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Comparative proteomic analysis of key proteins during abscisic acid-hydrogen peroxide-induced adventitious rooting in cucumber (*Cucumis sativus* L.) under drought stress

Li Changxia, Bian Biting, Gong Tingyu, Liao Weibiao*

College of Horticulture, Gansu Agricultural University, 1 Yinmen Village, Anning District, Lanzhou 730070, China

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ABSTRACT

Previous results have shown that hydrogen peroxide (H_2O_2) is involved in abscisic acid (ABA)-induced adventitious root development under drought stress. In this study, a comparative proteomic analysis was conducted to explore the key proteins during ABA-H_2O_2-induced adventitious rooting in cucumber (*Cucumis sativus* L.) under drought stress. The results revealed that 48 of 56 detected proteins spots were confidently matched to NCBI database entries. Among them, 10 protein spots were up-regulated while 4 protein spots were down-regulated under drought stress; 22 protein spots were up-regulated by ABA under drought stress; treatment with ABA plus H_2O_2 scavenger catalase (CAT) up-regulated 6 protein spots and down-regulated 6 protein spots under drought stress. The identified proteins were divided into three categories: biological process, molecular function, and cellular component. According to their functions, the 48 identified proteins were grouped into 10 categories, including photosynthesis, stress response, protein folding, modification, and degradation, etc. According to subcellular localization, about 24 proteins (half of the total) were predicted to be localized in chloroplasts. ABA significantly up-regulated the expression of photosynthesis-related proteins (SBPase, OEE1), stress-defense-related proteins (2-Cys-Prx, HBP2), and folding-, modification-, and degradation-related proteins (TPal) under drought stress. However, the effects of ABA were inhibited by CAT. The proteins were further analyzed at the transcription level, and the expression of four of five genes (except *2-Cys-Prx*) was in accordance with the corresponding protein expression. The protein abundance changes of OEE1 and SBPase were also supported by western blot analysis. Therefore, H_2O_2 may be involved in ABA-induced adventitious root development under drought stress by regulating photosynthesis-related proteins, stress defense-related proteins, and folding-, modification-, and degradation-related proteins.

1. Introduction

Drought is one of the most serious environmental stresses that affects global agriculture productivity. Thus, it is of interest to know how plants improve drought stress tolerance. To adapt to drought stress, plants have developed complicated and elaborate signaling networks. The reduction in plant growth under drought is controlled by a number of factors such as plant hydraulic status, phytohormones, osmotic adjustment, and reactive oxygen species (ROS) signaling (Khan et al., 2015). The development of enhanced drought resistance in plants requires, among other things, knowledge of physiological mechanisms and genetic control of the contributing traits at different plant developmental stages. A member of class III chitinases, DIP3, may function as a drought stress-induced protein and has been shown to be involved in the regulation of the stress response in upland rice (Guo et al., 2013). Zou et al. (2010) reported that calcium-dependent protein kinase, CPK10, plays an important role in response to drought stress in *Arabidopsis*.

Abscisic acid (ABA) is a small molecule classified as a plant hormone. ABA plays critical roles in plant growth and development as well as in adaptation to environmental stresses by increasing photosynthesis rates and antioxidative defense systems (Reddy et al., 2004). Our understanding of the molecular mechanisms underlying ABA signaling has been improved by a series of studies. Jia et al. (2011) found that ABA promoted strawberry ripening. The authors also reported that the putative ABA receptor, FaCHLH/ABAR, is a positive regulator of ripening in response to ABA. Several calcium-signaling proteins (Fujita et al., 2013), as well as sucrose non-fermenting 1-related protein kinases subfamily 2 (SnRK2s, Waadt et al., 2015), were also involved in ABA signaling. Some proteins related to energy metabolism, defense, and

* Corresponding author.
E-mail address: liaowb@gsau.edu.cn (W. Liao).

