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GABA enhances physio-biochemical metabolism and antioxidant capacity of germinated hulless barley under NaCl stress

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ABSTRACT

The effects of exogenous γ-aminobutyric acid (GABA) on the endogenous GABA metabolism and antioxidant capacity of germinated hulless barley (*Hordeum vulgare* L.) under NaCl stress were investigated. The results showed that all of the GABA treatments could alleviate the growth inhibition and oxidative damage by NaCl stress, with 0.5 mM being the most effective concentration. The GABA-treated barley seedlings exhibited a significantly higher content of endogenous GABA and other free amino acids, especially proline, which resulted from the changes in corresponding enzyme activity. The phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate coenzyme A ligase (4CL) activities also increased in GABA-treated barley, which led to higher total phenolic content and antioxidant capacity than that of the control barley. These results indicate that GABA treatment may be an effective way to relieve salt stress as it induces the accumulation of endogenous GABA and proline and total phenolic content, thus enhancing the antioxidant capacity.

1. Introduction

Salinity is considered an important abiotic stress factor that keeps a tight rein on the water absorption, root growth, and germination rate of plants, which in turn may lead to a decrease in fruit and vegetable production (Zahedi et al., 2011). Naturally occurring salt stress is generally due to NaCl (Foolad and Lin, 1997). 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 enzymes and genes involved in stress responses (Bartels and Sunkar, 2005). For barley grains, GABA (Widodo et al., 2009) and other amino acids (mainly proline) (Mazzucotelli et al., 2006) and phenolics (Ghafoor et al., 2014) were highly induced under stress, which played significant roles in resisting adversity.

GABA is a four-carbon non-protein amino acid that is metabolized via decarboxylation of glutamate or degradation of polyamine (Wan et al., 2014). There is evidence that GABA produced in non-transgenic plants in response to most stress conditions is derived from glutamate rather than polyamine, as the candidate genes for encoding 4-aminobutyraldehyde dehydrogenase are highly constitutive but not stress inducible (Shelp et al., 2012). The GABA shunt contains three enzymes: glutamate decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA; GABA transaminase (GABA-T), which catalyzes transamination conversion of GABA to succinic semialdehyde (SSA); and succinic semialdehyde dehydrogenase (SSADH), which catalyzes the irreversible NADP-dependent oxidation of SSR to succinate (Bouché and Fromm, 2004). Studies have clearly shown that GABA was involved in responses to NaCl stress by preventing reactive oxygen species (ROS) accumulation and cell death (Renault et al., 2010). Exogenous GABA application was also found to be an effective way to improve plant growth and relieve stress by modulating enzyme activities in nitrogen metabolic pathways (Barbosa et al., 2010), supplying NADH and/or succinate to mitochondrial metabolism through the TCA cycle (Bouché et al., 2003), increasing the accumulation of stress-protective alanine (Miyashita and Good, 2008), or improving polyamine biosynthesis and preventing polyamine degradation (Hu et al., 2015). At the same time, higher antioxidant capacity was also observed in GABA-treated plants

Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GABA-T, GABA transaminase; P5CS, pyrroline-5-carboxylate synthase; PDH, proline dehydrogenase; TCA, trichloroacetic acid; ABTS, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; GAE, gallic acid equivalents; FW, fresh weight of sample; DW, dry weight of sample; HPLC, high-performance liquid chromatography; PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate coenzyme A ligase

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and was thought to contribute to extending postharvest life of horticultural products under cold storage (Aghdam et al., 2015; Wang et al., 2014a,b). However, the mechanism of exogenous GABA on antioxidant capacity is still not completely understood.

In plants, proline is synthesized by pyrroline-5-carboxylate synthase (P5CS) from glutamate or by ornithine-δ-aminotransferase (OAT) from ornithine, and is degradated by proline dehydrogenase (PDH) (Verbruggen and Hermans, 2008). It has been reported that the accumulation of proline and other amino acids is one of the adaptations of plants subjected to NaCl stress (Kumar et al., 2003). The role of accumulated amino acids in plants includes acting as osmolytes, regulating ion transport, and modulating stomatal opening, as well as affecting synthesis and activity of some enzymes, gene expression, and redox-homeostasis (Rai, 2002). Proline alleviates NaCl stress by reducing oxygenase and carboxylase activities of Rubisco (Sivakumar et al., 2000), or quenching singlet oxygen (Matysik et al., 2002). Exogenous GABA application could induce proline accumulation to enhance freezing resistance of postharvest fruits and vegetables (Aghdam et al., 2015; Wang et al., 2014a,b). However, some studies did not observe any significant increase in free proline content (Jain et al., 1987; Dix and Pearce, 1981). In addition, others believed that elevated proline level was simply a stress effect rather than a cause of stress tolerance (Michel, 1987). Therefore, the proline metabolism in barley seedlings under salt stress with or without GABA treatment require further study.

As secondary metabolites of plants, phenolic synthesis and accumulation is generally stimulated in response to biotic/abiotic stresses such as salinity (Navarro et al., 2006). Indeed, salinity affects metabolic processes and induces physiological disorders, which lead to the inevitable production of reactive oxygen species (ROS) (Sreenivasulu et al., 2000). Therefore, phenolics are induced to defend against the ROS by scavenging free radicals, breaking radical chain reactions, and decomposing peroxides.

