African eggplant-associated virus: characterization of a novel tobamovirus identified from *Solanum macrocarpon* and assessment of its potential impact on tomato and pepper crops

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Abstract

A novel tobamovirus was identified in a fruit of *Solanum macrocarpon* imported into the Netherlands in 2018. This virus was further characterized in terms of host range, pathotype and genomic properties, because many tobamoviruses have the potential to cause severe damage in important crops. In the original fruit, two different genotypes of the novel virus were present. The virus was able to infect multiple plant species from the *Solanaceae* family after mechanical inoculation, as well as a member of the *Apiaceae* family. These species included economically important crops such as tomato and pepper, as well as eggplant and petunia. Both tomato and pepper germplasm were shown to harbor resistance against the novel virus. Since most commercial tomato and pepper varieties grown in European greenhouses harbor these relevant resistances, the risk of infection and subsequent impact on these crops is likely to be low in Europe. Assessment of the potential threat to eggplant, petunia, and other susceptible species needs further work. In conclusion, this study provides a first assessment of the potential phytosanitary risks of a newly discovered tobamovirus, which was tentatively named African eggplant-associated virus.

Introduction

The International Plant Protection Convention aims to safeguard agriculture and natural resources against the entry, establishment and spread of economically and environmentally significant pests (1). In this respect, import inspections are an important tool to prevent the introduction of regulated plant pests and may serve as an early warning system on emerging pests. In 2018, a phytosanitary inspector in the Netherlands intercepted a fruit of *Solanum macrocarpon* from Mexico, which showed diffuse chlorotic mottling (Fig 1). *Solanum macrocarpon*, also known as African eggplant, is a small tropical perennial originating from Africa that is nowadays cultivated in many countries for food, medicinal purposes and as an ornamental (2, 3). Since the observed symptoms on the fruit indicated a potential viral infection, samples of this fruit were analyzed by bioassay, serology and

high-throughput sequencing analysis. The results indicated the presence of a so far unknown tobamovirus.

Fig 1. *Solanum macrocarpon* fruit with diffuse chlorotic mottling.

The genus *Tobamovirus* (family *Virgaviridae*) currently contains 37 accepted species according to the 2021 release of the International Committee on Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/), including some devastating plant viruses. Tobamoviruses are highly stable, contagious and difficult to control. Tobamoviruses can rapidly spread locally by mechanical contact, while transmission via seeds enables them to spread over longer distances, as exemplified by the recent rapid spread of tomato brown rugose fruit virus (ToBRFV) (4-6) and tomato mottle mosaic virus (ToMMV) (7). Other tobamoviruses that can cause significant economic losses in crops if not controlled effectively, include cucumber green mottle mosaic virus (8), tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) (9).

The potential of tobamoviruses to spread rapidly and cause severe damage has prompted us to prioritize this novel virus for further characterization in terms of genomic properties, host range and pathotype. Tobamoviruses can be divided into at least eight subgroups based on their complete nucleotide sequence, the amino acid sequences of each individual viral protein and their host plant family (10). These subgroups consist of species that can infect plant species within the families

63 *Apocynaceae, Brassicaceae, Cactaceae, Cucurbitaceae, Leguminosae, Malvaceae, Passifloraceae,* and
64 *Solanaceae*.

65 To assess the biological properties and potential impact of the novel tobamovirus from *S.*
66 *macrocarpon*, host range studies focused on solanaceous crops, in particular tomato (*Solanum*
67 *lycopersicum*) and pepper (*Capscium* spp.). Both tomato and pepper germplasm are known to harbor
68 tobamovirus resistance genes. In tomato, the *Tm-1* gene, and the *Tm-2* and $Tm2^2$ alleles, originating
69 from wild tomato species, have been used extensively to protect against tobacco mosaic virus and
70 tomato mosaic virus (11). In pepper, the four *L* genes or alleles L^1, L^2, L^3 and L^4, each provide
71 resistance to an increasing number of tobamovirus pathotypes (12). Therefore, tomato and pepper
72 accessions harboring these resistances were tested to assess whether the novel tobamovirus poses a
73 threat to these crops.

74 The results of the molecular and biological characterization of this novel tobamovirus from *S.*
75 *macrocarpon*, which we tentatively named ''African eggplant-associated virus" contribute to the
76 assessment of its potential risks, so that authorities can determine the need for preventive control
77 measures.

78 Materials and Methods

79 Virus source, propagation and maintenance

80 A novel tobamovirus was identified from a fruit of *S. macrocarpon* imported from Mexico. The isolate
81 was propagated and maintained by successive mechanical passages in *Nicotiana occidentalis* P1.
82 Young leaves were harvested just after systemic symptoms appeared and stored at -80°C for further
83 experiments as well as long-term preservation.

