Specificity of resistance and tolerance to cucumber vein yellowing virus in melon accessions and evidence for resistance breaking associated with a single mutation in VPg

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1 **Specificity of resistance and tolerance to cucumber vein yellowing virus in melon accessions**
2 **and resistance breaking with a single mutation in VPg**

3

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16

17 **Abstract**

18 Cucumber vein yellowing virus (CVYV) is an emerging virus on cucurbits in the Mediterranean
19 Basin, against which few resistance sources are available, particularly in melon. The melon
20 accession PI 164323 displays complete resistance to isolate CVYV-Esp, and accession HSD 2458
21 presents a tolerance, i.e. very mild symptoms in spite of virus accumulation in inoculated plants.
22 The resistance is controlled by a dominant allele *Cvy-1¹*, while the tolerance is controlled by a
23 recessive allele *cvy-2*, independent from *Cvy-1¹*. Before introducing the resistance or tolerance in
24 commercial cultivars through a long breeding process, it is important to estimate their specificity
25 and durability. Upon inoculation with eight molecularly diverse CVYV isolates, the resistance was
26 found to be isolate-specific since many CVYV isolates induced necrosis on PI 164323, whereas the
27 tolerance presented a broader range. A resistance-breaking isolate inducing severe mosaic on PI
28 164323 was obtained. This isolate differed from the parental strain by a single amino acid change
29 in the VPg coding region. An infectious CVYV cDNA clone was obtained, and the effect of the
30 mutation in the VPg cistron on resistance to PI 164323 was confirmed by reverse genetics. This
31 represents the first determinant for resistance-breaking in an ipomovirus. Our results indicate
32 that the use of the *Cvy-1¹* allele alone will not provide durable resistance to CVYV and that, if used
33 in the field, it should be combined with other control methods such as cultural practices and
34 pyramiding of resistance genes to achieve long-lasting resistance against CVYV.

35

36 **Keywords:** infectious clone, ipomovirus, tolerance, resistance-breaking, VPg, fitness

37

38 **Introduction**

39 Cucumber vein yellowing virus (CVYV) is an emerging virus on cucurbits in the Mediterranean
40 Basin. CVYV belongs to the genus *Ipomovirus* in the family *Potyviridae*. Its genetic organization is
41 similar to that of viruses in the genus *Potyvirus*, except that it encodes no helper component
42 protein (HC-Pro) but a duplicated P1 protein that replaces the HC-Pro functionally as a silencing
43 suppressor (Valli et al. 2006). CVYV is transmitted by the whitefly *Bemisia tabaci* in a semi-
44 persistent manner (Lecoq et al. 2000; Desbiez et al. 2019). First described in Israel in 1960, CVYV
45 is now present throughout the Middle East and the western Mediterranean Basin (Velasco et al.
46 2016). Surveys in Sudan have shown that the virus has also been present for decades in Sub-
47 Saharan Africa (Desbiez et al. 2019). CVYV induces symptoms of vein clearing and yellowing of the
48 leaves, as well as discoloration and sometimes cracking on fruits that make them unmarketable
49 (Navas-Castillo et al. 2014). As for many other viruses, control of CVYV relies on the control of
50 whitefly populations and the use of genetic resistance when available (Janssen et al. 2003; Gomez
51 et al. 2009). The use of resistant cultivars is a sustainable and environmentally-friendly way of
52 controlling viruses but presents several limitations. First, resistance factors must be available in
53 the germplasm of the species of interest or wild relatives and introduced in high-yielding cultivars
54 through a time- and labor-consuming breeding process. Besides, upon large-scale deployment of
55 resistances in the field, their efficiency can be threatened because they happen to be too specific,
56 i.e. efficient only against a subset of the existing viral isolates, or broken through the evolution of
57 the pathogen (Garcia-Arenal and McDonald 2003; Kobayashi et al. 2014). Therefore, it is
58 important to estimate the potential durability of a resistance factor before using it in breeding
59 programs.

60 Resistance to CVYV has been described in wild *Cucumis* species (*C. prophetarum*, *C. africanus*, *C.*
 61 *dipsaceus*) (Marco et al. 2003) and in cucumber (*Cucumis sativus*) (Pico et al. 2003) with some
 62 strain variability (Galipienso et al. 2013). In melon (*Cucumis melo*), after screening 1188 melon
 63 accessions with a Spanish isolate of CVYV, five phenotypes were observed (Pitrat et al. 2012).
 64 Most accessions displayed either susceptibility (mosaic and vein clearing symptoms) or very high
 65 susceptibility (severe mosaic, yellowing and stunting), a few accessions showed systemic necrosis
 66 followed by rapid plant death, one accession was tolerant (very mild mottle despite virus
 67 accumulation in the plant), and one accession was resistant (no symptoms, no virus detection)
 68 (Pitrat et al. 2012) (Figure 1).

69 In this work, we studied the specificity of the resistance and tolerance phenotypes in melon
 70 cultivars against several CVYV isolates. We showed that the tolerance was efficient against a large
 71 number of isolates, whereas the resistance was very specific and easily overcome by the virus
 72 through an E to K point mutation at amino acid position 90 in the viral protein genome-linked
 73 (VPg). We also explored the possible impact of this mutation on VPg conformation.

74

75 **Materials and methods**

76 **Melon accessions**

77 Melon accessions representative of the five phenotypes observed after infection with CVYV-Esp
 78 (Pitrat et al. 2012) were used in the study: i) Védraçais, a Cantaloupe type from France, is
 79 susceptible to CVYV-Esp, showing symptoms of vein clearing and mosaic; ii) Ouzbeque 2 from
 80 Uzbekistan is highly susceptible with symptoms of severe mosaic, stunting and yellowing; iii)
 81 accession HSD 93-20-A from Sudan exhibits necrotic symptoms with rapid death upon infection;

82 iv) HSD 2458 from Sudan is tolerant to CVYV-Esp, i.e. the virus can be detected in inoculated plants
83 but the plants display only a very mild mottle ; and v) accession PI 164323 from India is resistant
84 to CVYV-Esp : no virus is detected and no symptoms are visible in inoculated plants. The resistance
85 was first observed after mechanical inoculation of the virus but it was shown to be also effective
86 after whitefly transmission (data not shown). Five to 10% of the mechanically infected PI 164323
87 plants developed a top necrosis after inoculation (Figure 1).

