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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Abstract

Cucumber vein yellowing virus (CVYV) is an emerging virus on cucurbits in the Mediterranean Basin, against which few resistance sources are available, particularly in melon. The melon accession PI 164323 displays complete resistance to isolate CVYV-Esp, and accession HSD 2458 presents a tolerance, i.e. very mild symptoms in spite of virus accumulation in inoculated plants. The resistance is controlled by a dominant allele *Cvy-1*^{1}, while the tolerance is controlled by a recessive allele *cvy-2*, independent from *Cvy-1*^{1}. Before introducing the resistance or tolerance in commercial cultivars through a long breeding process, it is important to estimate their specificity and durability. Upon inoculation with eight molecularly diverse CVYV isolates, the resistance was found to be isolate-specific since many CVYV isolates induced necrosis on PI 164323, whereas the tolerance presented a broader range. A resistance-breaking isolate inducing severe mosaic on PI 164323 was obtained. This isolate differed from the parental strain by a single amino acid change in the VPg coding region. An infectious CVYV cDNA clone was obtained, and the effect of the mutation in the VPg cistron on resistance to PI 164323 was confirmed by reverse genetics. This represents the first determinant for resistance-breaking in an ipomovirus. Our results indicate that the use of the *Cvy-1*^{1} allele alone will not provide durable resistance to CVYV and that, if used in the field, it should be combined with other control methods such as cultural practices and pyramiding of resistance genes to achieve long-lasting resistance against CVYV.

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

Introduction

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

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

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

Materials and methods

Melon accessions

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

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

F_1, F_2 and back-cross (BC) progenies were obtained between the above mentioned accessions in order to study the inheritance of the different phenotypes (Pitrat et al. 2012). Three loci appeared to be involved. At a first locus tentatively named *Cucumber vein yellowing resistance* (symbol *Cvy-1*), three alleles have been identified: *Cvy-1*$^+$ for susceptibility (present in Védrantais), *Cvy-1*^{1} controlling resistance in PI 164323 and *Cvy-1*^{2} controlling necrosis in HSD 93-20-A. At an independent second locus, the recessive allele *cvy-2* present in HSD 2458 controls the tolerance. And at a third locus, the allele *Cvy- 3*, present in Ouzbeque 2, controls the highly susceptible type of symptoms (Pitrat et al. 2012).

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

CVYV isolates and mechanical inoculation of melon accessions

Isolates CVYV-Esp, SU96-84, SU12-10, TN05-56, OM04-06, ISR, LIB and JOR are as described in Desbiez et al. (2019). They originate from Spain, Sudan, Tunisia, Oman, Israel, Lebanon, and 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 performedone 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.

Full-length sequencing of resistance breaking isolate CVYV-PI1

After mechanical inoculation of CVYV-Esp on melon PI 164323, symptoms of top necrosis were observedin 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.

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* DH5α 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 µl of plasmid DNA were submitted to rolling circleamplification (RCA) using the TempliPhi kit (GE Healthcare) according to the manufacturer's manual.One µl 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 µl of plasmid DNA, or 5 µl RCA product + 25 µl distilled water, were mixed with 30 µl of tungsten M20 at 50 mg/ml in 50% glycerol and 30 µl of $Ca(NO_3)_2$ 1.25M, pH 8. After 5 minutes at room temperature, a short centrifugation was performed and 60 µl of the supernatant were removed. Three µl of the remaining mix were used for the bombardment of

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

Site-directed mutagenesis in the VPg of CVYV-Esp

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

Since the expression level of CVYV-Esp infectious clone in *E. coli* was very low, clone 3.1 was submitted to RCA with the TempliPhi kit according to the manufacturer's manual before digestion with restriction enzyme *Nde*I 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* DH5α 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 µl DNA extracted by alkaline lysis) were amplified by RCA. One µl 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 potyvirid isolates.

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

of these isolates and of CVYV-PI1 were aligned with ClustalW. Alignments were also performed with the VPg amino acid sequences of other ipomoviruses: coccinia mottle virus (CocMoV, YP_009272707), squash vein yellowing virus (SqVYV, YP_001788991, AEV45694, ALN38790, AOY33888), cassava brown streak virus (CBSV, YP_007027011), Ugandan cassava brown streak virus (UCBSV, YP_004063980) and sweetpotato mild mottle virus (SPMMV, NP_734290).