84 ## Mechanical inoculation and growing conditions of test plants

85 Symptomatic tissue (fruit or leaf material) was used for mechanical inoculation of test plants
86 according to the procedure and conditions as described by Verhoeven and Roenhorst (2000) (13). All
87 test plants were grown from seed, except for *Solanum tuberosum* which was grown from tubers.
88 Tested plant species and varieties are specified in the Results section.

89 ## Host range and pathotype analysis

90 Nineteen species from six plant families were inoculated with the virus from *S. macrocarpon*. Per
91 species, four plants were inoculated with the virus and four plants with inoculation buffer only (mock
92 treatment). All plants were assessed for local and systemic disease symptoms twice a week for a total
93 duration of four weeks and tested by DAS-ELISA 10-25 days after inoculation. Symptoms in *S.*
94 *macrocarpon* were assessed until the fruits had matured. In addition, tomato and pepper accessions
95 with different tobamovirus resistance genes (tomato: *Tm1*, *Tm2* and $Tm2^2$; pepper: L^1, L^2, L^3 and L^4)
96 were tested in an experiment with the same set-up. These accessions and information on their
97 resistance genes were kindly provided by the Centre for Genetic Resources, the Netherlands (CGN).

98 ## Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA)

99 DAS-ELISA (14) was performed using polyclonal antisera raised against bell pepper mottle virus
100 (BPMoV), pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), and tomato mosaic virus
101 (ToMV-D) from Prime Diagnostics, the Netherlands. Host range and pathotype experiments were
102 conducted with PMMoV antiserum. Antisera were diluted at 1:1000 from a 1 mg/ml stock. Buffers
103 used were: coating buffer (15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, pH 9.6), homogenization buffer (2.9
104 mM KH_2PO_4, 2.7 mM KCl, 16 mM $Na_2HPO_4 \cdot 12H_2O$, 136.9 mM NaCl, containing Tween 20 at 0.5 ml/l,
105 polyvinyl pyrrolidone at 20 g/l and albumin from bovine serum at 2.0 g/l, pH 7.4), and substrate
106 buffer (p-nitrophenyl phosphate at 0.75 mg/ml in diethanolamine at 97.5 ml/l, pH 9.8). A volume of
107 200 µl was added to each well. Each sample was tested in duplicate and contained a pool of tissue
108 from four plants of the same combination of plant species and inoculum. Samples were taken from

109 non-inoculated young leaves or fruits and homogenized at an approximate ratio of 1:10 (w:v), using
110 extraction bags and a hand-held homogenizer (Bioreba). Coating and test sample incubations were
111 overnight at 4°C, substrate incubations for 1-2 hours at 37°C. Optical Density (OD) at 405 nm was
112 measured with a microplate reader (BioRad iMark) in duplicate wells from which an average OD
113 value was calculated. Samples were considered positive when the OD value was at least twice the
114 value of the negative controls (mock-inoculated healthy plants).

115 Illumina sequencing and analysis

116 Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Germany) following the
117 manufacturer's instructions. The RNA extract was sent to GenomeScan (The Netherlands) for
118 generation of 2 Gb of Illumina RNAseq 150PE (paired-end) data per sample. Sequencing was
119 performed on an Illumina NovaSeq6000. RNAseq data were analyzed in CLC Genomics workbench
120 v21.0.4 (Qiagen, Germany) and run in a custom workflow built for detection of *de novo* assembled
121 viral contigs (15). Consensus sequences (>100 nt; ARC>10) from *de novo* assembled contigs were
122 analyzed using megaBLAST and DIAMOND (16) with a local installation of the NCBI nr/nt database
123 downloaded from http://ftp.ncbi.nlm.nih.gov/. BLAST results were visualized in Krona (bitscore
124 threshold=25) (17). Putative viral sequences were further analyzed using Geneious Prime 2021.1.1
125 (Biomatters, New Zealand). Genomes of *Tobamovirus* species were aligned using MAFFT alignment
126 with automatic settings in Geneious, for the species of which a complete reference nucleotide
127 sequence was available in GenBank. A Neighbor-Joining tree was constructed using the Geneious
128 tree builder based on Tamura-Nei distances, bootstrap resampling, random seed 925,045, number of
129 replicates 1,000, and a support threshold at 70%.

130 Results

131 To determine whether the symptoms on the *S. macrocarpon* fruit could result from a viral infection,
132 a homogenate of symptomatic tissue was inoculated on a range of test plants routinely used as
133 indicators in virus diagnosis. The reaction on the inoculated test plants, six days post inoculation,

134 indicated the presence of a tobamovirus (Table 1). Therefore, symptomatic leaves of *N. occidentalis*
135 P1 were tested for different tobamoviruses by DAS-ELISA. Reactions were clearly positive for antisera
136 raised against BPMoV and PMMoV, but negative for TMV and ToMV-D (Table 2). Negative controls
137 (mock-inoculated plants) and homologous (positive) controls showed their expected outcomes.
138 Based on these results, the virus from *S. macrocarpon* appeared to be a tobamovirus that is
139 serologically related to BPMoV and PMMoV. The PMMoV antiserum was used to detect the virus in
140 all subsequent experiments.