88 F₁, F₂ and back-cross (BC) progenies were obtained between the above mentioned accessions in
89 order to study the inheritance of the different phenotypes (Pitrat et al. 2012). Three loci appeared
90 to be involved. At a first locus tentatively named *Cucumber vein yellowing resistance* (symbol
91 *Cvy-1*), three alleles have been identified: *Cvy-1*⁺ for susceptibility (present in Védreantais), *Cvy-1*¹
92 controlling resistance in PI 164323 and *Cvy-1*² controlling necrosis in HSD 93-20-A. At an
93 independent second locus, the recessive allele *cvy-2* present in HSD 2458 controls the tolerance.
94 And at a third locus, the allele *Cvy-3*, present in Ouzbeque 2, controls the highly susceptible type
95 of symptoms (Pitrat et al. 2012).

96 The five lines studied in this paper present the following genotypes: Védreantais [*Cvy-1*⁺ *cvy-2*⁺ *Cvy-*
97 *3*⁺], Ouzbeque 2 [*Cvy-1*⁺ *cvy-2*⁺ *Cvy-3*], HSD 2458 [*Cvy-1*⁺ *cvy-2* *Cvy-3*⁺], PI 164323 [*Cvy-1*¹ *cvy-2*⁺
98 *Cvy-3*], and HSD 93-20-A [*Cvy-1*² *cvy-2*⁺ *Cvy-3*] (Pitrat et al. 2012).

99

100 **CVYV isolates and mechanical inoculation of melon accessions**

101 Isolates CVYV-Esp, SU96-84, SU12-10, TN05-56, OM04-06, ISR, LIB and JOR are as described in
102 Desbiez et al. (2019). They originate from Spain, Sudan, Tunisia, Oman, Israel, Lebanon, and
103 Jordan, respectively, and are representative of the known molecular variability of CVYV (Desbiez

et al. 2019). All isolates were stored as dried leaf material on calcium chloride or as frozen leaf tissue in liquid nitrogen. For inoculations, dry or frozen plant tissues were first ground in 0.03 M Na_2HPO_4 + 0.2% diethyldithiocarbamate (DIECA). Activated charcoal and carborundum (75 mg/ml each) were added before rub-inoculating the two cotyledons of young plantlets of cucumber cf. Beit Alpha. Symptomatic cucumber leaves were used for mechanical inoculation of each melon accession with the same protocol, using 5 or 10 young plantlets of each accession. A second inoculation was performed one week later on the first leaf of the same melon plantlets to increase infection efficiency. Inoculated plants were kept in a quarantine greenhouse (C3 biosecurity level). The presence of the virus at the plant apex, six weeks after inoculation, was assessed by DAS-ELISA with a CVYV-specific antiserum (Desbiez et al. 2019) or by RT-PCR as described (Lecoq et al. 2007). The experiment was repeated three times.

115

116 **Full-length sequencing of resistance breaking isolate CVYV-PI1**

After mechanical inoculation of CVYV-Esp on melon PI 164323, symptoms of top necrosis were observed in 5 to 10% of the plants (Figure 1). In one instance, an axillary shoot developed that showed severe mosaic symptoms. Back inoculations were performed on PI 164323 from mosaic-showing plant material and the inoculated plants displayed mosaic symptoms. After five successive passages on PI 164323, the mosaic-inducing variant CVYV-PI1 was stored as dried material on calcium chloride and used for full-length sequencing. The complete sequence of CVYV-PI1 was determined through Sanger sequencing as described by Desbiez et al. (2019). Full-length sequences of CVYV-PI1 and CVYV-Esp were aligned with ClustalW included in MEGA6 (Tamura et al. 2013) and compared manually.

125

Cloning and inoculation of plants with an infectious cDNA clone of CVYV

Total RNA was extracted from a young plant of cucumber cv. Beit Alpha inoculated with CVYV-Esp using TRI-reagent (Molecular Research Center, Cincinnati, OH). The precise sequence of the 5' extremity from CVYV-Esp was reassessed by 5'-RACE (Ferriol et al. 2018). The genome of CVYV-Esp was amplified in five overlapping fragments of 1.2 to 1.8 kb through a two-step RT-PCR using a high-fidelity *Pfu* DNA polymerase for PCR and specific primers (Supplementary table S1) as described (Desbiez et al. 2012). Two introns were added by fusion PCR to fragments 2 and 3, in the P3 and CI coding regions, respectively. The five final fragments were purified from agarose gel, mixed with a linearized vector derived from pAGUS1 and used for transformation of competent *Saccharomyces cerevisiae* YHP 501 cells as described (Desbiez et al. 2012), before plating on CAU selective medium. The yeast colonies were then grown in CAU liquid medium, yeast DNA was extracted as described (Desbiez et al. 2012) and used to transform electrocompetent *Escherichia coli* DH5 α bacteria. Bacterial plasmid DNA was extracted by alkaline lysis and a screening was performed by restriction enzyme analysis. Since the yields of bacterial DNA were very low, 0.5 μ l of plasmid DNA were submitted to rolling circle amplification (RCA) using the TempliPhi kit (GE Healthcare) according to the manufacturer's manual. One μ l of RCA product was then used for restriction analysis. Inoculation was performed by biolistics using a GeneGun according to Gal-On et al. (1995), on the cotyledons of eight cucumber Beit Alpha plantlets. Briefly, 30 μ l of plasmid DNA, or 5 μ l RCA product + 25 μ l distilled water, were mixed with 30 μ l of tungsten M20 at 50 mg/ml in 50% glycerol and 30 μ l of Ca(NO₃)₂ 1.25M, pH 8. After 5 minutes at room temperature, a short centrifugation was performed and 60 μ l of the supernatant were removed. Three μ l of the remaining mix were used for the bombardment of

148 each plant. Three different DNA preparations were used: (1) plasmid DNA without RCA
 149 amplification (2) plasmid DNA amplified by RCA and digested with *SnaBI* that cuts only once in
 150 the vector, in order to inoculate monomers of the construct rather than the very long
 151 amplification products of the RCA reaction, and (3) plasmid DNA amplified by RCA without post-
 152 RCA treatment. The plants were kept in a quarantine greenhouse (C3 biosecurity level) until
 153 symptom development. The full-length sequence of clone 3.1 was established as described
 154 (Desbiez et al. 2019) and compared to the original sequence of CVYV-Esp.

