Differential Ampelovirus Multiplication in Plants May Explain its Relative Incidence in the Vineyards

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INTRODUCTION

Grapevine leafroll complex is composed by several species of the genus *Ampelovirus* and one *Closterovirus* (GLRaV-2). Within genus *Ampelovirus*, two of them, GLRaV-1 and GLRaV-3 are apparently more common in vineyards and are also included in the EU legislation as unauthorized in nursery stock. Other ampeloviruses (GLRaV-4, -5, -6, -9,-Pr,-CV, -Car) appear to be divergent variants of a single species, GLRaV-4 (Ghanem-Sabanadzovic *et al*., 2012; Martelli *et al*., 2012). Several GLRaV-4 related ampeloviruses have been identified recently in Spain and their incidence is apparently lower than that of GLRaV-1 and -3. The availability of RT-qPCR analysis for these viruses has allowed us to initiate studies to quantify the genome copies vines to try to correlate the differential concentration of the virus as a possible explanation for the differences in the relative incidence in field. In particular, we have used as model the ampeloviruses GLRaV-3, -4 and –5, present in different grapevine materials.

MATERIAL AND METHODS

Plant material and sampling. Plants of varieties Rome, Tintilla de Rota and Gorgollasa were field collected and canes rooted and kept in pots in an insect proof greenhouse. Sampling for RNA extraction was done in late April 2012.

Isolation of total RNA and cDNA synthesis. Total RNA (from 100 mg of medium leaf petioles) was extracted using Spectrum Plant Plant RNA kit (Sigma). RNA concentration were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to normalize the nucleic acid concentration for subsequent reverse transcriptions. First-strand cDNA synthesis in RT reactions was carried out using 200 ng of RNA, random nonamers (Takara) and Mu-MLV reverse transcriptase and following the manufacturer's instructions (Eurogentec).

Real-time PCR. Primers for the amplification of GLRAV-4 and GLRaV-5 RNA dependent RNA polymerase (*RdRp*) genes were designed using Primer3 on-line software tool. For GLRaV-4: LR4-RP_F2: GGCAGTGGAATTGGAAGTGT / LR4-RP_R2: CTGCACCTGTCCTCCTTTGT; and for GLRaV-5: LR5-RP_F1: ATCGAAATCTTGGCATCCAG / LR5-RP_R1: TCTCAGCTTT AGCTGCGTCA. For GLRaV-3 *RdRp* amplification we used primers LR3qrtF and LR3qrtR (Tsai *et al.,* 2011). For the internal control we used the specific primers for *Actin* gene amplification (Gutha *et al*., 2010). qPCR experiments were performed in white 96-well PCR plates using a Bio-Rad iQ5 Thermal cycler. One μL of cDNA template from each RT reaction were added to 10 μL of KAPA SYBR Green qPCR mix (KAPA Biosystems, Cape Town, South Africa), 500 nM of each pair of primers and sterile water to complete a final reaction volume of 20 μL. Samples were subjected to the following conditions: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 45 s. All qPCR assays were performed in duplicate. Specificity of the amplification products obtained were determined with software package Bio-Rad Optical System Software v.2.1 by melting-curve analysis of 60 s at 95°C, 60 s at 55°C, followed by fluorescence reading at 0.5 °C increments from 55 to 95°C.

Standard curves generation. Partial GLRaV-3, GLRaV-4 and GLRaV-5 *RdRp* genes were amplified for standard curves generation. A 1004 bp amplicon of GLRaV-4 encompassing partial *RdRp* gene was obtained with primers LR4-RP_T7F3: (T7promoter)-CTTTAGGGAGTGCTGGGTCA and LR4-RP_R1: GTATTGGCTGCACCTGTCCT, while a 936 amplicon was obtained for GLRaV-5 *RdRp* gene with primers LR5-RP_T7F2: (T7promoter)-CTGGTTTGATTGACGGTGTG and LR5-RP_R3: GCTGCCCAAGTGTCCAGTAT. Similarly was

obtained the GLRaV-3 *RdRp* amplicon following Tsai and coll. (2011). For RNA synthesis PCR amplicons were purified and used as templates using T7 RNA polymerase (MAXIscript SP6/T7 Kit, Invitrogen) according to the manufacturer instructions. Synthetic RNA obtained was quantified and serial diluted in virus free Sugar Seedless genomic RNA for reverse transcription and qPCR standard curve generation. Taking into account the molecular weight of the synthetic RNAs it was possible to calculate the number of genomic copies for each dilution and allowed to interpolate the GLRaV-3, GLRaV-4 or GLRaV-5 genome copy number for each sample.

RESULTS AND DISCUSSION

After obtaining standard curves from *in vitro* synthesized RNA of GLRaV-3, -4 and -5 *RdRp* genes we have calculated the relative and absolute concentration of viruses in leaf petioles. The efficiency of the amplification was determined for each primer pair and target resulting in 99.7% for GLRaV-3, 115.5 % for GLRaV-4 and 107.3% for GLRaV-5. For each variety, sample and virus the *Actin* gene was used for ΔCq_{virus} (Cq_{gene}-Cq_{ref}) determination and $\Delta\Delta Cq$ (ΔCq_{virusA}-ΔCq_{virusB}) was determined. In the plants analyzed, for GLRaV-4-GLRaV-3 $\Delta\Delta Cq$ averaged 2.4 and for GLRaV-5-GLRaV-3 averaged 3.3 which are around one magnitude order in the relative copy number. In addition, we could determine the absolute number of copies for GLRaV-3 and -5 in ten plants of the variety Tintilla Rota and eleven of the variety Rome which were infected by both viruses, resulting in the average number of copies per mg of tissue GLRaV-3 was 5.7×10^6. In contrast, the genome copy number of GLRaV-5 was significantly lower, averaging 1.60×10^5.mg^{-1}. On the other hand, we have analyzed plants of the variety Gorgollasa that were infected with GLRaV-4 alone or by GLRaV-3 and GLRaV-4, simultaneously. In five plants with single GLRaV-4 infection, the genome copy number per mg of tissue averaged 8.61×10^5. In the other five plants having double infection the number of copies of GLRaV-4 were similar to those having single infection averaging 6.31×10^5, while the number of copies of GLRaV-3 was greater and within the same range as in the varieties Tintilla de Rota and Rome: 5.24×10^6 copies.mg^{-1}. Data corresponding to those samples taken in mid fall 2011 and for a different gene (*hsp70*) showed equivalent relative differences (not shown). These preliminary results cannot discard interactions among ampeloviruses, but suggest that at least two variants of GLRaV-4 multiply less in plants than GLRaV-3, the most frequent in Spain, offering a possible explanation for the differences in the incidence of different *Ampelovirus* species. The relationship between symptom severity and ampelovirus concentration remains also to be investigated.

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