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Original Article

Ca^{2+} and aminoguanidine on γ-aminobutyric acid accumulation in germinating soybean under hypoxia–NaCl stress

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ABSTRACT

Gamma-aminobutyric acid (GABA), a nonproteinous amino acid with some benefits on human health, is synthesized by GABA-shunt and the polyamine degradation pathway in plants. The regulation of Ca^{2+} and aminoguanidine on GABA accumulation in germinating soybean (*Glycine max* L.) under hypoxia-NaCl stress was investigated in this study. Exogenous Ca^{2+} increased GABA content significantly by enhancing glutamate decarboxylase gene expression and its activity. Addition of ethylene glycol tetra-acetic acid into the culture solution reduced GABA content greatly due to the inhibition of glutamate decarboxylase activity. Aminoguanidine reduced over 85% of diamine oxidase activity, and 33.28% and 36.35% of GABA content in cotyledon and embryo, respectively. Under hypoxia–NaCl stress, the polyamine degradation pathway contributed 31.61–39.43% of the GABA formation in germinating soybean.

1. Introduction

Soybean (*Glycine max* L.) is an edible legumes consumed traditionally by Asians and also accepted in Western countries due to its cheap, healthy, and nutritional characters. Apart from dietary protein, carbohydrate, oil, vitamin, and mineral, soybean contains many other functional components (e.g., isoflavone and phosphatidylcholine) [1]. However, its nutritional value is limited by the presence of antinutritional factors such as tannins and enzyme inhibitors [2]. Previous research demonstrated that seed germination could effectively enhance the nutrient value of legumes [3,4]. During seed germination, endogenous enzymes are synthesized or activated to degrade biochemical components such as starch and protein in plant organs, resulting in a significant accumulation of micromolecules including γ-aminobutyric acid (GABA) [5].

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GABA exists widely in prokaryotic and eukaryotic organisms. It is a four-carbon nonproteinous amino acid that acts as a neurotransmitter in the brain and spinal cord of mammals [6]. Development of its functional food can benefit human health. In recent years, GABA-enriched foods have become popular due to its healthy functions for decreasing blood pressure, alleviating pain and anxiety, etc. [7] Seed germination is a simple way for GABA to accumulate. In plant tissues, GABA is biosynthesized through the GABA-shunt [8] and polyamine degradation pathway [9], where glutamate decarboxylase (GAD, EC 4.1.1.15) and diamine oxidase (DAO, EC 1.4.3.6) are the rate-limiting enzymes for GABA formation. In plant cells, GABA is synthesized via the α-decarboxy1ation of glutamate (Glu) in a nonreversible reaction that is cata1ysed by GAD. It is metabolized in the mitochondria to succinic semialdehyde and then succinate by GABA transaminase (EC 2.6.1.19) and succinate semialdehyde dehydrogenase (EC 1.2.1.16), respective1y [8]. This metabolic pathway is called the GABA shunt. GABA can also be formed via the γ-aminobutyraldehyde intermediate from the polyamine degradation reaction where DAO is the key enzyme [10]. A stressful environment such as hypoxia, salt stress, heat or cold shock, drought, and mechanical damage can strongly increase GAD and DAO activities for GABA accumulation [11].

GABA content was greatly enhanced in germinating soybean [12] under hypoxia conditions. Research has shown that hypoxia could lead the acidification of cytoplasm, while the optimum reaction pH of GAD for Glu decarboxylase is 5.5–6.0 [8]. Hence, hypoxia condition was helpful for GABA accumulation. NaCl stress also increased GABA content in germinating soybean [13]. GAD activity can be stimulated by Ca^{2+} [14] because it is a Ca^{2+}/calmodulin-binding protein. In addition, DAO also has a Ca^{2+} binding site [15]. Ethylene glycol-bis-(2-aminoethyl ether)-N,N′-tetra-acetic acid (EGTA) is a metal chelator that may affect GAD and DAO activity by chelating endogenous Ca^{2+}. Aminoguanidine (AG) is a specific inhibitor of DAO can effectively inhibit DAO activity and thus decrease GABA accumulation [13]. Using AG to inhibit DAO activity can investigate the interrelationships between the GABA-shunt and polyamine degradation pathway.

