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Phytoplasma adapt to the diverse environments of their plant and insect hosts by altering gene expression

Olga Makarova [a, 1], Allyson M. MacLean [b, 2], Mogens Nicolaisen [a, *]

[a] Department of Agroecology, Faculty of Science and Technology, Aarhus University, Forsøgsvej 1, Slagelse, DK-4200, Denmark
[b] Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, United Kingdom

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ABSTRACT

Phytoplasmas are intracellular insect-transmitted phytopathogenic bacteria with small genomes. To understand how Aster Yellows phytoplasma strain witches' broom (AY-WB) adapts to their hosts, we performed qRT-PCR analysis of 179 *in silico* functionally annotated AY-WB genes that are likely to have a role in host adaptation. 74 genes were up-regulated in insects and included genes involved in stress response, phospholipid synthesis, malate and pyruvate metabolism, hemolysin and transporter genes, multiple copies of thymidylate kinase, sigma factor and Zn-proteases genes. In plants, 34 genes encoding an immune dominant membrane protein, membrane-associated proteins, and multidrug resistance ABC-type transporters, were up-regulated. Differential regulation of gene expression thus appears to play an important role in host adaptation of phytoplasmas.

1. Introduction

Phytoplasmas are plant pathogenic cell-wall-less bacteria of the class *Mollicutes* that are responsible for causing considerable damage to crop production worldwide [1]. These phytopathogens are transmitted from plant to plant by sap-feeding insect vectors such as leafhoppers, psyllids, and planthoppers. Phytoplasmas reside in the phloem cells of infected plants and are acquired by insect vectors feeding from the plant sap. Following ingestion and entry into the insect midgut, the bacteria traverse the gut epithelium to enter the haemolymph, and colonize the salivary glands from where they are introduced with saliva to new plant hosts [2]. The ability to successfully colonize and replicate in organisms from two different kingdoms (Plantae and Animalia) is a remarkable trait that makes these bacteria of particular interest for studying the mechanisms involved in host adaptation. The genome sequences of several phytoplasma strains are currently available and have contributed significantly to our understanding of phytoplasma biology [3–7]. Analysis of those genomes has revealed that phytoplasmas lack intact metabolic pathways involved in the biosynthesis of various fatty acids, sterols, amino acids, and nucleotides as well as genes encoding various TCA cycle enzymes. Phytoplasma genomes likewise lack genes encoding F_0F_1-type ATP synthase genes, and a canonical phosphotransferase system (PTS), which facilitates the import of sugars in most other bacteria. Phytoplasma genomes do encode a variety of (mostly ABC-type) transport genes, suggesting that these pathogens rely heavily upon nutrients and metabolites extracted from the host cell. Considering their limited metabolic capacity, it is remarkable that they are able to colonize two highly dissimilar host environments.

A first attempt to systematically assess molecular events during phytoplasma host colonization was initiated by a proteome analysis of mulberry dwarf phytoplasma isolated from infected mulberry plants [8], followed by a microarray study of the OY-M phytoplasma gene expression in plants and insects [9]. Both studies confirmed the activity of fully operational cellular pathways involved in DNA replication, transcription, and translation. Interestingly, an analysis of gene expression data in OY-M revealed a distinct transcriptional shift in response to host switching [9], indicating that the phytoplasma transcriptome is greatly influenced by host background.

Although high-throughput, hybridization-based methods such as microarrays are inherently limited by cross-hybridization, variable incorporation rate of fluorescent dyes and issues with background and saturation signals. These experiments therefore require sophisticated normalization methods and allow only for a limited

* Corresponding author.
E-mail address: mn@agro.au.dk (M. Nicolaisen).
[1] Present address: Institute for Biology, Freie Universität Berlin, Königin-Luise-Strasse 1–3, 14195, Berlin, Germany.
[2] Present address: Boyce Thompson Institute for Plant Research, 533 Tower Road, Ithaca, NY, 14853-1801, USA.

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dynamic range of detection. The latter limitation is especially relevant for gene expression quantification in phytoplasmas considering that their RNA preparations contain large portions of host RNA and that bacterial RNA is known to be much less stable than eukaryotic RNA [10]. Most of these limitations can be overcome by using qRT-PCR. Indeed, qRT-PCR has been shown to be one of the most sensitive techniques for quantification of mRNA [11].

