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Proteomics analysis of Xiangcaoliusuobingmi-treated *Capsicum annuum* L. infected with *Cucumber mosaic virus*

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A B S T R A C T

Among different viruses, *Cucumber mosaic virus* (CMV) has the most extensive host range, being capable of infecting over 1200 species, and causes severe damage worldwide. Xiangcaoliusuobingmi (B1), a candidate plant immune activator drug, exhibited significant protective effects against CMV. However, its potential mechanism is still unknown. In this study, we found the defensive enzyme activities of peroxidase (POD), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD) and catalase (CAT) can be enhanced by B1. Meanwhile, we found RT-qPCR assay results of the defensive gene expression can be improved by B1 in capsicum. Moreover, we analyze the result of label-free proteomics, B1 could trigger abscisic acid (ABA) pathway. All data provide a more understanding about the response to infect CMV capsicum activeted by B1 in the level of the plant physiology and biochemistry, gene and protein.

1. Introduction

Qiemen sweet pepper (*Capsicum annuum L.*) is an economically important vegetable that belongs to the family *Solanaceae* and has been traditionally used as a spice for decades in China (Choi et al., 2005). Plant virus diseases, which are called 'plant cancers' by agricultural biologists, are major diseases that have caused enormous damage to pepper production. More than 30 virulent diseases are harmful to *C. annuum* L. production, including *Tobacco mosaic virus*, *Pepper mottle mosaic virus*, and *Cucumber mosaic virus* (CMV) (Azizan et al., 2017). Among them, CMV is the most abundant and well-studied agricultural virus on *C. annuum L.* that belongs to the genus *Cucumovirus* in the family *Bromoviridae* (Guo et al., 2017). CMV-infected *C. annuum L.* can cause serious systemic mosaic symptoms, such as leaf mottling, yellowing, stunting, and mosaic. In Bangladesh, the incidence of disease and loss of yield are 12%–56% and 28%–40%, respectively (Rahman et al., 2016). Relevant studies indicated that, as an isometric virus distributed over three icosahedral particles, CMV has a tripartite genome of three essential single-stranded plus RNA (RNA1, RNA2, and RNA3) (Jacquemond, 2012; Zeng et al., 2013; Revathy and Bhat, 2017). RNA1 encodes viral helicase, RNA2 encodes replicase, and RNA3 encodes movement and coat proteins (Murota et al., 2017). RNA2 encodes 2a and 2b protein, 2b protein is responsible for the inhibition of SA and JA, thus responsible for plant defense pathways (Mochizuki and Ohki, 2012). The CMV coat protein (CP) acts as a multifunctional factor for viral systemic movement, host range, and aphid transmission. Thus, CP is important for the regulation of antiviral silencing and viral suppressors of RNA silencing activity. CP also regulates the self-attenuation of the virus and the recovery of long-term symptoms (Zhang et al., 2017a). In addition, CMV is a severe agricultural virus that has the widest host range (> 1000 plants in 85 families) (Guo et al., 2017; Zeng et al., 2013; Roossinck, 2001), including monocots and dicots, herbaceous plants, shrubs, and trees (Ohshima et al., 2016). In addition, CMV can be divided into two primary subgroups (I and II). Subgroup I can be further subdivided into subgroups IA and IB based on the sequence similarity and serology (Guo et al., 2017; Zeng et al., 2013; Roossinck, 2002).

As an important natural product that possesses biological safety, simple structure, and desirable bioactivities, vanillin (4-hydroxy-3-methoxybenzaldehyde, CAS Registry Number: CN106467478A) is widely used as a lead compound in discovering novel medicines and in the field of agriculture (Qian et al., 2010; Seiber, 2011; Walton et al., 2003). In a previous study aiming to discover novel efficient antiviral agents, a bis(2-hydroxyethyl)dithioacetal fragment was introduced into vanillin to synthesize a series of title compounds (Sinha et al., 2008); strikingly, the target compound B1 2,2′-(((4-((4-chlorobenzyl)oxy)-3-

