and proteins, and activating the expression of genes involved in stress responses (Bartels and

Abbreviations: ADC, arginine decarboxylases; DAO, diamine oxidase; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; Glu, glutamate; ODC, ornithine decarboxylases; PA, polyamine; PAO, polyamine oxidase; PCA, perchloric acid; Put, putrescine; ROS, reactive oxygen species; RWC, relative water content; SAMDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; TCA, tricarboxylic acid cycle.

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Sunkar, 2005). Human interventions to prevent or alleviate salinization include advanced agronomic methods, importing new soils, and exogenous application of γ-aminobutyric acid (GABA) and polyamines (PAs). GABA is a four-carbon non-protein amino acid, which is produced in plants via decarboxylation of glutamate (Glu) by glutamate decarboxylase (GAD) (EC 4.1.1.15) or degradation of polyamine by diamine oxidase (DAO) (EC 1.4.3.6) and polyamine oxidase (PAO) (EC 1.5.3.3) (Wang et al., 2014). In plants, GABA is a metabolite involved in responses to biotic and abiotic stresses; it rapidly accumulates during stress responses and is involved in defense response systems (Shelp et al., 1999). Salt treatment of germinating soybean (*Glycine max* L.), tobacco (*Nicotiana tabacum*), and *Arabidopsis thaliana* induces GABA accumulation and activates GABA metabolism (Renault et al., 2010; Zhang et al., 2011; Yin et al., 2014). Barbosa et al. (2010) reported that exogenous GABA application improved plant growth, enhanced stress tolerance by modulating enzyme activities in nitrogen metabolic pathways, increased the accumulation of stress-protective alanine (Miyashita and Good, 2008), and prevented reactive oxygen species (ROS) accumulation and cell death.

Polyamines are low molecular weight aliphatic amines that have important functions in a wide range of processes, including growth, cell division, DNA replication, and protein synthesis (Roychoudhury et al., 2011). Spermidine (Spd), spermine (Spm), and putrescine (Put), are the major PAs in plants. These metabolites act as second messengers and mediate plant responses to several environmental stresses (Gill et al., 2010; Hu et al., 2012). Polyamine degradation pathways feed into the GABA biosynthesis pathway, and endogenous levels of PA and GABA are closely linked in plant cells (Wang et al., 2014). However, the effects of excessive [$Ca(NO_3)_2$] on coupled PA and GABA metabolism in muskmelon are unknown. We previously reported that foliar spraying with 50 mM GABA effectively improved muskmelon seedling tolerance to $Ca(NO_3)_2$ stress (Xu et al., 2015). The aims of the current study were to determine the effects of exogenous GABA application on PA and GABA metabolism under $Ca(NO_3)_2$ stress, and to elucidate the physiological mechanism of GABA-mediated tolerance to $Ca(NO_3)_2$ stress in muskmelon seedlings.

2. Materials and methods

2.1. Plant cultivation and experimental treatments

Experiments were performed at Northwest Agricultural and Forestry University research greenhouse, which is equipped with state-of-the-art environmental controls. Muskmelon seeds (*Cucumis melo* L., cv. Yipintianxia No. 208), which are sensitive to saline conditions (Zhao et al., 2014), were obtained from Shaanxi Qianpu Agricultural Development Co., Ltd, China. Seeds 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 2 d, 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. When the third leaves were fully expanded, all seedlings with equivalent growth and development were transplanted into troughs containing 40 L of half-strength Japan Yamazaki muskmelon special nutrient medium (pH 6.3 ± 0.1, electrical conductivity 1.3 ± 0.1 mS cm^{-1}), with 12 seedlings per trough and 24 thoughs.

Seedlings fully expanded with four true leaves were grown in the nutrient medium with or without 80 mM $Ca(NO_3)_2$, which was administered in two steps of 40 mM $Ca(NO_3)_2$ per day to prevent salt shock. The muskmelon seedlings were subjected to the following four treatments: (1) control, normal nutrient medium plus leaf spraying of 0 mM GABA; (2) control + GABA, normal nutrient medium plus leaf spraying of 50 mM GABA; (3) stress, nutrient medium containing 80 mM $Ca(NO_3)_2$ plus leaf spraying of 0 mM GABA; and (4) stress + GABA, nutrient medium containing 80 mM $Ca(NO_3)_2$ plus leaf spraying of 50 mM GABA. The GABA concentration was selected based on the results of our previous experiment (Xu et al., 2015). Seedlings were treated with daily foliar applications at 08.00 h. Shoots and roots were harvested at 0, 1, 3, 5, and 7 d for analysis of metabolite concentrations, enzyme activities related to PA and GABA metabolic pathways. After 7 d of treatment, 10 plants per treatment were harvested to measure plant growth and relative water content.

2.2. Growth parameters

Fresh weight was determined by collecting shoots and roots, washing in sterile distilled water, blotting dry, and weighing. Dry weight was determined in shoots and roots prepared as above, which were dried in an oven at 75 °C for 72 h, and then weighed. Leaf area was determined by scanning leaves with a desktop scanner (Epson Expression 1680, Heraeus Co. Ltd., Germany) and then using Image J soft to calculate the leaf area.

The seedling index was calculated according to a previously published method (Li and Zhang, 2009) using the following equation:

$$\text{Seedling index} = \text{Total dry weight}\{\text{Root dry weight}/(\text{Shoot dry weight} + \text{Stem diameter}/\text{Plant height})\}$$

The relative water content (RWC) was calculated using the following formula:

$$\text{RWC}(\%) = (\text{Fresh weight} - \text{Dry weight})/\text{Fresh weight} \times 100$$

2.3. Analysis of endogenous GABA content

The concentration of GABA in shoots and roots was estimated using the method of Zhang and Bown (1977) with some modifications. Frozen samples were ground to a fine powder in liquid nitrogen, and 2.0 g of the ground tissue was extracted in 1.0 mL lanthanum chloride. The sample was shaken at room temperature for 15 min, and centrifuged at 13,000× *g* for 5 min. The supernatant was removed to a new tube, treated with 200 μl of 1 M KOH, shaken for 5 min, and centrifuged at 13,000× *g* for 5 min. The resulting supernatant was transferred to a new tube and used for spectrophotometric determination of GABA using the following procedure: 2.0 mL of supernatant was added to a tube containing 100 μl of 1 M KOH, 100 μl of phosphate buffer (pH 10), 400 μl of 6% phenol, and 800 μl of 5% sodium hypochlorite. Samples were placed in a boiling water bath for 10 min, and immediately transferred to an ice bath for 5 min. Then, 800 μl of 60% alcohol was added to each sample, and absorbance was measured at 254 nm using a Shimadzu UV1800 spectrophotometer (Shimadzu Co. Ltd., Japan).