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Salt-induced hydrogen peroxide is involved in modulation of antioxidant enzymes in cotton

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ABSTRACT

Salt severely restricts cotton (*Gossypium hirsutum*) growth and production. The present study was undertaken to study the effect of salt-induced hydrogen peroxide (H_2O_2) on antioxidant enzymes in cotton. NaCl treatment or exogenous H_2O_2 was used to investigate the relationship between H_2O_2 content and levels of antioxidant enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT), as well as the transcriptional levels of corresponding genes. H_2O_2 content increased within 24 h following 200 mmol L^{-1} NaCl treatment. Both NaCl-induced and exogenous H_2O_2 increased the activity of antioxidant enzymes including APX and SOD and upregulated the transcriptional levels of *GhcAPX1*, *GhFeSOD*, and *GhchlCSD*. These increased activities and upregulated transcriptional levels were inhibited when the salt-induced H_2O_2 was scavenged by NAC. These results indicate that salt-induced H_2O_2 as a second signaling messenger modulates APX and SOD activities by regulating the transcription levels of corresponding genes, alleviating oxidative stress, and increasing salt tolerance in cotton.

1. Introduction

In arid and semiarid regions of the world, increased soil salinity impairs plant productivity in many different ways [1]. One of the damages induced by salt is the increase in reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) [2]. Higher plants produce ROS, which can be neutralized by intracellular antioxidants under normal conditions, but excessive accumulation during stress conditions can cause oxidative stress [2,3] and severely disrupt normal metabolism by peroxidation of membrane lipids [4,5], protein destruction [6], and nucleic acid mutation [4,7]. Such oxidative damages may result in diseases and degenerative processes. To maintain growth and

Abbreviations: SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; APX, ascorbate peroxidase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; O_2^-, superoxide anion; OH, hydroxyl radical; *GhFeSOD*, *G. hirsutum* FeSOD gene; *GhchlCSD*, *G. hirsutum* chloroplast Cu/ZnSOD gene; *GhcAPX1*, *G. hirsutum* cytosolic APX1 gene; *GhMnSOD*, *G. hirsutum* MnSOD gene; *GhCAT1*, *G. hirsutum* CAT subunit 1 gene; *GhPOD1*, *G. hirsutum* POD1 gene; *GhUBQ7*, *G. hirsutum* ubiquitin 7 gene; MDA, malondialdehyde; NAC, *N*-acetyl L-cysteine.

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productivity, plants have evolved antioxidant defense mechanisms, of which a well-known one is the antioxidant enzyme system. Superoxide dismutase (SOD; EC 1.15.1.1) is the major scavenger of O_2^- in this system. It converts O_2^- to H_2O_2, which can be scavenged by catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) [8], and peroxidase (POD; EC 1.11.1.7) [9]. These antioxidant enzymes are responsible for maintaining ROS at an appropriate level in the cell.

High accumulation of ROS has been considered for many years to be an undesirable and harmful event in stress metabolism [10]. However, several lines of evidence have suggested that ROS also act as signaling molecules [11–17], especially hydrogen peroxide, which plays an important role in responses to abiotic stress conditions [18–21]. In plants, H_2O_2 is produced in mitochondria, chloroplasts, peroxisomes, and at the plasma membrane or cell wall [22]. By comparison, H_2O_2 is a relatively long-lived (1 ms) molecule that is transported either by aquaporins [23,24] or by direct diffusion across membranes [25]. This characteristic is compatible with its role as a signaling molecule in plant growth, development, stomatal closure, root gravitropism, and abiotic stress [12,26–30]. A corresponding study has identified 175 expressed sequence tags (EST) of which 113 ESTs were induced and 62 ESTs were repressed by H_2O_2 in *Arabidopsis thaliana* [31].

Cotton fiber is the most natural material used in the textile industry; however, its yield is severely reduced by soil salinity [32]. In previous studies, the relationship between salt tolerance and antioxidant enzymes in cotton has been extensively investigated. Under salt stress, the balance between production and quenching of ROS was broken, with salt-tolerant cultivars upregulating their anti-oxidative enzymes at the cellular [33,34] or plant levels [35,36] more vigorously than salt-sensitive cultivars, suggesting that the antioxidant system was involved in the salt tolerance. There is a strong correlation between salt tolerance and antioxidant enzymes (such as CAT, POD, APX, and SOD), but the manner in which these enzymes are regulated in cotton remains unclear.