141 **Table 1. Symptomatology of indicator plants inoculated with the homogenate from the intercepted**
142 ***S. macrocarpon* fruit.**

	Symptoms	
Indicator species	**Local**	**Systemic**
Datura stramonium	NL (2)	-
Nicotiana glutinosa	NL (2)	-
N. occicentalis "P1"	-	~CV, G, LN (2)
N. tabacum "White Burley"	CL, NL (2)	-
Solanum lycopersicum "Moneymaker"	-	-

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144 -: no symptoms, ~: mild symptoms, CV: chlorotic vein banding, CL: chlorotic lesions, G: growth inhibition, LN:
145 leaf narrowing, NL: necrotic lesions. The first symptoms appeared six days post inoculation. Per species two
146 plants were inoculated. The number of plants showing specific symptoms is indicated in brackets.

147 **Table 2. Reaction of inoculated N. occidentalis P1 plants by DAS-ELISA.**

Antiserum	OD_{405}*
BPMoV	3.050
PMMoV	1.799
TMV	0.228
ToMV-D	0.269

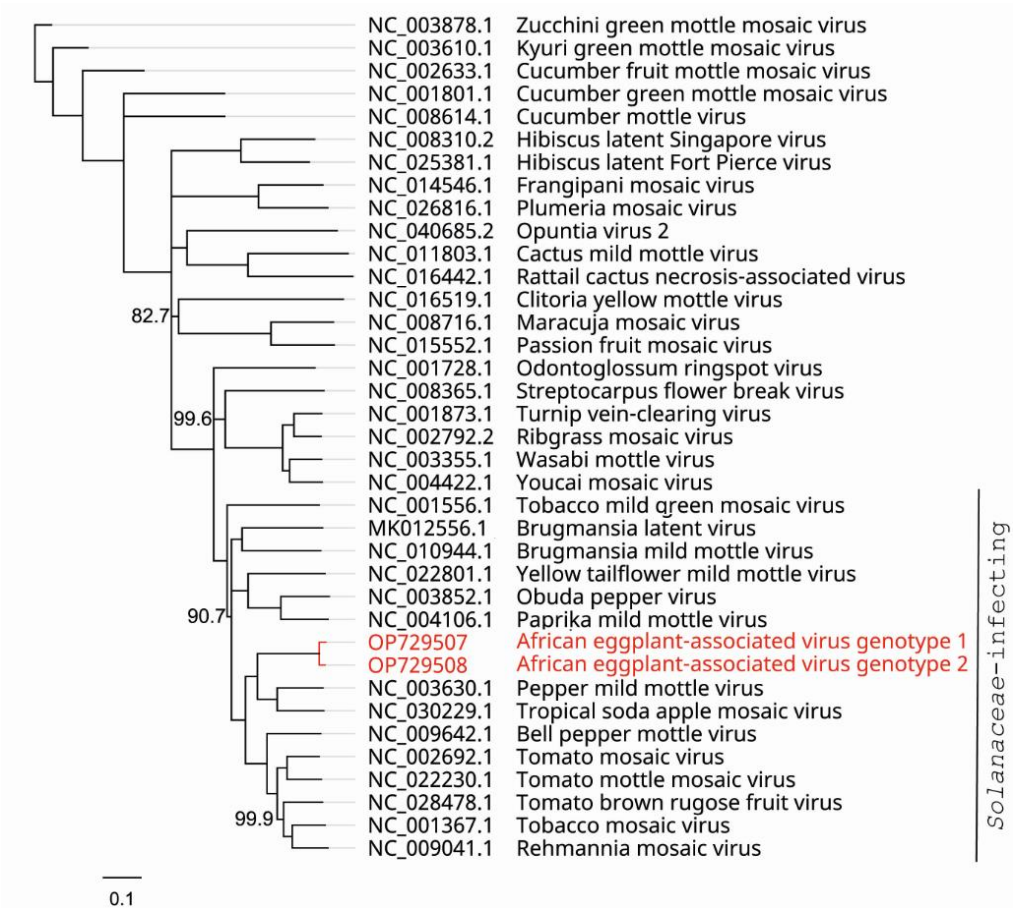
148 *Average OD values after 90 minutes of substrate incubation. OD values were considered positive (red) when ≥
149 2x the OD value of mock-inoculated controls, otherwise OD values were considered negative (green).

