Insights into salicylic acid responses in cucumber (*Cucumis sativus* L.) cotyledons based on a comparative proteomic analysis

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ABSTRACT

To investigate the response of cucumber seedlings to exogenous salicylic acid (SA) and gain a better understanding of SA action mechanism, we generated a proteomic profile of cucumber (*Cucumis sativus* L.) cotyledons treated with exogenous SA. Analysis of 1500 protein spots from each gel revealed 63 differentially expressed proteins, 59 of which were identified successfully. Of the identified proteins, 97% matched cucumber proteins using a whole cucumber protein database based on the newly completed genome established by our laboratory. The identified proteins were involved in various cellular responses and metabolic processes, including antioxidative reactions, cell defense, photosynthesis, carbohydrate metabolism, respiration and energy homeostasis, protein folding and biosynthesis. The two largest functional categories included proteins involved in antioxidative reactions (23.7%) and photosynthesis (18.6%). Furthermore, the SA-responsive protein interaction network revealed 13 key proteins, suggesting that the expression changes of these proteins could be critical for SA-induced resistance. An analysis of these changes suggested that SA-induced resistance and seedling growth might be regulated in part through pathways involving antioxidative reactions and photosynthesis.

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1. Introduction

Salicylic acid (SA) is considered to be an important signaling molecule, which is involved in local and endemic disease resistance in plants in response to various pathogenic attacks. One proposed mechanism by which plants defend themselves against these attacks is through the accumulation of large quantities of SA [1]. Further research indicates that increases in the endogenous levels of SA and its conjugates always coincide with the elevated expression of pathogenesis-related protein (*PR*) genes and the activation of disease resistance in pathogen-inoculated plants [2]. Exogenous application of SA or its synthetic functional analogs results in the activation of *PR* expression and enhanced resistance to pathogens [3]. With the assistance of SA-deficient mutants and -overexpressed transgenic plants, SA-mediated signaling pathways in response to biotic stresses have been extensively studied. Signaling downstream of SA is regulated by non-expressor of PR1 (NPR1)-dependent and -independent processes. Briefly, the accumulated endogenous SA or applied exogenous SA can induce changes in the cellular redox state, leading to the monomerization of NPR1. Then NPR1 proteins are translocated from the cytosol to the nucleus, where they interact with other transcription factors of TGA, WRKY and so on, and activate the expression of defense genes, such as *PR*, chitinases and glucanase [4,5].

Recently, an increasing number of reports have demonstrated that SA also plays an important role in provoking plant resistance to various abiotic stresses, such as high temperature [6], salinity [7], and drought stresses [8]. In order to reveal the action mechanism, lots of research focuses on the changes of antioxidative reactions induced by SA. They speculate that exogenous SA may improve the capability of antioxidative reactions through alter the activity of antioxidative enzymes, but the findings are somewhat inconsistent [9]. In addition, exogenous SA also influences a range of diverse processes in plants, including seed germination, stomatal closure,

ion uptake and transport, photosynthesis and growth rate [10,11]. The increased level of free and bound SA detected in plants growing from seeds soaked in SA solution before sowing is the product of de novo synthesis, rather than having been taken up and mobilized by the plants [12].

A survey of the current literature reveals that the analysis of SA-responsive physiological changes is not comprehensive, and the genomic analysis is somewhat incomplete because many gene products cannot be detected through transcriptome analyses resulted from the post-translation modification, so a few cases describing the physiological and transcriptional responses to SA cannot reveal the possible mechanism of SA action. Proteomics is becoming an increasingly important tool because proteins are directly linked to cellular functions. Proteomics, or the systemic analysis of proteins expressed by genome, is not only a powerful molecular tool used in describing complete proteomes at the organelle, cell, organ or tissue levels, but it can also compare proteomes affected by different physiological conditions, such as those resulting from the exposure to stressful conditions and exogenous induction [13]. The current status of SA-induced proteomic analysis has been reviewed recently. Twenty glutathione S-transferases (GSTs) at the level of protein expression in *Arabidopsis* cell induced by exogenous SA were identified using proteomic approaches and GSTs exhibited class specific responses to SA treatment, suggesting that several mechanisms were acting to induce GSTs upon SA treatment [14]. Proteins induced by antagonist yeast *Pichia membranefaciens* and SA in peach fruit were determined using proteome analysis, suggesting that antioxidant and PR proteins, as well as enzymes associated with sugar metabolism, were involved in resistance of peach fruit induced by *P. membranefaciens* and SA [15]. Antioxidant proteins were involved in the resistance against pathogen invasion of sweet cherry fruits after SA treatment at every maturity stage, while heat shock proteins and dehydrogenases might potentially act as factors only at later maturity stages [16]. During *Arabidopsis* seed germination, SA-responsive proteins have been detected by 2-DE and MS/MS methods. The processes affected by SA concerned the quality of protein translation, the priming of seed metabolism, the synthesis of antioxidant enzymes, and the mobilization of seed storage proteins [11]. These previous research on SA-induced proteome only focused on seed or fruit development and the response to biotic stresses, but little information is available about proteomic changes induced by exogenous SA during plant growth.

