1   Olive mild mosaic virus coat protein and p6 are suppressors of RNA silencing and their

2   silencing confers resistance against OMMV

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4   Running title: OMMV silencing suppressors confer resistance against OMMV

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6   Varanda, CMR[1#], Materatski, P[1], Campos, MD[1], Clara, MIE[3], Nolasco, G[2], Félix, MR[3]

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8   [1] ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e

9   Formação Avançada, Universidade de Évora, Pólo da Mitra, Ap.94, 7006-554 Évora, Portugal

10   [2] Meditbio - Universidade do Algarve, 8005-139 Faro

11   [3] Departamento de Fitotecnia, ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas,

12   Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, Ap.94, 7006-554 Évora,

13   Portugal

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15   #Corresponding author: CMR Varanda, carlavaranda@uevora.pt

16

Abstract

18

19   RNA silencing is an important defense mechanism in plants, yet several plant viruses

20   encode proteins that suppress it. Here the genome of Olive mild mosaic virus (OMMV)

21   was screened for silencing suppressors using a green fluorescent based transient

22   suppression assay. The full OMMV cDNA and 5 different OMMV open reading frames

23   (ORFs) were cloned into Gateway binary destination vector pK7WG2, transformed into

24   *Agrobacterium tumefaciens* C58C1 and agroinfiltrated into *Nicotiana benthamiana* 16C

25   plants. Among all ORFs tested, CP and p6 showed suppressor activity, with CP showing

26   a significant higher activity when compared to p6, yet lower than that of the full OMMV.

27   This suggests that OMMV silencing suppression results from a complementary action of

28   both CP and p6.

29   Such discovery led to the use of those viral suppressors in the development of OMMV

30   resistant plants through pathogen-derived resistance (PDR) based on RNA silencing. Two

31   hairpin constructs targeting each suppressor were agroinfiltrated in *N. benthamiana*

32   plants which were then inoculated with OMMV RNA. When silencing of both

33   suppressors was achieved, a highly significant reduction in viral accumulation and

34   symptom attenuation was observed as compared to that seen when each construct was

35   used alone, and to the respective controls, thus showing clear effectiveness against

36   OMMV infection. Data here obtained indicate that the use of both OMMV viral

37   suppressors as transgenes is a very efficient and promising approach to obtain plants

38   resistant to OMMV.

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40   **Importance**

41   OMMV silencing suppressors were determined. Among all ORFs tested, CP and p6

42   showed suppressor activity, with CP showing a significant higher activity when

43   compared to p6, yet lower than that of the full OMMV, suggesting a complementary

44   action of both CP and p6 in silencing suppression.

45   This is the first time that a silencing suppressor was found in a necrovirus and that two

46   independent proteins act as silencing suppressors in a member of the *Tombusviridae*

47   family.

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48   When silencing of both suppressors was achieved, a highly significant reduction in viral

49   accumulation and symptom attenuation was observed as compared to that seen when

50   each was used alone, thus showing clear effectiveness against OMMV infection. A high

51   percentage of resistant plants was obtained (60%), indicating that the use of both

52   OMMV viral suppressors as transgenes is a very efficient and promising approach to

53   obtain plants resistant to OMMV.

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57   **Introduction**

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59   RNA silencing is a gene inactivation mechanism identified in most eukaryotes that is

60   involved in several biological processes such as regulating endogenous gene expression,

61   maintenance of genome stability and defence against viruses (1, 2).

62   Amongst the several strategies plants have developed to counter virus infections, RNA

63   silencing is one of the most important (1, 3). In antiviral RNA silencing, double stranded

64   RNAs (dsRNAs) of sizes varying from 30 nucleotides (nt) to hundreds of nt (4) derived

65   from ssRNA virus replication intermediates, are recognized as foreign. Such dsRNAs in

66   plants are first processed by an RNase III-like nuclease (termed DICER-like or DCL) into

67   double stranded viral short interfering RNAs (siRNAs) of 21 to 22 nt long with 2 nt 3'

68   overhangs (1, 5). Plant virus infections are associated with the accumulation of these

69   virus specific siRNAs. The cleavage is then accomplished by members of the Argonaute

70   protein family (AGOs) (6) which recruit siRNA and associated proteins to form the RNA

71   induced silencing complex (RISC) with one of the siRNA strands (the guide strand) in a

72 process that is accompanied by the release/degradation of the other "passenger"

73 strand.

74 RISC possesses ribonuclease activity and is guided by the single stranded siRNAs to its

75 target based on sequence complementarity, resulting in binding and degradation of

76 homologous RNA molecules by the catalytic activity of the AGO (7, 8).

77 Cleavage products of the target RNAs serve as template of RNA dependent RNA

78 polymerases (RDR) to form dsRNAs, leading to secondary siRNA production (9), which

79 can again initiate silencing in a self-sustained manner.

80 To counteract host RNA silencing defense, viruses have evolved several strategies. One

81 of such involves viral proteins encoded in the genomes that suppress plant RNA

82 silencing, termed viral suppressors of RNA silencing (VSRs) (10, 11). Most viruses studied

83 have one VSR and many VSRs have been identified (12). These proteins are highly

84 divergent, appearing to have evolved independently in the different viruses and they

85 may interfere in different stages of the RNA silencing pathways, either binding dsRNAs

86 and inhibiting its processing into siRNAs; or sequestering viral siRNAs preventing their

87 incorporation into RISC or directly interfering with recognition of viral RNA, dicing and

88 RISC assembly (8, 13).

89 Viruses from different genera within *Tombusviridae* family, have different suppressors.

90 The p19 of tombusviruses *Tomato bushy stunt virus* (TBSV) and *Cymbidium ringspot*

91 *virus* (CymRSV), associated with long distance movement, is one of the most studied

92 viral suppressors (14-16). The only function of the p14 of the aureusvirus, such as *Pothos*

93 *latent virus*, seems to be to prevent silencing (17).

94 The CP of betacarmoviruses such as *Turnip crinkle virus*, as well the movement

95 proteins of some dianthoviruses also act as silencing suppressors (18-21). No silencing

96   suppressors are identified in the other genera in the family *Tombusviridae*, which would

97   help to elucidate the evolutionary progression of viruses within this family.