155

156 **Site-directed mutagenesis in the VPg of CVYV-Esp**

157 In order to validate the effect of the VPg mutation on CVYV infectivity in PI 164323, site-directed
 158 mutagenesis was performed on the CVYV-Esp infectious clone CVYV 3.1. RT-PCR was performed
 159 on the cloned DNA with mutagenic primers CV-mutaVPg2-F (5'-
 160 GGAGATTTCAAAGGGAAAGTCTGGGAAACAAAAGACATGGA-3') and CV-mutaVPg-R2 (5'-
 161 CCATGTCTTTTGTTCCTCCAGACTTCCCTTTGAAATCTCCAACG-3'). The underlined residue
 162 indicates the position of the mutation (position 5962 in the genome and 6355 in the intron-
 163 containing clone). Two fragments of 1300 nt and 1900 nt, respectively, overlapping at the
 164 position of the mutation, were amplified by RT-PCR with the following primers (1) CVYV-GRMKP-
 165 5' (5'- AGTGGATCCGGRAGGATGAARCCAGGNAC-3') + CV-mutaVPg2-R, and (2) CV-mutaVPg-F +
 166 CVYV-VVDNT-3' (5'- CCAGGATCCATGAGGGTATTGTCCACCAC-3').

167 Since the expression level of CVYV-Esp infectious clone in *E. coli* was very low, clone 3.1 was
 168 submitted to RCA with the TempliPhi kit according to the manufacturer's manual before
 169 digestion with restriction enzyme *NdeI* that cuts at positions 5210 and 8140 in the intron-

containing CVYV clone. The 12.7-kbp resulting vector DNA as well as the two PCR fragments were purified from gel and used for transformation of competent *S. cerevisiae* YHP 501 cells as described (Desbiez et al. 2012). After growth on CAU medium, yeast DNA was extracted and used to transform electrocompetent *E. coli* DH5 α bacteria. Bacterial plasmid DNA was extracted by alkaline lysis and a screening was performed by restriction enzyme analysis. The presence of the expected E90K mutation in the VPg was checked by amplification with primers CVYV-VPg-F (5'-GCAGGAGTCATCACGATAAC-3') and CVYV-6250-R (5'-ARCAATTGCGGTTTCAGCGAC-3') followed by Sanger sequencing of the targeted area. Positive clones (0.5 μ l DNA extracted by alkaline lysis) were amplified by RCA. One μ l of RCA product was used for inoculation by biolistics on the cotyledons of cucumber Beit Alpha plantlets.

Infectivity of CVYV-VPg-C1

One infectious clone containing the VPg mutation, CVYV-VPg-C1, was inoculated by GeneGun to five plantlets of cucumber cv. Beit Alpha. Leaf tissue of symptomatic plants was used for mechanical inoculation of 5 or 10 plantlets of cucumber Beit Alpha and melons cv. Védrantais, PI 164323, Ouzbeque 2, HSD 93-20-A and HSD 2458. The plants were kept in a quarantine greenhouse until symptom development. Six weeks after inoculation, all asymptomatic plants were tested by DAS-ELISA for the presence of CVYV. The experiment was repeated 3 times.

Comparison of VPg sequences and their disorder profiles among CVYV and potyvirus isolates.

The VPg sequences of 11 CVYV isolates were retrieved from GenBank (accessions AY578085, JF460793, KT276369, MK777988-MK777995). Nucleotide and translated amino acid sequences

192 of these isolates and of CVYV-PI1 were aligned with ClustalW. Alignments were also performed
 193 with the VPg amino acid sequences of other ipomoviruses: coccinia mottle virus (CocMoV,
 194 YP_009272707), squash vein yellowing virus (SqVYV, YP_001788991, AEV45694, ALN38790,
 195 AOY33888), cassava brown streak virus (CBSV, YP_007027011), Ugandan cassava brown streak
 196 virus (UCBSV, YP_004063980) and sweetpotato mild mottle virus (SPMMV, NP_734290).
 197 Four methods were used to predict the intrinsic disorder of CVYV VPg and the impact of the
 198 mutation on this disorder: (1) Disopred (<http://bioinf.cs.ucl.ac.uk/disopred>), (2) DisEMBL
 199 (<http://dis.embl.de>), (3) GlobProt (<http://globplot.embl.de>), and (4) PONDR®
 200 (<http://www.pondr.com>), as described by Desbiez et al. (2014). As a comparison, VPg sequences
 201 of three different ipomoviruses (SqVYV, CBSV and UCBSV) and of four potyviruses (potato virus Y,
 202 zucchini yellow mosaic virus, papaya ringspot virus and turnip mosaic virus) were analysed with
 203 PONDR®.

204

205 Results

206 Isolate specificity of CVYV resistance and tolerance

207 Upon inoculation of the reference susceptible accession Védraçais [*Cvy-1⁺ cvy-2⁺ Cvy-3⁺*], the
 208 eight CVYV isolates tested induced mosaic and vein banding symptoms (Table 1); isolates CVYV-
 209 JOR and CVYV-Su96-84 also induced some stunting. Accession Ouzbeque 2 [*Cvy-1⁺ cvy-2⁺ Cvy-3*],
 210 carrying the high susceptibility allele *Cvy-3*, displayed severe symptoms of mosaic and stunting
 211 upon inoculation with the different isolates. On HSD 93-20-A [*Cvy-1² cvy-2⁺ Cvy-3*] carrying the
 212 *Cvy1²* allele responsible for lethal necrosis upon inoculation with CVYV-Esp, all isolates except
 213 SU96-84 induced 100% top necrosis or systemic necrosis. Upon inoculation with SU96-84, in most