150

151 For further characterization, the viral genome sequence of the putative tobamovirus from *S.*
152 *macrocarpon* was determined by Illumina sequencing analysis of the original fruit sample. *De novo*
153 assembly resulted in a near-complete genome sequence of two genotypes, each consisting of a single
154 stranded RNA with four predicted open readings frames (ORFs), corresponding to the genome
155 organization of tobamoviruses (18). These respective ORFs encode a small replicase subunit, an RNA-
156 dependent RNA polymerase (translated through ribosomal readthrough at the stop codon of the
157 small replicase subunit), a movement protein, and a coat protein. Genotype 1 (GenBank accession

158 number OP729507) and genotype 2 (GenBank accession number OP729508) contained 6228 and
159 6356 nucleotides respectively, with 96.1% nucleotide similarity. No other viruses were detected.

160 NCBI BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that both genotypes of the
161 virus showed most resemblance to isolates of PMMoV, with a maximum query coverage of 97% and
162 a nucleotide identity of 75%. Since the nucleotide identity with PMMoV is considerably lower than
163 the tobamovirus species demarcation threshold of 90% (18), the virus should be considered as a
164 member of a new species, for which we propose the name "African eggplant-associated virus". A
165 phylogenetic analysis including genome sequences of other tobamoviruses indicated that African
166 eggplant-associated virus is most closely related to members of species infecting solanaceous plants,
167 in particular PMMoV and tropical soda apple mosaic virus (Fig 2).



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169 **Fig 2. Unrooted neighbor-joining tree with (near) complete nucleotide sequences of the novel virus**
170 **(red) and other members of the genus *Tobamovirus* including their GenBank accession numbers.**
171 The bar represents 0.1 substitutions per site. This tree was constructed by the neighbor-joining
172 algorithm in Geneious software, using the genetic distance model Tamura-Nei, with 1,000 bootstrap

replicates and the support threshold set at 70%. Support values (%) that were less than 100% are indicated next to the supported internal branches.

BLAST searches also indicated a close resemblance to a bacteriophage isolate (GenBank accession number BK018885), which aligned with the first 3,874 nucleotides of the novel virus, corresponding to a query coverage of 60%. The nucleotide identity was 95.7% and 99.5% for genotype 1 and genotype 2, respectively. Most probably, this sequence was incorrectly assigned to a bacteriophage.

To gain more insight into the potential host range of the novel virus, it was mechanically transferred from *N. occidentalis* P1 to test plants (Table 3). Nine out of 19 tested species, including *S. macrocarpon*, were susceptible based on symptomatology (i.e. systemic symptoms) and/or detection by DAS-ELISA, of which *Ammi majus* was the only non-solanaceous plant. *A. majus*, *S. lycopersicum* "Moneymaker" and *Solanum melongena* "Violetta Lunga 2" showed asymptomatic or latent infections. The tested *Amaranthaceae, Brassicaceae*, *Cucurtitaceae*, and *Fabaceae* species remained uninfected. Negative and positive controls showed their expected outcomes. Figure 3 illustrates the most obvious symptoms per species.

Table 3. Host range study: symptomatology and DAS-ELISA results of test plants after inoculation with the novel tobamovirus.

		symptoms		OD_{405}
Family	**Species**	**local**	**systemic**	**PMMoV**
Amaranthaceae	*Chenopodium album*	CL (4)	-	0.022
	Chenopodium quinoa	CL (4), ~CR (2)	-	0.135
Apiaceae	*Ammi majus*	- / -*	-; -*	1.169 / 0.681*
Brassicaceae	*Brassica rapa* subsp. *pekinensis* "Granaat"	-	-	0.105
Cucurbitaceae	*Cucumis sativus* "Chinese Slangen"	-	-	0.000
Fabaceae	*Pisum sativum* "Kelvedon Wonder"	-	-	0.052
	Vicia faba "Witkiem"	-	-	0.071
Solanaceae	*Capsicum annuum* "Westlandse Grote Zoete"	-	C, M, Ms (4)	1.353
	Datura stramonium	NL (4)	-	0.149
	Nicotiana glutinosa	NL (4)	-	0.048
	Nicotiana hesperis "67A"	-	C, G, NLE, R (4)	0.768
	Nicotiana occidentalis "P1"	-	C, G, LC, NLE, R (4)	1.653
	Nicotiana tabacum "White Burley"	CL (4), NL (1)	-	0.101
	Petunia hybrida	NL, NV (1)	LC (3) G, M, R (4)	0.989
	Solanum lycopersicum "Moneymaker"	-	-	0.631
	Solanum macrocarpon	-	CV, ~M (2)	2.340
	Solanum melongena "Violetta Lunga 2"	- / -*	- / -*	0.763 / 1.788*
	Solanum nigrum	-	CL (3) R (2)	0.647
	Solanum tuberosum "Nicola"	-	-	0.090

Per species four plants were inoculated; the number of plants showing specific symptoms is indicated in brackets. Average OD values after two hours of substrate incubation were considered positive (red) when ≥ 2x the value of mock-inoculated controls, otherwise values were considered negative (green). -: no symptoms, ~: mild symptoms, C: chlorosis, CL: chlorotic lesions, CR: chlorotic rings, CV: chlorotic vein banding, G: growth inhibition, LC: leaf curling, M: mottling, Ms: mosaic, NL: necrotic lesions, NLE: necrotic leaf edge, NV: necrotic vein banding, R: rugosity. * results of a repeated experiment on four new plants.