98   OMMV is a member of the genus *Alphanecrovirus* within the *Tombusviridae* family,

99   being one of the most spread viruses in olive orchards (22-24). Its genomic RNA is 3683

100   nt long with 5 open reading frames (ORFs) and the virus is likely to have resulted from

101   recombination events between two other necroviruses OLV-1 and TNV-D, based on the

102   high amino acid identity with the RNA dependent RNA polymerase of OLV-1 and of the

103   CP of TNV-D (25). ORF1 (p23), pre-readthrough, and ORF2 (p82), predicted as the RdRp,

104   are involved in RNA replication, ORF3 (p8) and ORF 4 (p6) are predicted to be involved

105   in virus movement, and ORF5 (p29) is predicted as the CP and it is involved in capsid

106   assembly, systemic movement and vector transmission (26). To the best of our

107   knowledge, no other functions of the gene products such as RNA silencing suppression

108   were ever identified in any alpha- or betanecrovirus.

109   In this study, OMMV encoded proteins were examined to identify suppressors of RNA

110   silencing and two were found, the CP and p6. OMMV silencing suppression ability seems

111   to result from a coordinate and complementary action of both. In addition, resistance

112   to OMMV in *N. benthamiana* was achieved using hpRNA constructs containing both CP

113   and p6.

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119   **Results**

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121 **Determination of OMMV silencing suppressors**

122 To identify potential OMMV RNA silencing suppressors, all proteins encoded by the

123 genome were tested for their ability to suppress silencing by using a GFP reporter gene

124 in plant tissues. Individual OMMV ORFs were cloned into a binary vector (pk7wg2)

125 driven by the CaMV 35S promoter in *A. tumefaciens*. For comparison, a full-length cDNA

126 clone of OMMV that generates a full infection in *N. benthamiana* was also cloned.

127 Transient expression assays were performed on transgenic 16C *N. benthamiana* plants

128 by co-infiltrations of each clone plus pK_GFP, a construct that expresses a transcript

129 homologous to the transgene of 16C plants.

130 Infiltrated *N. benthamiana* leaf patches showed GFP fluorescence under UV light at 2

131 dpi in all samples (data not shown). Leaves infiltrated with the silencing inducer pk_GFP

132 showed a weak GFP fluorescence at 3 dpi (Figure 1, GFP 3dpi) and at 5 dpi fluorescence

133 disappeared (Figure 1, GFP 5 dpi) and silencing began, as seen by the development of a

134 red colour under UV light on the infiltrated region.

135 In the positive control, leaves co-infiltrated with pk_GFP and the strong suppressor Tav-

136 2b presented bright fluorescence at 3 dpi (Figure 1, Tav 3 dpi) and fluorescence was

137 maintained for the next 7 days, although less intense (Figure 1, Tav-2b 10 dpi).

138 In the co expression of OMMVp23, OMMVp52 and OMMVp8, at 3, 5 and 10 dpi, no

139 fluorescence was observed under UV light and silencing began at 5 dpi and was

140 maintained at 10 dpi.

141 At 3, 5 and 10 dpi, OMMVp6, OMMVCP and OMMV were able to suppress GFP silencing,

142 as seen by the green fluorescence under UV light.

143 OMMVCP and OMMV showed the most intense suppressor activity at 5 dpi, whereas

144 OMMVp6 showed the most intense suppressor activity at 3 dpi. However, OMMVp6

145 green fluorescence showed lower intensity at all times when compared to OMMVCP

146 and OMMV and was almost undetectable at 10 dpi.

147 GFP fluorescence in the presence of pk_OMMV was reproducibly stronger than with

148 each of the viral genes and similar to the levels observed for Tav-2b. However, in

149 contrast to Tav-2b, where higher levels were observed at 3 dpi, a brighter fluorescence

150 was observed at 5 dpi.

151 Monitoring of upper non infiltrated leaves showed systemic silencing at 15 dpi in all

152 samples, suggesting that OMMVp6 and OMMVCP possess suppressor local RNA

153 silencing activity but cannot suppress systemic silencing.
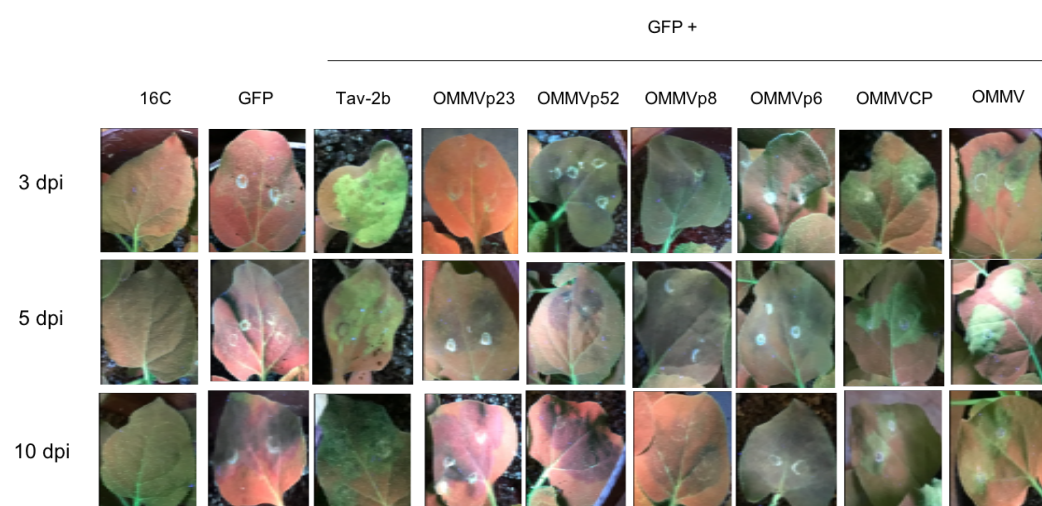
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Figure 1. Visual observation of leaves from transgenic *N. benthamiana* 16C plants under UV light at 3, 5 and 10 days postinfiltration (dpi). Non infiltrated (16C), infiltrated with Agrobacterium tumefaciens harbouring pK-GFP alone (GFP) and co infiltrated with pK-GFP plus: Tav-2b, OMMVp23, OMMVp52, OMMVp8, OMMVp6, OMMVCP and OMMV.

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165   RT-qPCR showed that all samples presented C_T values within the linear calibration

166   curves. Two reference genes (PP2 and FBox) were used to normalize target genes

167   expression. The amplification efficiency and correlation coefficient (R^2) of their

168   calibration curves were 107.25% and 0.9943 for PP2 and 99.62% and 0.9978 for FBox.