214 cases no symptoms were visible and no virus was detected by DAS-ELISA but occasionally
215 systemic necrosis was observed. Accession HSD 2458 [*Cvy-1⁺ cvy-2 Cvy-3⁺*] carrying the *cvy-2*
216 allele conferring tolerance to CVYV-Esp was tolerant and displayed only very mild mottle
217 symptoms upon inoculation with the different isolates, even though the Jordanian and Oman
218 isolates were more severe than the others. The melon accession PI 164323 [*Cvy-1¹ cvy-2⁺ Cvy-3*]
219 carrying the resistance allele *Cvy-1¹* displayed complete resistance with no visible symptoms and
220 no virus detection by DAS-ELISA to isolate CVYV-SU12-10. Upon inoculation with CVYV-Esp and
221 CVYV-Isr, most inoculated plants (>90%, n>30) displayed no symptoms and no virus accumulation
222 at the systemic level (Table 1), even though a low proportion of the inoculated plants (5 to 10%)
223 developed top necrosis, followed by plant death. For the five other isolates CVYV-JOR, CVYV-
224 SU96-84, CVYV-TN05-56, CVYV-OM04-06 and CVYV-LIB, nearly all inoculated plants showed lethal
225 top necrosis (Table 1 ; Figure 1).

226

227 **Full-length sequencing of CVYV-Esp and the resistance-breaking variant CVYV-PI1**

228 An evolved isolate CVYV-PI1, developing severe mosaic symptoms on PI 164323, was obtained
229 after mechanical inoculation of CVYV-Esp. The full-length sequences of CVYV-PI1 and CVYV-Esp
230 were obtained(Desbiez et al. 2019). The accurate sequence of the 5' extremity of CVYV-Esp was
231 obtained by using 5'-RACE, indicating that the CVYV sequence available before was truncated and
232 the first 17 nt were missing. The sequence of CVYV-Esp sequence presented an A-rich 5' extremity
233 as expected for a potyvirus, and the first 15 nt were identical to those of the SqVYV reference
234 isolate (accession NC_010521). Compared to CVYV-Esp, three mutations were found in CVYV-PI1,
235 at positions 5055, 5962 and 7620. Mutations 5055 (TTA/TTG) and 7620 (CGC/CGT) were

236 synonymous, whereas mutation 5962 (GAA/AAA, G₅₉₆₂A) caused an amino acid change at position
 237 90 in the viral VPg, from glutamic acid (E) to lysine (K) (E₉₀K).

238

239 **Infectious cDNA clone of CVYV-Esp**

240 After amplification of the CVYV-Esp genome and yeast transformation, clones with the
 241 expected size and restriction profiles were obtained but the level of expression of plasmid DNA
 242 in *E. coli* was very low, and RCA amplification was performed to enhance the plasmid
 243 concentration. After GeneGun inoculation on cucumber cv. Beit Alpha, 100% of inoculated plants
 244 with clone 3.1 showed symptoms of vein clearing two weeks after inoculation with the RCA-
 245 amplified plasmid, whether or not it was digested with *Sna*BI, showing that direct GeneGun
 246 inoculation of very long RCA products was as efficient as inoculation with the 15 kbp linearized
 247 plasmid. In contrast, no infection was observed with inoculation of the plasmid without RCA
 248 amplification. The full-length sequence of clone 3.1 was determined, and it differed from the
 249 sequence obtained ten years earlier for the original field isolate by 26 mutations including 7 non-
 250 synonymous located in the P1 (3 mutations), NIb (2 mutations), CI and CP (1 mutation each).
 251 There was no non-synonymous mutation in the VPg.

252

253 **Effect of the VPg E₉₀K mutation on CVYV infectivity and symptom expression**

254 The G₅₉₆₂A (VPg E₉₀K) mutation was introduced in the CVYV 3.1 clone by PCR mutagenesis, yielding
 255 the mutated clone CVYV-VPg-C1. The presence of the mutation in CVYV-VPg-C1 clone was
 256 confirmed by partial sequencing. After amplification by RCA and inoculation by GeneGun of clone
 257 CVYV-VPg-C1, 5/5 inoculated Beit Alpha cucumber plants showed symptoms of vein clearing and

mosaic indistinguishable from those of CVYV 3.1. After mechanical inoculation on PI 164323, all inoculated plants displayed severe mosaic and yellowing symptoms similar to those induced by the natural resistance-breaking isolate CVYV-PI1 (Table 1). Both CVYV-PI1 and CVYV-VPg-C1 induced similar symptoms as CVYV-Esp on the melon accessions HSD 2458 (tolerance), Ouzbeque 2 (severe symptoms of mosaic and stunting) and HSD93-20-A (lethal top necrosis) (Table 1). On Védtrantais, CVYV-PI1 induced mottle or mosaic symptoms milder than those of CVYV-Esp and CVYV 3.1, and the cloned mutant CVYV-VPg-C1 induced only very mild mottle (Table 1) although the virus was readily detected using DAS-ELISA.

Diversity and structure of the VPg coding region of CVYV and other ipomoviruses

The amino acid VPg sequences of 11 CVYV isolates and the evolved variant CVYV-PI1 were aligned and compared. There was no extensive sequence polymorphism in the VPg coding region for CVYV (183 aa for all isolates) (Figure 2a). Most CVYV isolates had an E at amino acid position 90 in the VPg, except CVYV-ISR and CVYV-ISM (sharing identical VPg sequences) that had a D. Among the 11 CVYV sequences excluding CVYV-PI1, 14 non-synonymous mutations were present in the VPg coding region, nine of which were observed in only one isolate. Ten mutations were in the central part of the VPg between aa 90 and 137 out of 183. CocMoV, closely related molecularly to CVYV, displayed ~75% amino acid identity in the VPg with different CVYV isolates. It had a Q at the position corresponding to CVYV VPg₉₀, immediately followed by an E, and a K at position VPg₉₃ instead of the E present in all CVYV isolates (Figure 2a). Melon accession PI 164323 was susceptible to the Su12-25 isolate of CocMoV in mechanical inoculation (H. Lecoq, unpublished). The other ipomoviruses displayed between 30% and 50% aa conservation in the VPg with CVYV,

without conservation at or around the E₅₉₆₂ position (Figure 2a).