To the best of our best knowledge, there has been no report on the effect of exogenous GABA on NaCl stress in germinated hulless barley. The objective of this study was to investigate how plant growth and oxidative damage changes in germinated barley under NaCl stress with or without exogenous GABA. The content of endogenous GABA and proline, as well as dynamic changes in total phenolic content and antioxidant capacity of barley, were also studied. We expect the results to determine a possible relationship between GABA accumulation and antioxidant system enhancement, and to elucidate the physiological mechanism of GABA-mediated tolerance to NaCl stress in germinated hulless barley.

2. Materials and methods

2.1. Plant material and experimental design

The barley (*Hordeum vulgare* L.) seeds were purchased from Institute of Agricultural Sciences of the Yangtze River Bank (Jiangsu, China). The cultivar was grown in 2016 and stored at -20 ℃ until the time of steeping and germination.

Barley seeds were soaked in 0.5% sodium hypochlorite for 15 min and then washed with distilled water. After that, they were immersed in distilled water with a ratio of 1:5 (w/v) for 6 h at 25 °C. Soaked seeds were placed in the automatic germination machines (BX-801, Beixin Hardware Electrical Factory, Zhejiang, China), which provided a 2-min mist every 1 h and incubated in darkness (25 °C). The experiments were performed at Nanjing Agricultural University research germination house, which was equipped with high-precision temperature equipment. After two days of germination in distilled water, the seeds were transferred to different cultivating solutions. The treatments in the incubators were as follows: (1) CK: the barley was sprayed with distilled water. (2) NaCl stress: the barley was sprayed with 60 mM NaCl and marked as N. (3) NaCl + 0.25 mM GABA: the barley was sprayed with 60 mM NaCl plus 0.25 mM GABA and marked as NG1. (4) NaCl + 0.5 mM GABA: the barley was sprayed with 60 mM NaCl plus 0.5 mM GABA and marked as NG2. (5) NaCl + 5.0 mM GABA: the barley was sprayed with 60 mM NaCl plus 5.0 mM GABA and marked as NG3.

The hulless barley was germinated for six days. A part of the samples was freeze-dried, milled, and passed through a 0.5-mm sieve. The other part was immediately frozen in liquid nitrogen and stored at −80 °C. For each treatment, three replicates were taken for analysis.

2.2. Determination of respiratory rate, electrolyte leakage, MDA content, $O_2^{-}\bullet$ production, and H_2O_2 content

The respiratory rate was measured according to a previously established method (Yin et al., 2014) and was expressed as mg $CO_2\,g^{-1}\,h^{-1}$. The electrolyte leakage was determined with a conductivity meter (DDS-307, China). MDA content was measured according to the method of Madhava Rao and Sresty (2000). $O_2^{-}\bullet$ production was measured based on the method of Elstner and Heupel (1976). $O_2^{-}\bullet$ production was calculated against the standard curve using sodium nitrite as a standard and expressed as nM $[NO_2]\ g^{-1}\ min^{-1}$. The H_2O_2 content was measured according to the method of Patterson et al. (1984) and was expressed as μM/g fresh weight, FW.

2.3. Microtomy sectioning

Root segments (10 mm) were immediately immersed in cold formaldehyde-acetic acid-ethanol for 24 h at 4 °C. The fixed materials were dehydrated with 70% ethanol for 30 min, 85% for 30 min, 95% for 30 min, 100% for 20 min, and 100% ethanol for 20 min, successively. Sections were prepared using a rotary microtome (RM2016, Leica Biosystems, Germany) set at 10 μm. Finally, the slicing was dewaxed with dimethylbenzene, dyed with safranin and fast green, and sealed with optical gum.

2.4. Determination of free amino acid content

Proline and total amino acid content were quantified by the ninhydrin method (Bates et al., 1973). The freeze-dried barley flour was incubated at 25 °C for 1 h with 20 mL of 4% (w/v) trichloroacetic acid (TCA) for 1 h. After centrifugation (12,000 × g, 4 °C, 30 min), the supernatant was used for the enzyme assay. The supernatant was filtered with a 0.45-μm organic filter membrane, and 20 μL of samples were injected into a Hitachi Amino Acid Analyzer (L-8500-PH-KIT) equipped with a HITACHI Ion-Exchange column (#2622 SC). The experiments were performed at Central Laboratory of the College of Food Science and Technology. Elution proceeded at a flow rate of 25 mL/h of ninhydrin and 35 mL/h of buffer solution under a constant temperature of 135 °C. Peaks were integrated by area. Results were expressed as mmol/g DW.

2.5. Determination of P5CS and PDH enzymes

The activities of P5CS and PDH were determined as described by Shang et al. (2011) with some modification. The fresh barley seedlings (0.5 *g*) were ground with 5 mL 50 mM Tris−HCl buffer (pH 7.4) containing 7 mM $MgCl_2$, 0.6 M KCl, 3 mM ethylene diamine tetraacetic acid (EDTA), 1 mM DTT, and 5% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C, and the supernatant was used for the enzyme assay. For P5CS, 0.5 mL of crude enzyme extraction solution was combined with 3 mL Tris−HCl buffer (100 mM, pH 7.2, containing 25 mM $MgCl_2$, 75 mM sodium glutamate, 5 mM ATP). The reaction was initiated by the addition of 0.4 mM NADPH. For PDH, 0.5 mL of crude enzyme extraction solution was combined with 2.5 mL Na_2CO_3−HCl buffer (150 mM, pH 10.3, containing 2.67 mM proline and 10 mM NAD^+). One unit of all of the three enzymes activities was defined as the change in absorbance per hour