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Contribution of GABA shunt to chilling tolerance in anthurium cut flowers in response to postharvest salicylic acid treatment

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ABSTRACT

Due to the sensitivity of anthurium flowers to chilling injury (CI), its favorable temperature storage is 12.5–20 °C. There is evidence that maintaining the functional γ-aminobutyric acid (GABA) shunt pathway is crucial for tolerance to postharvest chilling stress by providing energy (ATP) and reducing molecule (NADH) and minimizing reactive oxygen species (ROS) accumulation. In this experiment, the impact of salicylic acid (SA) treatment applied by postharvest stem-end dipping (2 mM, 15 min at 20 °C) on GABA shunt pathway activity of anthurium cut flowers (cv. Sirion) storage at 4 °C for 21 days were investigated. The anthurium cut flowers in response to 2 mM SA treatment displayed significantly higher GABA transaminase (GABA-T) activity during storage at 4 °C for 21 days, which coincided with lower GABA content, leading to flowers with lower spathe browning. SA treatment enhanced GABA shunt pathway activity, by enhancing GABA-T activity, during storage at 4 °C, lead to consumption of GABA for providing sufficient ATP content associated with the lower H_2O_2 content. Also, anthurium cut flowers in response to SA treatment displayed significantly higher unsaturated/saturated fatty acids (unSFA/SFA) ratio, which can be results not only from higher energy content but also from lower phospholipase D (PLD) and lipoxygenase (LOX) activities. These findings showed that SA treatment at 2 mM maintained functional GABA shunt pathway activity which leads to providing higher ATP, lower H_2O_2 accumulation, higher unSFA/SFA ratio, proposed that the SA can be applied as an effective procedure for improving anthurium cut flowers tolerance to postharvest chilling stress, by enhancing membrane fluidity.

1. Introduction

Salicylic acid (SA), as a safe signaling molecule can be applied commercially as a postharvest technology for amelioration of chilling injury in horticultural crops (Asghari and Aghdam, 2010). Recently, Cai et al. (2014) reported that the postharvest SA treatment ameliorated chilling injury in grape berries, which was associated with lower electrolyte leakage. Cai et al. (2014) suggested that the amelioration of chilling injury in grape berries by postharvest SA treatment may be results from (1) enhancing sucrose consumption by promoting glycolysis and tricarboxylic acid cycle (TCA) activity, (2) enhancing energy, ATP, providing, (3) enhancing antioxidant system activity, and (4) enhancing HSPs accumulation.

In plants, the γ-aminobutyric acid (GABA) shunt pathway consist of three enzymes which are responsible for biosynthesis of GABA from glutamate and back to TCA cycle in succinate form by bypassing two steps of the TCA cycle (Bouché and Fromm, 2004). Cytosolic glutamate decarboxylase (GAD), a pyridoxal phosphate (PLP) dependent enzyme, is responsible for irreversible converting of glutamate to GABA, which is associated with H^+ consumption and CO_2 releasing (Shelp et al., 2012). The optimum activity of GAD is at pH 5.8 (Shelp et al., 2012). H^+ consumption by GAD for the biosynthesis of GABA in the cytosol, which led to cytosolic pH adjustment, can be act as a defense mechanism overcomes chilling stress which leads to cytosol acidification (Fait et al., 2007). GAD activity is under regulation by pH and calcium/calmodulin (Ca^{2+}-CaM), independently. When pH reduce, GAD activity increase, but at neutral pH, GAD activated by Ca^{2+}-CaM binding (Shelp et al.,

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2012). Michaeli et al. (2011) reported that GABA permease (GABP), as a mitochondrial GABA carrier, transport GABA from the cytosol into mitochondria, where succinic semialdehyde (SSA) produces from GABA by the action of GABA transaminase (GABA-T). Subsequently, by mitochondrial NAD^+ dependent succinic semialdehyde dehydrogenase (SSADH) enzyme, SSA is converted to succinate, which can enter to TCA cycle and act as the electron donor to the mitochondrial electron transport system (Shelp et al., 2012; Michaeli and Fromm, 2015). It has been reported that GABA shunt pathway, GABA-T and SSADH activity, have the ability to provide NADH and succinate for TCA cycle and mitochondrial electron transport chain for ATP production and prevent accumulation of H_2O_2 under abiotic stress. So it could be suggested that degradation of GABA by GABA-T and SSADH enzymes alleviates oxidative stress by providing sufficient energy associated with declining ROS accumulation and is pivotal for overcome oxidative stress (Bouché et al., 2003; Fait et al., 2007; Michaeli and Fromm, 2015; Gilliham and Tyerman, 2016).

In this experiment, the impact of postharvest SA treatments on the (1) GABA shunt pathway activity, GABA content and its biosynthesis GAD and degradation GABA-T enzymes activity, and its possible contribution to ATP providing and H_2O_2 scavenging and (2) PLD and LOX enzymes activity coincided with unSFA/SFA ratio in anthurium cut flowers during storage at 4 °C were evaluated. We propose SA treatment promoted GABA degradation by increasing GABA-T activity, which alleviate the chilling damage by providing sufficient energy coincided with scavenging of H_2O_2 in anthurium cut flowers during storage at 4 °C. Also, higher unSFA/SFA ratio results from lower PLD and LOX enzymes activity can be a defense mechanism for alleviation the chilling damage in anthurium cut flowers in response to SA treatment during storage at 4 °C.