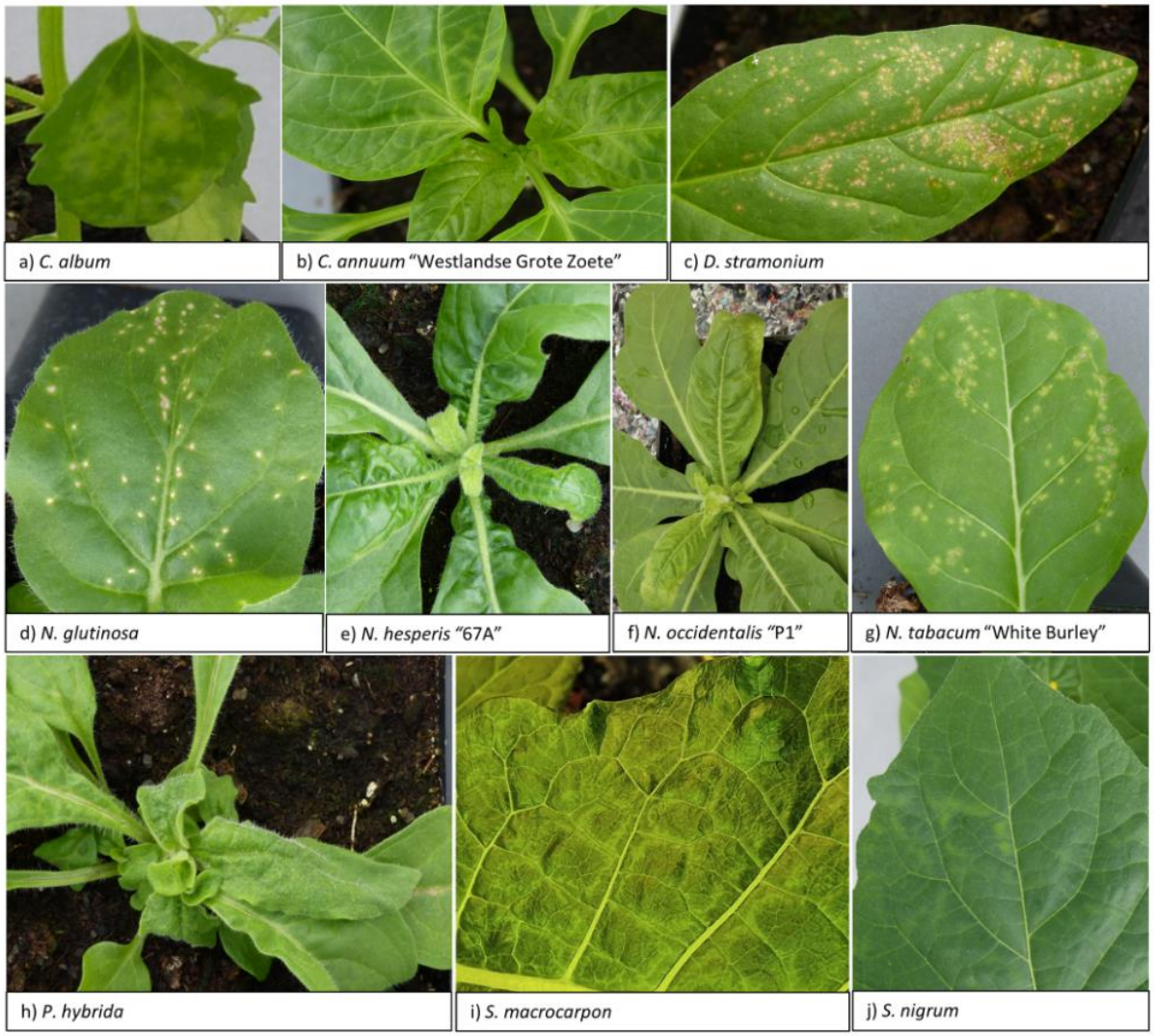

Fig 3. Symptoms on test plants upon inoculation with the novel tobamovirus.
a) *Chenopodium album*: local chlorotic lesions, b) *Capsicum annuum* "Westlandse Grote Zoete": systemic chlorosis, c) *Datura stramonium*: local necrotic lesions, d) *Nicotiona glutinosa*: local necrotic lesions, e) *Nicotiana hesperis* "67A": systemic chlorosis, growth inhibition, rugosity, f) *Nicotiana occidentalis* "P1": systemic chlorosis, growth inhibition, leaf curling, rugosity, g) *Nicotiana tabacum* "White Burley": local chlorotic lesions, h) *Petunia hybrida*: local necrotic lesions, systemic leaf curling, growth inhibition, rugosity, i) *Solanum macrocarpon*: systemic chlorotic vein banding, j) *Solanum nigrum*: systemic chlorotic lesions, rugosity.