169   As expected by visual observations, RT-qPCR at 3 dpi showed higher GFP mRNA levels

170   on plants co inoculated with pk_GFP +Tav-2b, +OMMVp6, +OMMVCP and +OMMV as

171   compared to single inoculated with pk_GFP and non-inoculated 16C leaves (Figure 2),

172   reaching the highest levels with the strong suppressor Tav-2b. Plants co infiltrated with

173   pK_GFP and OMMVp23, OMMVp52 and OMMVp6 showed GFP mRNA levels higher than

174   non-infiltrated 16C plants and similar to those obtained with single pK_GFP infiltrations.

175   At 5 dpi, RT-qPCR showed a great reduction in GFP mRNA levels of GFP alone or in the

176   presence of OMMVp23, OMMVp52 and OMMVp8 and at 10 dpi GFP mRNA levels

177   decreased to almost undetectable levels.

178   Consistent with visual observations, at 5 dpi, high GFP transcript levels were observed

179   in patches expressing OMMVp6, OMMVCP, OMMV and Tav-2b, and at 10 dpi, all GFP

180   levels decreased, nevertheless with higher levels than non-infiltrated 16C plants (from

181   0.3 in OMMVp6 to 1-fold greater in OMMV). GFP mRNA accumulation in OMMVp6

182   infiltrated patches was lower compared to OMMVCP and OMMV, suggesting that

183   OMMVp6 is a weaker suppressor of silencing than OMMVCP.

184   The co infiltration of pk_GFP and OMMV reached the highest relative mRNA GFP levels

185   (1.6-fold greater than non-infiltrated 16C at 5 dpi), and was higher than the levels

186   obtained with the co infiltration with OMMVCP and OMMVp6 at all times tested,

187   suggesting an enhanced suppressor activity resulting from the combined action of p6

188   and CP.

189   Plants co-infiltrated with pk_GFP and OMMVCP and pK_GFP and OMMV maintained the

190   greenish patch for 11 and 14 days, respectively (data not shown).

191   At 3 and 5 dpi, expression in leaves co-infiltrated with pk_GFP and each OMMVp23,

192   OMMVp52, OMMVp8 and OMMVp6, was similar to that of the strong silencing

193   suppressor OMMVCP. This result confirms that proteins were being expressed and

194   demonstrated that weak or no silencing suppression was not due to low protein
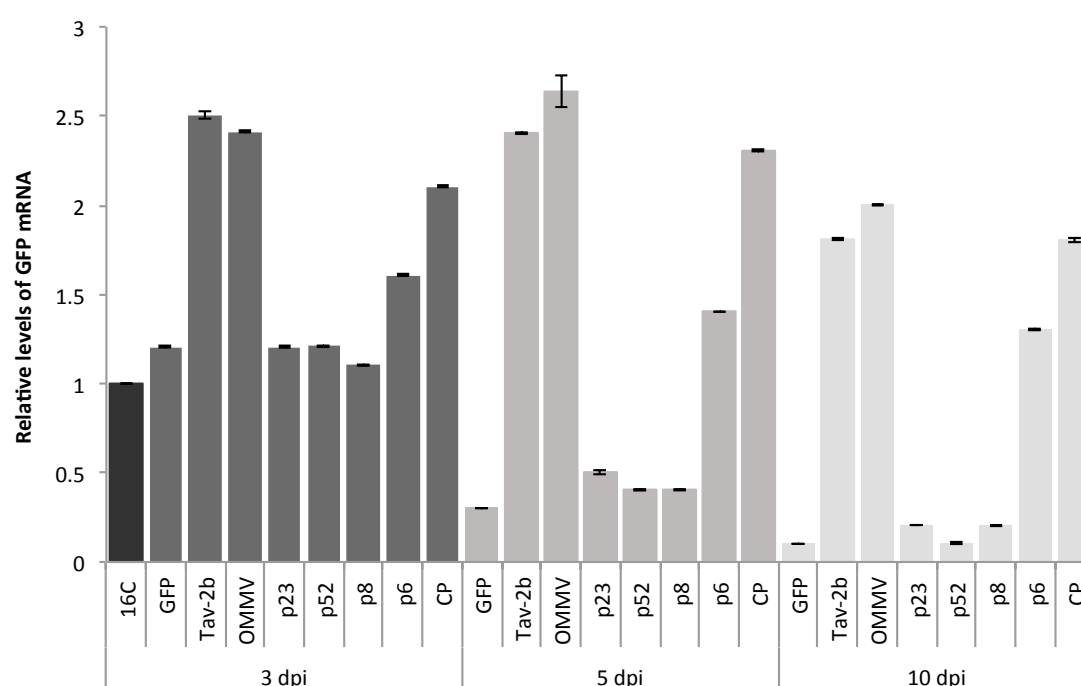
195   expression.

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200   Figure 2. Mean relative levels of GFP mRNA ± standard error (SE) in infiltrated 16C *N. benthamiana* leaves

201   at 3 dpi, 5 dpi and 10 dpi, determined by RT-qPCR and normalized by the levels of PP2 and F-box reference

202   genes. 16C: 16C non-inoculated plants; GFP: single infiltration with pk_GFP; Tav-2b, OMMVp23,

203   OMMVp52, OMMVp8, OMMVp6, OMMVCP and OMMV: co-infiltration of pk_GFP with the corresponding

204   constructs. The GFP transgene level in a non-infiltrated *N. benthamiana* 16C plant was used as a standard

205   and given a value of 1.

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207   PERMANOVA analysis revealed significant differences in the factors "ORFs" and "Time"

208   (p < 0.0001). In addition, a significant interaction occurred between the two factors

209   ("ORFs" and "Time") (p < 0.0001). At 3 dpi the highest values (mean ±SE) of relative GFP

210   mRNA abundance were 2.50 ± 0.01 in Tav-2b gene, followed by 2.41 ± 0.01 in OMMV,

211   2.10 ± 0.005 in CP and 1.60 ± 0.004 in p6 (Figure 2). Individual pairwise comparisons at

212   3 dpi revealed high variability of relative GFP mRNA abundance with significant

213   differences between most ORFs (p < 0.05) (Table 3). Individual pairwise comparisons

214   showed no significant differences between: *p* GFP *vs.* p23 > 0.9153, *p* p23 *vs.* p52 >

215   0.6178 and *p* p23 *vs.* p52 > 0.088.

216   At 5 dpi the highest values (mean ±SE) of relative GFP mRNA abundance were 2.64 ±

217   0.09 in OMMV followed by 2.40 ± 0.005 in Tav-2b, 2.30 ± 0.004 in CP and 1.40 ± 0.002

218   in p6 (Figure 2). Individual pairwise comparisons at 5 dpi revealed high variability of

219   relative GFP mRNA abundance with significant differences between most of the ORFs (p

220   < 0.05) (Table 3). Individual pairwise comparisons revealed no significant differences

221   between: *p* Tav-2b *vs.* OMMV > 0.0518 and *p* p52 *vs.* p8 > 0.9977.

