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Use of quantitative real time PCR for a genome-wide study of AYWB phytoplasma gene expression in plant and insect hosts

Olga MAKAROVA[1], Allyson M. MACLEAN[2], Saskia A. HOGENHOUT[2], Mogens NICOLAISEN[1]
[1]*Department of Agroecology, Faculty of Science and Technology, Aarhus University, Slagelse, DK-4200, Denmark*
[2]*Department of Disease and Stress Biology, The John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK*

Abstract

Phytoplasmas are obligate parasites of plants and insects and cause significant crop yield losses worldwide. A number of microarray gene expression studies have been performed to understand better the effects of phytoplasma infection on plant physiology. However, little effort has been made for the study of changes in gene expression patterns of the pathogen itself. Quantitative real time PCR in combination with the delta delta Ct method has been shown to be a relatively inexpensive and simple alternative to microarrays. We employed this method to explore whether it is possible to apply this technique for reliable gene expression quantification of phytoplasmas on a large scale. In our experimental setup, 242 genes of aster yellows phytoplasma strain witches' broom (AY-WB) were tested for differences in expression in plant and insect host environments, and were shown to be predominantly expressed in the plant or insect hosts. *In silico* operon prediction corroborated the experimental data. Our findings suggest that the delta delta Ct method can be used to study the physiology of this pathogen.

Key words: AY-WB, plant, insect vector, gene expression, relative quantification.

Introduction

Phytoplasmas are plant pathogenic bacteria that are transmitted by sap-feeding leafhopper vectors. Due to the economic impact phytoplasmas incur via crop loss, there is an increased interest in studying the effects of the pathogen on its plant hosts. A number of gene expression and protein profiling studies were performed on agronomically important crops severely affected by phytoplasma diseases, including grapevine (Margaria and Palmano, 2011). However, there are few reports of gene expression profiling of the pathogen itself (Ji *et al*., 2010), reflecting the major difficulties in molecular characterization of this intracellular pathogen. Indeed, phytoplasmas are obligate parasites, which means that RNA preparations contain large portions of host nucleic acids, and hence it is required to utilize highly sensitive methods to quantify phytoplasma transcript levels.

Reverse transcription coupled with real-time PCR (quantitative real-time PCR, qRTPCR) has been shown to be the most sensitive technique for quantification of mRNA, especially for low-abundance transcripts or tissues with low RNA concentrations (Pfaffl *et al*., 2002). There are two major approaches to gene expression quantification. The absolute quantification method is considered to be a "golden standard" of gene quantification, however, it requires use of calibration curves with a template of known concentration, which is both laborious and costly. The relative quantification method allows avoidance of the use of standards, as it relies on the relative quantification of a target gene versus normalization genes, in which the latter are constitutively expressed genes involved in the general metabolism of the organism.

Here we report that the relative delta delta (dd)Ct method can overcome many problems encountered in phytoplasma gene expression quantification. Considering that the phytoplasma genomes are relatively small, the ddCt method may be successfully applied for studying genome-wide gene expression of phytoplasmas in various hosts and over time.

Materials and methods

Aster yellows phytoplasma strain witches' broom (AY-WB) – infected *Macrosteles quadrilineatus* and *Arabidopsis thaliana* were used for this experiment. Healthy *M. quadrilineatus* and *A. thaliana* were used as negative controls. Total RNA was extracted with TRIzol reagent (Carlsbad, CA, USA), treated with DNaseI (Invitrogen) and used for cDNA synthesis (High Capacity RNA-to-cDNA Kit, Applied Biosystems) according to the manufacturers' protocols. To check for efficiency of genomic DNA removal, we included a control in which reverse transcription was omitted. Power SYBR Green Master Mix (Applied Biosystems) and ABI PRISM 7900HT sequence detection system (Applied Biosystems) were used for running qRT-PCR reactions. Primers were designed with Primer Express software (Applied Biosystems).

Two technical replicates were run for each biological sample (5 *Arabidopsis* and 3 populations of *M. quadrilineatus*) and results were expressed as threshold cycle (Ct) values. Replicative DNA helicase *dnaB*, DNA gyrase subunit A *gyrA*, pyruvate kinase *pykF*, 6-phophofructokinase *pfkA* and docking protein *FtsY* were used as controls for normalization. The ddCt method (Applied Biosystems, User Bulletin 2, 1997) implemented in the REST 2009 software (QIAGEN and Pfaffl) was used for data analysis. OperonDB online tool (Ermolaeva *et al*., 2001) was used for operon prediction.