Based on PONDR prediction, the central part of CVYV VPg where the resistance-breaking mutation was located appeared as a highly ordered region, and the E₉₀K mutation, despite the change from a negatively charged glutamic acid to the positively charged lysine, did not have a strong effect on its disorder profile (Figure 2b). The VPg disorder profile of CVYV was very different from that of the potyviruses PVY, ZYMV, PRSV and TuMV (Supplementary figure S2), where the central region of the VPg was confirmed as an intrinsically disordered domain (Supplementary figure S2). The disorder profiles of the ipomoviruses CVYV, SqVYV, CBSV, and UCBSV were very variable, the central domain being either ordered or disordered, with contrasted situations even for the closely related CBSV and UCBSV (Supplementary figure S2), suggesting that the VPg interaction patterns between potyviruses and ipomoviruses, and also between different ipomoviruses are not conserved.

Discussion

In this work, we found that resistance to CVYV present in melon accession PI 164323 and conferred by the allele *Cvy-1*¹ was isolate-specific since only some isolates failed to infect, whereas others induced a rapid and lethal top necrosis. Besides, the resistance to CVYV-Esp could be overcome through a point mutation in the virus genome, resulting in an E to K amino acid change (E₉₀K) in the viral VPg. We validated the effect of that mutation by reverse genetics using a newly-developed infectious cDNA clone of CVYV. Previous attempts to obtain infectious clones of CVYV were unsuccessful, probably due to the fact that the sequences available so far lacked 17 nts at their 5' extremity, including the first four "As" required for potyvirid infectivity (Simon-

302 Buela et al. 2000). Despite the presence of two introns in the P3 and CI coding regions of the
303 cDNA, the yield of CVYV-containing plasmids were very low in *E. coli*, probably in relation to the
304 toxicity of P1a or P1b proteins in bacteria. No infection was obtained after direct biolistic
305 inoculation of the plasmid, indicating that the threshold concentration of plasmid DNA for
306 infectivity was not reached. For potyviruses, less than 0.1 ng plasmid DNA can be sufficient for
307 high infectivity after bombardment (Gal-On et al. 1995), but it seems more DNA may be required
308 for CVYV. Indeed, CVYV is generally less efficiently transmitted than cucurbit-infecting potyviruses
309 in mechanical inoculation (H. Lecoq, unpublished). The use of RCA prior to inoculation improved
310 the infectivity of the plasmid. Resistance-breaking mutants, both the natural CVYV-PI1 and the
311 cloned CVYV-VPg-C1, induced severe mosaic symptoms on PI 164323. Such severe symptoms
312 could be expected since PI 164323, besides the broken *Cvy-1*¹ resistance allele, possesses the
313 *Cvy-3* “high susceptibility” allele also present in Ouzbeque 2 (Pitrat et al. 2012).

314 The E to K mutation at aa position 90 in the VPg of CVYV-VPg-C1 was sufficient to induce resistance
315 breaking in PI 164323. Indeed, contrary to other pathogens, most situations of resistance breaking
316 in plant viruses are related to point mutations rather than indels or loss of expression (Sacristan
317 and Garcia-Arenal 2008). This is probably due to the constraints of virus genomes where all
318 proteins are required for infectivity and are frequently multifunctional (Revers and Garcia 2015).

319 The E₉₀K mutation in the VPg resulted in charge change in the protein, which probably affects its
320 interaction with so far undetermined plant or virus factors. The area where the mutation took
321 place appears as highly ordered, and the mutation does not seem to modify deeply the level of
322 disorder of the protein (Figure 2b).

323 Surprisingly, although the resistance related to *Cvy-1*¹ was efficient only against some CVYV

324 isolates that display an E or a D at position 90 in the VPg, all the other isolates tested had an E at
 325 position 90 and presented few mutations in the direct vicinity of this site. CVYV-JOR had a V to I
 326 mutation at position 91, and OM04-06 had an E to G mutation at position 101, but no common
 327 mutation was detected. Top necrosis was frequently observed upon infection with different CVYV
 328 isolates, which might be considered as a systemic hypersensitive response (Abebe et al. 2021). As
 329 described for cucumber mosaic virus, a small decrease in the level of resistance induction could
 330 change a hypersensitive response (not visible in PI 164323 but that may take place at the cellular
 331 level) to systemic necrosis that may account for the low and variable frequency of top necrosis in
 332 PI 164323 upon inoculation with CVYV-Esp. Determinants for the top necrosis response may thus
 333 be different from those involved in the *bona fide* resistance breaking observed for CVYV-PI1. PI
 334 164323 was also observed to display occasionally top necrosis even in the absence of virus
 335 infection, although the mechanisms involved are not known (M. Pitrat, unpublished).

336 Mutations in the VPg have been associated with resistance-breaking virus isolates in several plant-
 337 virus interactions (Truniger and Aranda 2009), notably among potyviruses where direct
 338 interaction between the VPg and the plant translation initiation factors eIF4E and/or eIF(iso)4E is
 339 required for infectivity (Le Gall et al. 2011; de Oliveira et al. 2019). In these cases, the resistance
 340 is recessive (Truniger and Aranda 2009 ; Revers and Garcia 2015), and mutations in the highly
 341 disordered central region of the VPg affect the direct interaction with eIF4E (Ala-Poikela et al.
 342 2019 ; Charon et al. 2018). Melon lines silenced for eIF4E showed resistance to several potyviruses
 343 and also to CVYV (Rodriguez-Hernandez et al. 2012), suggesting that the resistance mechanisms
 344 could be related in both cases to a lack of functional interaction between virus VPg and plant
 345 eIF4E. Cucumbers with the eIF4E gene disrupted by CRISPR/Cas9 technology were also immune