Four methods were used to predict the intrinsic disorder of CVYV VPg and the impact of the mutation on this disorder: (1) Disopred (http://bioinf.cs.ucl.ac.uk/disopred), (2) DisEMBL (http://dis.embl.de), (3) GlobProt (http://globplot.embl.de), and (4) PONDR® (http://www.pondr.com), as described by Desbiezet al. (2014). As a comparison, VPg sequences of three different ipomoviruses (SqVYV, CBSV and UCBSV) and of four potyviruses (potato virus Y, zucchini yellow mosaic virus, papaya ringspot virus and turnip mosaic virus) were analysed with PONDR®.

Results

Isolate specificity of CVYV resistance and tolerance

Upon inoculation of the reference susceptible accession Védrantais [*Cvy-1*$^{+}$ *cvy-2*$^{+}$ *Cvy-3*$^{+}$], the eight CVYV isolates tested induced mosaic and vein banding symptoms (Table 1); isolates CVYV-JOR and CVYV-Su96-84 also induced some stunting. Accession Ouzbeque 2 [*Cvy-1*$^{+}$ *cvy-2*$^{+}$ *Cvy-3*], carrying the high susceptibility allele *Cvy-3*, displayed severe symptoms of mosaic and stunting upon inoculation with the different isolates. On HSD 93-20-A [*Cvy-1*^{2} *cvy-2*$^{+}$ *Cvy-3*] carrying the Cvy1^{2} allele responsible for lethal necrosis upon inoculation with CVYV-Esp, all isolates except SU96-84 induced 100% top necrosis or systemic necrosis. Upon inoculation with SU96-84, in most

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

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

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

synonymous, whereas mutation 5962 (GAA/AAA, $G_{5962}A$) caused an amino acid change at position 90 in the viral VPg, from glutamicacid (E) to lysine (K) ($E_{90}K$).

Infectious cDNA clone of CVYV-Esp

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

Effect of the VPg $E_{90}K$ mutation on CVYV infectivity and symptom expression

The $G_{5962}A$ (VPg $E_{90}K$) mutation was introduced in the CVYV 3.1 clone by PCR mutagenesis, yielding the mutated clone CVYV-VPg-C1. The presence of the mutation in CVYV-VPg-C1 clone was confirmed by partial sequencing. After amplification by RCA and inoculation by GeneGun of clone 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 Védrantais, 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 VPg_{90}, immediately followed by an E, and a K at position VPg_{93} 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 E_{5962} 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 $E_{90}K$ 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 ($E_{90}K$) 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-

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

The E to K mutation at aa position 90 in the VPg of CVYV-VPg-C1 was sufficient to induce resistance breaking in PI 164323. Indeed, contrary to other pathogens, most situations of resistance breaking in plant viruses are related to point mutations rather than indels or loss of expression (Sacristan and Garcia-Arenal 2008). This is probably due to the constraints of virus genomes where all proteins are required for infectivity and are frequently multifunctional (Revers and Garcia 2015). The $E_{90}K$ mutation in the VPg resulted in charge change in the protein, which probably affects its interaction with so far undetermined plant or virus factors. The area where the mutation took place appears as highly ordered, and the mutation does not seem to modify deeply the level of disorder of the protein (Figure 2b).

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

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

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

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

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

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

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Ethical statement:

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

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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-1^1</u>, cvy-2$^+$, Cvy-3)	HSD 93-20-A (<u>Cvy-1^2</u>, 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 $E_{90}K$ 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.