AY-WB is transmitted by the polyphagous leafhopper *Macrosteles quadrilineautus* and is capable of infecting many plant species, including lettuce, aster, periwinkle, and the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*. When infected by AY-WB, plants exhibit symptoms such as yellowing, stunting, phyllody and witches' broom [12,13]. AY-WB phytoplasma belongs to *Candidatus* Phytoplasma asteris, the largest known group of phytoplasmas that are associated with more than 100 economically important diseases of plants [14]. The small genome of AY-WB has been shaped by reductive evolution and encodes potential mobile units (PMUs) [4] and a large repertoire of effector proteins [15]. PMU1 has been shown to excise from the AY-WB genome to form a high copy-number circular plasmid in bacteria that colonize the insect host, and expression of PMU1 genes is up-regulated in the infected insects [16]. These observations suggest that this genetic element contributes towards AY-WB adaptation to its leafhopper vector [16]. Further experimental characterization of AY-WB has allowed the identification of candidate effector proteins, of which most were differentially expressed in the plant or insect host [13]. Two of these, SAP11 and SAP54, have been shown to modulate plant host morphology and development [13,17,18]. Moreover AY-WB infection of a plant host can have a positive impact on insect vector fitness via the down-regulation of jasmonate-dependent plant defense mechanisms by the AY-WB effector protein SAP11 [17]. These findings recommend AY-WB as a highly successful pathogen that is well adapted to its plant and insect hosts. On this basis, and for other reasons (*i.e.* availability of a genome sequence and a broad plant host range that includes Arabidopsis), AY-WB is an attractive model organism for studying host adaptation of phytoplasmas.

In this study, we hypothesized that phytoplasmas possess diverse mechanisms that help the pathogen adapt to the various environments encountered within its hosts. Herein, we report the results of a large-scale screen of AY-WB gene expression by qRT-PCR in plant and insect hosts. We focused on those genes that were most likely to be involved in metabolism and interactions with the environment. These results demonstrate that AY-WB responds to host switching by a distinct shift in gene expression patterns.

2. Materials and methods

2.1. Biological material

AY-WB was originally isolated from infected lettuce in Celeryville, OH in 1998 [12]. The phytoplasma isolate was maintained in its insect host *Macrosteles quadrilineatus* L. (Hemiptera: *Cicadellidae)* and in *Callistephus chinensis* (Asterales: *Asteraceae*) in a greenhouse at 23 °C, with a 16/8 h light/dark cycle. The presence of AY-WB phytoplasma in China aster and in the insect vector was confirmed by TaqMan qPCR as described previously [19]. Three insect (*M. quadrilineatus*) and five plant (*A. thaliana*) biological replicates were used in the experiment. To generate infected insect material, three independent populations of insects were established by maintaining non-infected insects on AY-WB-infected China aster for 4 weeks. Insects from each population were collected in 20 ml plastic containers, filling approximately ¼ of the container, thus, each containing approximately 300 individuals. This pool of insects was used for RNA extraction. Plant biological replicates comprised the above ground vegetative portion (scion) of individual plants. To produce infected plant material, five 3-week-old Arabidopsis (Col-0) plants were exposed to AY-WB-infected *M. quadrilineatus* for one week (two adult males were added per plant), and tissues were harvested four weeks following exposure to insects. Controls consisted of uninfected *M. quadrilineatus* adult leafhoppers and 3-week-old Arabidopsis plants that were exposed to uninfected leafhoppers for a one-week period. Arabidopsis plants were grown in a growth chamber under a short-day photoperiod (10/14 h light/dark cycle) at 22 °C. All biological material was immediately frozen in liquid nitrogen upon collection and stored at −80 °C.

2.2. Primers

Fourteen functional COG (Clusters of Orthologous Groups of proteins) [20] categories (Fig. 1) and a group of twenty predicted SAMPs (Secreted AY-WB Membrane Proteins) [15] that were likely to include genes involved in metabolism and interactions with the environment were selected for the analysis (Table S1a and b). Full length nucleotide sequences were retrieved from the NCBI GenBank-deposited AY-WB complete genome sequence [4] (GenBank accession number CP000061.1). Primers were designed for all genes present in each group using the Primer Express software (Applied Biosystems, Foster City, CA) to generate PCR products between 50 and 80 bp. To optimize gene assays, primer pairs were checked for secondary structure formation and for regions containing low sequence complexity. To ensure that each primer pair would amplify one unique gene target, a BLAST search was performed against the AY-WB phytoplasma genome. Primer sequences and information on primer specificity are summarized in Supplementary Table S2.

2.3. RNA extraction, DNAseI treatment, and cDNA synthesis

The frozen material (plant tissue or pooled insects) was placed in 20 ml pre-cooled plastic containers along with eight 4 mm steel balls and disrupted in a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ) homogenizer as follows: 1200 strokes per minute for 30 s, repeated 6 times. Total RNA was extracted from 50 mg of the homogenized material with TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, ethanol precipitated overnight at −20 °C, and dissolved in 10 μl of RNase-free water. The integrity, concentration, and purity of RNA were assessed on a 1% EtBr-stained agarose gel visualized under UV light and on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA samples were treated with *DNaseI* (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. All RNA samples were tested for presence of contaminating DNA by running a PCR reaction with primers for *AYWB_254* gene without a reverse transcription step. In a few instances, it was considered necessary to repeat the *DNaseI* treatment to eliminate genomic DNA. One microgram of total RNA per reaction was used for cDNA synthesis using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Plant and insect cDNA samples were diluted in RNase-free water five and two times, respectively.

2.4. qPCR

Two μl of template cDNA, 1x Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 10 mM of each primer were mixed together in a total volume of 15 μl, and PCR reactions were run as follows: 50 °C 2′/95 °C 10′/40 × (95 °C 15”/60 °C 1′) using an ABI PRISM 7900HT sequence detection system (Applied