346 to CVYV, the resistance being recessive as for potyviruses (Chandrasekaran et al. 2016). Contrary
347 to most potyvirus resistance, resistance to CVYV in melon PI 164323 appeared to be controlled
348 by a dominant allele *Cvy-1*¹ (Pitrat et al. 2012), which does not fit with a hypothesis of the
349 resistance allele being a plant component, e.g. eIF4E, required for infectivity. There was no amino
350 acid difference in the eIF4E between Védrintais and PI 164323 (C. Dogimont, pers. comm.). PI
351 164323 presented a nonsynonymous mutation in the eIF(iso)4E protein but the same mutation
352 was also present in accession 90625 (C. Dogimont, pers. comm.) that shows no resistance to
353 CVYV-Esp (Pitrat et al. 2012). Thus, there was no obvious correlation between eIF4E or eIF(iso)4E
354 coding sequences and resistance to CVYV. Besides eIF4E, the VPg of potyviruses interacts with
355 several host and virus proteins (Jiang and Laliberté 2011). Potyvirus VPg contributes directly to
356 inhibiting plant antiviral silencing response (Rajamäki et al. 2014 ; Cheng and Wang 2017), and it
357 also interacts with the viral silencing suppressor HC-Pro (Jiang and Laliberté 2011). CVYV VPg may
358 play a role in silencing suppression as well, either by itself or cooperatively through interaction
359 with other viral products including the silencing suppressor P1b that functionally replaces the
360 potyviral HC-Pro (Valli et al. 2006). These properties might be affected in the resistance-breaking
361 mutant. Alternatively, CVYV VPg may be recognized as an avirulence factor by a host resistance
362 gene in PI 164323, inducing an effector-triggered immune response leading to complete
363 resistance or to top necrosis depending on the timing or efficiency of the reaction. More studies
364 will be needed to understand the nature of the *Cvy 1* gene and all the details of the interaction
365 between CVYV and melon.

366 Since the resistance conferred by *Cvy 1*¹ is isolate-specific, it should not be used in the field
367 in areas where necrosis-inducing isolates are present. A single mutation, corresponding to an A

368 to G transition in the virus genome, was sufficient to induce resistance breaking in PI 164323.
369 This constitutes the least favorable situation for resistance durability (Fabre et al. 2009).
370 Resistance breaking might have a fitness cost, as observed for other plant-virus interactions
371 (Desbiez et al. 2003 ; Janzac et al. 2010), which could contribute to preserving resistance
372 durability through optimized management of the resistance deployment (Rimbaud et al. 2021).
373 However, this may not be sufficient to ensure satisfactory durability of the resistance in the field,
374 and thus a durable control of CVYV, without being associated with other control measures.
375 Pyramiding the resistance and tolerance genes (Mundt 2018) and eliminating the Cvy-3 “super-
376 susceptibility” allele may lead to more efficient and durable control to this damaging virus
377 against which very few resistances are available so far in melon.

378

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383

384 **Ethical statement:**

385 This research did not involve any human participants and/or animals. The authors declare that
386 they have no conflict of interest

387

388 **References**

389 Abebe, D. A., van Bentum S., Suzuki, M., Ando, S., Takahashi, H., and Miyashita, S. 2021. Plant

390 death caused by inefficient induction of antiviral R-gene-mediated resistance may function as a
391 suicidal population resistance mechanism. *Comm. Biol.* 4 :947.

392 Ala-Poikela, M, Rajarnaki, M-L, and Valkonen, J. P. T. 2019. A Novel Interaction Network Used by
393 Potyviruses in Virus-Host Interactions at the Protein Level. *Viruses-Basel* 11: 1158.

394 Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., Sherman, A.,
395 Arazi, T.,and Gal-On, A. 2016. Development of broad virus resistance in non-transgenic cucumber
396 using CRISPR/Cas9 technology. *Mol. Plant Pathol.* 17: 1140-1153.

397 Charon, J., Barra, A., Walter, J., Millot, P., Hebrard, E., Moury, B., and Michon, T. 2018. First
398 Experimental Assessment of Protein Intrinsic Disorder Involvement in an RNA Virus Natural
399 Adaptive Process. *Mol. Biol. Evol.* 35: 38-49.

400 Cheng, X., and Wang A. 2017. The Potyvirus Silencing Suppressor Protein VPg Mediates
401 Degradation of SGS3 via Ubiquitination and Autophagy Pathways. *J. Virol.* 91: e01478-16.

402 de Oliveira, L. C., Volpon, L., Rahardjo, A. K., Osborne, M. J., Culjkovic-Kraljacic, B., Trahan, C.,
403 Oeffinger, M., Kwok, B. H., and Borden, K. L. B. 2019. Structural studies of the eIF4E-VPg complex
404 reveal a direct competition for capped RNA: Implications for translation. *Proc. Natl. Acad. Sci. USA*
405 116: 24056-24065.

406 Desbiez, C, Gal-On, A., Girard, M., Wipf-Scheibel, C., and Lecoq, H. 2003. Increase in *Zucchini*
407 *yellow mosaic virus* symptom severity in tolerant zucchini cultivars is related to a point mutation
408 in P3 protein and is associated with a loss of relative fitness on susceptible plants. *Phytopathology*
409 93:1478-1484.

410 Desbiez, C., Caciagli, P., Wipf-Scheibel, C., Millot, P., Ruiz Garcia, L., Marian, D., Dafalla, G., and
411 Lecoq, H. 2019. Evidence for long-term prevalence of cucumber vein yellowing virus in Sudan and

- 412 genetic variation of the virus in Sudan and the Mediterranean Basin. *Plant Pathol.* 68:1268-1275.
- 413 Desbiez, C., Chandeysson, C., Lecoq, H., and Moury, B. 2012. A simple, rapid and efficient way to
414 obtain infectious clones of potyviruses. *J. Vir. Meth.* 183: 94-97.
- 415 Desbiez, C., Chandeysson, C., and Lecoq, H. 2014. A short motif in the N-terminal part of the coat
416 protein is a host-specific determinant of systemic infectivity for two potyviruses. *Mol. Plant*
417 *Pathol.* 15:217-221.
- 418 Fabre, F., Bruchou, C., Palloix, A., and Moury, B. 2009. Key determinants of resistance durability
419 to plant viruses: Insights from a model linking within- and between-host dynamics. *Virus Res.* 141:
420 140-149.
- 421 Ferriol, I., Domingo-Calap, M.-L., Desbiez, C., and Lopez-Moya, J.-J. 2018. Determination of the
422 complete 5'-UTR of the ipomovirus Cucumber vein yellowing virus (CVYV), and its relevance for
423 infectivity in cucurbit plants. in: XIX Congreso de la Sociedad Española de Fitopatología, Toledo,
424 Spain.
- 425 Gal-On, A., Meir, E., Huet, H., Hua, W.J., Raccach, B., and Gaba, V. 1995. Particle bombardment
426 drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. *J. Gen. Virol.*
427 76:3223-3227.
- 428 Galipienso, L., Janssen, D., Rubio, L., Aramburu, J., and Velasco, L. 2013. Cucumber vein yellowing
429 virus isolate-specific expression of symptoms and viral RNA accumulation in susceptible and
430 resistant cucumber cultivars. *Crop Protect.* 43:141-145.
- 431 Garcia-Arenal, F., and McDonald, B. 2003. An analysis of the durability of resistance to plant
432 viruses. *Phytopathology* 93:941-952.
- 433 Gomez, P., Rodriguez-Hernandez, A.M., Moury, B., and Aranda, M.A. 2009. Genetic resistance for