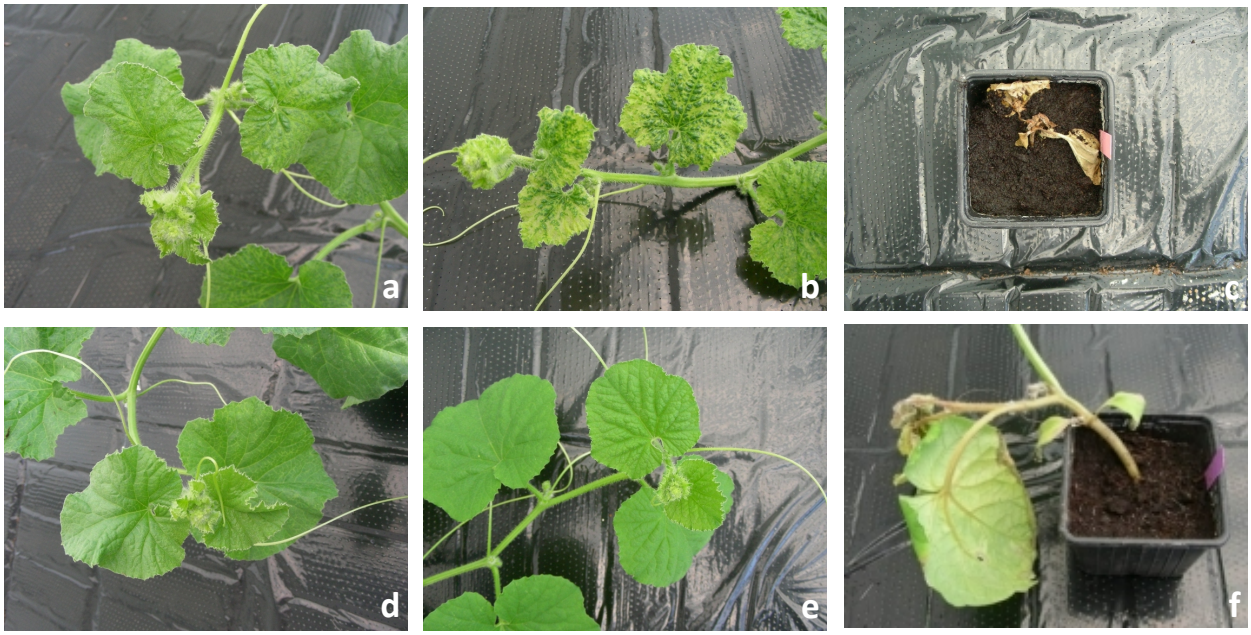


Figure 1

```
CVYV-Esp  ---GKVGYRR DKRVGRFVFD GPDEDIIENF GVEYSHDVVT KKMSKAQKLK QAKEKGWKIG KVDRPKKIFR QLYGVNPLEF DEVYLTVGDF KGEVWETKD-
CVYV-PI1  ---..... .......... .......... .......... .......... .......... .......... .......... .......... ..K.....-
TN05-56   ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
LIB       ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
ALM32     ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
SU96-84   ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
SU12-10   ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
JOR       ---..... .......... .......... .......... .......... ....R..... .........K .......... .......... ...I.....-
IR02-106  ---..... .......... .......... .......... .......... .......... .........K .......... .......... .........-
ISR       ---..... .......... .......... ........I. .......... .......... .......... .......... .......... ..D.....-
ISM       ---..... .......... .......... ........I. .......... .......... .......... .......... .......... ..D.....-
OM04-06   ---..... .......... .......... .......... .......... .......... .......... .......... .......... .........-
CocMoV    ---.....K. ...TA.Y... ...Q.MV.A. ........MS G.......T. K..DA..... .........S .......... ....M....C ..QE.K.T.-
SqVYV-FL  -GRNNLRFK. ...TA..... .E.Q.MV.T. .I...D..IQ ...T.R..QR A.SNS..... ....V.RV.H .......... .K..M....L M.NE.S.NEK
SqVYV-cHe -GRNNLRFK. ...TA..... .E.Q.MV.T. .I...D..IQ ...T.R..QR A.SNS..... ....V.RV.H .......... .K..M....L M.NE.S.NEK
SqVYV-IL  -GRSNLKFK. ...TA..... .D.Q.MV.T. .....D..IQ ...T.R..QR A.SNS..... ....V.RV.H .......... .K..M..... I.SE.S.NEK
SqVYV-Ir  HASNTLRSTS .T.PA.S.S. AD.Q.ML.TL .I...D..IH .T.TTRHTQR A.SHS..T.. T.H.VTRV.H .S.......S .T..M..R.L MRTECS.N.K
CBSV      ---A.HK.N. ...T..LM.. YS.Q.TV.T. .....DA.I. G.......ER ESRK...... ..N..MRV.H .......... ...IMR...W ATDP.TA..-
UCBSV     ---A.HK.N. ...T..LM.. MD.QETY... .P..TD..IS A..T....ER DSRK....A. .IN..MRV.H .......... ...VMR..KL ET.P.DV.E-
SPMMV     HG-K.IRLN. ...NAC...S .T.DAMV.EY .....Q..IH GR......AR .M.L..K.P. SDT.-V.P.K V...ID.NDY .T.A.SA.GL TT.AVPVGE-

          MDIDEMYSDL YSDFNLGNR- KGYSKDVYLV FSKKDSDIEA VIDLQPHRSK MASSMSLNPM GFPEEEGRWR QSGDVKMRKR IEEE------ -VEVQ
          .......... .........- .......... .......... .......... .......... .......... .......... ....------ -....
          .......... .........- .......... .......... .V........ .......... .......... .......... ....------ -....
          .......... .........- .......... .......... .V........ .......... .......... .......... ....------ -....
          .......... .........- .......... .......... .V........ .......... .......... .......... ....------ -....
          .......... .........- .......... .......V.. .V........ .......... .......... .......... ....------ -....
          .......... ....D....- .......... .......... .V........ .......... .......... .....R.... ....------ -....
          .......... .........- .......... .......... .V........ .......... .......... .......... ....------ -....
          .......... .........- N.......... .......... .V........ .......... .......... .......... ....------ -....
          .......... ..E......- .......... .......... .V........ .......... .......... .......... ....------ -....
          .......... ..E......- .......... .......... .V........ .......... .......... .......... ....------ -....
          ....G..A.. .........- .......H.. .......... .V........ .......... .......... .......... ....------ -....
          L.....FEEI .D..S.A..F .TVP...H.. .K.SG..E.S .VT......R .......... .......... ...E.RR... ...D------ -....
          WTVEDLIV.M DDE.GV.R.G DLETEV.QIH .KRD..NE.K .VT.T..... ...C...... .......... .T.KPVDCV. VKKDESPG-- KI.L.
          WTVEDLIV.M DDE.GV.R.G DLETEV.QIH .KRD..NE.K .VT.T..... ...C...... .......... .T.KPVDCV. .KKDESPG-- KI.L.
          WTVEDLIV.M DDE.GV.R.G DLETEVIQIH .KRDN.NE.K .VT.T..K.. ...C...... .......... .T.QPVDCV. V.K.KSSG-- QI.L.
          WTVEDLIV.M HD.SGV.R.G DLDTEL.HIH SKRD..NEDK .VT.T....T ...C...... .L..D.R... .T.KPVD.V. VKKDESPA-- TL.LH
          VNV.G.LIE. DD.YHILKDD RMLG.R.E.A .T.SG.SD.T .VQ.T....R .......S.. .......... .T.SPVVQ.. T.SGH----- T..M.
          LNV.A.MIE. DD.YHILRDD RMFG.K.S.A .R.EGA.E.T IVN.T..... .T.....A.. .......... .T.APLI-.K T.K.D----- E....
          ASLIDLMLE. DDETGIFR-- .QVVNELK.K YTNNANGEQ. MVR.T..D.R R.TIG.FM.S ...DHH.E.. .T.AAEII.N VAVDSHVGTP T.DAE
```