Results

To study whether a relative gene expression quantification method could be applied to phytoplasmas in a high throughput manner, an AY-WB pathosystem was used. AY-WB phytoplasma has a unique life cycle that includes both plant and insect hosts. We hypothesised that the adaptation to such different habitats would be reflected in gene expression in the respective hosts, which in turn could be detected by the ddCt method.

Hence, 242 annotated AY-WB genes belonging to different COG groups, and representing 36% of the total number of AY-WB phytoplasma ORFs were selected for the study. These genes also included 56 effector and 20 secreted membrane proteins, which were believed to be potentially involved in phytoplasma – host interactions (Bai *et al*., 2009). To ensure that each primer pair would amplify only one target we performed a search for homologs for each gene and designed the primers in a gene-specific manner. No amplification was found in healthy samples. Dissociation curves were run after completion of PCR and presence of a single product for each gene assay was confirmed.

Additionally, to increase reliability of the data analysis, amplification of serial dilutions for each gene was performed. REST (Pfaffl *et al*., 2002) software was used for statistical data analysis. This tool performs sophisticated data analysis, which allows the use of multiple normalization genes and can correct for differences in amplifications efficiencies if standard curve data are available.

The majority of the 242 AY-WB genes tested yielded relative expression values in plant versus insects or insects versus plants between >1 and 3 (not considered significantly regulated), while 47 genes (or 19% of all genes tested) had a significant level of up-regulation, ranging from relative expression values of 3 to 29. These highly expressed genes included members of the COG functional groups: energy production and conversion; nucleotide transport and metabolism; transcription; and posttranslational modification. Several effector and secreted membrane protein genes were also highly differentially expressed.

Discussion

Phytoplasmas are intracellular parasites, unable to grow on an artificial media. This limitation has prevented a rapid progress in understanding the pathogen's biology. However, recent advances in sequencing technology allowed the completion of genome sequencing projects of four phytoplasmas, including AY-WB (Bai *et al*., 2006). This prepared a framework for functional large-scale studies of this particular phytoplasma. However, there are a few obvious problems encountered during phytoplasma gene expression quantification. Inability to determine pathogen's titre in a high host RNA background makes it impossible to use absolute RT-PCR quantification and the normalization of microarray data is difficult. Relative quantification (ddCt method) overcomes these problems. Our results show that there are differences in expression of AYWB phytoplasma genes in plant and insect hosts, suggesting that this method can be robustly used for study phytoplasma physiology and host adaptation. The experimental data were corroborated by *in silico* operon prediction, as genes predicted to belong to the same operon exhibited similar levels of expression. This method of gene expression analysis opens new possibilities for studying biology of this pathogen on a large scale, for example by comparing phytoplasma gene expression between different plant hosts, different tissues of the same plant or by performing genome-wide gene expression studies.

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References

BAI X., ZHANG J., EWING A., MILLER S. A., RADEK A. J., SHEVCHENKO D. V., TSUKERMAN K., WALUNAS T., LAPIDUS A., CAMPBELL J. W., HOGENHOUT S. A., 2006.- Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts.– *Journal of Bacteriology*, 188(10): 3682-3696.

BAI X., CORREA V., TORUÑO T., AMMAR E. D., KAMOUN S., HOGENHOUT S. A., 2009.- AY-WB phytoplasma secretes a protein that targets plant cell nuclei.– *Molecular Plant-Microbe Interactions*, 22(1): 18-30.

ERMOLAEVA M., WHITE O., SALZBERG S., 2001.- Prediction of operons in microbial genomes.- *Nucleic Acids Research*, 29: 1216-1221.

JI X., GAI Y., LU B., ZHENG C., MU Z., 2010.- Shotgun proteomic analysis of mulberry dwarf phytoplasma.- *Proteome Science*, 8: 20.

MARGARIA P., PALMANO S., 2011.- Response of the *Vitis vinifera* L. cv. 'Nebbiolo' proteome to flavescence dorée phytoplasma infection.- *Proteomics*, 11(2): 212-224.

PFAFFL M. W, HORGAN G., DEMPFLE L., 2002.- Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.- *Nucleic Acids Research*, 30(9): e36.

Corresponding author: Olga MAKAROVA (e-mail: olga.makarova@agrsci.dk), Department of Agroecology, Faculty of Science and Technology, Aarhus University, Forsoegsvej 1, Slagelse, DK-4200, Denmark.