Horizontal and vertical transmission of the hypovirulence-associated mycovirus *Fusarium oxysporum* f. sp. dianthi virus 1.

Carlos G. Lemus-Minor¹, M. Carmen Cañizares², M. D. García-Pedrajas² & Encarnación Pérez-Artés¹

¹ Department of Crop Protection, Instituto de Agricultura Sostenible, Consejo Superior de Investigaciones Científicas (IAS-CSIC), Alameda del Obispo s/n, 14004 Córdoba, Spain

² Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora”, Universidad de Málaga, Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Estación Experimental “La Mayora”, 29750 Algarrobo-Costa, Málaga, Spain.

e-mail: eperezartes@ias.csic.es

Telephone: +34 957 499223

Fax: +34 957 499252

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Abstract

Mycovirus *Fusarium oxysporum* f. sp. *dianthi* virus 1 (FodV1) has been recently described infecting isolate *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi* (*Fod*). FodV1 is a new member of the family *Chrysoviridae*, and its genome consists of 4 dsRNA segments ranging from 2.6 to 3.5 kb. Presence of high levels of FodV1 in its original fungal host correlated with alteration of some phenotypic traits, including virulence. In this work we have analysed if FodV1 can be transferred horizontally to another *Fod* isolate by hyphal anastomosis, and vertically through conidiation, and if the mycovirus accumulates in the recipient isolate at similar levels that those found in the donor one. Moreover, we have investigated if the new virus-infected isolate reproduces the same phenotypic alterations that the original virus-infected does. Results indicated that FodV1 transfers horizontally between compatible isolates by hyphal anastomosis, reaching a high level of accumulation in the recipient isolate, and vertically during sporogenesis. Presence of FodV1 in the new fungal host reduced the growth rate and altered the morphology of the colony on solid medium, and diminished the conidiation rate in liquid medium. More interestingly, FodV1 induced hypovirulence in its new fungal host. Results contained in this work constitute the basis for further research on the application of mycovirus FodV1 to the control of *Fusarium* wilt diseases.

Keywords Biological control, carnation, *Chrysoviridae*, *Fusarium* wilt

Fusarium wilt, caused by *Fusarium oxysporum* f. spp., affects to a wide number of economically important crops worldwide. The systemic nature of these diseases makes chemical treatments ineffective once symptoms are detected, thus enhancing the need for alternative strategies of control. In the last years, interest in mycoviruses has increased because their potential contribution to sustainable agriculture as biological control agents of their fungal hosts (reviewed in: Ghabrial and Suzuki 2009; Ghabrial et al. 2015; Pearson et al. 2009; Xie and Jiang 2014). The use of mycoviruses as biocontrol agents requires, in the first place, the identification of mycoviruses that reduce the virulence of the fungus towards its host plant(s).

We have recently published the first report of a mycovirus associated to hypovirulence in the important species *Fusarium oxysporum* (Lemus-Minor et al. 2018). Mycovirus *Fusarium oxysporum* f. sp. dianthi virus 1 (FodV1) was isolated from strain *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi* (*Fod*), the forma specialis of *F. oxysporum* that infects carnation. The genome of FodV1 has been fully sequenced, and it consists of four dsRNA segments ranging from 2,646 to 3,555 base pairs (Lemus-Minor et al. 2015). FodV1 is a new member of the family *Chrysoviridae*, closely related to other “chryso-like” viruses previously reported (Darissa et al. 2012; Lee et al. 2014; Lemus-Minor et al. 2015; Urayama et al. 2012; Urayama et al. 2014). Comparison of the naturally infected isolate *Fod* 116 (*Fod* 116V⁺) and another selected version of the same isolate with only a residual level of viral dsRNA (*Fod* 116V⁻) evidenced that presence of high levels of FodV1 significantly reduced the fungal mycelium growth rate, the conidiation rate, and the virulence on carnation plants of its fungal host (Lemus-Minor et al. 2018). Alteration of all these hypovirulence-associated phenotypic traits in the presence of mycovirus FodV1 would open the possibility to its use as a biological control agent of Fusarium wilt of carnation. The successful application of mycoviruses to the control of phytopathogenic fungi is dependent on: i) their ability to stably accumulate in the fungal hosts, and ii) their efficient transmission among isolates in the natural fungal populations. Mycoviruses are transmitted by hyphal anastomosis (horizontal transmission), and during sporogenesis (vertical transmission). Horizontal transmission occurs naturally between individuals that belong to the same or closely related vegetative compatibility group (VCG). Vertical transmission through asexual spores also occurs commonly, but its frequency varies greatly depending on the virus and host strains involved (Lee et al. 2014).

To determine the potential of FodV1 of being used as a biological control agent, in this work we have analyzed if this mycovirus can be transferred horizontally through hyphal anastomosis between vegetatively compatible isolates, and vertically during conidiation, as well as if the new recipient isolate reproduces the same phenotypic alterations that the donor does.