Figure 2a

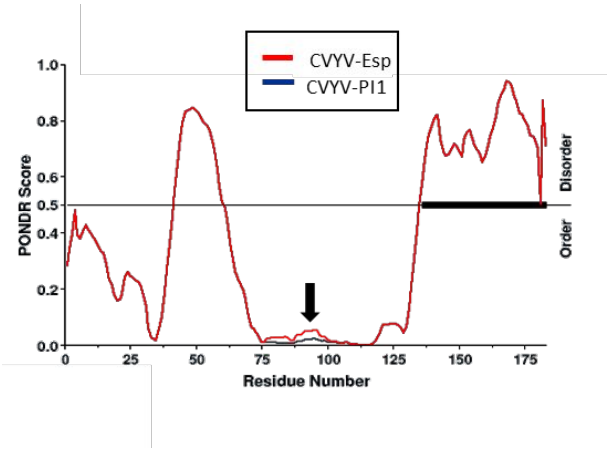
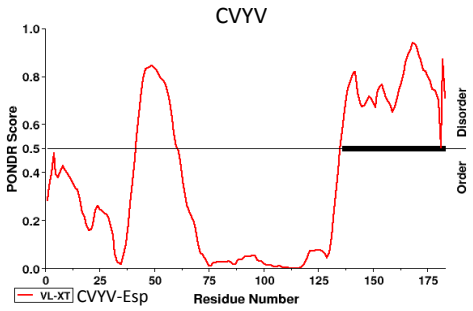

CVYV-Esp
CVYV-PI1
PONDR Score
1.0
0.8
0.6
0.5
0.4
0.2
0.0
0
25
50
75
100
125
150
175
Residue Number
Disorder
Order

