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Different Proteomics of Ca^{2+} on SA-induced Resistance to *Botrytis cinerea* in Tomato

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Abstract

This study aims to comprehensively study the effects of Ca^{2+} on the SA-induced resistance *Botrytis cinerea* in tomato through proteomics analysis. A proteomic approach was used to uncover the inducible proteins of tomato in the susceptible tomato cultivars 'L402' against *Botrytis cinerea* after salicylic acid (SA) and a combination treatment of $CaCl_2$ and SA. The results showed that the use of combination treatment of $CaCl_2$ and SA significantly enhanced tomato resistance against *Botrytis cinerea*. In total, 46 differentially expressed protein spots from 2-DE gel maps were detected, of which 41 were identified by mass spectrometry. All the identified proteins were categorized into eight groups according to their putative functions: defense response (14.00%), antioxidative protein (9.75%), photosynthesis (24.39%), molecular chaperone (4.88%), energy (17.01%), metabolism (21.95%), protein synthesis (4.88%) and signal transduction (0.2%). Of the proteins in the eight function groups, the effect of stress/defense and reactive oxygen species on Ca^{2+}-regulated SA-induced resistance may be the most important one in induced resistance by RT-PCR. The expression level of pathogenesis-related proteins (PRs) and chitinase was upregulated by a combination treatment of $CaCl_2$ and SA. The characterization of these proteins greatly helped to reveal the induced proteins involved in the regulation of Ca^{2+} on SA-induced resistance to *Botrytis cinerea*. In the combination treatment of $CaCl_2$ and SA, the defense response and antioxidative protein were clearly upregulated much more than SA alone or the control treatment by the method of proteomics and RT-PCR. The present findings suggest that susceptible tomato cultivars treated by the combination treatment of $CaCl_2$ and SA might possess a more sensitive SA signaling system or effective pathway than SA treatment alone. In addition, results indicated that SA could coordinate other cellular activities linked with photosynthesis and metabolism to facilitate defense response and recovery, indicating that the self-defense capability of tomato was improved by the combination treatment of $CaCl_2$ and SA.

Keywords: tomato; calcium; salicylic acid; *Botrytis cinerea*; proteomic; induced resistance

1. Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop, and worldwide is the second most important vegetable crop next to potato. Present world production is about 100 million tons of fresh fruit from 3.7 million ha (Food and Agriculture Organization of the United Nations, 2001). However, its yield and quality are seriously compromised by infectious diseases caused by various fungal, bacterial, and viral diseases. Gray mold (https://en.wikipedia.org/wiki/Botrytis_cinerea), caused by the necrotrophic fungus *Botrytis cinerea*, is one of the most serious diseases of tomato. In China, gray mold is responsible for losses of over 30% of tomato yield in processing facilities (Yao et al., 2011). Control of the disease mainly depends on breeding resistant cultivars and using chemical fungicides. Breeding for resistance is the most economical method by which to control infection (Basnet et al., 2013). However, the frequent occurrence of new races of *Botrytis cinerea* has reduced the effectiveness of resistant cultivars in the greenhouse. Meanwhile, environmental concerns call for strict regulations on the use of chemical fungicides (Wahab, 2015). Development of new disease control strategies based on innate plant defense mechanisms may offer a promise for less crop loss due to *Botrytis cinerea*.

Plants have evolved a wide variety of inducible defense mechanisms that can be activated by a variety of biotic and abiotic stimuli aside from their basal physical and chemical barriers (Bari and

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Jones, 2009; Justyna and Ewa, 2013). Salicylic acid (SA) is one of the signaling molecules associated with hypersensitive response (HR) and systemic acquired resistance (SAR); it can effectively induce the enhancement of enzymatic activity related to defensive processes (e.g. β-1, 3-glucanase and chitinase activities) (Vlot et al., 2009), increase total phenolic content, and cause immediate production of reactive oxygen species (ROS) (Yun and Chen, 2011), all three of the prior items to enhancement of plant resistance against a wide range of pathogens (Torres, 2009; Liu et al., 2012). In particular, the genetic role of SA in the activation of defense responses against biotrophic and hemibiotrophic pathogens has been widely recognized (Guru et al., 2007; Loake and Grant, 2007; Pieterse et al., 2009; Yuan et al., 2013), but the reports on the genetic role of SA in necrotrophic pathogens have been limited. Calcium is one of the important 'second messages'; some studies have shown that induced resistance of plants under biotic stress can be alleviated by exogenous Ca^{2+} (Lecourieux et al., 2006; Shao et al., 2008; Serrano et al., 2012; Downie, 2014). Treatment with Ca^{2+} can enhance the peroxidase activities, suggesting a physical cell wall strengthening and/or the generation of antimicrobial compounds that limit fungal development (Anna et al., 2005; Clark, 2013; Xu et al., 2013).

