New Insights in Plant Response to Viroid Infection by Differential Expression Analysis

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ABSTRACT. Viroids are small circular RNA molecules, 246 to 400 nt long, which infect several crop plants and can cause diseases of economic importance. *Citrus* spp. are the hosts in which the highest number of viroids has been recovered. *Citrus exocortis viroid* (CEVd), causal agent of citrus exocortis disease, is responsible for several losses in citrus crops. Little is known about the molecular and cellular mechanisms by which viroids infect plants and produce symptoms. To deepen this knowledge, changes in the gene-expression profile during the early (pre–symptomatic) and the last (post-symptomatic) stage of Etrog citron infection by *Citrus exocortis viroid* (CEVd) were investigated using a citrus cDNA microarray. MaSigPro analysis was performed, and based on their expression profile along time, genes were divided into five clusters. These results will allow us to have a clearer idea about the changes in the plant transcriptome that are associated with symptom expression and/or with specific plant defense mechanisms.

Citrus exocortis viroid (CEVd) is a well-known pathogen described first in 1972 (5) and which is widespread all over the citrus-growing areas of the world. It is the causal agent of the exocortis disease which causes stunting and bark scaling symptoms in trifoliate orange and its hybrids and specific symptoms on the indicator plant Etrog citron, namely a severe epinasty, stunting or bark cracking (3).

The molecular basis of the interaction between viroids and their host plants are mostly unknown. Furthermore, research on citrus gene expression is still limited, as well as studies on the role of viroids in modulating gene expression of host plants. In order to investigate this aspect and also to identify the genes activated in response to CEVd infection, plants of the indicator host Etrog citron inoculated with a severe CEVd isolate were analyzed using a genome-wide 20K cDNA microarray developed under the Citrus Functional Genomic Project (CFGP; http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) that includes 21,081 putative unigenes of citrus (6).

Moreover, since most of the studies on plant viroid response have always been accomplished by comparing healthy with infected/symptomatic plants, the temporal response of the genes is lost and this makes it difficult to differentiate whether changes reflect symptom expression or are just a consequence of the plant decline. So, the effect of the severe CEVd isolate on gene expression of Etrog citron plants was analyzed during the early (pre-symptomatic) and the last (post-symptomatic) stage of infection, because it is well known that gene expression is not constant, but variable with time, and this change controls the cellular physiology and, ultimately, the phenotype.

Eight plants were each graft-inoculated with two bark pieces from the CEVd-infected source, and the same number of plants were left as healthy controls. Material was collected from infected plants before they showed symptoms, soon after the detection of viroid infection 30 days post-infection (dpi), and after symptom expression approximately 2 mo later (90 dpi). CEVd infection was confirmed by Northern hybridization analysis (7). Total RNA from control and infected plants was extracted using the protocol described by Ancillo et al. (2), amplified, labelled and

hybridized to the microarray (6). Four replicates for each timepoint were analysed using a global reference experimental design as following: a) timepoint 0: healthy plants vs common reference; b) timepoint 1: infected but still asymptomatic plants vs common reference; c) timepoint 2: infected and symptomatic plants vs common reference. Reference consisted of a pool of all the samples included in the experiments.

