Physiological and Molecular Plant Pathology 86 (2014) 43–48

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Identification of genes associated with phytoplasma resistance through suppressive subtraction hybridization in Chinese jujube

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ARTICLE INFO

Article history:
Accepted 3 March 2014

Keywords:
Chinese jujube
Jujube witches' broom disease
Phytoplasma
Suppression subtractive hybridization

ABSTRACT

Jujube witches' broom (JWB) is a destructive disease for Chinese jujube caused by phytoplasma. A suppression subtractive hybridization library of resistant cultivar 'Xingguang' was constructed under phytoplasma stress to identify genes related to JWB resistance. 77 of 200 unique expressed sequence tags had significant sequence homologies and were classified into 10 functional groups. The most abundant group was disease/defense (20.8%), which was consistent with the phytoplasma stress. These differentially expressed genes provide the groundwork for addressing the plant–phytoplasma interaction. Meanwhile, the expression of five selected genes (*TLP*, *PR10*, *HSP70*, *ERF*, *kinase-related protein*) was confirmed to upregulate at different infection periods.

1. Introduction

Chinese jujube (*Ziziphus jujuba* Mill.), a native fruit tree of China, ranks the first among the dry fruits in terms of production. Jujube witches' broom (JWB), caused by phytoplasma, is the most serious and destructive disease for jujube tree cultivation in Asian. Phytoplasma causes various symptoms, including yellowing, phyllody (floral organs becoming leaf-like), small leaves and witches'-broom in Chinese jujube. The disease leads to tree death within a few years after the first appearance of symptoms [1]. Phytoplasmas are important agricultural pathogens [2], have been identified in over 1000 plant species worldwide, and are responsible for plant diseases which result in significant economic impact [3].

The plant response to phytoplasma infection has been studied mainly at the metabolite level, which has shown that phytoplasmas co-opt the activities of plant enzymes involved in carbohydrate metabolism for their own energy and growth requirements, and that they are spread by using the host plant's phloem system [4–7]. Recently, physiological and biochemical analyses in lime plants infected with '*Ca.* Phytoplasma aurantifolia' have demonstrated that phytoplasmas caused decreases in the contents of soluble proteins, pigments, soluble sugars and auxin, as well as increases in starch content and activities of antioxidant enzymes in leaves [8].

The molecular mechanisms involved in the phytoplasma–plant interaction have been examined in a limited number of studies [9]. Only a few studies have been conducted to identify genes that are differentially expressed during the interaction of phytoplasmas with host plants [10]. However, to our knowledge there is no information reported on the differential gene expression in resistant materials under phytoplasma stress and no studies were carried out on the differential gene expression in Chinese jujube infected with phytoplasma.

Z. jujuba 'Xingguang', bred and released by our research team, is an excellent cultivar with very high resistance to JWB [11]. In the field, it always kept healthy growth after grafted onto different cultivars with JWB. Seriously diseased jujube trees could recover to set fruits normally by crown reconstruction via top-grafted with 'Xingguang' scions. In this study, a suppression subtractive hybridization (SSH) library of 'Xingguang' was constructed under phytoplasma stress for identifying JWB resistance-related genes which will enhance our understanding of the molecular mechanisms of plant resistance to phytoplasma.

2. Materials and methods

2.1. Plant materials

The scions of 'Xingguang' were grafted on three healthy jujube trees (non-infected) and another three trees with JWB disease

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http://dx.doi.org/10.1016/j.pmpp.2014.03.001
0885-5765/

Abbreviations

JWB	jujube witches' broom
SSH	suppression subtraction hybridization
CK	control check
DAGI	days after grafting inoculation
ESTs	expressed sequenced tags
AP2	APETALA2
rRT-PCR	relative reverse-transcription polymerase chain reaction
TLP	thaumatin-like protein
ERF	ethylene response factor
EREBP	ethylene-responsive element binding protein
PR	pathogenesis-related
bZIP	basic region/leucine zipper
HSP	heat shock protein
MAPK	mitogen-activated protein kinase
PTO	*Pseudomonas* tomato resistance
PTI	PTO-interacting

(infected), respectively. Susceptible cultivar 'Pozao' was used as control check (CK). The root stock cultivar was 'Zanhuangdazao'. All grafting treatments were conducted with three replicates in each tree. The fresh leaves of treatments (both non-infected and infected) were collected at five stages (40 days, 60 days, 75 days, 95 days and 115 days after grafting inoculation (DAGI)), then frozen by liquid nitrogen rapidly and kept at −80 °C.

2.2. Extraction of DNA and detection of phytoplasma

Total DNA of leaves was extracted by modified CTAB method [12]. And then the P1/P7 PCR method [13] was used to identify the JWB phytoplasma in those leaves at different stages after grafting.