The role of Ca^{2+} in regulating SA in induction of tomato innate resistance against *Botrytis cinerea* infection has been widely documented in our lab (Li et al., 2012, 2015). However, there is little published information available on the mechanism of Ca^{2+} on SA in induction of disease resistance in the level of proteomic (Torres, 2009; Vlot et al., 2009; Liu et al., 2012; Yuan et al., 2013). In this study, we were interested in the identification and functional analysis of differently expressed proteins by various treatments. Thus, we investigated and analyzed the difference of the expressed proteins in the control, SA treatments, and a combination treatment of $CaCl_2$ and SA using 2-DE followed by MALDI™ TOF/TOF. The aim was to further explain the molecular mechanism of the biological process at the proteomic level. The present study offers new insights into the physiological mechanism involved in the regulation of Ca^{2+} on SA-induced resistance to *B. cinerea* and provides theoretical evidence for better disease control of tomato and other vegetables.

2. Materials and methods

2.1. Plant materials and treatment

The seeds of the tomato cultivar *S. lycopersicum* L. 'L402', a popular variety in Northeast China, are susceptible to *B. cinerea*. *S. lycopersicum* L. 'L402' seeds were germinated and grown in 12 cm^2 nutrition pots in heated greenhouses (average day/night temperatures, 25 °C /15 °C) with natural light and a relative humidity of 60% during April 2014 at the Shenyang Agricultural University. The plants were watered according to normal cultivation management.

The tomato plants were divided into three groups at the five-leaf stage, wherein each group contained 30 pots (3 biological replications with 10 plants per replication). Tomato 'L402' plants were given three treatments: the control, the SA treatment, and the combination treatment of $CaCl_2$ and SA (Ca + SA). The control treatment was foliar-sprayed ddH_2O; the SA treatment was foliar-sprayed exogenous 2 mmol · L^{-1} SA; and the Ca + SA treatment was foliar-sprayed ddH_2O and 8 mmol · L^{-1} $CaCl_2$ immediately followed by application of exogenous 2 mmol · L^{-1} SA. After three days of treatments, all the seedlings were inoculated with *B. cinerea* spores by placing 5 mL of a suspension of 10^6 spores · mL^{-1} in 2% glucose solution. Plants tissue samples were frozen using liquid nitrogen at 48 h days postinfection (DPI) and stored at −86 °C before being used to extract protein and mRNA. The disease survey was performed according to the methods of Fang (1998).

2.2. Extraction of secreted protein and mRNA from treated leaves

Proteins were extracted from leaves according to the method described by Lu et al. (2013). Three replicates of the treated seedlings were placed in liquid nitrogen, transferred to a pre-chilled mortar and under liquid nitrogen, and were ground into a fine powder using a pestle. Subsamples, weighing 3 g, were extracted using 30 volumes of pre-chilled 10% TCA–0.07% DTT/acetone buffer and precipitated overnight at −20 °C. Centrifugation was performed at 13 000 g for 60 min at 4 °C, then the supernatant was removed, and 30 volumes of pre-chilled 10% TCA–0.07% DTT/acetone buffer were added. The mixture was allowed to precipitate for 60 min, and then centrifuged at 13 000 g for 60 min at 4 °C. This step was repeated a minimum of four times. At last, the precipitant was simply air-dried at 4 °C for 5 min.

2.3. RNA isolation and validation of expression profiles reverse transcription

Total RNAs were extracted using TRIzol® reagent (Thermo Fischer Scientific, Waltham, MA, USA). The reverse transcription (RT) primers were designed following Chen et al. (2005) and Varkonyi-Gasic et al. (2007). The RT reactions were performed using M-MLV Reverse Transcriptase (Takara Bio Inc., Tokyo, Japan) as directed by the manufacturer. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the QuaniFast SYBR Green PCR Master Mix kit (Qiagen, Valencia, CA, USA) for qRT-PCR analysis using Applied Biosystems™ ABI PRISM® 7500 Sequence Detection System and its associated software (Thermo Fischer Scientific, Waltham, MA, USA). Primers were designed from the peptide sequences obtained after mass analysis according to NCBI and tomato databases (https://solgenomics.net/). Amplification was initiated with a denaturation step of 10 s at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. All reactions were performed in triplicate, and negative controls (no template and no RT) were included for each gene. The relative mRNA levels for each miRNA gene from various treatment samples were quantified with respect to the internal *Q96483* RNA standard. At least two independent RNA isolations were used for cDNA synthesis, with two biological replicates and three technical replicates for qRT-PCR analysis of each cDNA sample.

2.4. 2-DE analysis

All proteins were redissolved in rehydration buffer [9 mol · L^{-1} urea, 2 mol · L^{-1} thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 1% PMSF] for 2-DE analysis. The protein content in the extracts was measured by the Bradford method using bovine serum albumin as the standard. The volume containing 1 mg total soluble protein