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Research article

Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants

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ABSTRACT

Cold acclimation increases plant tolerance to a more-severe chilling and in this process an accumulation of H_2O_2 in plants is often observed. To examine the role of H_2O_2 in cold acclimation in plants, the accumulation of H_2O_2, antioxidant metabolism, the glutathione redox state, gas exchange and chlorophyll fluorescence were analyzed after cold acclimation at 12/10 °C and during the subsequent chilling at 7/4 °C in tomato (*Solanum lycopersicum*) plants. Cold acclimation modestly elevated the levels of H_2O_2, the gene expression of *respiratory burst oxidase homolog 1* (*Rboh1*) and NADPH oxidase activity, leading to the up-regulation of the expression and activity of antioxidant enzymes. In non-acclimated plants chilling caused a continuous rise in the H_2O_2 content, an increase in the malondialdehyde (MDA) content and in the oxidized redox state of glutathione, followed by reductions in the CO_2 assimilation rate and the maximum quantum yield of photosystem II (F_v/F_m). However, in cold-acclimated plants chilling-induced photoinhibition, membrane peroxidation and reductions in the CO_2 assimilation rate were significantly alleviated. Furthermore, a treatment with an NADPH oxidase inhibitor or H_2O_2 scavenger before the plants subjected to the cold acclimation abolished the cold acclimation-induced beneficial effects on photosynthesis and antioxidant metabolism, leading to a loss of the cold acclimation-induced tolerance against chilling. These results strongly suggest that the H_2O_2 generated by NADPH oxidase in the apoplast of plant cells plays a crucial role in cold acclimation-induced chilling tolerance.

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1. Introduction

Plants have evolved various ways to cope with their changing surroundings, especially the unfavorable environmental stresses. A better understanding of the biochemical changes in plants under stresses should enable the genetically engineered plants to be developed with enhanced resistance to abiotic stresses and even those resulted from agricultural practices (e.g., herbicides and insecticides). Chilling is a major abiotic stress that limits the productivity and geographical distribution of many plant species. However, many temperate plants have the ability to sense low temperatures and to activate mechanisms leading to an increase in the chilling or freezing tolerance, a phenomenon known as cold acclimation [1,2]. Cold acclimation is associated with numerous biochemical alterations, including changes in the membrane lipid composition [3], calcium fluxes [4] and changes in cell wall properties [5]. These changes are accomplished through the expression of a number of cold-regulated genes (*COR*) and proteins [6].

H_2O_2 as a relative stable oxygen species (ROS) was accumulated during the cold acclimation [7]. ROS are often induced in plants under various stresses and play a dual role: at mild concentrations, ROS act as signal molecules involved in acclamatory signaling, triggering the tolerance against various stresses; and, at high concentrations, ROS orchestrate programmed cell death [8,9]. H_2O_2 accumulation, mostly in the cell walls of mesophyll cells facing intercellular spaces, was triggered by many environmental stimuli or hormones, such as abscisic acid (ABA), jasmonic acid (JA), polyamines (PAs) and brassinosteroids (BRs) through enhancing NADPH oxidases [9,10], which are encoded by *respiratory burst oxidase homologs* (*Rbohs*) in plants. The role of different *Rboh* genes in plant growth and stress responses has been well-studied in *Arabidopsis*,

Abbreviations: A_{sat}, the light-saturated CO_2 assimilation rate; F_v/F_m, the maximum quantum yield of PSII; GSH, reduced glutathione; GSSG, oxidized glutathione; NPQ, non-photochemical quenching of chlorophyll fluorescence; Φ_{PSII}, quantum efficiency of electron transfer at PSII; qP, photochemical quenching; *Rbohs*, respiratory burst oxidase homologs.

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but only two Rboh genes, *Rboh1* and *Wfi1*, have been identified in tomato [11]. Among them, *Rboh1* showed high sequence similarity to *AtrbohD*, which plays a predominant role in both stress and adaptation responses in *Arabidopsis* [12].

H_2O_2 has been suggested to be a signal molecule in defense and adaptive responses, such as increase tolerance to chilling in maize [7], tolerance to paraquat in cucumber seedlings [9], and tolerance to pathogen challenge in transgenic tobacco and potato plants [13]. It has been shown that H_2O_2 enhances the antioxidant capacity of cells by increasing the activities of antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) [9]. In addition, cold acclimation also induces a high glutathione content and glutathione reductase (GR) activity, which are associated with chilling tolerance in several plant species, while both exogenously applied and endogenously produced H_2O_2 play a significant role in regulating cellular GSH levels [14,15]. An increased antioxidant capacity, in terms of enzymatic and non-enzymatic antioxidants, is often correlated with enhanced tolerance to chilling stress. Changes in the relative redox state of PSII affect both chilling tolerance and acclimation of the photosynthetic apparatus to avoid low temperature-induced photoinhibition. Plants that were able to increase their resistance to photoinhibition were also able to increase chilling tolerance during cold acclimation [16]. In fact, cold acclimation is frequently accompanied by an increased tolerance to photoinhibition and an increase in the photosynthetic capacity by minimizing the potential for photo-oxidative damage [16].