Cotton cultivar Xinluzhong 31 (XLZ31), used as the experimental material in our study, is a hybrid developed from *Gossypium hirsutum* × *G. herbaceum*in Xinjiang, China. In preliminary studies, this cultivar showed higher activity of antioxidant enzymes and salt tolerance than did others [37]. Our hypothesis was that salt-induced H_2O_2 in XLZ31, as a signaling molecule, activates the antioxidant system to alleviate oxidative stress on the membrane and improve salt tolerance. In this study, we investigated the role of H_2O_2 in regulating the activity of antioxidant enzymes and transcripts of these corresponding genes. The results suggested that H_2O_2 might modulate APX and SOD activities by upregulating the transcription of the *GhcAPX1*, *GhchlCSD*, and *GhFeSOD* genes in cotton to improve salt tolerance.

2. Materials and methods

2.1. Plant materials and treatments

Seeds of cotton cultivar XLZ31 were sown in pots containing perlite:vermiculite (1:3) in a 16 h:8 h light/dark cycle under a temperature regime of 20–29 °C and 40–60% relative humidity. Seedlings after emergence for 1 week were transferred to a 250 mL bottle containing 100 mL Hoagland solution (pH 6.0). Nutrient solution was added every other day and replaced every week. After 20-day cultivation, young plants with four leaves and growth vigor were transferred into nutrient solution containing 200 mmol L^{-1} NaCl, 200 mmol L^{-1} NaCl with NAC (0, 1, and 5 mmol L^{-1}), or H_2O_2 (0, 0.05, 0.1, 0.5, 1, and 10 mmol L^{-1}) for 24 or 12 h. Leaves at the same positions on cotton seedlings were harvested in three replicates and stored in liquid nitrogen for later use.

2.2. H_2O_2 and lipid peroxidation determination

Measurement of H_2O_2 followed Hu et al. [38] and Xue et al. [39]. Fresh leaves (200 mg) were homogenized in 2 mL cold acetone and centrifuged at 12,000×*g* for 10 min at 4 °C. Then 1 mL of supernatant was transferred into a 1.5-mL microtube containing 0.1 mL titanium sulfate (5%, w/v) and 0.2 mL strong aqua ammonia. After thorough mixing and centrifugation at 9,000×*g* for 5 min, the precipitate was washed with acetone twice or more until no chlorophyll remained. The resulting titanium–peroxide complex was dissolved in 5 mL H_2SO_4 (2 mol L^{-1}). The content of H_2O_2 was measured by the absorbance at 415 nm. The amount was calculated from a standard curve of H_2O_2 and expressed as nmol g^{-1} fresh weight. Lipid peroxidation was estimated from the formation of malondialdehyde (MDA), which was assayed according to Parida et al. [40].

2.3. Determination of enzyme activities

To extract total protein, 200 mg fresh leaves were homogenized in liquid nitrogen, and 2 mL extraction buffer was added. The buffer contained 50 mmol L^{-1} sodium phosphate (pH 7.8), 0.5 mmol L^{-1} EDTA, 1% polyvinylpyrrolidone-40 (PVP, w/v), 1 mmol L^{-1} ascorbic acid (ASA), and 20% glycerol (v/v). The homogenate was centrifuged at 15,000×*g* for 15 min, and the resulting supernatant was immediately used for the enzyme assay [41].

APX activity was measured by oxidation of ASA at 290 nm, following Tseng et al. [41] and Batisha et al. [42]. Some changes were made as follows: 3 mL reaction mixture contained 50 mmol L^{-1}sodium phosphate buffer (pH 7.0, 1.8 mL), 15 mmol L^{-1} ASA (0.1 mL), 0.3 mmol L^{-1} H_2O_2 (1 mL), and 0.1 mL enzyme extract. CAT activity was determined spectrophotometrically by H_2O_2 destruction at 240 nm following Jung [43]. The reaction mixture (3 mL) contained 1.95 mL deionized water, 100 mmol L^{-1} H_2O_2 (1 mL), and 50 μL enzyme extract. POD activity was estimated following Kim et al. [44]. The reaction buffer contained 100 mmol L^{-1} H_2O_2 (1 mL), 0.2% guaiacol (1 mL, w/v), 50 mmol L^{-1} sodium phosphate buffer (pH 7.0, 0.95 mL), and 50 μL enzyme extract. The activity of SOD was determined by the inhibition of photochemical reduction of nitro blue tetrazolium following Desingh and Kanagaraj [36].

2.4. mRNA quantification by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from 100 mg fresh leaves of each sample using an RNA kit (Bioteke, Beijing, China) according to the instructions of the manufacturer. After digestion with DNaseI