222   At 10 dpi the highest values (mean ±SE) of relative GFP mRNA abundance were 2.00 ±

223   0.005 in OMMV followed by 1.81 ± 0.005 in Tav-2b, 1.80 ± 0.01 in CP and 1.30 ± 0.003

224   in p6 (Figure 2). Individual pairwise comparisons at 5 dpi revealed high variability of

225   relative GFP mRNA abundance with significant differences between most of the ORFs (p

226   < 0.05) (Table 3). Individual pairwise comparisons revealed no significant differences

227   between: *p* GFP *vs.* p52 > 0.3574, *p* p23 *vs.* p52 > 0.6807, *p* p23 *vs.* p8 > 0.6569, *p* p52

228   *vs.* p8 > 0.6799 and p Tav-2b vs. CP > 0.8368.

229

230 Table 3: Details of the two-factor PERMANOVA Pair-Wise tests with "ORFs" (8 levels, fixed) and

231 "Time" (3 levels, fixed) for all variables analysed. Bold values highlight significant effects (p <

232 0.05).

Pair-Wise tests ORFs	3 dpi	5 dpi	10 dpi
GFP *vs* Tav-2b	**0.0001**	**0.0001**	**0.0001**
GFP *vs* OMMV	**0.0001**	**0.0001**	**0.0001**
GFP *vs* p23	0.9153	**0.0001**	**0.0001**
GFP *vs* p52	0.6178	**0.0001**	0.3574
GFP *vs* p8	**0.0002**	**0.0001**	**0.0001**
GFP *vs* p6	**0.0001**	**0.0001**	**0.0001**
GFP *vs* CP	**0.0001**	**0.0001**	**0.0001**
Tav-2b *vs* OMMV	**0.0129**	0.0518	**0.0001**
Tav-2b *vs* p23	**0.0001**	**0.0001**	**0.0001**
Tav-2b *vs* p52	**0.0001**	**0.0001**	**0.0001**
Tav-2b *vs* p8	**0.0001**	**0.0001**	**0.0001**
Tav-2b *vs* P6	**0.0001**	**0.0001**	**0.0001**
Tav-2b *vs* CP	**0.0001**	**0.0004**	0.8368
OMMV *vs* p23	**0.0001**	**0.0001**	**0.0001**
OMMV *vs* p52	**0.0001**	**0.0001**	**0.0001**
OMMV *vs* p8	**0.0001**	**0.0001**	**0.0001**
OMMV *vs* p6	**0.0001**	**0.0002**	**0.0001**
OMMV *vs* CP	**0.0001**	**0.0134**	**0.0001**
p23 *vs* p52	0.6137	**0.0016**	0.6807
p23 *vs* p8	**0.0001**	**0.0017**	0.6569
p23 *vs* p6	**0.0001**	**0.0001**	**0.0001**
p23 *vs* CP	**0.0001**	**0.0001**	**0.0001**
p52 *vs* p8	**0.0001**	0.9977	0.6799
p52 *vs* p6	**0.0001**	**0.0001**	**0.0001**
p52 *vs* CP	**0.0001**	**0.0001**	**0.0001**
p8 *vs* p6	**0.0001**	**0.0001**	**0.0001**
p8 *vs* CP	**0.0001**	**0.0001**	**0.0001**
p6 *vs* CP	**0.0001**	**0.0001**	**0.0001**

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235 **OMMV resistance challenge**

236 The presence of each construct was confirmed by RT-qPCR at 3dpi in plants infiltrated

237 with Agrobacterium cultures carrying the constructs.

238 The appearance of disease symptoms was monitored at 2, 5, 10 and 16 dpi and recorded

239 as a disease index scale. As shown in figure 3, at 2 dpi, plants expressing OMMV CP,

240 OMMV p6 and both OMMV CP and p6 did not show any symptoms, whereas, 2 plants

241   in negative control showed mild symptoms (DSI, 5%). At 5 dpi, plants from all groups

242   showed symptoms, but only few plants of negative control group reached the disease

243   index scale of 2, presenting the highest DSI (32.5%), followed by plants expressing p6

244   alone (22.5%). At 10 dpi and 16 dpi, control plants showed severe symptoms (DSI 75%

245   and 100%, respectively). Plants expressing OMMV p6 showed a slight lower DSI at 10

246   dpi (60%) but also reached 100% at 16 dpi showing only a delay in the appearance of

247   symptoms. At the same time points, plants expressing OMMV CP presented

248   considerably lower DSIs (32.5% and 57.5%), indicating a tolerance to OMMV infection.

249   In contrast, plants expressing both OMMV CP and p6 showed a maximum DSI of 20% at

250   16 dpi, indicating a very high tolerance to OMMV infection. Additionally, in the

251   experiments, an average of 60% of the plants expressing OMMV CP and p6 did not

252   present any viral symptoms at 16 dpi, suggesting a resistance to OMMV. Plants

253   expressing OMMV CP also showed the existence of resistant plants, though in a lower

254   level (20%). Plants were monitored until 30 dpi and the highest DSI was 60% in plants

255   expressing OMMV CP and 30% in plants expressing both OMMV CP and p6, no plants

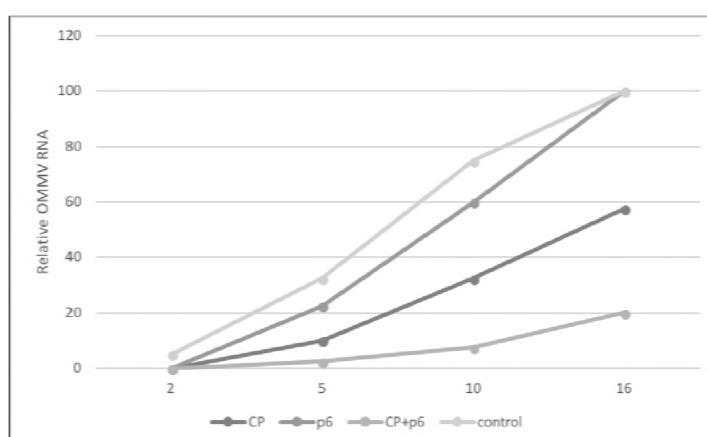256   reaching the maximum disease index.