To fulfill Koch's postulates, *S. macrocarpon* plants were observed until fruits had matured, 147 days after inoculation. The initial virus symptoms on the leaves had disappeared completely ten days after their first observation. For mature plants and fruits, the virus was still detected by DAS-ELISA, but there were no phenotypic differences compared to mock-inoculated plants and fruits. Therefore, associating the novel virus with the symptoms observed on the original fruit (Fig 1), according to Koch's postulates, was not possible.

219 Furthermore, in the inoculated *S. macrocarpon* plants only genotype 2 was detected by Illumina
220 sequence analysis. To trace the disappearance of genotype 1, subsequent passages in *N. occidentalis*
221 P1 used for virus propagation were analyzed. Both genotypes were still detected in all passages and
222 their nucleotide sequence remained exactly the same, though the relative frequency of genotype 1
223 decreased with each passage. Based on sequence depth, the approximate ratios of genotype 1 to
224 genotype 2 were 2:3 for the original *S. macrocarpon* fruit, 1:4 for *N. occidentalis* P1 (passage 1), and
225 1:14 for *N. occidentalis* P1 (passage 2) (S1 Fig).

226 To assess whether the novel tobamovirus poses a threat to tomato and pepper crops, accessions
227 harboring different tobamovirus resistances were inoculated (Table 4). These included (combinations
228 of) *Tm1*, *Tm2*, and $Tm2^2$, and L^1, L^2, L^3 and L^4 resistance genes/alleles for tomato and pepper,
229 respectively. Even though all tomato accessions remained asymptomatic, only the four accessions
230 harboring the combination of *Tm1* and $Tm2/Tm2^2$ tested negative by DAS-ELISA. Interestingly, the
231 accessions that harbored *Tm1*, *Tm2* or $Tm2^2$ separately tested positive by DAS-ELISA. Therefore, a
232 combination of these *Tm* resistance genes seems to account for resistance against the novel
233 tobamovirus.

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Table 4. Pathotype analysis: symptomatology and DAS-ELISA results of tomato (S. lycopersicum) and pepper (Capsicum spp.) accessions harboring different (combinations of) tobamovirus resistance genes after inoculation with the novel tobamovirus.

			symptoms		DAS-ELISA
Family	**Species**	**Tobamovirus resistance**	**local**	**systemic**	**PMMoV**
Solanaceae	*Capsicum annuum* "Westlandse Grote Zoete"		CL, R(2)*	~C (2)*	0.371 (local), 2.156 (systemic)
	Capsicum annuum "CGN19226"	L1	CL, NL, R (4)	-	0.109
	Capsicum frutescens "CGN21546"	L2	CL, NL, R (4)	-	0.240
	Capsicum chinense "CGN21557"	L3	NL (4)	-	0.102
	Capsicum chacoense "CGN21477"	L4	LA, NL, NR (4)	-	0.165
	Solanum lycopersicum "Moneymaker"		-	-	2.118
	Solanum lycopersicum "CGN24212"	Tm1	-	-	1.975
	Solanum lycopersicum "CGN24213"	Tm2	-	-	2.131
	Solanum lycopersicum "CGN24214"	$Tm2^2$	-	-	2.047
	Solanum lycopersicum "CGN24218"	Tm1, $Tm2/Tm2^2$	-	-	0.084
	Solanum lycopersicum "Arbason"	Tm1, $Tm2/Tm2^2$	-	-	0.109
	Solanum lycopersicum "Cindel"	Tm1, $Tm2/Tm2^2$	-	-	0.096
	Solanum lycopersicum "Halay"	Tm1, $Tm2/Tm2^2$	-	-	0.069

Per species four plants were inoculated; the number of plants showing specific symptoms is indicated in brackets. Average OD_{405} values after 2 hours of substrate incubation were considered positive (red) when ≥ 2x the value of mock-inoculated controls, otherwise values were considered negative (green). -: no symptoms, ~: mild symptoms, C: chlorosis, CL: chlorotic lesions, LA: leaf abscission, NL: necrotic lesions, NR: necrotic rings, R: rugosity. *two plants showed local symptoms only, two other plants showed systemic symptoms only, DAS-ELISA was performed separately on pooled samples of young leaves of the two plants with local symptoms and systemic symptoms, respectively.

