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Combination of iTRAQ proteomics and RNA-seq transcriptomics reveals jasmonate-related-metabolisms central regulation during the process of Jujube witches' broom recovery by tetracycline treatment

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ABSTRACT

Witches' broom disease, caused by phytoplasma, is currently the most destructive disease of jujube. Tetracycline derivatives have been used to treat witches' broom, and can allow recovery from the phytoplasma infection. Genes related to jasmonic acid (JA) biosynthesis and JA-induced protein-like play roles in phytoplasma infection. Jujube shoots (*Ziziphus jujuba* Mill. 'Huizao') infected by phytoplasma were excised and grown *in vitro* before treatment with tetracycline. RNA-Seq and iTRAQ analyses of samples treated for different lengths of time were performed during recovery from jujube witches' broom (JWB) symptoms and diminishing presence of phytoplasma. Phytoplasma was not detected by PCR in the shoots after 6 months of tetracycline treatment (MTT). RNA-Seq and iTRAQ analyses identified 26,790 genes and 6184 proteins from jujube shoot samples. There were 272 differentially expressed genes (DEGs) and 20 differentially expressed proteins (DEPs) between the 3 and 6 MTT samples, respectively. The largest number and the greatest changes of DEGs and DEPs were for those related to alpha-linolenic acid metabolism, jasmonate biosynthesis, and jasmonate induced protein-like (JIPs). JA content slightly decreased at the sixth month compared with the third month. The research avenues explored here showed that genes in the JA biosynthesis pathway, proteins that respond to JA (JIPs), and JA content itself were concurrently regulated during JWB recovery. Alpha-linolenic acid metabolism pathway had higher RichFactor in comparisons between the 3 and 6 MTT samples during JWB recovery, which suggesting JA played vital roles during JWB recovery. The results in this study will help us to understand the roles JA plays in host-phytoplasma interactions during recovery and infection of JWB.

1. Introduction

Phytoplasma-associated diseases occur worldwide in more than 700 plant species, including several important horticultural, ornamental, and crop plants (Askari et al., 2011). Jujube witches' broom (JWB), caused by '*Candidatus Phytoplasma ziziphi*', is currently the most destructive and devastating disease of jujube in Asia (Lee et al., 2012; Liu et al., 2014). The phytoplasma, transmitted by grafting and insects, induces drastic changes in jujube morphology, such as the witches' broom deformity, phyllody, and virescence.

Phytoplasmas are biotrophic bacterial pathogens that are restricted to the cytoplasm of sieve cells in the plant phloem, which depend on insect vectors for transmission (Sugio et al., 2011; Tomkins et al., 2018). Currently, phytoplasmas cannot be cultured on artificial media nor are there effective methods or chemicals to completely control and kill phytoplasmas. In previous reports, shoot tip culture (Wang and

Abbreviations: JA, jasmonic acid; JWB, jujube witches' broom; MTT, month of tetracycline treatment; DEGs, differentially expressed genes; DEPs, differentially expressed proteins; JIPs, jasmonate induced protein-like; ssDNA, Single strand DNA; DNBs, DNA nanoballs; RCR, rolling circle replication; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SCX, strong cation exchange; TEAB, triethylammonium bicarbonate; KEGG, Kyoto Encyclopedia of Genes and Genomes; LOX, lipoxygenase; OPRs, 12-oxophytodienoic acid reductase; AOC, allene oxide cyclase; JMT, jasmonate *O*-methyltransferase; PLA1/2, phospholipase A1/2

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Valkonen, 2008), cryopreservation (Wang et al., 2015), and treatment with antibiotics (Zaim and Samad, 1995; Askari et al., 2011) were shown to suppress phytoplasma infection in explants. Tetracycline derivatives can be injected into the stem or seeds to treat witches' broom, but only provide temporary recovery from the phytoplasma infection (Zaim and Samad, 1995; Askari et al., 2011). Host responses to the antibiotics and the subsequent interaction between the phytoplasma and the host are completely unknown during this recovery process.

In recent years, most studies have focused on phytoplasma infecting process and compared physiological changes between healthy and infected plants. The results indicate that phytoplasma infection affects photosynthetic activity (Liu et al., 2016), increases secondary metabolites, and reduces the contents of chlorophyll, total soluble sugars, auxin, and jasmonic acid (JA) (Tai et al., 2013; Minato et al., 2014; Ye et al., 2017; Wang et al., 2018; Paolacci et al., 2018). Among of various factors, JA was found to play a vital role during the phytoplasma-infected plants. For instance, in phytoplasma-infected Arabidopsis plants, Secreted AY-WB Protein 11 (SAP11) interferes with JA biosynthesis *via* targeting and destabilizing transcription factors class II TCP (Sugio et al., 2011; Tomkins et al., 2018). Similarly, the phytoplasma-secreted TENGU effector leads to Arabidopsis sterility by down-regulating the JA and auxin pathways (Minato et al., 2014). In contrast, biotrophic pathogens independent of insect vector transmission often produce effectors that activates the JA pathway, and then increase colonization themselves (Tomkins et al., 2018). However, in biotrophic phytoplasma-infected jujube plants (dependent of insect vector transmission), it was also found that JA related metabolisms, including both JA-induced proteins and JA biosynthesis, were activated accompanied by JA increasing during the process of phytoplasma infection in our recent study (Ye et al., 2017; Wang et al., 2018). Moreover, jasmonate contents as well as jasmonate biosynthesis and signaling genes were up-regulated both in phytoplasma- recovered and diseased grape plants compared with the healthy grape plants (Paolacci et al., 2018). Although all the above research suggests that JA regulation plays an important role after phytoplasma infection, it seemed to be different results of JA content in JWB-infected jujube plants compared with other phytoplasma-infected plants. Therefore, it is necessary to known how and when JA regulation occurs, and is it actually up- or down- regulation of JA-related metabolisms during jujube phytoplasma infection?