2. Materials and methods

2.1. Flowers and treatments

Based on our previous study (Aghdam et al., 2016b), 2 mM salicylic acid for postharvest treatment was used. For postharvest salicylic acid treatment, 180 anthurium flowers cv. Sirion were harvested in the morning when 40–50% of the true flowers on the spadix had fully opened and placed in water at the growers' property and transported at 12 °C in water to the laboratory. Then, the flower stems were recut to 30 cm length and divided into 2 lots of 90 flowers for the following treatments in triplicate (30 flowers per replicate) by dipping of individual flower stems at 0 (control) and 2 mM SA solution for 15 min at 20 °C and then removed from the SA solution and were allowed to air-dry at room temperature and individually placed in water and stored at 4 °C (85–90% RH) for 21 days. After evaluation of chilling injury every 7 days during storage at 4 °C by determining the 10 individual flower spathes browning, GABA content and its biosynthesis GAD and degradation GABA-T enzymes activity, and its possible contribution to energy (ATP) providing coincided with H_2O_2 scavenging, associated with PLD and LOX enzymes activity and unSFA/SFA ratio were evaluated.

2.2. Spathe browning

The browning of anthurium cut flowers spathe was assessed using visualizing the total brown area on the spathes of 10 individual flowers using a scale from 1 to 5; 1 = no chilling injury; 2 = mild injury (1–20% of spathe affected); 3 = moderate injury (21–50% of spathe affected); 4 = severe injury (51–80% of spathe affected); 5 = very severe injury (81–100% of spathe affected). CI was calculated as $\sum$ (number on CI scale × number of flowers at that number on the CI scale)/total number of flower in each group, according to Promyou et al. (2012).

2.3. GABA content and GAD and GABA-T enzymes activity

GABA content in anthurium cut flowers spathe was measured according to Deewatthanawong et al. (2010). Spathe tissue (0.5 g) was homogenized in 1 ml of methanol for 10 min at room temperature. After vacuum dried, dissolved in 1 ml 70 mM lanthanum chloride followed by 15 min of shaking, and centrifugation at 13,000*g* for 15 min. Then, 800 μl supernatant was mixed with 160 μl 1 M KOH. After shaken for 5 min, and centrifugation at 13,000*g* for 10 min, the supernatant was used for GABA determination. The GABA content was measured enzymatically using a *Pseudomonas fluorescens* GABase (Sigma), a commercial mix of GABA-T and SSADH. The GABase assay mixture contained 75 mM potassium pyrophosphate (pH 8.6), 3.3 mM β-mercaptoethanol, 10 mM 2-oxoglutarate, 1.25 mM $NADP^+$, 0.016 unit GABase. The absorbance at 340 nm was read before and 10 min after adding α-ketoglutarate using the 96-well plate reader, and GABA was determined by comparison with a standard curve of GABA and expressed as μmol g^{-1} spathe fresh weight (FW).

For GAD and GABA-T extraction, 2 g of spathe was homogenized with 6 ml of extraction buffer containing 0.1 M Tris–HCl (pH 9.1), 10% (v/v) glycerol, 1 mM DTT, 5 mM EDTA, 0.5 mM PLP and 1 mM PMSF. After centrifugation at 20,000*g* for 30 min at 4 °C, the supernatant was used for GAD and GABA-T assay.

GAD activity was assayed based on GABA production, according to Bartyzel et al. (2003). GAD activity was determined by incubating 20 μl of enzyme extract in the assay mix with 150 mM potassium phosphate (pH 5.8), 0.1 mM PLP and 20 mM glutamate in a final volume of 200 μl. Blanks were without glutamate. After the incubation at 30 °C for 1 h, 0.6 M perchloric acid was added to stop the reaction, and neutralized immediately with 3 M KOH. For compute of GAD activity the GABA content in the neutralized samples was measured by GABase assay. GAD activity expressed as nmol GABA per mg^{-1} protein in min.

GABA-T was assayed using pyruvate as an amino acceptor, according to Ansari et al. (2005). GABA-T activity was determined by incubating 25 μl of enzyme extract in the assay mix with 50 mM Tris–HCl (pH 8.0), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP, 10% (v/v) glycerol, 16 mM GABA and 4 mM of pyruvate in a final volume of 500 μl. Blanks were without substrates, GABA and pyruvate. After the incubation at 30 °C for 1 h, 4 mM sulfosalicylic acid was added to stop the reaction. For computed of GABA-T activity the alanine synthesized was measured by alanine dehydrogenase (ADH) assay. The ADH assay was performed in the assay mix with 150 mM Tris–HCl (pH 9.0), 1.0 mM NAD^+ and 0.02 units *Bacillus subtilis* ADH (Sigma). The absorbance at 340 nm was read using the 96-well plate reader, and alanine content was determined by comparison with a standard curve of alanine. GABA-T activity expressed as nmol alanine per mg^{-1} protein in min.

2.4. Assays of ATP/ADP/AMP content

ATP, ADP, and AMP contents in anthurium cut flowers spathe were measured according to Yi et al. (2008). Spathe tissue (2 g) was homogenized with 6 ml of 0.6 M perchloric acid. After centrifugation at 16,000*g* for 15 min at 4 °C, the supernatant (3 ml) was quickly neutralized to pH 6.5–6.8 using 1 M KOH, diluted to 4 ml and passed through a 0.45 μm filter. ATP, ADP and AMP were analyzed by HPLC with a 4.6 mm × 250 mm C18 column and an UV detector at 254 nm. Mobile phase A consisted of 0.06 M K_2HPO_4 and 0.04 M KH_2PO_4 dissolved in deionized water and adjusted to pH 7.0 with 0.1 M KOH. Mobile phase B was acetonitrile. ATP, ADP, and AMP contents were computed based external standard and expressed as μg^{-1} of spathe