Abbreviations: CMV, *Cucumber mosaic virus*; B1, Xiangcaoliusuobingmi; *C. annuum L.*, *Capsicum annuum L.*; ABA, abscisic acid; SA, salicylic acid; FDR, false discovery rate; iBAQ, intensity-based absolute quantification; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; POD, peroxidase; SOD, superoxide dismutase; CAT, catalase; PAL, phenylalanine ammonia-lyase; RT-qPCR, quantitative real-time PCR; ABR1, ABA-responsive 1; ROS, reactive oxygen species

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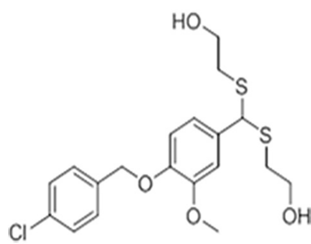

Fig. 1. Structure of Xiangcaoliusuobingmi.

methoxyphenyl) methylene) bis (2-hydroxyethyl) dithioacetal (Fig. 1), named Xiangcaoliusuobingmi, had remarkable protective activities against CMV (Zhang et al., 2017b).

In plants, resistance to plant activators, including dufulin (DFL), chitosan oligosaccharide (COS), and cytosinpeptidemycin, can protect the plant against pathogens. This resistance elicits defensive responses, such as 'oxidative burst', cell death, and phytoalexin synthesis. Two modes of plant immunity, namely, pattern-triggered immunity and effector-triggered immunity, have been used as indicators of pathogen attack, which is defined by the forms of molecules recognized by plants (Jones and Dangl, 2006). Typically, pathogen-associated molecular patterns are perceived by surface-localized pattern recognition receptors of plants (Zipfel, 2014). Salicylic acid (SA), ABA, and ethylene are plant hormones that are useful in mediating ROS and temperature stress signals. DFL is related to the SA signaling pathway by up-regulating the expression of HrBP1, resulting in the up-regulation of PR-1a and PR-5 (Chen et al., 2012). COS can strengthen the defensive enzyme activities, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), to enhance the rice disease resistance through the mitogen-activated protein kinase (MAPK) signaling cascade pathway (Yang et al., 2017). Cytosinpeptidemycin can up-regulate serine/threonineprotein kinase SAPK7 to induce ABA response and can also up-regulate some resistance genes (Yu et al., 2017). However, the potential mechanism of B1-induced immunity in capsicum is still unclear.

In the study, we first analyzed the variation of plant physiology and biochemistry studies caused by B1- triggered, at the same time, some resistance genes can be up-regulated. We used label-free proteomics technology coupled with bioinformatics to analyze the differentially expressed proteins (DEPs) and elucidate the mechanism of B1. The result showed that B1 can control the capsicum virus caused by CMV by improving the plant host resistance.

2. Materials and methods

2.1. Capsicum growth promotion experiments

Qiemen sweet pepper (*C. annuum L.*) seeds were randomly divided into three groups: control (CK), COS, and B1. Every group was divided into five concentration, 0.0125, 0.025, 0.050, 0.100, and 0.200 mg/ml. A solvent (10 ml) was added to the CK group, and 10 ml of COS and B1 aqua was added to the other groups. The capsicum seeds were soaked for 24 h and then washed with distilled water for five to six times to remove the solution. The germination rate, root length, and bud length of each group were then counted. Each experimental group was repeated three times.

2.2. Plant material and sampling

Qiemen sweet pepper (*C. annuum L.*) plants were cultivated in a greenhouse at 26 °C–30 °C and 60% relative humidity with a 16 h day/8 h night cycle. A total of 500 μg/ml of COS and B1 were sprayed on the pepper plants of the treatment group until the four- to six-leaf stage. B1 belongs to the treatment group, whereas water and COS belongs to the negative (CK) and positive controls, respectively. After 24 h, *C. annuum L.* was infected with CMV by artificial friction. The aggressive isolate CMV-Fny, belonging to the subgroup IA (Giner et al., 2017). Biological tissue samples were collected at 0, 1, 3 and 5 days after the inoculation treatment for tests on chlorophyll content, fluorescent quantitation of defense genes, and defensive enzyme activity assay. The samples were collected and preserved at −80°°C. Measurements were performed in triplicate.