434 the sustainable control of plant virus diseases: breeding, mechanisms and durability. Eur. J.
435 Plant Pathol. 125 :1-22.

436 Janssen, D., Ruiz, L., Cano, M., Belmonte, A., Martin, G., Segundo, E., and Cuadrado, I.M. 2003.
437 Physical and genetic control of *Bemisia tabaci*-transmitted cucurbit yellow stunting disorder virus
438 and cucumber vein yellowing virus in cucumber. Bull. OILB/SROP 26:101-106.

439 Janzac, B, Montarry, J., Palloix, A., Navaud, O., and Moury, B. 2010. A point mutation in the
440 polymerase of Potato virus Y confers virulence towards the Pvr4 resistance of pepper and a high
441 competitiveness cost in susceptible cultivar. Mol. Plant-Microbe Interact. 23:823-830.

442 Jiang, J., and Laliberté, J.-F. 2011. The genome-linked protein VPg of plant viruses: a protein with
443 many partners. Curr. Opin. Virol. 1 :347-354.

444 Kobayashi, K., Sekine, K.T., and Nishiguchi, M. 2014. Breakdown of plant virus resistance: can we
445 predict and extend the durability of virus resistance? J. Gen. Plant Pathol. 80 :327-336.

446 Le Gall, O., Aranda, M.A., and Caranta, C. 2011. Plant Resistance to Viruses Mediated by
447 Translation Initiation Factors. In: Recent Advances in Plant Virology, pp. 177-194.

448 Lecoq, H., Desbiez, C., Delecolle, B., Cohen, S., and Mansour, A. 2000. Cytological and molecular
449 evidence that the whitefly-transmitted Cucumber vein yellowing virus is a tentative member of
450 the family Potyviridae. J. Gen. Virol. 81: 2289-2293.

451 Lecoq, H., Dufour, O., Wipf-Scheibel, C., Girard, M., Cotillon, A.-C., and Desbiez, C. 2007. First
452 report of Cucumber vein yellowing virus in melon in France. Plant Dis. 91: 909.

453 Marco, C.F., Aranda, M.A., Montoro, T., and Gómez-Guillamón, M.-L. 2003. Evaluation of several
454 accessions and wild relatives of *Cucumis melo* against Cucumber Vein Yellowing Virus (CVYV).
455 Cucurbit Genet. Coop. Ann. Rep. 26: 7-8.

- 456 Mundt, C. 2018. Pyramiding for resistance durability: theory and practice. *Phytopathology*
 457 108:792-802.
- 458 Navas-Castillo, J., Lopez-Moya, J.J., and Aranda, M.A. 2014. Whitefly-transmitted RNA viruses that
 459 affect intensive vegetable production. *Ann. Appl. Biol.* 165:155-171.
- 460 Pico, B., Villar, C., and Nuez, F. 2003. Screening *Cucumis sativus* landraces for resistance to
 461 cucumber vein yellowing virus. *Plant Breeding* 122:426-430.
- 462 Pitrat, M., Wipf-Scheibel, C., Besombes, D., Desbiez, C., and Lecoq, H. 2012. Resistance of melon
 463 to Cucumber Vein Yellowing Virus (CVYV). *Cucurbitaceae 2012: Proceedings of the Xth Eucarpia*
 464 *Meeting on Genetics and Breeding of Cucurbitaceae*, 157-164.
- 465 Rajamaki, M. L., Streng, J., and Valkonen, J. P. T. 2014. Silencing Suppressor Protein VPg of a
 466 Potyvirus Interacts With the Plant Silencing-Related Protein SGS3. *Mol. Plant-Microbe Interact.*
 467 27:1199-1210.
- 468 Revers, F., and Garcia, J.A. 2015. Molecular biology of potyviruses. *Adv Virus Res* 92:101-199.
- 469 Rimbaud, L., Fabre, F., Papaix, J., Moury, B., Lannou, C., Barrett, L.G., and Thrall, P.H. 2021. Models
 470 of plant resistance deployment. *Ann. Rev. Phytopathol.* 59, doi: 10.1146/annurev-phyto-020620-
 471 122134.
- 472 122134.
- 473 Rodriguez-Hernandez, A.M., Gonsalvez, B., Sempere, R.N., Burgos, L., Aranda, M.A., and Truniger,
 474 V. 2012. Melon RNA interference (RNAi) lines silenced for Cm-eIF4E show broad virus resistance.
 475 *Mol. Plant Pathol.* 13:755-763.
- 476 Sacristan, S., and Garcia-Arenal, F. 2008. The evolution of virulence and pathogenicity in plant
 477 pathogen populations. *Mol. Plant Pathol.* 9:369-384.
- 478 Simon-Buela, L., Osaba, L., Garcia, J.A., and Lopez-Moya, J.J. 2000. Preservation of 5'-end integrity

479 of a potyvirus genomic RNA is not dependent on template specificity. *Virology* 269:377-382.

480 Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular

481 Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.

482 Truniger, V., and Aranda, M.A. 2009. Recessive resistance to plant viruses. *Adv. Virus Res.* 75:119-

483 159.