In contrast to the previous experiment (Table 3) in which all plants of the pepper cultivar "Westlandse Grote Zoete" showed systemic symptoms, only two out of four inoculated plants showed local symptoms, whereas the other two plants showed systemic symptoms only. However, since cultivar "Westlandse Grote Zoete" does not harbor any tobamovirus resistance genes the presence of the virus was expected in all plants, which was indeed confirmed by DAS-ELISA (Table 4). Furthermore, all pepper accessions harboring a tobamovirus resistance gene/allele showed local but no systemic symptoms (S2 Fig), and tested negative by DAS-ELISA (Table 4). Therefore, it appears that each individual L^1, L^2, L^3 and L^4 gene/allele provided resistance to the novel tobamovirus. Overall,

259 these results showed that resistance against the novel virus is present in both the tomato and pepper
260 germplasm.

261 Finally, to gain more insight into the geographic distribution and the host range of the novel virus,
262 both symptomatic and asymptomatic fruits of *S. macrocarpon* and other *Solanaceae* imported into
263 the Netherlands in 2021, were subjected to Illumina sequencing analysis. This analysis included 19
264 samples from four countries, but no further material from Mexico: *Solanum betaceum* from
265 Colombia (2 samples), *S. macrocarpon* from Colombia (1 sample), *S. macrocarpon* from Surinam (10
266 samples), *S. melongena* from Malaysia (1 sample), and *Solanum torvum* from Thailand (5 samples).
267 The novel virus was not detected in any of these samples.

268 Discussion

269 This study assessed the potential risks of a newly discovered tobamovirus, tentatively named
270 "African eggplant-associated virus", from *S. macrocarpon*. The virus was easily transmitted
271 mechanically, and showed the ability to infect economically important crops, such as eggplant,
272 pepper, petunia, tomato, and other species including *Ammi majus, Nicotiana hesperis, N. occidentalis*
273 and *Solanum nigrum*. Overall, it was able to infect representatives of two out of six tested plant
274 families.

275 Sequence analysis indicated that the novel virus had a genome organization characteristic for
276 tobamoviruses, with less than 75% nucleotide identity to other tobamoviruses, which is significantly
277 lower than the tobamovirus species demarcation threshold of 90% nucleotide identity (18).
278 Therefore, it should be considered as a novel species within the genus *Tobamovirus*. Two genotypes
279 of the novel virus were present in the intercepted *S. macrocarpon* fruit. Although passages in *N.*
280 *occidentalis* "P1" of the novel virus did not change the nucleotide sequence of either genotype, a
281 decrease in relative frequency of genotype 1 was observed which led to genotype 2 taking over
282 completely in a subsequent passage to *S. macrocarpon.* This might be explained by adaptation to the
283 new host, as fitness cannot be maximized simultaneously in all potential hosts (19, 20).

284 Remarkably, an NCBI BLAST with the novel virus sequences resulted in a close hit with an alleged
285 bacteriophage isolate, which we expect to be incorrectly assigned and to actually belong to the same
286 novel tobamovirus. This “bacteriophage” sequence was part of a study that analyzed read data from
287 human metagenomic samples (21). Even though the query cover of the alignment with the novel
288 virus was only 60%, the high nucleotide identity with genotype 1 and genotype 2, 95.7% and 99.5%
289 respectively, suggests that the same virus was partially present in a human metagenomic sample.
290 Possibly, food or plant material contaminated with the virus had been consumed or touched by a
291 human.

292 A central paradigm in plant pathology is to demonstrate disease causation from a candidate
293 pathogen based on Koch’s postulates. The novel virus was consistently detected in inoculated *S.*
294 *macrocarpon* plants, but we were not able to replicate the symptoms found on the original fruit.
295 Difficulties in demonstrating causal association are not uncommon in plant virology and the
296 limitations of Koch’s postulates have been widely discussed (22). A potential explanation for the lack
297 of fruit symptoms could be the absence of genotype 1 in the inoculated *S. macrocarpon* plants.
298 Additionally, the development of symptoms after infection is influenced by environmental
299 circumstances and genotype of the plant, or a combination of these factors. Symptom variability as a
300 result of environmental or cultivar effects can be considered as a bottleneck for associating a novel
301 virus with a disease (23). Alternatively, the diffuse chlorotic mottling observed on the intercepted
302 fruit may have had a non-viral cause and we may have accidentally discovered an asymptomatic
303 infection.

304 A host range and pathotype study was conducted to assess the potential impact of the novel virus.
305 The infection of multiple solanaceous species was not unexpected, because the novel tobamovirus
306 was discovered in a host from this family. Additionally, *Ammi majus* from the *Apiaceae* family was
307 identified as a host. Although host ranges of tobamoviruses are usually quite narrow in nature, they
308 can be moderate to wide under experimental conditions (18). Wild species like *Ammi majus* and *S.*

309 *nigrum* that were infected experimentally might act as virus reservoirs, in which a virus can evolve
310 and spread to other plants and crops (24), for instance to one of the domesticated solanaceous
311 hosts. Such virus reservoirs complicate disease management. Vice versa, infected crops could pose a
312 risk to natural vegetation (25, 26).