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261 Figure 3: Disease severity of plants expressing OMMV CP, OMMV p6, OMMVCP+p6 and

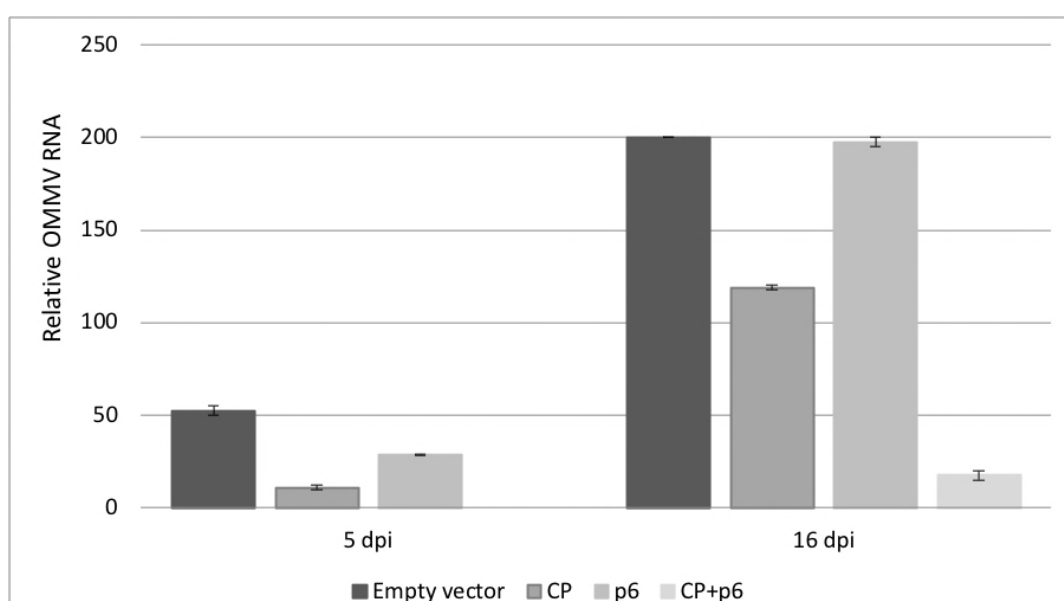262 negative control at 2, 5, 10 and 16 days after inoculation of OMMV

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268 Figure 4: Viral accumulation levels in inoculated leaves at 5 and 16 days after OMMV inoculation

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270 Viral accumulation levels were determined by real time RT-qPCR and results are

271 consistent with disease severity symptoms observed. Plants expressing OMMVCP and

272 OMMVp6 individually resulted in significantly less virus accumulation in inoculated

273 leaves at 5 dpi than when the empty vector was used. At 5 dpi no virus was detected in

13

274   plants expressing both OMMV CP and p6. At 16 dpi, plants transformed with OMMV CP

275   and plants transformed with both OMMV CP and p6, showed significant less OMMV

276   accumulation than plants transformed with OMMV p6 and the negative control. Plants

277   expressing both OMMV CP and p6 showed significant lower accumulation values than

278   plants expressing OMMV CP. Although a slight lower viral accumulation was obtained in

279   plants expressing OMMV p6 and empty vector at 16 dpi, differences were not

280   significant.

281   Both visual observations and quantitative RT-PCR indicate that OMMV CP, and

282   remarkably OMMV CP together with p6, attenuate viral symptoms and reduce viral

283   accumulation levels.

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286   **Discussion**

287   Most plant viruses have evolved by encoding one or more silencing suppressors as a

288   counter-response to the antiviral plant gene silencing defence mechanism (11).

289   Members of the *Tombusviridae* family share common features, but, show different

290   strategies to suppress silencing. Tombusvirus p19, the equivalent p14 of the

291   aureusvirus, the replicase proteins of the dianthovirus and the CP of the carmoviruses

292   (alpha- and beta-) have been identified as Tombusviridae silencing suppressors.

293   The high diversity of viral suppressors within *Tombusviridae* family did not anticipate a

294   more likely suppressor for the alphanecrovirus OMMV. The closest related genera, the

295   carmoviruses, use the CP as silencing suppressor, however, in opposition to the

296   carmoviruses CP, the CP of necroviruses lacks a protruding domain and has low similarity

297   to the CP of carmoviruses. In addition, the genome of OMMV does not present proteins

298   equivalent to tombusvirus p19 and aureusvirus p14 and the necroviruses replicase has

299   very low similarity to the replicase of the Dianthovirus (39).

300   In this study, the complete OMMV genome was screened for the presence of a gene

301   with potential RNA silencing suppression activity. Coinfiltration assays using *N.*

302   *benthamiana* 16C line, GFP-transformed, revealed that the full genome of OMMV has a

303   suppressor activity similar to the strong suppressor Tav-2b. When each of the individual

304   proteins were tested separately, CP and p6 were capable of inhibiting dsGFP-induced

305   local silencing, at different levels, as seen by enhanced GFP fluorescence and GFP mRNA

306   levels, suggesting a coordinate and complementary action of both CP and p6 as silencing

307   suppressors that result in an enhanced activity when CP and p6 are co expressed. The

308   analysis of each protein separately demonstrated that most suppressor activity is due

309   to the CP, with p6 showing a much lower inhibition of local RNA silencing.

310   This finding is in line with previous data on OMMV mutants containing several changes

311   in CP sequence, which exhibited different levels of symptoms in indicator plants,

312   suggesting a clear role of that protein in symptom modulation (26).

313   The combination of several functions in the same protein may be advantageous for the

314   virus. In particular, the silencing suppression function in the CP may have advantage

315   since it is the viral genome most exposed to host defence machinery, due to the mode

316   of virus replication (40).

317   This is the first time that two independent proteins were found to act as silencing

318   suppressors in a member of Tombusviridae family similarly to that shown in some

319   members of Closteroviridae (41) and Fimoviridae (42).

320   The existence of different viral suppressor proteins in one virus seems to be an

321   advantage in targeting different pathways of host defence at cellular level as it allow for

322   a more successful host infection. Viruses with single suppressors may only gain this

323   advantage in mixed infections with others that possess distinct silencing suppressors

324   with different targeting modes thus causing a more effective interference with the plant

325   defence mechanism that result in increased symptoms, higher viral accumulation and

326   favour cell to cell movement (43-45). This has been observed with PVX and CMV that

327   encode distinct suppressors that target both intracellular and intercellular silencing

328   leading to enhanced viral accumulation and symptoms characteristic of synergistic

329   effect, in double infections (46).