Cucumber (*Cucumis sativus* L.) is one of the most important economical vegetables in the world. As cucumber seedlings developing, they become more susceptible to be infested by pathogens [17]. Furthermore, cucumbers require appropriate environment to grow and are easy to be subjected to environmental stresses, especially low temperature or weak light in protected cultivation. The application of exogenous SA may alleviate the damage resulted from biotic and abiotic stresses, but the action mechanism is still unclear. Thus, it is imperative to screen the proteome pattern of cucumber cotyledons in response to exogenous SA, in order to gain a better understanding of the molecular mechanism for regulating plant development and resistance.

2. Materials and methods

2.1. Plant material and SA treatments

Cucumber (*C. sativus* L. cv. Zhongnong 203) seeds were sown on 0.7% agar medium with half-strength Hoagland nutrient solution (pH 6.3) in square plastic petri plates (12.5 cm × 12.5 cm). An SA solution was prepared with half-strength Hoagland nutrient solution and neutralized to pH 6.3 with 1 N NaOH. After 9

days, germinated seedlings with completely expanded cotyledons were transferred to a hydroponic culture system containing half-strength Hoagland nutrient solution (pH 6.3) supplemented with 0 (control), 0.01, 0.05, 0.1 or 0.5 mM SA, and the seedlings were treated for 9 days. The nutrient solutions were replaced every two days. The experiment was carried out in a controlled environmental chamber with a 10 h/14 h light/dark cycle (8:00–18:00 was light, provided by fluorescent lamps with a light intensity of approximately 300 μmol m^{-2} s^{-1} and other periods were dark), 27 °C/16 °C day/night temperature, and relative humidity of 80–85%.

2.2. Measurements of growth parameters and photosynthesis

After treatment with different concentrations of exogenous SA for 1, 5, and 9 days, the phenotypic changes in the cucumber seedlings were observed and photographed. For the dry mass assay, five seedlings from every treatment were divided into shoots and roots, dried at 80 °C to a constant dry mass, and measured.

The net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate of the cucumber cotyledons from untreated and 0.05 mM SA-treated seedlings for 1, 5, and 9 days were measured using a LI-COR 6400 portable gas analysis system with a light-emitting diode light source (LI-COR Inc., Lincoln, NE) at 10:00 am. At least five seedlings from each treatment were selected, and one cotyledon per seedling was measured.

2.3. Protein extraction

The proteins from the cucumber cotyledons were extracted using a trichloroacetic acid/acetone method as previously described [18] with some modifications. Approximately 0.5 g of cotyledon tissue from cucumber seedlings exposed to 0 or 0.05 mM SA for 5 days was ground in liquid nitrogen and suspended in 10% (w/v) TCA in acetone with 0.07% (w/v) DTT at −20 °C overnight. After centrifugation at 35,000 × g for 30 min at 4 °C, the pellets were washed with 10 mL ice-cold acetone containing 0.07% DTT, incubated at −20 °C for 1 h and centrifuged again at 4 °C. This step was repeated three times, and then the pellets were lyophilized with N$_2$ to remove any remaining acetone. The sample powders were then solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer pH 4–7, 50 mM DTT). After incubation at 25 °C for 3 h, the suspension was centrifuged at 16,000 × g for 30 min at 25 °C to remove the insoluble material. The supernatants were immediately frozen in liquid nitrogen and then stored at -80 °C in aliquots.

2.4. 2-DE, staining, image acquisition and analysis

Two-dimensional gel electrophoresis was performed according to Jorge et al. [19] with slight modifications. The acetone-precipitated proteins were redissolved in IEF rehydration solution (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer pH 4–7, 65 mM of DTT, and a trace of bromophenol blue). For isoelectric focusing, the Ettan IPGphor3 system (Amersham Biosciences, Uppsala, Sweden) and pH 4–7 IPG strips (24 cm, linear) were used according to the manufacturer's recommendations. A 450 μL sample (1.2 mg protein) of extracted proteins was loaded onto a commercially available precast IPG strip with a 24 cm linear pH 4–7 gradient and actively rehydrated at 30 V for 14 h at 20 °C. Focusing was performed in three steps: 200 V for 1 h, 500 V for 1 h, 1000 V for 2 h, and 8000 V for 10 h. Then, the gel strips were equilibrated for 15 min in 10 mL equilibration buffer (50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and 0.002% (w/v) bromophenol blue) and for another 15 min in alkylating equilibration buffer that contained 2.5% (w/v)