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A microarray analysis highlights the role of tetrapyrrole pathways in grapevine responses to "stolbur" phytoplasma, phloem virus infections and recovered status

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ABSTRACT

After providing a picture of the global transcriptomic changes of grapevine responses to "stolbur" phytoplasma, the recovery status and molecular responses to the phytoplasma and virus co-presence were analyzed. NimbleGen® *Vitis vinifera* genome arrays were used. Lower transcript abundance of the genes involved in photosynthesis, trehalose, phospholipids was observed in response to the presence of "stolbur" phytoplasma. The expression of the genes involved in tetrapyrrole increased. The recovered plants showed that the transcripts involved in ATP synthesis and amino acid metabolism, secondary metabolism and biotic stress-related pathways increased. Recovery was associated with tetrapyrrole pathway repression. Co-infection with viruses induced the genes involved in the hormone categories (cytokinin, gibberellin, salicylic acid and jasmonates).

1. Introduction

Vitis vinifera is severely affected by "grapevine yellows" (GY) disease, a syndrome associated with the different phytoplasmas related with diseases that are associated with several agricultural crops. They are wall-less prokaryotes that belong to *Mollicutes*, and they survive in phloem plant tissues and insects [1]. The main grapevine phytoplasma diseases are "flavescence dorée" (FD - groups 16SrV–C and 16SrV-D) and "stolbur" (STOL - group 16SrXII-A), the phytoplasma involved in "bois noir" disease (BN) [2]. FD is a quarantine pathogen that is restricted to a number of European grape-producing countries, whereas "bois noir" is distributed worldwide and causes serious epidemics in several susceptible grapevine varieties.

The symptoms of the two phytoplasma diseases are similar and depend on grape varieties, environmental conditions and agronomic practices. Symptoms may appear on the whole plant or are limited to a sector or cane. They consist in downwardly rolled leaves with yellowing in the white-berried varieties, and a purple-reddish coloring in the red-berried varieties. Discolorations may affect some sectors or the whole blade, which becomes thicker and brittle. Internodes may shorten or new leaves are produced in summer months. Berries may ripen unevenly and be dry [3].

Vineyard management against "bois noir" vector *Hyalesthes obsoletus* Signoret is not effective because this insect spends most of its life cycle on the wild plants that grow around vineyards. Different strategies have been followed; e.g. the specific elimination of the host wild plants, infected grapevine eradication and using healthy propagation material.

In some phytoplasma-infected plant hosts, recovery implies the complete remission of symptoms in previously symptomatic plants. This phenomenon is linked with the disappearance of

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phytoplasmas from the crowns of infected trees. Cytochemical analyses have shown that recovery is associated with biochemical changes in the phloem [4]. Recovery has been reported in several grapevine varieties affected by FD and/or BN in different viticultural regions, and depends on environmental conditions, grape varieties, rootstocks and agronomic practices. It can be induced when grapevines are subjected to abiotic stress, such as uprooting plants followed by immediate transplanting, partial uprooting or plant pulling, and also by pruning or pollarding [4]. Although recovered plants show overproduced hydrogen peroxide in phloem tissue [5], the physiological causes of the recovery process have still not been elucidated. "Stolbur" infection negatively regulates key primary pathways, such as photosynthesis, carbohydrate and lipid metabolism [6,7]. It also induces the genes involved in defense mechanisms, represses cell wall degradation and alters the balance of growth regulators [8–10]. The physiological response of grape plants to phytoplasma infection can be strongly modified by co-infection with one virus or more. Viral infections are very common in all grape varieties, and can either induce specific symptoms or correlate with a latent infection state. Some of the commonest viruses that affect grape plants are phloem-limited, which therefore make plant responses to phytoplasma infection more complex and significantly influence host/pathogen interactions.

This paper presents a picture of the global transcriptomic changes for grapevine leaf responses to "stolbur" infection before and after symptoms appear, and in the recovery status and with the co-presence of the phloem virus.