2.3. Extraction of total RNA and construction of SSH library

Total RNA was extracted by modified CTAB method [14]. A SMART™ PCR cDNA Synthesis Kit (Clontech, USA) was used in the synthesis of double-stranded cDNA according to the manufacturer's instructions. Mixed cDNA of five stages from infected 'Xingguang' was used as the 'tester', and that from non-infected 'Xingguang' was used as 'driver'. A forward subtracted library was constructed using the PCR-selected cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's instructions.

2.4. Identification of the insert size of cDNA by PCR amplification

PCR amplification was performed with the M13 primer provided by the Kit. The length of subtractive cDNA fragment was identified by electrophoresis on 1.0% agarose gel.

2.5. Sequencing of the positive clones and analysis of the sequences

Plasmid DNA was extracted (San Prep Column Plasmid Mini-Preps Kit, SANGON) from 470 recombinant colonies and sequenced by Shanghai Sangon bio-company. The DNA sequences were edited to remove the vector and adaptor sequences and then checked for redundancy using the DNAMAN (LynnonBiosoft, USA). All unique expressed sequenced tags (ESTs) were annotated according to NCBI (http://www.ncbi.nlm.nih.gov) using BLASTN program.

2.6. Gene expression analysis by relative reverse transcription polymerase chain reaction (rRT-PCR)

Total RNA extracted from infected and non-infected 'Xingguang' leaves (each 0.2 g) at 40 d, 60 d, 75 d, 95 d and 115 d after grafting were used to synthesize cDNA by the SMART PCR cDNA Synthesis Kit (Clontech). Leaf samples of different phases were collected respectively from three trees. Five differentially expressed genes, included kinase-related protein, ethylene response factor (*ERF*), thaumatin-like protein (*TLP*), pathogenesis-related 10 (*PR10*) and heat shock protein 70 (*HSP70*), were analyzed by rRT-PCR. Quantification of the rRT-PCR product was performed as described by Dean et al. [15]. The gene-specific primers used in this study were designed by Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA), the ESTs name and primers sequences listed in Table 1. *ZjHis3* was used as an internal standard [16]. The rRT-PCR of each gene was repeated three times, and the PCR products were resolved by 2.0% agarose gel. The band was determined with the Doc-it gel image system (UVP, LLC, Upland, CA).

3. Results

3.1. The symptom of 'Xingguang' and 'Pozao' under JWB phytoplasma stress and detection of phytoplasma

'Xingguang' and 'Pozao' scions sprouted 30 d after grafted on rootstocks. The JWB symptoms of yellowing, small leaves and witches'-broom in susceptible cultivar 'Pozao' were observed at 60 DAGI by the diseased rootstock (Fig. 1a). But the resistant cultivar 'Xingguang' scions only showed symptom slightly at initial stage after grafting inoculation and then reversed to normal growth (Fig. 1a). Moreover JWB phytoplasma presence was also verified by PCR method in those 'Xingguang' scions which grafted on diseased trees only at first two stages i.e. 40 and 60 DAGI (Fig. 2a). The JWB phytoplasma in infected 'Xingguang' scions was suppressed and eliminated eventually. In contrast to 'Xingguang', the specific band of JWB phytoplasma was found in infected 'Pozao' scions at all five stages (Fig. 2b). 'Xingguang' and 'Pozao' scions which grafted on the healthy jujube rootstock always behaved healthy (Fig. 1b) and no DNA fragments of the phytoplasma were detected (Fig. 2a and b).

3.2. Construction of suppression subtracted cDNA library and identification of insert size

Total RNAs from the non-infected (driver) and the infected 'Xingguang' (tester) scions were used to construct the SSH library. Finally, 1300 clones were obtained and 500 clones were randomly selected from the library to detect insert size by PCR with the M13 primer. 470 positive clones carrying single exogenous fragment (94.0%) were detected and the length of the inserted fragments ranged from 200 bp to 1000 bp (Fig. 3).

3.3. Analysis of EST sequences

200 unigenes were obtained from above 470 positive clones sequenced in the library. Of those, 77 ESTs (38.5%) were homologous to known genes and other 123 ESTs (61.5%) were homologous to genes with unknown function or without matches in the database (Fig. 4). By consulting several articles in the past few years (Liu and Jiang 2010; Fu et al., 2005; Zhang et al., 2001), these highly expressed 77 ESTs in resistance process to JWB were grouped into 10 functional categories (Table 2). The most abundant group with known function in the library was disease/defense (20.8%), followed by transcription (7.0%) and metabolism (7.0%). Those disease/defense genes such as *Peroxidase*, *Thaumatin-like protein, PR10 and*