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Effects of potato spindle tuber viroid infection on tomato metabolic profile

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ABSTRACT

Viroids are the smallest plant pathogens consisting of a single stranded circular RNA molecule with a strong secondary structure, lacking a coat protein or any other proteins. The mechanism of viroid pathogenicity has remained unclear. Recent advances in instrumentation and data mining have made it possible to study the effects of various stresses on primary and secondary metabolisms. Here, we have utilized metabolic profiling approach to show how PSTVd infection alters tomato metabolic profile and the related pathways. Three terminal leaflets of third true leaf of 20-day-old tolerant tomato cultivar 'Moneymaker' were mechanically inoculated by PSTVd intermediate variant cDNAs and samples were taken from eighth leaf, 19 days post-inoculation. Metabolites were extracted and analyzed by gas chromatography/mass spectrometry (GC/MS) and subjected to statistical data analysis. Affected pathways were identified by Pathway Tools program and were compared with microarray data previously reported. The study showed that 79 metabolites changed significantly and 23 pathways were identified in relation to these metabolites. Fourteen of these pathways were similar to those reported in other works. The altered pathways in PSTVd infected tomato leaves included, eight cutin and wax biosynthesis, seven pathways that produce defense related compounds, two energy generator pathways, three hormone biosynthesis pathways, two signal transduction pathways, and one nucleotide biosynthesis pathway. Our data on up/down-regulation of pathways supported the data produced on their corresponding gene(s) up/down-regulation.

1. Introduction

Viroids, are the smallest known pathogens consisting of small circular single-stranded RNAs that express biological functions directly without encoding proteins (Hadidi et al., 2003). A small sequence variation in a viroid genome can affect its biological activity (Tabler and Tsagris, 2004). The stunted growth of PSTVd infected tomato plants results from restricted cell growth but not cell division or differentiation (Qi and Ding, 2003). Growth reduction in citrus induced by infection with citrus exocortis viroid (CEVd) was associated with reduced levels of gibberellin 20-oxidase mRNA (Vidal et al., 2003).

The interaction of circular PSTVd with several different tomato proteins and the ability of RNA polymerase II to interact with PSTVd terminal loops has been demonstrated (Owens and Hammond, 2009). PSTVd infection transcriptionally activates a host kinase gene, *pkv*, in tomato. Infection by severe and intermediate strains of PSTVd causes high level of *pkv* expression while the mild strain induces a lower level of expression of this gene (Hammond and Zhao, 2000).

According to the recent studies viroid infection is associated with the appearance of viroid-specific small RNA with sizes similar to the endogenous small interfering RNA and microRNA and thus might alter the normal gene expression in the host plant. The ability of viroid-derived sRNAs to cleave plant mRNAs via an RNA silencing mechanism has been shown in recent works (Navarro et al., 2012; Avina-Padilla et al., 2015; Adkar-Purushothama et al., 2015). A positive correlation has also been found between the levels of viroid-derived (vd) siRNAs and symptom severity for PSTVd (Itaya et al., 2001). Large majority of vd-siRNAs originated from "hotspots" on the genomic RNA especially in the pathogenicity domain. PSTVd-siRNAs can directly target host mRNAs for RISC-mediated degradation (Itaya et al., 2007) or act indirectly by altering levels of host siRNA metabolism (Martin et al., 2007).

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Metabolomics has allowed relatively rapid and high-throughput detection of a vast range of metabolites, thus providing an in-depth analysis of the total metabolome of biological processes in plants (Bino et al., 2004; López-Gresa et al., 2012).

Some characteristic metabolites of tomato plants infected with CEVd were identified by nuclear magnetic resonance (NMR)-based metabolomics. In this study, glycosylated gentisic acid was the most important metabolite induced in CEVd infected tomatoes and production of small amounts of glucose and malic acid was also stimulated by CEVd infection (López-Gresa et al., 2010). In another study, reduction in apoplast phenolics (such as caffeic acid −4-*O*-b-D-glucoside) and rutin (quercetin-*gluco*-rhamnoside) was observed but in cases higher levels of some metabolites were also observed. Gentisic acid (2, 5-dihydroxybenzoic acid) was shown to be induced in tomato in response to CEVd and tomato mosaic virus (ToMV) (Belles et al., 1999). Oil composition analysis of leaves and fruit peels of CEVd pre-infected citrus determined by GC/MS also showed that the amount of these compounds were reduced in leaves and the variety of these compounds in fruit peel changed significantly (Vekiari et al., 2002). The tomato and pospiviroid PSTVd pathosystems constitutes another model plant-viroid interaction system.

Recently, Bagherian et al. (2014) compared the metabolic profiles of susceptible tomato cultivar 'Rutgers' infected by mild variant of PSTVd with those of mock inoculated plants. They showed that 40 unique metabolites changed significantly over their controls at $P \leq 0.2$, as a result of up/down regulation in 14 metabolic pathways. In the current study, the tolerant tomato cultivar 'Moneymaker' infected by PSTVd intermediate variant was studied at 19 days post-inoculation in order to extend our knowledge on changes of metabolites in plant-viroid interactions. Using the *Solanum lycopersicum*-PSTVd interaction as a basic model system, the role of defense mechanisms involved in pathogenesis was investigated by GC/MS analysis. As part of an ongoing global investigation on the metabolic state of tomato plants under different biotic or abiotic stresses, the main objective of this study was geared to increase our knowledge on the PSTVd infected tomato leaf metabolome. A better understanding of the PSTVd-tomato interaction at the biochemical levlel might also provide relevant data for metabolic engineering of tomato plants with higher resistance toward viroids (López-Gresa et al., 2012).