2.3. Protein extraction and trypsin digest

The extraction of all pepper proteins were performed based on the methods by Mehta with slight modification (Mehta and Rosato, 2003; Qian et al., 2013). Approximately 1.0 g of leaf samples were frozen in liquid nitrogen (N_2) until fine powders were produced. Then, the samples were homogenized in ice-cold sucrose lysis buffer containing 500 mM Tris–HCl (pH 7.5), 700 mM sucrose, 100 mM KCl, 0.05 M EDTA, and 0.04 M dithiothreitol (DTT) at room temperature for 60 min. A total of 5 ml of Tris–phenol was added under sustained shaking at 4 °C. After 30 min, the mixture was centrifuged at 4500 rpm for 20 min. The upper phenol layer was collected, and 10 ml of 100 mM ice-cold ammonium acetate in 100% methanol was added. The sample was placed in a refrigerator at −20 °C overnight. Then, the mixture was centrifuged at the same rotation speed. The precipitate was collected and cleaned thrice with pre-cold acetone (Kim et al., 2006). Finally, the pellets were dried in Speed Vac (SIM International Group Co., Ltd., Newark, NJ, USA) for 3 h and then dissolved in 1 ml of 8 M UA buffer (8 M urea, 100 mM Tris, 0.04 M DTT, pH 8.5) for 45 min at 37 °C. The total protein concentration was confirmed using the Bradford method (Sengupta et al., 2011). Afterward, 100 μg of proteins were collected and alkylated with 55 mM iodoacetamide to inhibit the reduction in the cysteine residues. Then, the sample was incubated in the dark at room temperature for 45 min. The sample was centrifuged for 20 min at 12000 rpm at 4 °C by using 50 mM UA with 3 kDa Millipore, ultrafiltration was repeated. Next, the protein was digested in 40 μl of 25 mM NH_4HCO_3 with 3 μg of trypsin (Promega, Madison, WI, USA) overnight at 37 °C and 900 rpm (Sun et al., 2016). The suspension was centrifuged at 12000 rpm and at 4 °C for 40 min. Afterward, the filtrates of the final peptides were collected and vacuum dried. The samples were solubilized in 40 μl of H_2O (LC/MS grade) consisting of 0.1% formic acid for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

2.4. LC-MS/MS analyses

The peptide of each sample was analyzed by the NanoLC · 1DTM plus system (Eksigent, Dublin, CA) coupled with a triple time-of-flight (TOF) 5600 mass spectrometer (Foster City, CA, USA). First, 8 μl of crude polypeptides were injected using a full loop injection. A ChromXP Trap column (Nano LC TRAP Column, 3 μm C18-CL, 120 Å, 350 μm × 0.5 mm; Foster City, CA, USA) was used to desalinate the peptides at a flow rate of 2 μl/min for 10 min. Then, the peptides were eluted into a second analytical column: NanoLC C18 reversed phase column (3C18-CL, 75 μm × 15 cm, Foster City, CA, USA). The mobile phases comprised two phases, A (2% ACN and 0.1% FA) and B (98% ACN and 0.1% FA), separated with a linear gradient at 300 nL/min for over 120 min. Triple TOF 5600 MS directly eluted the peptides in a data-dependent way to automatically switch between TOF-MS and Product Ion acquisition by using Analyst (R) Software (TF1.6) (AB SCIEX, Foster City, CA, USA). Every two samples were calibrated by β-galactosidase digest. Elution lasted for 10 min and identification for 30 min.

2.5. Data analysis and results of label-free quantitation

MS data were processed using the MaxQuant software version 1.5.2.8 by Andromeda search engine (Cox and Mann, 2008; Cox et al.,

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