Fusarium oxysporum f. sp. *dianthi* strains *Fod* 116V⁺, the strain originally infected with the mycovirus, and *Fod* 77Hyg^R, a vegetatively compatible virus-free strain that had been previously transformed with a hygromycin resistance gene (*hph*), were used as donor and recipient isolates, respectively (Fig. 1). Both *Fod* 116 and *Fod* 77 were race 2 representatives belonging to VCG 0021, and had been collected from the same geographic area (Chipiona) but in different years (2008 and 2000, respectively) (Gómez-Lama et al. 2012; Gómez-Lama and Pérez-Artés, 2014). Donor and recipient isolates were grown together in a Potato Dextrose Agar (PDA) plate at 25°C until the mycelium from both isolates came in contact. Tips of mycelium from the contact zone were picked up and transferred to PDA plates with hygromycin (75 µg*mL⁻¹), and the grown colonies were subjected to single conidia selection in water-agar (WA) plates (Fig. 1a). To check for the presence of viral dsRNA in recipient isolate *Fod* 77Hyg^R, the resultant monoconidial cultures were used to perform dsRNA purification analyses by cellulose column chromatography, as described in Lemus Minor et al. (2018). Results from dsRNA purifications showed that some of the *Fod* 77Hyg^R monoconidial cultures analysed contained dsRNA segments similar to those found in donor isolate *Fod* 116V⁺ (Fig. 1b). To prove that these dsRNA segments corresponded to mycovirus FodV1, total RNA extracts were obtained using TRIzol[®] Reagent (Ambion[®], Thermo Fisher Scientific, Waltham, MA USA) and then used to perform a reverse transcription (RT) and a polymerase chain reaction (PCR) using specific primers for the RNA-dependent RNA polymerase (RdRp) sequence of FodV1 (Lemus Minor et al. 2018). Results obtained demonstrated that the dsRNA segments found in isolate *Fod* 77Hyg^R corresponded to mycovirus FodV1 (Fig. 1c). One of these mycovirus-infected monoconidial cultures (*Fod* 77Hyg^RV⁺) was selected and used for further assays.

An interesting characteristic of mycovirus FodV1 is the unusual high level of accumulation detected in isolate *Fod* 116V⁺, its original fungal host (Lemus-Minor et al. 2018). In order to know if this is a characteristic of the mycovirus itself or a circumstance determined by the genetic background of the fungal host, we analyzed the relative quantity of FodV1 dsRNA in the new recipient isolate *Fod* 77Hyg^RV⁺ compared to the donor *Fod* 116V⁺. To do that, a RT followed by a quantitative PCR targeting the

RdRp segment of FodV1 was carried out using total RNA extracts from each virus-infected isolate. The relative amount of the RdRp segment was calculated as a proportion of the transcripts from the fungal genes actin beta/gamma1 and β -tubulin. Results showed that, although the proportional quantity of FodV1 dsRNA determined in isolate *Fod* 116V⁺ was assessed as ≈ 2.83 times (mean of both reference genes) higher to that found in isolate *Fod* 77 Hyg^RV⁺, both the donor and the recipient isolates exhibited unusual high levels of accumulation of viral dsRNA (Table 1).

As already mentioned, previous studies carried out with isolate *Fod* 116V⁺ showed that a high titer of mycovirus FodV1 correlated with alterations in a number of phenotypic traits of its fungal host. Once the mycovirus was transferred from isolate *Fod* 116V⁺ to isolate *Fod* 77Hyg^R, it was necessary to prove that these alterations were also expressed in the new recipient isolate. To do that, a comparison of isolates *Fod* 77Hyg^R (virus-free), and *Fod* 77Hyg^RV⁺ (virus-infected) was done. Previously, we checked that introduction of the *hph* gene did not alter the morphology and growth in solid and liquid medium of the wild type isolate *Fod* 77 (not shown). Effect of FodV1 on growth on solid medium, and conidiation rate in liquid medium, was estimated by growing isolates *Fod* 77Hyg^R and *Fod* 77Hyg^RV⁺ on PDA or casein hydrolyzed liquid medium, respectively, as described in Lemus-Minor et al. (2018). Results showed that both phenotypic traits were affected by the presence of mycovirus FodV1 (Fig. 2). The virus-infected isolate *Fod* 77Hyg^RV⁺ had a significantly reduced growth rate on solid medium (Fig. 2b) and conidiation rate in liquid medium (Fig. 2c), and the pigmentation and the fluffy appearance of the colonies were also modified (Fig. 2a).

Induction of hypovirulence by FodV1 in the new recipient *Fod* 77Hyg^R isolate was also demonstrated. First, we tested the virulence of wild type isolate *Fod* 77 compared with isolate *Fod* 77Hyg^R to discard any effect of the introduced *hph* gene on the virulence (Table 2). Then, two pathogenicity tests were performed, using four susceptible carnation cultivars that were inoculated with either isolate *Fod* 77Hyg^R or *Fod* 77Hyg^RV⁺. Inoculation of carnation cuttings and culture conditions were as previously described (Gómez-Lama Cabanás et al. 2012). Disease severity values were used to calculate the percentage of the standardized area under the disease progress curve (sAUDPC). Factorial analysis of the data evidenced a significant reduction in the disease severity values obtained with all carnation cultivars when the *Fod* isolate was infected with mycovirus FodV1 (Table 2).