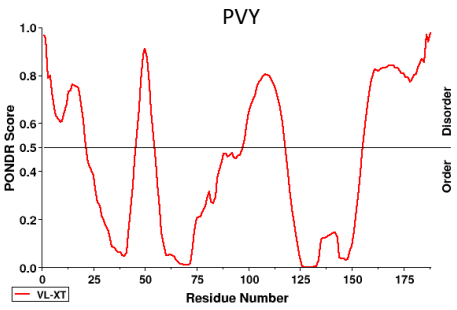
Figure 2b

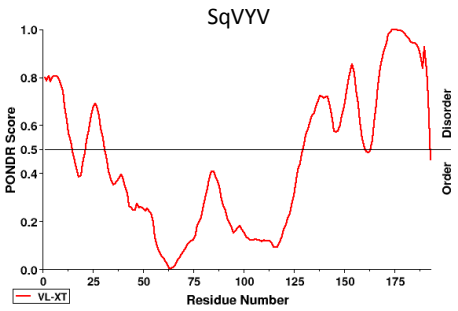
Name	Position	Sequence
CVYV-1F	1-25	*ATATAAGGAAGTTCATTTCATTTGGAGAGG*AAAATAAACATTACATGAAATTACA
CVYV-1R	2823-2847	CTCTACATCTTCTAACAAACTTAA
CV-P3-intron1-F	2823-2847	TTAAGTTTGTTAGAAGATGTAGAGGTAAGTTTCTGCTTCTACC
CV-P3-intron1-R	2848-2870	TGTGAAAACTCAATGCACGCATCCTGCATATCAACAAATTTTG
CVYV-2F	2848-2870	GATGCGTGCATTGAGTTTTCACA
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	*GCGAATCTAGA*TTTTTTTTTTTTTTTTTTTTATAACTTTACGCATAAAGG

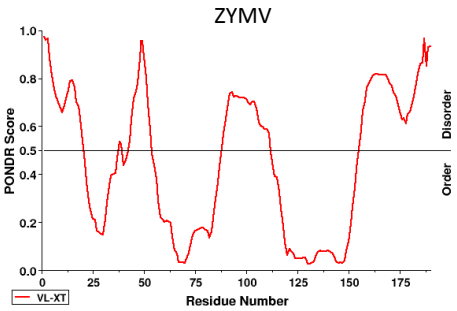
Supplementary table S1. Primers used for PCR amplification of CVYV-Esp in order to obtain the full-length infectious clone containing two introns.

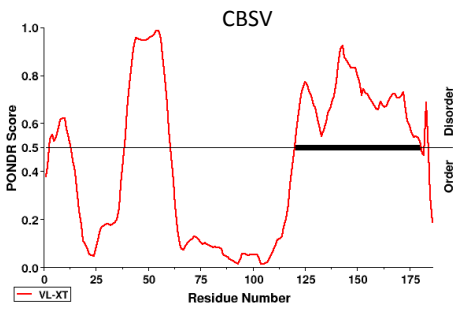
Underlined sequences correspond to the extremities of the introns. Sequences in italics correspond to the extremities of the vector

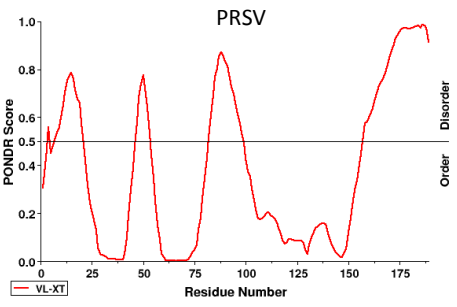


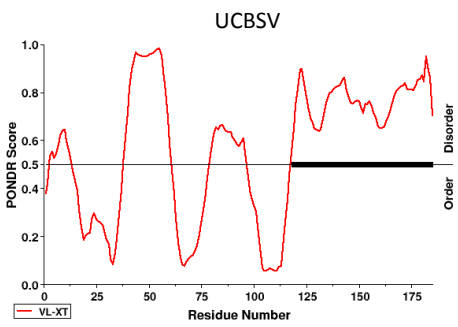


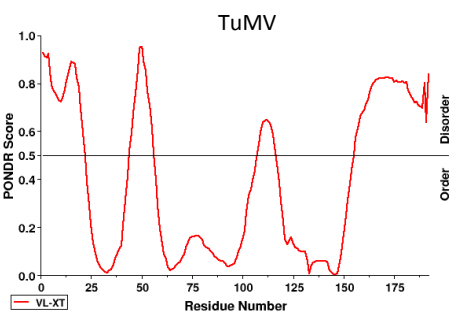












Supplementary figure S2