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Study of sponge gourd ascorbate peroxidase and winter squash superoxide dismutase under respective flooding and chilling stresses

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ABSTRACT

The objectives of this work were to study the responses of ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and physiological parameters of bitter melon (BM), sponge gourd (SG), and winter squash (WS) under waterlogged and low temperature conditions. The BM and SG plants were subjected to 0–72 h flooding treatments. Moreover, BM and WS plants were exposed to chilling at 12/7 °C (day/night) for 0–72 h. The results show that different genotypes responded differently to environmental stress according to their various antioxidant enzymes and physiological parameters. The activity of APX in roots and leaves of SG plants significantly higher than that of BM plants during continuous flooding. Significant increases in SOD activity in leaves of WS plants were also observed throughout the entire chilling duration compared to BM plants. On the basis of our observations, we conclude that increased APX and SOD activities provide SG and WS plants with increased waterlogging and chilling stress tolerance, respectively. Both APX and SOD activities can be used for selecting BM lines with the best tolerances to water logging and chilling stresses.

1. Introduction

The Cucurbitaceae (Cucurbit) family includes around 825 species derived from tropical and subtropical regions, including 26 species cultivated as vegetables (Henriques et al., 2012). Among the cultivated cucurbits, bitter melon (BM, *Momordica charantia* L.) is one of the most important vegetables. It is a white-to-green-colored immature fruit with a warty appearance and as the name suggests, it is valued for its unique bitter flavor. In addition, it is considered a prized vegetable because of its high nutritive value and medicinal properties. BM extract partitions are reported to show many pharmacological activities, including anti-inflammatory, antioxidant, and anti-radical activities (Lii et al., 2009).

Flooding conditions cause oxygen starvation, which arise from the slow diffusion of gases in water and from oxygen consumption by plant root. Problems caused by flooding may be solved by growing flood-tolerant crops or grafting intolerant plants onto tolerant ones. Sponge gourd (SG, *Luffa cylindrical*) is an annual upland crop vegetable originating in India and southern Asia, and is distributed mainly in tropical to warm-temperate areas. This species is flood tolerant in comparison to BM. In Taiwan, the yield of BM is increased by grafting with *Luffa* spp., which allows BM to survive in flooded soils (Shimamura et al., 2007). BM grows well in warm temperatures similar to those preferred for squash, but is chill-sensitive. Winter squash (WS, *Cucurbita moschata* L.) is a creeping, climbing, herbaceous, annual, monoecious plant. It has been used successfully in Taiwan as a cold-hardy rootstock that is resistant to chilling stress for winter production of BM.

Environmental stresses induce the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH^-$). Oxygen deprivation stress in plant cells is distinguished by three physiologically different states: transient hypoxia, anoxia and reoxygenation. Generation of ROS is characteristic for hypoxia and especially for reoxygenation (Blokhina et al., 2003). Toxic radicals can be removed by both enzymes and non-enzymatic compounds to protect plant cells against oxygen toxicity and counter the hazardous effects of ROS. The activities of antioxidant enzymes, which reflect the ROS pool, are often used as a measure of ROS-mediated oxidative stress. Antioxidant enzyme levels change differentially

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in response to environmental constraints, depending on the magnitude of the stress and species-specific sensitivity to the stress. A coordinated increase in the activities of oxygen-detoxifying enzymes is necessary to protect plant leaves from the accumulation of oxygen radicals as a result of environmental stress. The complex antioxidant defense system that has evolved in plants is composed of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), and peroxiredoxins (PRX). High levels of some antioxidant enzymes, either constitutive or induced, were found to be important in the pea (Moskova et al., 2009), mustard greens (Kumar et al., 2010), tobacco (Gechev et al., 2003), cucumber (Zhou et al., 2009), clover (Simova-Stoilova et al., 2012), corn (Chugh et al., 2011), sweet potato (Lin et al., 2006), and eggplant (Lin et al., 2004) in order to survive oxidative stress after being subjected to different flooding and chilling conditions. Nevertheless, there are few studies on the responses of antioxidant enzymes of BM, SG, and WS in terms of their ability to survive flooding and chilling stresses. The effects of flooding and chilling on the antioxidant activity of BM, SG, and WS, and the roles played by antioxidant enzymes in protecting plant cells from damage occurring due to flooding and chilling stresses were thus examined in this study.

The breeding of BM with broad abiotic stress resistance is hampered by the lack of practical selection tools like genetic markers because knowledge about genetic and physiology behind a successful rootstock is still very limited. It is necessary to identify physiological characteristics that reflect the complex underlying genetic make-up. These physiological biomarkers, ideally easy to measure, could be used as generic tools to develop a reliable method to support the selection of a BM having flooding and chilling-stress resistances. With this approach, BM and its specific stress-tolerant rootstocks, SG and WS, were studied with regard to efficient use of time, labor, and space. Because SG and WS have been used as rootstocks for anoxic and chilling stresses, respectively, to avoid reduced BM production, the hypothesis of this research was that increases in the activities of APX and SOD were part of the reason for the higher flooding and chilling tolerance in these rootstocks. The long-term goal of our work is to help breed a flood- and chill-tolerant BM to be grown in summer and winter seasons. The present research project studied the antioxidant enzymes of BM, SG, and WS under waterlogged and low temperature conditions.