2. Materials and methods

2.1. cDNA synthesis and inoculation

PSTVd infectious clones used in this study were made according to the method oriented by Gora et al. (1994). A full length (359 base pairs) of PSTVd intermediate variant (accession no.: AY937179) cDNA was synthesized and inserted at *Sma* I restriction site in pUC19 cloning vector by Biomatic Corporation (Cambridge, Ont., Canada). Lyophilized recombinant vector was resuspended in nuclease free water (to have a working stock of 10 ng/μL). For amplification of PSTVd cDNA monomer, ten nano-grams of working stock was subjected to PCR by specific primers (Gora et al., 1994). The amplified cDNAs were separated by electrophoresis in a 1.5% agarose gel in 1 x TAE (Tris-acetate-EDTA) buffer and the full-length product was isolated from the gel using QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's instructions. As excised PSTVd cDNA from vector has higher infectivity than recombinant vector (Tabler and Sänger, 1984), aliquot of recovered PSVd cDNA monomer (2 μg in 10 μL of 20 mM NaHPO4, pH 7) were rubbed on the carborundum-dusted three terminal leaflets of the third true leaf of 20-day-old tomato plants. Negative controls, were rubbed with sample buffer.

2.2. Plant growing conditions

Seeds of tomato (*Solanum lycopersicum* L.) cultivar 'Moneymaker' (Meissner et al., 1997; West Coast Seeds Ltd., Delta, BC, Canada) were planted in 15 cm pots containing peat, perlite, and vermiculite (0.8:1:1), watered regularly and supplied with an all-purpose fertilizer (20-20-20, Plant Products Co. Ltd., Ontario, Canada) once a week. For each negative and positive control, three biological replicates in completely randomized design were considered.

Inoculated plants were kept under controlled greenhouse conditions at 30/25 °C (16 h day/8 h night) with 70% relative humidity under 3500 Lux light for development of disease symptoms. Under these conditions 19 days post-inoculation the eighth leaf (from plant base) of control and infected tomato plants were collected (Owens et al., 2012). Plants were maintained under greenhouse conditions to guarantee high rate of viroid replication and development of symptoms. Leaves were harvested 30 days after inoculation and their veins and surrounding tissues were collected and subjected to RNA extraction and RT-PCR followed by sequencing of PCR product.

2.3. RNA extraction, RT-PCR and sequencing

The veins and surrounding tissues were used for nucleic acid extraction by phenol-chloroform protocol as described by Bagherian et al. (2009). The extracts were subjected to RT-PCR using specific primers p1 (GGGGATCCCTGAAGCGC) and p2 (GGGGAAACCTGGAGCGA) following the method described by Bernad and Duran-Vila (2006). The PCR products were purified using High Pure Plasmid Extraction Kit (Fermentas). Bidirectional sequencing of all samples using specific primers was performed by Tech Dragon Inc. (Hong Kong). Sequences were analyzed and compared to those available in GenBank, using NCBI/BLAST to search for related sequences.

2.4. Metabolite extraction and GC/MS analysis

Metabolite extraction was performed according to the method described by Fiehn et al. (2000) with modifications in the amount of injection and split ratio. Briefly, leaflets of plants (300 ± 30 mg) were crushed in liquid nitrogen to quench any hydrolytic activity, freeze-dried and stored at −80 °C. Freeze-dried tissue was extracted with methanol-water and then with chloroform (Fiehn et al., 2000). Supernatants (polar fraction) were separated from the pellet (non-polar fraction) using Ultrafree-CL 0.22 μm microfilters. The extracts were subsequently methoximated to prevent cyclization of carbonyl moieties in the β-position of reducing sugars, and derivatized with MSTFA. At the outset, 50 μL of ribitol (0.2 mg mL^{-1} in water) and nonadecanoic acid (2 mg mL^{-1} in chloroform) were added to the methanol-water and chloroform fractions, respectively, to serve as internal standards (Hamzehzarghani et al., 2005, 2008a,b).

Aliquots of 0.2 μL of both phases (polar and non-polar) were analyzed separately using Agilent 7000 Triple Quadrupole GC/MS System in splitless mode. Chromatography was performed with a DB-1 capillary column (Non-polar with 100% dimethylpolysiloxane, 0.30 μm film thickness, 0.25 mm in diameter and 24 m in length, Agilent Technologies) with a helium flow rate of 1 mL/min. The injection port temperature was maintained at 230 °C. After 5 min solvent delay time at 70 °C, the column temperature was programmed to rise by 5 °C/min to 310 °C, followed by a 1 min isocratic. The ion source temperature was 200 °C, and mass ions in the range of 45–600 *m*/*z* were scanned at a rate of 1.1 scans/sec.