In order to further reveal roles of JA biosynthesis and signaling during the interaction between jujube and phytoplasma, *in vitro* jujube (*Ziziphus jujuba* Mill. 'Huizao') shoots infected with phytoplasma were treated with tetracycline to heal JWB symptoms, and RNA-Seq and iTRAQ analyses of samples treated for different lengths of time were performed during the plant's recovery from JWB symptoms. This result will contribute to understand JA roles in an inverse method compared with phytoplasma infecting process.

2. Materials and methods

2.1. Plant materials

Phytoplasma-infected shoots with axillary buds were collected as explants and washed under running tap water for half an hour. Explants were then sterilized by immersion in 1% NaClO for 20 min, followed by three rinses with sterile distilled water. Sterilized explants were placed on MS medium containing 30 g L^{-1} sucrose and 6 g L^{-1}agar and adjusted to pH 5.95–6.0 before autoclaving at 118 kPa for 20 min in culture jars. These 3- to 4-leaved shoots, which remained infected with phytoplasma, were subcultured once every four weeks. After three or four subcultures, each single shoot with about 4–6 cm in height and 15 leaves was placed on MS media containing 25 mg/L tetracycline, to suppress phytoplasma growth. The shoots from three jars ((9–12 shoots) were sampled each month during tetracycline treatment until symptoms of JWB completely disappeared and phytoplasma was not detected by PCR. All cultures were maintained in a growth chamber with a 14-h photoperiod provided by cool-white fluorescent tubes (30 μmol m^{-2} s^{-1} photon flux density) at a temperature of 25 ± 1 °C.

2.2. PCR analysis

Each PCR sample was composed of all explants (9–12 shoots) from three different jars. Three PCR samples were prepared for each treatment (with or without tetracycline) at each sampling date (0, 3, and 6 months of tetracycline treatment, MTT). DNA was extracted from *in vitro* shoots using Trizol (DP441, TianGen, China). Primers targeting the 16S rRNA (F1: CGCTAAAGTCCCCACCATTA, R1: CACATTGGGACTGA GACACG) were used to detect JWB phytoplasma (Ye et al., 2017). PCR reactions contained 1 μL primer mix (0.5 μM each), 2 μL DNA (100 ng), 10 μL PCR Master Mix (TaKaRa, Dalian, China), and 12 μL purified ddH_2O in a total volume of 25 μL. PCR reactions were conducted using the following procedure: 94 °C for 6 min, followed by denaturation for 45 s at 94 °C with 35 cycles, annealing for 45 s at 56 °C, extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min. PCR products were detected using 1.5% agarose gel electrophoresis.

2.3. RNA-seq on BGISEQ-500 platform

To obtain gene and protein expression information of jujube plantlets during the process of recovery, six plantlet samples at two time points (3 MTT: positive PCR detection of phytoplasma with three-month tetracycline treatment, weaker JWB symptom; 6 MTT: negative PCR detection of phytoplasma with six month tetracycline treatment, no detected JWB symptom) were collected. Samples from 3 jars of *in vitro* shoots after 3, and 6 months of tetracycline treatment (MTT) were prepared for RNA-Seq. These samples were sent to BGI for RNA-Seq using their method. Briefly, total RNA was extracted from samples using the RNAprep Pure Plant Kit (DP441, TianGen, China). The poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads and fragmented into small pieces using divalent cations. The cleaved RNA fragments were transcribed into cDNA using reverse transcriptase and random N6 primers. These cDNA fragments have an addition of a single 'A' base and subsequent ligation of an adapter. The products were purified and enriched with PCR amplification. PCR yield was quantified by Qubit Single strand DNA (ssDNA) circles were obtained from pooled samples by heat separation, which yielded the final library. DNA nanoballs (DNBs) were generated from the ssDNA circle by rolling circle replication (RCR) to increase the fluorescent signals generated at the sequencing process. The DNBs were loaded into the patterned nanoarrays, and paired-end reads of 100 bp were processed using the BGISEQ-500 platform (BGI, Wuhan, China).

2.4. Protein extraction and digestion

Explants from three jars as one biological sample were ground into powder in liquid nitrogen, mixed with a lysis buffer (8 M Urea, 40 mM Tris-HCl, pH 8.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA. After placing on ice for 5 min, 10 mM dithiothreitol (DTT) was added. The homogenate was centrifuged at 25,000 *g* and 4 °C for 20 min. The supernatant was incubated at 56 °C for 1 h, and then alkylated with 55 mM iodacetamide (IAM) for 45 min in darkness. After centrifugation at 25,000 *g* and 4 °C for 20 min, the protein was quantified using the Bradford method (Bradford, 1976). The protein quality was checked using SDS-PAGE. Totally six protein samples at 2 time points were extracted.

The protein solution (100 μg) was digested using Trypsin Gold (Promega, Madison, WI, USA) with the protein: trypsin ratio of 40: 1 at 37 °C overnight. After digestion, the peptides were desalted with a Strata X C18 column (Phenomenex, CA, USA) and dried under vacuum centrifugation.