Previous studies mainly focused on either hypoxia or NaCl stress alone. Limited information is available on the comparative studies on an addition of Ca^{2+} and AG for GABA accumulation in germinating seeds under hypoxia-NaCl stress. This scientific research is designed to explicate the influence mechanism of Ca^{2+} and AG on GABA accumulation in germinating soybean, investigate the effects of Ca^{2+} and AG on GAD and DAO activities, and evaluate their gene expression levels under hypoxia–NaCl stress.

2. Materials and methods

2.1. Materials and reagents

Soybean seeds (cultivar *Yunhe*, obtained from Jilin Province of China in 2012) were stored in polyethylene containers at −20°C. Standard samples of GABA (99% in purity), AG, EGTA, and dimethylaminoazobenzene sulfonyl chloride (dabsyl chloride, 99% in purity) were purchased from Sigma Chemical Co. Ltd. (St Louis, MO, USA). Acetonitrile was high-performance liquid chromatography (HPLC) grade. Other chemicals and reagents were of analytical grade.

2.2. Material treatment and experimental design

Soybean seeds were surface sterilized with 1% of sodium hypochlorite for 30 minutes, washed and steeped with distilled water at 30°C for 4 hours. After germinating for 48 hours in a dark incubator at 30°C, they were placed in cultivating pots with lids (φ 6.0 cm × 18.5 cm) containing 10 mM citrate acid buffer (pH 4.1) by the following treatments based on previous experiments [12,16]. Control: citrate acid buffer as the culture solution (pH 4.1); $CaCl_2$: Control + 6.0mM $CaCl_2$; $CaCl_2$+AG: Control + 6mM $CaCl_2$ + 2.5mM AG; EGTA: Control + 5.0mM EGTA; and EGTA + AG: Control + 5.0mM EGTA +2.5mM AG.

Dissolved oxygen concentration of the culture solution was kept at 5.46 ± 0.02 mg/L by an aerated pump (Yuyao Jintai Meter Ltd., Zhejiang, China) with an airflow rate of 0.9 L/min. After 48 hours of germination under the above treatments, the germinated seeds were washed with distilled water and dried on filter paper, then frozen in liquid nitrogen for further analyses.

2.3. Determination of GABA and Glu

GABA and Glu were extracted and purified according to Bai et al [14]. The residues were dissolved with 2 mL of 1M $NaHCO_3$ (pH 9.0) and centrifuged at 6000 × *g* for 10 minutes. GABA and Glu were determined by HPLC (Agilent 1200; GMI, Ramsey, MN, USA) with a ZORBAX Eclipse AAA reversed-phase column (3.5 μm), 4.6 mm × 150 mm inner diameter as described by Syu et al [17]. The amino acid solution (1 mL, pH 9.0) was mixed with 1 mL of dabsyl chloride (2 mg/mL, in acetone) and reacting at 67°C for 10 minutes. After that, the reaction was stopped by putting the tubes into an ice bath and then was detected at 425 nm using UV–vis diode-array absorbance detection. The mobile Phase A was acetonitrile and the mobile Phase B was 0.045M CH_3COONa (pH 4), the allowed time of separation of GABA and Glu was within 30 minutes at a constant temperature of 30°C.

2.4. Determination of GAD activity

GAD activity was determined according to Bai et al [14]. One gram of germinated soybean was homogenized on an ice bath with 6 mL of potassium phosphate buffer (70mM, pH 5.8), which contained 2mM β-mercaptoethanol, 2mM ethylene diamine tetraacetic acid (EDTA), and 0.2mM PLP. The homogenate was centrifuged at 10,000 × *g* for 20 minutes at 4°C, and the supernatant was collected for enzyme assay. The reaction mixture consisted of 200 μL of crude enzyme liquid and 100 μL of substrate (1% of Glu, pH 5.8), incubated at 40°C for 2 hours and then terminated at 90°C for 5 minutes. The centrifugal suspension was filtered through a 0.45-μm membrane filter. The filtrate was analyzed for GABA content. One unit of enzyme activity was defined as the release of 1 μmol of GABA produced per hour at 40°C.