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Accumulation of transcription factors and cell signaling-related proteins in the nucleus during citrus–*Xanthomonas* interaction

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ABSTRACT

The nucleus is the maestro of the cell and is involved in the modulation of cell signaling during stress. We performed a comprehensive nuclear proteome analysis of *Citrus sinensis* during interaction with host (*Xanthomonas citri* pv. *citri*-*Xcc*) and non-host (*Xanthomonas oryzae* pv. *oryzae*-*Xoo*) pathogens. The nuclear proteome was obtained using a sequential method of organelle enrichment and determined by nano-LC–MS/MS analysis. A total of 243 proteins accumulated differentially during citrus–*Xanthomonas* interaction, belonging to 11 functional groups, with signaling and transcription-related proteins dominating. MADS-box transcription factors, DEAD-box RNA helicase and leucine aminopeptidase, mainly involved in jasmonic acid (JA) responses, were in high abundance during non-host interaction (*Xoo*). Signaling-related proteins like serine/threonine kinase, histones (H3.2, H2A), phosphoglycerate kinase, dynamin, actin and aldolase showed increased accumulation early during *Xoo* interaction. Our results suggest that there is a possible involvement of JA-triggered defense responses during non-host resistance, with early recognition of the non-host pathogen.

1. Introduction

Plants evolved sophisticated mechanisms to restrict the great majority of microbes they encounter through a broad-range form of immunity called non-host resistance (NHR). The NHR is the immunity displayed by an entire plant species against all genetic variants of a pathogen (Thordal-Christensen, 2003) and is considered a general defense mechanism (Nürnberger and Lipka, 2005). NHR is genetically complex and involves several components of constitutive and inducible plant defenses (Uma et al., 2011).

The NHR is a quantitative trait controlled by more than one gene. In most cases silencing or mutating of gene(s) that possibly have a role in NHR compromise it partially (Sharma et al., 2003; Uma et al., 2011). Efficient signal perception and robustness of individual pathogen recognition events are characteristics of NHR (Nürnberger and Scheel, 2001). The activation of defense responses in plants depends on the timely recognition of the invading pathogen. This recognition leads to a rapid activation of signal transduction cascades that may involve protein phosphorylation, ion fluxes, reactive oxygen species (ROS), and transcriptional reprogramming (Eulgem, 2005; Oh et al., 2006). Transcriptome analyses of gene expression have greatly contributed to the understanding of NHR in plants (Daurelio et al., 2011, 2013; Tao et al., 2003; Zimmerli et al., 2004). However, proteome analysis endeavor the complete set of proteins encoded by the genome, thus complementing transcriptome studies (Gygi et al., 1999; Tian et al., 2004).

Immunity of plants against microbes involves multiple organelles. Sub-organellar proteomics reduces the complexity of the total cellular proteome, enabling the visualization of low abundance proteins and allows studying specific groups of proteins that are central to a certain function. Moreover, it provides suitable information regarding where and how these proteins exert their particular functions (Dreger, 2003). The nucleus is a key repository of important host components, including transcription factors (TFs) and regulators, and is essential for plant immunity (Rivas, 2012). Analysis of the changes in the nuclear proteome, therefore, will allow identification of essential components of NHR. Nuclear proteins have been implicated in different cellular processes such as cell signaling, gene regulation, differentiation, translation, proteolysis, physiological responsiveness and a variety of RNA-associated functions (Khan and Komatsu, 2004). Nuclear proteomes were analyzed in normal and abiotic stress conditions in model and crop plants (Narula et al., 2013), and in soybean-rust interaction (Cooper

Abbreviations: hpi, hours post infiltration; HR, hypersensitive response; JA, jasmonic acid; NHR, non-host resistance; PR, pathogenesis-related protein; ROS, reactive oxygen species; SA, salicylic acid; TF, transcription factor; *Xcc*, *Xanthomonas citri* pv. *citri*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.

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et al., 2011). No such attempts have been reported with respect to NHR in trees.

Citrus plants are susceptible to a number of diseases with various degrees of economic impact. Citrus canker A (referred as Asiatic citrus canker) is caused by *Xanthomonas citri* pv. *citri* (*Xcc* strain A) and highly affects yields of many important citrus species. Citrus canker types B and C (referred as South American canker) are caused by *X. citri* pv. *aurantifolii*, which has a lower incidence on citrus (Schaad et al., 2005; Schubert et al., 2001). The transcriptome of citrus during citrus–*Xcc* compatible interaction has been well studied (Cernadas et al., 2008; Gandia et al., 2007; Kim et al., 2009; Mozoruk et al., 2006), however, the citrus non-host response has been explored to a lesser degree.

The non-host response of citrus against *X. campestris* pv. *vesicatoria* (*Xcv*) was analyzed through cDNA microarray hybridization and compared with Asiatic citrus canker infection (Daurelio et al., 2013). Hypersensitive response (HR) symptoms were reported in key lime upon *Xcc* strain Aw inoculation (Rybak et al., 2009). We have characterized the non-host response of citrus against rice pathogen (*Xanthomonas oryzae* pv. *oryzae-Xoo*) at the biochemical and proteomic (cellular and extracellular matrix-associated proteome changes) level (Rani and Podile, 2014; Rani et al., 2014). Citrus leaves challenged *with Xoo* showed HR. Citrus extracellular marix-associated proteome analysis indicated accumulation of pathogenesis-related (PR) proteins and jasmonic acid-triggered miraculin-like proteins (Kunitz-type of trypsin inhibitor proteins) during *Xoo* interaction (Rani and Podile, 2014). Further, global citrus leaf proteome changes during host (*Xcc*) and non-host (*Xoo*) pathogen interactions revealed that proteins associated with ROS-metabolism and cell wall strengthening were involved in NHR. Here, we analyzed citrus leaf nuclear proteome changes during host (*Xcc*) and non-host (*Xoo*) pathogen interactions through a high-throughput LC–MS/MS approach at 2, 8, 16 and 48 h post inoculation (hpi). We report that proteins related to signaling and JA-induced defense responses were induced in citrus NHR against *Xoo*.