As aforementioned an accumulation of H_2O_2 induced by cold acclimation has been frequently observed, however, the physiological relevance of the H_2O_2 accumulation and the pathway for its generation have still remained poorly understood. In this work, we hypothesized that plant cells may sense cold conditions at the plasma membrane by the generation of H_2O_2 as a signaling molecule. H_2O_2 activates the downstream defense response by increasing the transcription of defense-related genes and the activation of their respective enzymes, leading to an enhanced tolerance to photoinhibition. Accordingly, we analyzed the H_2O_2 accumulation, transcript levels of *Rboh* genes, the activity of the corresponding proteins and antioxidant metabolism in cold-acclimated or non-acclimated tomato plants, a thermophilic plant species that is sensitive to chilling. Furthermore, the changes in gas exchange, chlorophyll fluorescence quenching, and the glutathione content were also determined to examine the influence of cold acclimation on the cellular redox state and chilling tolerance.

2. Results

2.1. Effects of cold acclimation on H_2O_2 accumulation

To examine the possible role of H_2O_2 in cold acclimation, the accumulation of H_2O_2 in the leaves was determined by both DAB-staining and spectrophotometric methods. As shown in Fig. 1, cold acclimation resulted in a significant increase in H_2O_2 accumulation, and this increase was accompanied by significant

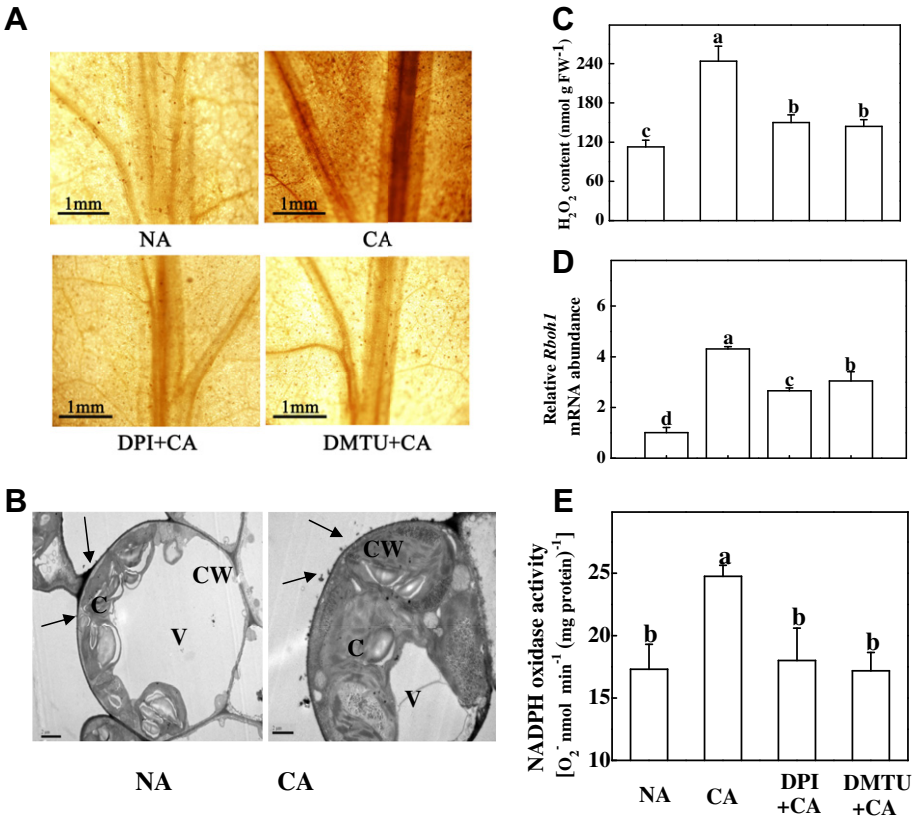

Fig. 1. Changes in H_2O_2 accumulation, *Rboh1* expression and NADPH oxidase activity, as influenced by cold acclimation. A, The *in situ* detection of H_2O_2 in leaves. The leaf segments were loaded with DAB and incubated for 6 h. The H_2O_2 accumulation was detected by an Olympus motorized system microscope (BX61, Olympus Co., Tokyo, Japan). Bar = 1.0 mm. B, The cytochemical localization of H_2O_2 accumulated in the mesophyll cells of leaves detected with $CeCl_3$ staining and transmission electron microscopy. Arrows, $CeCl_3$ precipitates; C, chloroplast; CW, cell wall; V, vacuole; IS, intercellular space. C, The H_2O_2 content in tomato leaves after 3 d of cold acclimation. D, The transcript level of *Rboh1* in tomato leaves after 3 d of cold acclimation. E, The NADPH oxidase activity in tomato leaves after 3 d of cold acclimation. The data shown are the average of three replicates, with the standard errors shown by vertical bars. Means denoted by the same letter did not significantly differ at $P < 0.05$, according to Tukey's test. NA, non-acclimated; CA, cold-acclimated; FW, fresh weight.

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