330   Synergistic effects have been seen between OMMV and the close related OLV-1 during

331   co-infection. OMMV is only acquired from the soil when OLV-1 is present, in which case

332   both viruses are able to invade the plant and spread throughout the plant causing

333   systemic symptoms instead of the typical local lesions induced by each virus separately

334   (47). In fact, it is interesting to notice that both OMMV silencing suppressors are

335   predicted to be involved in movement, p6 in cell to cell movement and CP in systemic

336   movement (48).

337   The discovery of OMMV viral suppressors have prompted us to explore their use in the

338   development of OMMV resistant plants through pathogen-derived resistance (PDR)

339   based on RNA silencing.

340   The development of viral resistant plants has been a matter of study for many years and

341   different levels of resistance have been obtained varying with the type of molecules and

342   viral genome region used to trigger RNA silencing (49-51). As an alternative to transgenic

343   plants and to overcome their associated biosafety and legislative constrains, transient

344   RNA silencing systems have been developed consisting on the direct delivering of

345   different RNA silencing molecules into plants (52-54).

346   The expression of self-complementary hairpin-RNA, in opposition to single sense or

347   antisense constructs, induces a high level of RNA silencing in plants due to the

348   production of dsRNA through the transcription of the hairpin structure; in addition, the

349   presence of an intron in between the complementary regions stabilizes the construct

350   and enhances silencing efficiency (55-57).

351   Tests were conducted to find out whether expression of artificial RNAs targeting the

352   suppressors CP and p6 could counteract the suppressor function and confer resistance

353   to OMMV using hairpin constructs. Two hairpin constructs targeting each the p6 and the

354   CP were agroinfiltrated in plants to transiently express long pathogen related dsRNAs

355   and preactivate plant RNA silencing machinery through the procession of dsRNAs into

356   siRNAs responsible for the degradation of invading viral RNA.

357   For the construction of the p6 hairpin construct, the full p6 was used; for the CP hairpin

358   construct, the first 141 nt of the CP were removed (OMMV CPminus141) in order to

359   reduce the potential biosafety risks associated to the production of CP molecules as well

360   as to exclude CP mediated resistance events, still a long fragment was used so that a

361   higher number of potentially active virus-specific sRNAs would be present and

362   specifically target the coding region of CP.

363   This study shows that the expression of OMMV CPminus141 and OMMV p6 interfered

364   with the multiplication of OMMV as plants expressing them resulted in a highly

365   substantial reduction in viral accumulation and symptoms attenuation, proving their

366   effectiveness against OMMV infection. The percentage of resistant plants obtained here

367   (60%) is similar to the highest percentages of viral resistance obtained in other studies

368   (54, 57, 58).

369   Plants transiently expressing OMMV CPminus141 interfered with the multiplication of

370   OMMV however it was not as effective against OMMV infection as plants expressing

371   both that and OMMV p6 as just 20% of the plants were OMMV resistant. No resistant

372   plants were obtained when OMMV p6 was being expressed however a slight delay in

373   the appearance of symptoms occurred when compared to control. These results show

374   that OMMV can counteract these mechanisms by inhibiting silencing. Only when plants

375   expressed both OMMV suppressors, CP and p6, a high efficient RNA silencing

376   mechanism was triggered. This has also been observed for CTV where resistance is only

377   achieved when the three viral silencing suppressors were targeted simultaneously (59).

378   Findings obtained here contribute to increase the knowledge on resistance induction

379   frequencies and are of great importance for the future development of OMMV resistant

380   plants, either transgenic or, in alternative, in transient systems by directly delivering

381   RNA silencing molecules into plants, as to overcome the lack of regulatory framework

382   and strong opposition associated to transgenic plants. Additionally, resistance to other

383   close related viruses may be achieved as viruses with less than 15-20% differences at

384   sequence level may confer protection to each other (60, 61). Such small differences exist

385   between OMMV and other necroviruses such as TNV-D and OLV-1, whose co infections

386   are frequent in nature (62). It is common for plants to be invaded by several viruses,

387   and this study shows the most efficient OMMV transgenes that confer resistance to

388   OMMV, which may be used in multiple combined sequences assembled with other

389   viruses expected to infect the crop to achieve protection and a broader resistance

390   against a wide range of viruses.

391   Besides helping to understand the interaction between viruses and hosts, the

392   knowledge on viral suppressors may also have other commercial impacts as in the

18

393   production of heterologous proteins using bioreactor plants. Host RNA silencing has

394   shown to reduce the efficiency of gene expression in plants (63). In this way, the co-

395   expression of a silencing suppressor and the target gene may be an attractive option to

396   reduce problems in transgene expression. This is essential to respond to the continuous

397   increase in the demand to produce large amounts of recombinant proteins for the

398   industry for which plants are one of the most effective and safe systems for large scale

399   production.

400

Materials and Methods

402

403   **OMMV silencing suppressor genes**

404

405   <u>Generation of constructs</u>

406   Plasmid DNA containing the viral full-length of an OMMV clone (27) was used for

407   amplification of OMMV p23, p52, p8, p6 and CP ORFs as well as full-length OMMV. Each

408   amplified sequence was cloned into pDONR™221 (Invitrogen) through a Gateway

409   recombination reaction in accordance to manufacturer's instructions. Primers used in

410   the reactions are listed in table 1. Genes were transferred from pDONR221 to pK7WG2

411   binary vector (28), under the promotor CaMV 35S, through LR recombination.

412   Confirmation of the correct sequences was done by sequencing the constructs.

413   The 2b suppressor gene of *Tomato aspermy virus* (TAV), used as positive control, and

414   green fluorescent protein (GFP) (m-gfp5-ER) (29), used as a silencing inducer, were

415   cloned as described previously (30).

416   Binary vectors were transformed into competent *Agrobacterium tumefaciens* strain

417   GV3101/C58C1, carrying pMP90 Ti plasmid which confers resistance to gentamycin. *A.*

418   *tumefaciens* cultures were grown individually on Luria-Bertani (LB) medium

419   supplemented with gentamycin, spectinomycin and rifampicin at 50 μg mL^{-1} each,

420   10mM MES and 20 μM acetosyringone at 28 °C, and 200 rpm until reaching an OD$_{600}$ of

421   0.5. Cells were precipitated, re-suspended in 10 mM MgCl$_2$ (pH 5.6), 10 mM MES and

422   100 μM acetosyringone, and kept in the absence of light at room temperature for 1 h

423   before infiltration (31).

424

425

426   Table 1: Primers used in the Gateway recombination reactions. Recombination

427   sequences specific for the gateway system are underlined.