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primary metabolism were up-regulated by ABA under abiotic stress (Zhu, 2016). ABA up-regulates photosynthesis-related and stress-defense-related genes in land plants (Stevenson et al., 2016).

Hydrogen peroxide (H_2O_2) as a central redox-signaling molecule is involved in oxidative stress. H_2O_2 was shown to modulate the activity of related antioxidant defense enzymes in cucumber leaves under heat stress (Gao et al., 2010). It also greatly reduced degradation of photosynthetic pigments and increased activities of different antioxidant enzymes in maize under drought stress (Ashraf et al., 2015). Increasing evidence now indicates that H_2O_2 regulates some specific proteins and genes in plants under abiotic stresses. H_2O_2 regulated the expression of some photosynthesis-related genes in *Microcystis aeruginosa* under oxidative stress (Qian et al., 2010), and was inferred to play a key role in the synthesis of heat-shock protein 70 (HSP70) in maize during drought and heat stress (Hu et al., 2010). Some studies have shown that H_2O_2 crosstalks with ABA to regulate various physiological and molecular processes. H_2O_2-induced HSP70 accumulation through ABA-independent pathway was reported in grafted cucumber plants (Li et al., 2014). Under salt stress, H_2O_2 improved cotton seed germination by mediating the down-regulation of ABA biosynthesis genes *NCED5* and *NCED9* (Kong et al., 2017).

Adventitious root development is a complex process influenced by multiple environmental and endogenous factors. Some environmental factors have been identified, such as temperature, drought, light condition, etc. Recently, many researchers have focused on understanding adventitious rooting response signaling pathways. Several plant hormones and signal molecules have been shown to control adventitious root formation (Niu et al., 2017). Studies also found that some signal molecules are involved in auxin-induced adventitious root development, e.g. H_2O_2 (Bai et al., 2012), nitric oxide (NO, Wen et al., 2016), carbon monoxide (CO), hydrogen sulfide (H_2S), and hydrogen gas (H_2, Zhu et al., 2016). Proteomics is becoming an increasingly important tool because proteins are directly related to function. The high resolution of 2-DE makes it a powerful tool for separating complex protein mixtures. This methodology has been employed to analyze plant proteins in response to both biotic and abiotic stress. In our lab, Li et al. (2016) reported that H_2O_2 was involved in ABA-induced adventitious root development in cucumber (*Cucumis sativus* L.) under drought stress. Our previous results confirmed that H_2O_2 participated in adventitious rooting induced by ABA under drought conditions via stimulating water and chlorophyll content, chlorophyll fluorescence, carbohydrate and nitrogen content, and some enzyme activities. However, the molecular mechanisms of ABA-H_2O_2-induced adventitious rooting under stress conditions are still unknown. In this study, we used two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) to investigate comparatively ABA-H_2O_2-induced alterations of protein profiles in cucumber during adventitious rooting under drought stress.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatments

Cucumber seeds (PanAmerican Seed, USA) were kindly supplied by Gansu Academy of Agricultural Sciences, Lanzhou, China. Selected identical seeds were surface-sterilized in 5% (w/v) sodium hypochlorite for 10 min, and then soaked in distilled water for 6 h. The seeds were germinated on filter paper containing distilled water in Petri dishes (15-cm diameter, 2.5 cm deep) and maintained at 25 ± 1 °C for 5 d with a 14-h photoperiod (photosynthetically active radiation = 200 μmol s^{-1} m^{-2}). The 5-d-old cucumber seedlings with primary roots removed were used as explants, and then the explants were maintained under the same temperature and photoperiod conditions for 2 d. Drought stress was administrated by polyethylene glycol 6000 (PEG, Shanghai Zhanyun Chemical Co. Ltd., Shanghai, China). Every 10 cucumber explants were placed in Petri dishes containing 60 mL of distilled water (control), 60 mL of PEG (0.05%, w/v), 51.7 mL of PEG plus 8.3 mL ABA (0.5 μmol L^{-1}, Sigma, St. Louis, MO, USA), and 49.2 mL of PEG plus 8.3 mL ABA plus 2.5 mL catalase (100 μmol L^{-1}, CAT, Sigma). For each treatment, three replicates were performed (10 explants per replicate). The concentrations of these chemicals were selected based on the results of a preliminary experiment.