2. Materials and methods

2.1. Plant materials, cultural practices, and stress treatments

Seeds of BM (*Momordia charanthia* L. cv. Yu-Hwa), SG (*Luffa cyllindria* Roem cv. Son-Yi), and WS (*C. moschata* cv. Gon-Zon) were purchased from Known-You seed company, the largest seed company in Taiwan. Bitter melon is a flood- and chill-sensitive variety, and requires optimum growing temperatures for satisfactory production. However, the SG and WS varieties are more flood- and chill-tolerant than BM, respectively, and are used as rootstocks during summer and winter in Taiwan. Seeds were surface-sterilized with 0.1% mercuric chloride for 5 min and washed with double-distilled water before use. Seeds were then sown in a commercial potting soil mixture, and seedlings were transplanted into 15.4-cm diameter plastic pots and placed in a growth chamber under 400 $\mu mol\ m^{-2}\ s^{-1}$ light with a 14-h photoperiod provided by fluorescent and incandescent light. The temperature of the BM plants was maintained at 30 °C and 25 °C (day and night), with the SG and WS plants kept at 25 °C and 20 °C (day and night), both at a relative humidity (RH) of 70%. Plants were watered with a half-strength Hoagland solution (Hoagland and Arnon, 1950) every other day to maintain optimal irrigation and growth for 30 days before imposition of flooding and chilling stresses.

Pots of BM and SG plants were divided into control groups receiving no flooding treatment and flooding treatment groups wherein plants were subjected to five flooding treatments for periods of 6, 12, 24, 48, and 72 h. For each treatment, three replications were used. Pots were randomly placed in 28 cm × 14 cm × 14 cm plastic buckets and subjected to flooding by filling the buckets with tap water to 5 cm above the soil surface. Pots were removed from the buckets at different times following flooding, and plants were removed and their roots rinsed with tap water. Roots and leaves from each plant were clipped, frozen in liquid nitrogen, and stored at −80 °C in an ultrafreezer until used. Three plants from each flooding period were harvested at the same time of the day and used for the enzyme measurements. Moreover, pots of BM and WS plants were randomly placed in a growth chamber under a 14 h photoperiod with an irradiance of 400 $\mu mol\ m^{-2}\ s^{-1}$ and an RH of 70%. All of the plants were exposed to chilling at 12/7 °C (day/night) for 0, 6, 12, 24, 48, and 72 h. Three replicates of each treatment were randomly placed in a growth chamber. The experiment was performed twice independently for a randomized design of growth environment, sampling day, and biochemical analysis. Following each treatment, young, fully expanded leaves from each plant were clipped to measure enzyme activities.

2.2. Enzyme extraction and activity determination

Samples were prepared for SOD (EC 1.15.11), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), and GR (EC 1.6.4.2) activity analyses by homogenizing 0.2 g of each frozen leaf in 990 μl of ice-cold 100 mM HEPES buffer (pH 7.0) containing 1 mM phenylmethysulfonyl fluoride and 0.03 g polyvinylpyrrolidone. The extracts were centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatants were then collected in a fresh tube for enzyme assays. Enzyme activities were determined using a spectrophotometer. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Hwang and VanToai, 1991). Two milliliters of the CAT assay reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 20 μl of the enzyme extract. The decrease in H_2O_2 followed the decline in optical density at 240 nm, and activity was calculated with the extinction coefficient (40 $mM^{-1}\ cm^{-1}$ at 240 nm) for H_2O_2. Meanwhile, GR activity was measured by the GSH-dependent oxidation of NADPH. The reaction mixture contained 25 mM Tris–$MgCl_2$ (pH 7.6), 5 mM NADPH, 50 mM GSSG, and 1 ml of the enzyme extract (Foyer et al., 1997). The change in absorption at 340 nm (NADPHε = 6220 $M^{-1}\ cm^{-1}$) was recorded over 2.5 min. The assay for APX activity was carried out in a reaction mixture containing 166 mM HEPES (pH 7), 1.5 mM sodium ascorbate, 1 mM H_2O_2, and 40 μl of the enzyme extract. The change in absorption at 290 nm was recorded 80 s after the addition of H_2O_2 (Nakano and Asada, 1981). SOD activity was determined using the SOD Assay Kit – WST (Dojindo Molecular Technology, Gaithersburg, MD, USA). The SOD assay kit utilizes the WST working solution 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-tetrazolium, which produces a water-soluble formazan dye upon reduction with superoxide anions ($^{\bullet}O_2^-$). The rate of the reduction with O_2^- is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, 50% of the SOD inhibitory activity can be measured at an absorbance of 450 nm. The specific activity of SOD (inhibition rate) was calculated using the equation described in the protocol of the kit. One unit of enzyme was defined as the amount of enzyme required to decompose 1 μmol of substrate [$min^{-1}\ g^{-1}$ fresh weight (FW)].