428

Primer name	Primer (5' - 3')	Region amplified	Location in OMMV genome	Fragment length (bp)
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT	-	-	-
attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT			
OMMVattB1	AAAAAGCAGGCTAGTATACATACCAAGTATA	OMMV	1 - 19	3707
OMMVattB2	AGAAAGCTGGGTGGGGTCGGGCAAAGGCC		3667 - 3683	
OMMVp23attB1	AAAAAGCAGGCTAGGATAAAATGGAGCTCAC	p23	52 - 70	642
OMMVp23attB1	AGAAAGCTGGGTCCTATTTGGCCCGGAAGGCC		650 - 669	
OMMVP52attB2	AAAAAGCAGGCTGGGGCTTAATGGAAAGAGT	p52	844 - 862	1416
OMMVP52attB2	AGAAAGCTGGGTATCAGTTTGGTAATCCATTG		2216 - 2235	
OMMVp8attB1	AAAAAGCAGGCTTTTAATCAATGGATTACCA	p8	2210 - 2228	267
OMMVp8attB2	AGAAAGCTGGGTACACAGCCATAACTCAAAG		2433 - 2452	
OMMVp6attB1	AAAAAGCAGGCTCTTTTGAGTTATGGCTGTGT	p6	2433 - 2452	208
OMMVp6attB2	AGAAAGCTGGGTTGTCTATTTTGCGATCG		2600 - 2616	
OMMVp8attB1	AAAAAGCAGGCTTTTAATCAATGGATTACCA	p8 - p6	2210 - 2228	431
OMMVp6attB2	AGAAAGCTGGGTTGTCTATTTTGCGATCG		2600 - 2616	
OMMVCPattB1	AAAAAGCAGGCTACCAAAACATGCCTAAGAG	CP	2628 - 2646	842
OMMVCPattB2	AGAAAGCTGGGTTCAAACGTTAATGGTAGGG		3427 - 3445	

429

430

431   Agrobacterium co-infiltration assays and GFP imaging

432   Silencing suppressor assays were based on the previously described system (31). Briefly,

433   when leaves of *N. benthamiana* 16C line plants carrying a copy of 35S:GFP:TNos

434   transgene are agroinfiltrated with *A. tumefaciens* carrying the construct 35S GFP, the

435   green fluorescent signal disappears under UV light due to GFP silencing. However, if the

436   construct 35S GFP is co infiltrated with a silencing suppressor, the fluorescence does not

437   disappear and may even become more intense due to the inhibition of gene silencing

438   caused by the suppressor.

439   For transient expression assays, Agrobacterium cultures carrying each construct were

440   infiltrated into leaves of 4-week-old *N. benthamiana* 16C line plants, gently provided by

441   David Baulcombe (University of Cambridge, UK). Single and co-infiltrations assays were

442   performed using a 5 mL needleless syringe. Single infiltration consisted on pK_GFP in a

443   15 mL suspension of *A. tumefaciens*. For co-infiltration assays, Agrobacterium cultures

444   containing each construct individually, including Tav-2b, and GFP were mixed in 1:1 v/v

445   ratio before agroinfiltration, centrifuged and ressuspended in a final volume of 15 mL.

446   Three leaves per plant and ten plants per each construct were infiltrated. Plants were

447   observed during 12 days post infiltration (dpi). Each experiment was repeated three

448   times.

449   GFP fluorescence of infiltrated leaves and whole plants was examined using a long-

450   wavelength UV lamp (Blak-Ray B-100AP, UVP) and photographed with a digital camera

451   (Sony α100 DSLR-A100K).

452

453   RNA extraction and Real-Time RT-PCR

454   Total RNA was extracted from randomly selected agroinfiltrated leaves at 3, 5 and 10

455   dpi, using RNeasy Plant Mini Kit (Qiagen) in accordance with the manufacturer's

456   instructions. The quality and concentration of all RNA preparations were determined by

457   Nanodrop 2000c (Thermo Scientific).

458   For reverse transcription, 1 µg of total RNA was used in a 20 µL reaction using Maxima®

459   First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) in accordance to

460   manufacturer's instructions.

461   Primers were designed using Primer Express 3.0 software for Real-Time PCR (Applied

462   Biosystems) using the default parameters for the software (Table 2). Quantitative assays

463   of GFP mRNA were performed by real time RT-PCR (RT-qPCR), carried out on a 7500 Real

464   Time PCR System (Applied Biosystems).

465   A mRNA GFP 147-bp fragment, OMMV ORFs (p23, p52, p8, p6, CP) as well as full length

466   OMMV, were amplified. The protein endogenous control genes phosphatase 2A (PP2)

467   and F-box protein (F-box) were used as internal standards.

468   RT-qPCRs were carried out with 12.5 µL of 2 × SYBR Green PCR Master Mix, 0.3 µM of

469   each primer and 12.5 ng of cDNA per sample, prepared in 96-well plates and run for 40

470   amplification cycles comprising a 15 s denaturation at 95 °C, followed by a 1 min at 60 °C

471   step. A negative control with no template and three technical replicates were

472   considered.

473   Cycle threshold (C_T) values were determined using the fit-point method and the Applied

474   Biosystems 7500 software with a fluorescence threshold arbitrarily set to 0.1. The

475   relative level of GFP mRNA was determined using the amount of GFP mRNA from 16C

476   non-inoculated plants as reference level. At the end of the qPCR, melt curve analysis

477   was conducted to validate the specificity of the primers. A standard curve for each gene

22

478   was constructed for relative expression level estimation and data was normalized by PP2

479   and F-box (reference genes).

480   To exclude the possibility of weak or no silencing suppression being due to low levels of

481   respective protein expression in infiltrated leaves, quantitative assays of mRNA of each

482   the proteins were performed by RT-qPCR as described previously for GFP.

