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Effect of genotype and exogenous application of glycinebetaine on antioxidant enzyme activity in native gels of 7-day-old salt-stressed tomato (*Solanum lycopersicum*) seedlings

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ABSTRACT

Tomatoes are classified as moderately salt-tolerant crops, but they are unable to synthesize glycinebetaine (GB), a compound that alleviates salinity-induced inhibition of plant growth. We investigated the effects of salinity on the antioxidative system of tolerant/susceptible genotypes of tomato. In the susceptible genotype's (F144) hypocotyls and radicles, the activities of superoxide dismutase (SOD), L-ascorbate oxidase (L-AAO), ascorbate peroxidase (APX) and catalase increased under salt stress, whereas that of peroxidase (POD) II decreased slightly. In the relatively tolerant genotype Patio, salt stress made little difference in the hypocotyls, whereas the activities of SOD, L-AAO, APX and POD increased significantly in the radicles. The effect of exogenous application of GB under salt stress on antioxidative enzyme activity was also investigated. The results constitute an important step in the elucidation of mechanisms underlying resistance to salinity.

1. Introduction

Soil salinity poses a severe constraint on the distribution and productivity of major crops worldwide. It is estimated that about 20% of the earth's land mass and nearly half of all irrigated land are affected by salinity (Zhu, 2001). Salinity effects are more prominent in arid and semiarid regions, where limited precipitation, high evapotranspiration and high temperatures contribute to the problem which is highly relevant for agricultural production in these regions. High concentrations of salt ions in plant cells often cause osmotic stress, ion imbalance and nutrient deficiency.

Salt stress may lead to oxidative stress, as suggested by the increased activity of antioxidant enzymes in response to high salinity, and by the correlation of salt tolerance with antioxidant enzyme levels (Camp et al., 1996; Xie et al., 2008). Reactive oxygen species (ROS) are produced under salinity stress and are responsible for the damage to membranes and other essential macromolecules such as photosynthetic pigments, proteins, DNA and lipids (Sairam et al., 2005). To prevent excessive accumulation of ROS, plant species have developed enzymatic as well as non-enzymatic antioxidative systems.

Numerous studies have indicated that antioxidant enzyme activities are correlated with plant tolerance to abiotic stresses, and that these enzymes are required to maintain redox homeostasis. High activities of some antioxidant enzymes have been found to be important in pea, rice, tomato and olive (Hernandez et al., 2000; Shalata et al., 2001; Prashanth et al., 2008; Melgar et al., 2009), among others. The antioxidants help maintain ROS at low concentrations, thereby preventing oxidative damage while they play crucial functions in signal transduction (Matamoros et al., 2010). Efficient destruction of ROS requires the coordinated action of several antioxidants. Key enzymes involved in ROS detoxification are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase. The primary scavenger is SOD, presenting the first line of defense by converting O_2^- to hydrogen peroxide (H_2O_2) and O_2 (Scandalios, 1997; Møller, 2001). SODs are a group of metalloenzymes present in various cellular compartments in three isoforms: Cu/ZnSOD, FeSOD and MnSOD (Asada, 1994; Foyer et al., 1994). SOD is eliminated by APX in association with dehydroascorbate reductase and glutathione reductase (GR), and regenerates ascorbic acid (AA). The redox status of the extracellular AA pool is regulated by ascorbate oxidase (AAO). This enzyme is considered the first step in the AA-degradation pathway in the apoplast (Pignocchi et al., 2006). CAT, a heme-containing enzyme, breaks down H_2O_2 to O_2 and H_2O (Sairam et al., 2005). APX is widely distributed in the cytosol and other organelles, especially in

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chloroplasts where CAT is virtually absent (Cavalcanti et al., 2007). Glutathione peroxidases are a family of isozymes which catalyze the reduction of H_2O_2 and organic and lipid hydroperoxides by reduced glutathione to water or alcohols, respectively (Eshdat et al., 1997). GR is a flavoenzyme which has great importance in cell metabolism due to its involvement in redox processes and in the transfer of oxidized glutathione to reduced glutathione with the accompanying oxidation of NAD(P)H (Smith et al., 1989).

Tomatoes, one of the world's most important and widely grown crops, are classified as moderately salt tolerant. Salinity has been shown to reduce tomato yield but improve fruit-quality traits, such as total soluble solids, acid contents and color (Chen et al., 2009). Tomatoes are very sensitive to salt stress during the germination and seedling stages (Foolad, 1997). Salt-induced changes in the antioxidative system of tomatoes have been reported in leaves and roots (Shalata and Tal, 1998; Koka et al., 2006). The higher salinity tolerance of the wild-type *Lycopersicon pennellii* has been correlated with lower lipid peroxidation. Zushi and Matsuzoe (2009) showed that tomato fruits have antioxidant systems to protect themselves from salt-induced oxidative stress. This finding was supported by lipid peroxidation and H_2O_2 levels, which remained unchanged under saline conditions. However, all available data are from either mature plants or fruits. There is no information on the antioxidant system under salinity in the early stages of root initiation. Moreover, there are no reports on the response of antioxidant enzymes in fresh market tomatoes exposed to high salinity. Recently, an investigation aimed at better understanding the molecular mechanisms underlying adaptation to salt stress was carried out in 7-day-old tomato cultivars, using a proteomics approach (Chen et al., 2009). Analysis of biochemically isolated proteins from salt-stressed tomato seedlings showed that the differential protein profile is comprised of 40 distinct proteins, subdivided into six functional categories. One of the major regulatory networks was found to involve the SOD pathway, suggesting that all the antioxidative system is involved in the response of tomato genotypes to salinity. It was also found that exogenously applied glycinebetaine (GB) could alleviate the inhibition of tomato growth induced by salt stress by changing protein-expression levels. However, the nature of the mechanisms controlling these processes is poorly understood. Consequently, in the present study, tomato seedlings' strategy and tolerance under salinity were studied through an analysis of the expression patterns of antioxidative enzymes (SOD, L-AAO, APX, POD and CAT) and their isozymes and enzymatic activity in gels in the presence or absence of GB.