Besides the horizontal transmission, another factor that could contribute to the spread of the mycovirus in the fungal population is the efficiency in the vertical transmission through asexual spores. To assess

the frequency of vertical transmission of FodV1 in both *Fod* 116V⁺ and *Fod* 77Hyg^RV⁺ virus-infected isolates, 50 and 30 single conidia, respectively, were obtained, and the resultant monoconidial cultures were analysed for the presence of the mycovirus by performing total RNA purifications with TRizol, followed, only in the case that the viral dsRNA was not visible after agarose gel electrophoresis of the total RNA extracts, by a RT-PCR using specific primers for the RdRp segment of FodV1. In the case of isolate *Fod* 116V⁺, agarose gel electrophoresis of total RNA extracts showed that only 2 of the 50 (4%) monoconidial cultures exhibited visible levels of FodV1 dsRNA (Fig. 3a). Subsequent RT-PCR amplifications revealed 10 additional monoconidial cultures (total 24%) infected with low levels of mycovirus FodV1 (Fig. 3b). In the case of isolate *Fod* 77Hyg^RV⁺, agarose gel electrophoresis of the total RNA extracts showed presence of high levels of FodV1 in all the 30 monoconidial cultures (100%) analyzed (Fig. 3c). Differences in the efficiency of vertical transmission among fungal isolates have been reported for other fungal species-mycovirus combinations (Lee et al. 2014). This evidence supports the hypothesis that selection of a specific virus-infected isolate could influence transmission and dissemination of hypovirulence.

All the above results indicate that mycovirus FodV1 could be a good candidate to be used as a biological control agent against Fusarium wilt of carnation. In this regard, demonstration that FodV1 can spread through different isolates inducing hypovirulence in the new infected host is relevant. Although mycovirus FodV1 has been found in the forma specialis *dianthi*, its use could be extended to other formae speciales of *F. oxysporum* by transfection via protoplast fusion (Lee et al. 2011). Success in transfection would open the possibility of using FodV1 not only for the biological control of Fusarium wilt of carnation but also for the control of other agronomically important Fusarium wilts. Therefore, results contained in this paper encourage continuing research on the utilization of FodV1 for the biological control of Fusarium wilts.

Compliance with Ethical Standards

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Fig. 1 Horizontal transference of mycovirus FodV1 from isolate *Fod* 116V⁺ to isolate *Fod* 77Hyg^R by hyphal anastomosis. a) Hyphal anastomosis on PDA. D: donor isolate *Fod* 116V⁺; R: recipient isolate *Fod* 77Hyg^R. 1H and 2H: points in the contact zone where the mycelial tips were taken. b) Agarose gel electrophoresis of cellulose purified dsRNA extracts from isolates *Fod* 77Hyg^RV⁺ (monoconidial culture obtained after hyphal anastomosis between isolates *Fod* 77Hyg^R and *Fod* 116V⁺), *Fod* 77Hyg^R (recipient), and *Fod* 116V⁺ (donor). c) Agarose gel electrophoresis of the RT-PCR products obtained from the above isolates using specific primers to detect the RdRp segment of FodV1

Fig. 2 Effect of mycovirus FodV1 on particular phenotypic traits of its fungal host. a) Colony morphology of *Fod* 77Hyg^R (virus-free strain), and *Fod* 77Hyg^R V⁺ (virus-infected strain), on PDA after 9 days of growth. b) Two dimensional colony growth rate. The colony area was measured at days 5 to 7, and 9, of growth on PDA with hygromycin at 25 °C in the dark. Values are the average area of 5 colonies. Different letters indicate statistically significant differences according to Fisher's Least significant difference (LSD) ($P \leq 0.05$). c) Conidiation rate in liquid medium. Isolates were cultured in casein hydrolyzed medium, and conidia counted at 3, 4, 5, 6 and 7 days of growth (three replicates per time). Number of conidia was expressed as conidia·mL⁻¹, and used to do a two factors (day and isolate) analysis of variance (ANOVA). Significant differences among means for total conidia count values with each isolate were determined using the Tukey's honestly Significant Difference (HSD $P \leq 0.01$) test. ANOVA analysis was performed using the Statistix 10.0 program (Analytical software). Vertical lines in each point represent the standard error.

Fig. 3 RNA analysis of monoconidial cultures to assess the vertical transmission of mycovirus FodV1. a) and c): Agarose gel electrophoresis of total RNA extracts (TRIzol[®] Reagent) from different monoconidial cultures obtained from the virus-infected isolates *Fod* 116V⁺ (a), and *Fod* 77Hyg^R V⁺ (c). b) Agarose gel electrophoresis of the RT-PCR products obtained using specific primers for the RdRp segment of FodV1. M: molecular weight marker λ Hind III