484 Valli, A., Martin-Hernandez, A.M., Lopez-Moya, J.-J., and Garcia, J.A. 2006. RNA silencing

485 suppression by a second copy of the P1 serine protease of *Cucumber vein yellowing ipomovirus*,

486 a member of the family *Potyviridae* that lacks the cysteine protease HCPro. *J. Virol.* 80 :10055-

487 10063.

488 Velasco, L., Salem, N., Willemsen, A., Lapidot, M., Mansour, A.N., Rubio, L., and Galipienso, L.

489 2016. Genetic variation and evolutionary forces shaping Cucumber vein yellowing virus

490 populations: risk of emergence of virulent isolates in Europe. *Plant Pathol.* 65:847-856.

491

Table 1 : Symptoms induced by CVYV isolates on melon accessions.

Strain	Melon accession and genotype				
	Védrantais (Cvy-1 ⁺ , cvy-2 ⁺ , Cvy-3 ⁺)	PI 164323 (<u>Cvy-1</u> ¹ , cvy-2 ⁺ , Cvy-3)	HSD 93-20-A (<u>Cvy-1</u> ² , cvy-2 ⁺ , Cvy-3 ⁺)	HSD 2458 (Cvy-1 ⁺ , <u>cvy-2</u> , Cvy-3 ⁺)	Ouzbeque 2 (Cvy-1 ⁺ , cvy-2 ⁺ , <u>Cvy-3</u>)
CVYV-Esp	M	0 (TN)	TN	mo	M+
CVYV-ISR	M, VB	0 (TN)	M nec (TN)	mo	M (nec)
CVYV-JOR	M+, St	TN	TN	M, St	M+
CVYV-SU96-84	M, VB, St	TN	0 (nec)	mo	M+ (nec)
CVYV-SU12-10	M	0	nec	mo	M+, St
CVYV-TN05-56	M	TN	nec	mo	M (nec)
CVYV-OM04-06	M, VB	TN	TN	M	M+ (nec)
CVYV-LIB	M	TN	TN	mo	M+, VB
CVYV-PI1	M-	M+	TN	mo	M+
CVYV 3.1 (clone)	M	0	TN	mo	M+
CVYV-VPg-C1	mo	M+	TN	mo	M+

M : mosaic; M+ : severe mosaic ; M- : mild mosaic; mo = very mild mottle; St: stunting ; VB : vein banding; TN : top necrosis followed by plant death; nec : systemic necrosis; 0 : no symptoms and no virus accumulation. The genotype of each accession is indicated for the 3 genes Cvy1, cvy2 and Cvy3. The alleles involved in the accession behaviour upon inoculation with CVYV-Esp are underlined.

Symptoms indicated in brackets were observed occasionally on the inoculated plants. The dashed line separates the field isolates from the evolved CVYV-PI1 and the viruses obtained from infectious clones (CVYV 3.1 and CVYV-VPg-C1).

Figure legends

Figure 1: Symptoms induced by CVYV-Esp on different melon accessions (a) mosaic on Védrantais; (b) severe mosaic on Ouzbeque 2; (c) lethal necrosis on HSD 93-20-A; (d) very mild mottle on HSD 2458, (e) lack of infection on PI 164323; (f) occasional necrosis on PI 164323.

Figure 2a: Alignment of VPg amino acid sequences of twelve CVYV isolates and eight other ipomoviruses. The “K” mutation present in CVYV-PI1 is indicated in bold.

Figure 2b : Overlay of predictions of disorder for the VPg of CVYV-Esp (in red) and CVYV-PI1 (in dark blue).

Regions of the VPg with a predicted PONDR score above 0.5 are considered as disordered. The thick black line indicates a propensity of the region to fold in a secondary structure, maybe in contact with an interactor. The arrow indicates the position of the E₉₀K mutation.

Supplementary material

Supplementary table S1: Primers used to obtain the infectious clone of CVYV

Supplementary figure S2: PONDR prediction of disorder for the VPg of CVYV-Esp, three ipomoviruses -squash vein yellowing virus (SqVYV), cassava brown streak virus (CBSV) and Uganda cassava brown streak virus (UCBSV)- and four potyviruses potato virus Y (PVY), zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV) and turnip mosaic virus (TuMV). Regions of the VPg with a predicted PONDR score above 0.5 are considered as disordered. The thick black line indicates a propensity of the region to fold in a secondary structure, maybe in contact with an interactor.

Figure 1

Figure 2a

Figure 2b

Name	Position	Sequence
CVYV-1F	1-25	ATATAAGGAAGTTCA TTTCATTTGGAGAGG AAATAAACATTACATGAAA TTACA
CVYV-1R	2823-2847	CTCTACATCTTCTAACA AACTTAA
CV-P3-intron1-F	2823-2847	TTAAGTTTGTAGAAAGATGTAGAGGTAAGTTTCTGCTTCTACC
CV-P3-intron1-R	2848-2870	TGTGAAAAC TCAATGCACGCATCCTGCATATCAACAAATTTG
CVYV-2F	2848-2870	GATGCGTGCATTGAGTTTTCACA
CVYV-2R	4023-4048	CTGTTCTAACTCTCCCTCTGTACGC
CV-CI-intron2-F	4023-4048	GCGTACAGAGGGAGAGTTAGAACAGGTAAGTATGCACTTAAAGA
CV-CI-Intron2-R	4049-4068	AACCGTGATGGGTTGCGTTCCTGCATAATTTCAAAGATTG
CVYV-3F	4049-4073	AATTATGCAGGAACGCAACCCATCACGGTTATGAC
CVYV-3R	5746-5765	ATRATGTCCACATCAGGTCC
CVYV-4F	5356-5375	TTGATGGTTGGTTGGATACC
CVYV-4R	7309-7328	TAGAGAGCTCCCATTGCGGC
CVYV-5F	7105-7124	TATGGYCCAAGTGTGATGAC
CVYV-5R	9733-9751	GCGAATCTAGATTTTTTTTTTTTTTTTTTATAACTTTACGCATAAAGG

Supplementary table S1. Primers used for PCR amplification of CVYV-Esp in order to obtain the full-length infectious clone containing two introns.

Underlined sequences correspond to the extremities of the introns. Sequences in italics correspond to the extremities of the vector

Supplementary figure S2