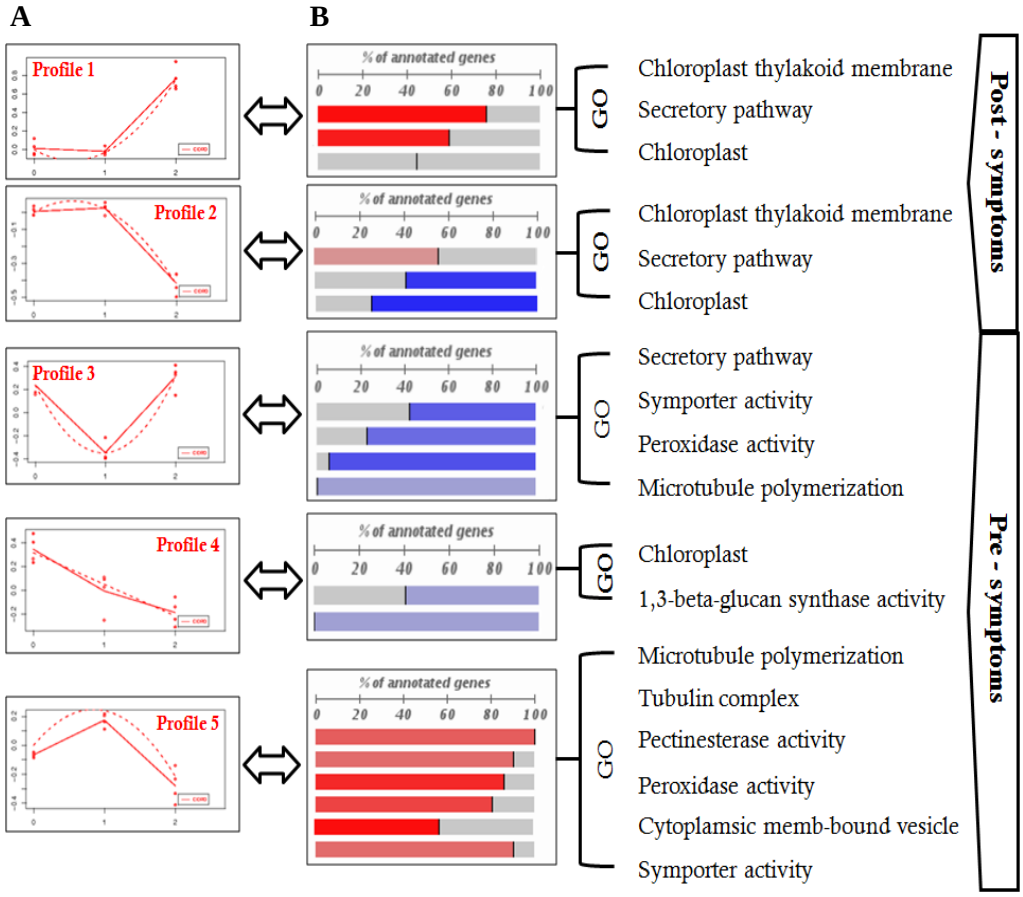

Fig. 1. (A) Gene expression profiles obtained by maSigPro analysis. (B) Fatiscan gene set enrichment results. Shown are significant coordinately expressed GO terms within whole gene sets. The normalized percentage of genes annotated with a specific term is indicated for each group. Red indicates positive correlation to the cluster expression profile and blue the negative one. Color intensity denotes how strongly the term is correlated.

In order to find significant expression profile differences between the experimental groups, MaSigPro analysis was performed (4). Results revealed that 132 genes changed expression over time and, based on their time profiles, genes were divided into five clusters (Fig. 1A). Worthy of note, in the first two clusters, the gene expression level was the same at timepoint 0 and timepoint 1, whereas it changed only at timepoint 2 coinciding with the symptom expression stage, and therefore with the plant decline. On the other hand, genes belonging to the other three clusters started to change their expression level at timepoint 1 which

corresponds to the pre-symptomatic stage. These are probably the most interesting genes considering that to reach the symptomatic stage, genes should start to change expression at an earlier stage.

In order to understand how cellular functions are activated and/or deactivated along the infection course, FatiScan analysis (6) was performed to identify functional categories associated with the specific time profiles. The functional annotation scheme used is the Gene Ontology (GO) which characterizes genes on the basis of their molecular functions, biological processes, and cellular components. Over-represented GO functional terms positively or negatively correlating to the expression profile of the five clusters are shown in Fig. 1B.

Differential expression achieved with microarray analysis was confirmed calculating the relative accumulation of six genes (pyruvate phosphate dikinase chloroplast precursor, putative callose synthase 1 catalytic subunit, auxin transporter protein 1, cell division protein FtsH isolog, peroxidase precursor, xyloglucan transferase) by quantitative real-time RT-PCR, using the SYBR Green assay and the Light-Cycler System (Roche), obtaining similar results.

cDNA microarrays allowed us to determine differentially expressed functional categories in Etrog citron in response to CEVd infection and more interesting, to analyze the evolution of gene expression over time in the pre-symptomatic stage and after symptom expression. The results showed here indicate that chloroplast, chlorophyll, auxin transport, callose and pectin deposition, peroxidase activity and other defense genes are altered, and probably these changes might allow the plants to live through the infection and slow down the disease decline.

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