2. Materials and methods

2.1. Plant material and pathogen inoculations

The procedures followed for maintenance of the host and cultures of pathogenic bacteria, and for pathogen inoculation were described by Rani and Podile (2014). *Citrus sinensis* (L.) Osbeck (sweet orange) grafted on Rangpur lime (*C.* × *limonia* Osbeck) plants obtained from a commercial Government certified nursery (Shiridi Sai Baba Nursery, Sangareddy, Andhra Pradesh, India) were maintained in the green house at 26 °C with a photoperiod of 16 h and controlled relative humidity. For all the experiments new flush of 22–28 d-old leaves were used. *X. citri* pv. *citri* strain 306 *and X. oryzae* pv. *oryzae* strain BXO43 *cultures* were grown in peptone/sucrose broth (20 g/L sucrose, 5 g/L peptone, 0.5 g/L K_2HPO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, pH-7) at 28 °C for 24 h. Log phase cells were harvested. The cell pellet was washed twice with 10 mM $MgCl_2$, and a dilution of optical density (OD_{600}) 0.2 was used for leaf inoculation. *Xcc* or *Xoo* suspensions or 10 mM $MgCl_2$ (as mock) were infiltrated into the abaxial surface of the leaf on both sides of the midrib with a needle less 1 mL tuberculin syringe. Mock-treated and pathogen-challenged (*Xcc* and *Xoo*) leaves of 2, 8, 16 and 48 hpi were collected in biological triplicates and stored at −80 °C until further use.

2.2. Isolation of nuclear proteins

Nuclear proteins were isolated from citrus leaves as described by Pandey et al. (2008). In brief, 20 g of citrus leaves were finely powdered in liquid nitrogen with 0.3% (W/W) polyvinylpyrrolidone and transferred into a beaker containing 200 mL of ice-cold hyperosmotic buffer (10 mM Trizma base, 80 mM KCl, 10 mM EDTA, 1 mM spermidine and 0.5 M sucrose, pH 9.5), 0.15% 2-mercaptoethanol and 0.5% triton X-100 and gently stirred for 30 min. The suspension was filtered through four layers of cheese cloth. Later the homogenate was centrifuged in a fixed-angle rotor at 1800 × *g* at 4 °C for 20 min. The pellet was gently resuspended in 30 mL of ice-cold wash buffer (hyperosmotic buffer without Triton X-100). The resuspended nuclei were filtered through two layers of Miracloth and the nuclei were pelleted by centrifugation at 1800 × *g* at 4 °C for 15 min in a swinging bucket rotor. The pellet was washed twice with ice-cold wash buffer by repeating the above step.

2.3. Assessment of integrity, enrichment and purity of nuclear fraction

The nuclear fraction was stained for 15 min with 0.1 μg/mL 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) in 0.1 M potassium phosphate buffer (pH 7.4) and washed twice with phosphate buffer saline. For microscopy, a small volume of the suspension was placed on a slide and covered with a cover glass. The images were taken with and without a UV filter under a Leica microscope.

To check the enrichment and purity of the nuclear fraction, cytosolic and nuclear protein samples were suspended in SDS sample buffer. Samples were heated at 70 °C for 10 min in the SDS sample buffer to dissolve proteins, separated on a 12.5% polyacrylamide gel with 4.5% stacking gel, and electrophoresis was carried out at 100 V for 4–5 h. To check for enrichment of nuclear proteins, 30 μg of cytosolic and 10 μg of nuclear fraction proteins were run on SDS–PAGE. Protein bands were visualizes by Coomassie staining.

The purity of the nuclear fraction was assessed by protein blot analyses. An equal amount of cytosolic and nuclear proteins was profiled on SDS–PAGE and immunoblotting was performed with rabbit polyclonal FBPase antibody (Agrisera, UK).

2.4. Extraction of nuclear proteins

The nuclear proteins were extracted by using Tri-reagent as per manufacturer's guidelines. The final pellet was resuspended in isoelectric focusing (IEF) sample buffer [8 M urea, 2 M thiourea, and 4% (w/v) CHAPS]. The concentration of protein was determined by the amidoblack method (Henkel and Bieger, 1994).

2.5. Sample preparation for nano-LC–MS/MS analysis

Approximately 5 μg protein in 10 μL of sample buffer was mixed with equal volume of SDS sample buffer (2% [w/v] SDS, 50 mM Tris–HCl [pH 6.8], 6% v/v β-mercaptoethanol, 10% [w/v] glycerol and bromophenol blue) and heated at 95 °C for 20 min to dissolve proteins. Protein samples were separated on 7.5% polyacrylamide pre-cast gels (PAGEL NPU-7.5L; ATTO Corporation, Tokyo, Japan) at 100 V until the upper end of sample dye band enters 2 mm from the well. Gel slices from the well to 2 mm in front of the dye were cut into four equal pieces and in gel tryptic-digestion for nano-LC–MS/MS was performed according to Takahashi et al. (2013). Briefly, gel pieces were fixed in 40% ethanol and 10% acetic acid. Subsequently, gels were dehydrated in acetonitrile and vacuum-dried. Later, the proteins in the gel piece were reduced with 10 mM DTT for 45 min at 56 °C and alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min in the dark at ambient temperature. The gel pieces were sequentially rehydrated and dehydrated with 100 mM NH_4HCO_3 and acetonitrile, respectively. The gels were vacuum-dried and incubated in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/mL trypsin