2.2. Preparation of the proteins from cucumber explants

We extracted total proteins from cucumber explants exposed to four different treatments for 2 d. The samples of cucumber explants were finely powdered in a mortar with 0.04 g polyvinyl-polypyrrolidone (PVPP) and liquid N_2. Then, the samples were extracted with Tris−HCl pH 7.9 buffer. The extract was centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was collected and then mixed with five-fold ice-cold extraction buffer [10% (w/v) tricarboxylic acid (TCA) in acetone with 0.07% (v/v) β-mercaptoethanol] overnight at −20 °C. Homogenates were centrifuged at 20,000*g* for 30 min at 4 °C. The pellets were washed with cold acetone containing 0.07% β-mercaptoethanol, incubated at −20 °C for 1 h and centrifuged at 20,000 *g* for 20 min at 4 °C. The process was repeated two or three times until the supernatant was colorless. Next, the pellets were washed twice with ice-cold 80% acetone containing 0.07% β-mercaptoethanol, centrifuged as described earlier, and then dried by lyophilizer (Nibo SCIENTZ Biotechnology Co. Ltd., Zhejiang, China). The remaining pellets were dissolved in plant protein lysis buffer [7 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 4% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammo-nio]-1-propanesulfonate), 0.5% (v/v)] at room temperature for 2 h and then centrifuged at 20,000*g* for 30 min at 4 °C. The protein concentration of each pellet was determined according to the method described by Peterson et al. (1977). Bovine serum albumin (BSA) was used as a standard.

2.3. 2-DE and staining

Proteins were separated by 2-DE. The first dimension separates proteins according to their isoelectric point. Isoelectrofocusing (IEF) was carried out on 17-cm-long immobilized pH gradient (IPG) strips, providing a nonlinear pH 4–7 gradient (Bio-Rad, Hercules, CA, USA). Strips were rehydrated in rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.001% (w/v) bromophenol blue and 0.2% (w/v) Bio-Lyte] containing protein sample. Based on the following considerations, 600 μg was the suitable sample load for our analysis. Next, immobilized linear gradient strips were rehydrated at 50 V for 14 h at 20 °C. Electrofocusing was performed using PROTEAN® i12™IEF Cell (Bio-Rad) at 20 °C using a four-step process: 250 V for 1.5 h with a rapid ramp; 1000 V for 2.5 h with a linear ram; 9000 V for 5 h with a linear ramp; and 9000 V for 90,000 V-h with a rapid ramp. After the first dimension separation, focused strips were equilibrated for 15 min with equilibration buffer I [6 M urea, 2% sodium dodecyl sulphate (SDS), 0.375 M Tris−HCl (pH 8.8), 20% glycerol, and 130 mM DTT] and then for another 15 min with equilibration buffer II [6 M urea, 2% SDS, 0.375 M Tris−HCl (pH 8.8), 20% glycerol, and 135 mM iodoacetamide]. The second dimension separated proteins according to molecular weight. After equilibration, the strips were directly applied to 12% polyacrylamide SDS gel and separated at 100 V/gel for 1 h and at 250 V/gel for about 6 h using PROTEAN® II Xi Cell (Bio-Rad). After electrophoresis, gels were stained with CBB G-250.

2.4. Image and data analysis

The 2-DE gels were scanned using GS 800 Calibrated Densitometer scanner (Bio-Rad). Image and data analyses of the gels were performed using the PDQuest software (Version 8.0; Bio-Rad). CBB-stained gels were selected for the profile analysis. Spots with more than a 1.5-fold change were analyzed, and $P < 0.05$ was considered to indicate significant changes in abundance.