483

484   Table 2: Primers used in RT-qPCR

Gene	Primer name	Primer (5' - 3')	Reference
Protein phosphatase 2A	PP2ArtFW	GACCCTGATGTTGATGTTCGCT	(32)
	PP2ArtREV	GAGGGATTTGAAGAGAGATTTC	
F-box protein	FBOXrtFW	GGCACTCACAAACGTCTATTTC	(32)
	FBOXrtREV	ACCTGGGAGGCATCCTGCTTAT	
GFP	GFP-ER Taq-F	GCCAACACTTGTCACTACTTTCTC	(30)
	GFP-ER Taq-R	GTAGTTCCCGTCGTCCTTGAAG	
OMMV p23	OMMVP23rtFW	CGAGTCCGCAAGCAGAAGAAG	This study
	OMMVP23rtREV	GGGTAGACCAAACTCGGCA	
OMMV p52	OMMVP52rtFW	TCCAAGACGCCCCGAAAC	This study
	OMMVP52rtREV	TGGTTACAGGGGAATGACGC	
OMMV p8	OMMVP8rtFW	GCTCAGAAATCGCAGCAAGG	This study
	OMMVP8rtREV	TGTCACGGTAATGGTCTGTTCT	
OMMV p6	OMMVP6rtFW	TGTGTCGCTGCTGTGATACTT	This study
	OMMVP6rtREV	TTGCAAGGATGAGGATGAGAAT	
OMMV CP	OMMVCPrtFW	TGTCCAGCCACAGCTCTCAT	This study
	OMMVCPrtREV	TTCGATGAACTCAATCTCATATCGC	

485

486   Data analysis

487   Univariate and multivariate analyses were performed using the PRIMER v6 software (33)

488   with the permutational analysis of variance (PERMANOVA) add-on package (34), to

489   detect significant differences (p < 0.05) in the relative GFP mRNA abundance between;

490   "ORFs" p23, p52, p8, p6, CP, OMMV, GFP and Tav-2b, and "Time" day 3, day 5 and day

491   10. A two-way PERMANOVA was applied to test the null hypotheses that no significant

23

492   differences existed between "ORFs" and "Time". PERMANOVA analyses were carried

493   out with the following two-factor design, ORFs; p23, p52, p8, p6, CP, OMMV, GFP and

494   Tav-2b (eight levels, fixed); and Time; day 3, day 5 and day 10 (three levels, fixed). The

495   data were square-root transformed in order to scale down the importance of highly

496   values of relative GFP mRNA abundance. The PERMANOVA analysis was conducted on

497   a Bray-Curtis similarity matrix (35). If the number of permutations was lower than 150,

498   the Monte Carlo permutation p was used. Whenever significant interaction effects were

499   detected, these were examined using a posteriori pairwise comparisons, using 9999

500   permutations under a reduced model.

501

502

503   **OMMV resistance challenge**

504

505   A full length cDNA of OMMV (36) was used for in vitro transcription using RiboMax^{TM}

506   Large Scale RNA Production System-T7 (Promega). Following transcription, DNA

507   templates were removed by digestion with DNase and transcripts were purified by

508   extraction with phenol:chloroform (5:1) acid equilibrated (pH 4.7) (Sigma) and ethanol

509   precipitated.

510   Synthesized RNA was mechanically inoculated onto 6-8 leaf stage *N. benthamiana*

511   plants, maintained in a growth chamber at 23 °C with a 16 h photoperiod, for viral

512   propagation. OMMV infected leaves were then ground in cold 0.1 M, sodium phosphate

513   buffer, pH 6.0 (1:3w/v), filtered, clarified in the presence of organic solvents,

514   concentrated by differential centrifugation and further purified by ultracentrifugation

515   through sucrose density gradients (37). The concentration of viral preparations was

516   determined by Nanodrop 2000c (Thermo Scientific) prior to inoculation.

517   Hairpin constructs of CP and p6 were constructed based on the CP without the first 141

518   nt to exclude CP mediated resistance events (using OMMVCP-141attB1: 5'

519   <u>AAAAAGCAGGCT</u>ATCCTAGATCTTCTGGGCTAAGC and OMMVCPattB2) and on the full p6

520   (using OMMVp6attB1 and OMMVp6attB2), respectively. hpRNA-CP and hpRNA-p6

521   constructs were obtained through LR recombination from each pDONR221-CP and

522   pDONR221-p6, as described previously, to pHELLSGATE12 (38), placed in sense and

523   antisense directions to produce self-complementary dsRNAs. Confirmation of the

524   correct sequences was done by sequencing of the constructs after linearization with *Cla*I

525   which cleaves within the intron.

526   15 μg of purified OMMV were inoculated onto two fully expanded carborundum dusted

527   leaves of 4-week-old *N. benthamiana* 16C line plants 3 days after infiltration with

528   Agrobacterium cultures carrying pHELLSGATE12-CP and pHELLSGATE12-p6, as described

529   for transient expression assays. Inoculated plants were grown in the conditions

530   mentioned above. The presence of each construct was confirmed by RT-qPCR at 3dpi as

531   shown previously.

532   Ten plants were infiltrated with pHELLSGATE12-CP, ten with pHELLSGATE12-p6, ten with

533   both constructs and ten with the empty vector to be used as negative control. All 40

534   plants were mechanically inoculated with OMMV. Experiments were repeated two

535   times.

536   Plants were monitored daily for 30 days for symptom development. A four-grade

537   disease scale was adopted to describe OMMV symptoms along time: 0, no symptoms;

538   1, mild chlorotic mosaic; 2, intense chlorotic mosaic; 3, necrotic mosaic; 4, pronounced

25

539   leaf necrosis and deformation. Disease severity was evaluated on 2 dpi, 5 dpi, 10 dpi and

540   16 dpi for each batch of plants infiltrated with the different constructs, as follows:

541   Disease Severity Index (DSI)= (SUM of all disease ratings/(Total number of

542   ratings*Maximum disease grade))*100.

543   Total RNAs were extracted from inoculated leaves from 3 randomly selected plants, at

544   5 dpi and 16 dpi. Virus accumulation was determined by quantitative real time PCR

545   analysis using OMMVp23 primers (Table 2) as described above.

546   Univariate and multivariate analyses were performed as described above, using the

547   PRIMER software to detect significant differences (p < 0.05) in the virus accumulation

548   between; "ORFs" p6, CP and p6+CP, and "Time" 5 dpi and 16 dpi. A two-way

549   PERMANOVA was applied to test the null hypotheses that no significant differences

550   existed between "ORFs" and "Time". PERMANOVA analyses were carried out as

551   previously, with the following two-factor design, ORFs; p6, CP and p6+CP (three levels,

552   fixed); and Time; 5 dpi and 16 dpi (two levels, fixed). The data were square-root

553   transformed in order to scale down the importance of highly values of virus

554   accumulation.

555

556

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557

558

571 CV, GN and MRF designed the studies. CV, PM and MRF designed the experiments. PM

572 performed the statistical analyses. MDC and MIC contributed to discussions. CV, PM, GN

573 and MRF contributed to the interpretation of the results. CV and PM performed the

574 figures and drafted the manuscript. All authors contributed for the writing of the final

575 manuscript.

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577
578

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