2. Materials and methods

2.1. Plant material, growth conditions and stress treatments

Seeds of a salt-sensitive tomato genotype (*Solanum lycopersicum* L. cv. F144 from Hazera, Israel) and the relatively salt-tolerant *S. lycopersicum* L. cv. Patio (Tomato Growers, USA), (Alian et al., 2000), were germinated in a growth chamber in the dark at $25 \pm 2\,^{\circ}C$ on Petri dishes ($\phi = 9$ cm) containing two layers of wet filter paper until radicle initiation. Eight uniformly germinated seeds were transferred to new Petri dishes on filter paper imbibed with 2.5 ml of one of four different Hoagland solutions (A–D); the dishes were then sealed with parafilm, and the seedlings were grown under a 12-h photoperiod.

The treatments imposed consisted of four different solutions. Treatment solution A consisted of half-strength Hoagland solution (Arnon, 1938), serving as the control treatment. Solution B contained half-strength Hoagland and 120 mM sodium chloride; solution C was solution B plus 5 mM GB, and solution D was half-strength Hoagland and 5 mM GB. The GB concentration of 5 mM was selected following some preliminary experiments (Chen et al., 2009). The length and fresh weight of both, radicles and hypocotyls of 64 seedlings of each osmotic solution were measured after 7 days. Membrane stability was determined by measuring Relative stress injury (RSI%) using the formula: $RSI\% = (ECa/ECb) \times 100$, where ECa denotes electrical conductivity taken at room temperature after 5 h incubation in distilled water and ECb denotes electrical conductivity after autoclaving the tissues (Dionisio-Sese and Tobita, 1998). The entire experiment was repeated three times, each time with eight seedlings from each treatment.

2.2. Protein extraction and determination of total protein content

Samples of tomato radicles and hypocotyls (1 g each) were homogenized in liquid nitrogen with 2 ml of extraction buffer (0.1 M Tris-pH 8.0, 5% sucrose, 2 mM PMSF and 40 μl complete inhibitor [1 tablet of complete protease inhibitor (from Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in 1 ml DD water], cooled on ice for 10 min and centrifuged at 10,000 rpm for 30 min. Protein content was measured in the supernatant following the standard procedure for microtiter plates in the Sigma protein assay instruction manual (Bicinchoninic acid protein assay kit, product code BCA-1 and B9643). Total protein was not changed by the treatments imposed (data not shown). The supernatant was then transferred to fresh tubes to determine antioxidase activities.

2.3. Gel electrophoresis and detection of SOD activity in gels

The advantage to determining activity in native gels lies in the fact that sodium dodecyl sulfate (SDS) is absent, and as a result, proteins are not denaturized prior to loading. The analyzed proteins thus carry their native charge at the pH of the gel and separate according to their different electrophoretic mobilities and the sieving effects of the gel. Samples were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under nondenaturing, nonreducing conditions, essentially as described by Laemmli (1970) except that the SDS was omitted. Electrophoretic separation was performed at 4 °C. Electrophoretic separation was performed at 4 °C. Negative staining detected the activities of three types of SODs (Cu/ZnSOD, MnSOD and FeSOD) on a non-denaturing 12% polyacrylamide gel. The three bands of SOD activity were identified according to Brown et al. (2008). The gel was incubated for 10 min with gentle shaking in 50 mM phosphate buffer (pH 7.8), then for 10 min in nitro-blue-tetrazolium solution (1 mg/ml) and for another 10 min in 50 mM phosphate buffer (pH 7.8) containing 0.01 mg/ml riboflavin (Sigma Aldrich) and 3.25 mg/ml *N′,N′,N′,N′*-tetramethylenediamine (TEMED) at room temperature. Areas of SOD activity remained clear when the gel was exposed to light. The gel was scanned immediately after the photochemical reaction and the data were quantified using ChemImager 4400 software. This procedure was true for all the other gels of all the enzymes measured. Three replicates were performed in all the experiments.

2.4. Detection of L-AAO activity in gels

Negative staining detected the activities of two types of L-AAO on a non-denaturing 10% polyacrylamide gel according to Manchenko (1994). Following electrophoresis, the gels were incubated in solution I (20 mg AA in 100 ml 0.1 M Tris–HCl buffer, pH 8.0) at 30 °C for 15 min. Then solution I was discarded and the surface of the gel were blotted to remove excess liquid. A piece of filter paper saturated with solution II (2.5 mg 2,6-dichlorophenol indophenol in 10 ml H_2O) was placed on the gel. Achromatic bands of L-AAO activity developed on a blue background after 5–10 min. These bands are ephemeral and were therefore immediately photographed.