2. Materials and methods

2.1. Plant material and experimental design

The analysis was performed on the grapevine cultivar Montepulciano. Analyzed plants were located in a vineyard in Giulianello (province of Latina, Latium, central Italy; GPS coordinates: 41.683, 12.867), and showed typical yellows symptoms for 4 sequential years (Fig. 1).

From monitoring year 1, two vineyard rows (128 plants) were analyzed by nested-PCR to verify presence of phytoplasma, as described in the literature [11,12]. Absence of "flavescence dorée" (FD) was observed in all the samples. Serological tests (ELISA) were also performed to detect phloem viruses: *Arabis mosaic virus* - ArMV; *Grapevine Leafroll-associated Virus* types 1, 2 and 3 -GRLaV-1, GLRaV-2, GLRaV-3; *Grapevine virus A* - GVA; *Grapevine virus B* - GVB; *Grapevine Fleck virus* - GFkV; and *Grapevine Fanleaf virus* - GFLV.

Fig. 1. Symptoms on "bois noir"-infected 'Montepulciano' plants. Leaves show reddish discoloration and downwardly rolled margins.

During year 1, four symptomatic plants, solely infected by the "stolbur" phytoplasma, were uprooted and replanted to induce recovery [4]. They were classified as recovered because they had never shown any symptoms, and "stolbur" phytoplasma was never detected during a 4-year period. The plant material analyzed herein was collected between the end of August and September and was divided into the following plant categories: healthy (He; no phytoplasma and viruses - three replicates); asymptomatic plants infected only by "stolbur" (Phy AS - two replicates); symptomatic plants infected only by "stolbur" (Phy SY - two replicates); symptomatic plants co-infected by "stolbur and GLRaV-3 and GVA viruses (Phy plus virus - two replicates); recovered plants (Re - two replicates). Each replicate comprised six medial leaves from two branches of three plants. The petioles and leaf midribs of each sample were frozen in liquid nitrogen and maintained at −20 °C.

2.2. Microarray and functional analyses

RNA was extracted with the RNeasy Plant mini kit (Qiagen Inc., Valencia, CA). NimbleGen® *V. vinifera* genome array (Roche NimbleGen® Inc., Madison, WI, USA) was used for the microarray analysis. All the procedures for labeling and microarray hybridizations were performed following the NimbleGen® Inc. kit instructions. To retrieve additional functional annotations, probesets were associated with their homologous *Arabidopsis thaliana* genes. Robust Multiarray Average (RMA) data were analyzed with an additional normalization step (cubic spline) to remove the batch effect, such as the non linear bias between duplicated arrays from different batches. Microarray data were submitted to NCBI's Gene Expression Omnibus (GEO), under accession number GSE52540.

Functional classifications were based on those found in the MapMan software [13]. Differentially expressed genes were viewed using a mapping file constructed for the *Vitis* genome sequence [14]. Differentially regulated genes ($\log_2 > 1$ and $\log_2 < -1$; p-value < 0.05) in each pair-wise comparison were shown in a different color based on a gradient legend of green (down-regulated) and red (up-regulated). The Pageman analysis was run with the list of the differentially regulated genes with a Wilcoxon test (ORA cut-off value = 1).

2.3. Array validation

Quantitative SYBRGreen real-time PCR (qRTPCR) was performed to validate the expression profiles obtained by the NimbleGen® Chip V. vinifera genome array. Five genes were selected from the panel of the differentially expressed genes in the pair-wise comparison: Phy/He SY and Re/He. The housekeeping gene used to normalize gene expression was V. vinifera actin (AF369524). The primers for each assayed gene were designed using the Primer3 software (http://primer3.sourceforge.net/). The Welch two-sample t-test was employed to compare the relative expression ratios of the infected and healthy samples (p-value < 0.05). RNAs were extracted from three replicates from each experimental condition using the RNeasy Plant mini kit (Qiagen Inc., Valencia, CA). Amplifications were performed with 20 ng/μl of RNA extracted by the SensiMixTM one-step kit with ROX (Bioline - UK) according to the manufacturer's instructions. The primer sequences are provided in Table S1. The C_t value of each gene was normalized with actin to obtain the ΔC_t value.