313 For tomato four resistant accessions were identified which all harbored the combination of *Tm1* and
314 *Tm2*/*Tm2*^{2}, whereas accessions with only one of these genes were tolerant, *i.e*. no symptoms were
315 observed while infection was detected by DAS-ELISA. This suggests that a combination of these *Tm*
316 genes is required for resistance. Most commercial tomato cultivars on the European market that are
317 grown in greenhouses harbor this combination of resistances. For open field crops, the use of these
318 resistances is less common, but tobamovirus infections are less likely to spread (27). Therefore, the
319 impact of the novel virus on tomato cultivation in Europe is likely to be low. It cannot be excluded
320 that another factor in the genetic background of the resistant accessions is involved. Allele dosage
321 might play a role as well, as it is not clear whether the resistances were homozygous or heterozygous
322 in the tested accessions. Even though *Tm2*^{2} has historically been the most durable tobamovirus
323 resistance, it has previously been broken by ToBRFV (5) and partially broken by ToMMV (28). In a
324 screening of 160 tomato genotypes for ToBRFV resistance, only a single resistant genotype was
325 identified. A locus controlling tolerance mapped to chromosome 11 and was found to interact with a
326 locus on chromosome 2, at the *Tm1* region, to confer resistance (29). It would be interesting to
327 identify the genetics behind tolerance and resistance to African eggplant-associated virus in tomato
328 as well.

329 The breaking of *Tm2*^{2} resistance by ToBRFV has been attributed to a replacement of the amino acid
330 cysteine (C68) by histidine (H67) in its movement protein. C68 is conserved among all other
331 *Solanaceae*-infecting tobamoviruses, and is involved in *Tm2*^{2} activation and viral movement (30). The
332 novel tobamovirus contains C68, but surprisingly also infected a tomato accession harboring *Tm2*^{2}.

333 The breaking of this resistance is therefore not due to the replacement of C68 as was shown for
334 ToBRFV.

335 For pepper, accessions harboring either of the L^1, L^2, L^3 or L^4 genes/alleles were resistant, whereas a
336 cultivar without any of these resistance genes was susceptible. Since most commercial pepper
337 varieties grown in the EPPO region harbor *L* resistance genes/alleles (27), the impact on pepper in
338 Europe is also likely to be low. However, it cannot be excluded that pepper plants harboring these
339 resistances are susceptible to African eggplant-associated virus under different conditions. For
340 example, an isolate of ToBRFV was able to infect pepper plants harboring $L^{1,3,4}$ at high temperatures,
341 whereas they were otherwise resistant (5).

342 Due to the advance of high throughput sequencing, novel virus species are being identified at a rapid
343 pace, but biological characterization is often lacking due to resource constraints (23, 31). However,
344 such experiments are crucial to determine the biological significance and predict the potential impact
345 of a novel virus. The current study identified a novel tobamovirus, tentatively named African
346 eggplant-associated virus. This virus experimentally infected multiple plant species, mainly
347 *Solanaceae*, thus being potentially harmful to several economically important crops including
348 eggplant, pepper, petunia and tomato. This study indicated that potato is not a host, while resistance
349 is present in both tomato and pepper germplasm. As most commercial tomato and pepper varieties
350 grown in European greenhouses harbor the relevant resistances, the risk of infection of these crops
351 in Europe is likely to be low. The non-resistant tomato accessions showed no symptoms under the
352 applied experimental conditions, indicating that the economic impact of the novel virus may be low
353 in this crop, even in the absence of resistance. However, the durability of these resistances against
354 the novel virus and their effectiveness under different environmental conditions remain unknown.

355 In conclusion, the present distribution of the novel virus and its prevalence are unknown. The virus
356 was not detected in a limited survey of solanaceous fruits imported into the Netherlands and did not
357 include further samples from Mexico. At present, we cannot predict the likelihood of transmission of

358 African eggplant-associated virus to other plant species. In general, hygiene measures and the use of
359 genetic resistance are the main strategies to prevent transmission of tobamoviruses. In this respect,
360 the observed latent infections in multiple experimental host species underline the merit of screening
361 symptomless plants for viral infections (32). Therefore, additional monitoring of *S. macrocarpon* and
362 other *Solanaceae* around the world, including non-symptomatic material, is needed to get a better
363 understanding of the incidence, distribution and phytosanitary risk of African eggplant-associated
364 virus and other potentially harmful viruses.

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448 Supporting information

449 **S1 Fig. Sequence depth of both virus genotypes in the original sample and after passages in *N.***
450 ***occidentalis* "P1" and *S. macrocarpon.***

451 **S2 Fig. Symptoms on pepper accessions (*Capsicum spp.*) inoculated with the novel tobamovirus.** a)
452 *Capsicum annuum* "Westlandse Grote Zoete": chlorotic lesions, b) *C. annuum* "CGN19226": chlorotic lesions,
453 leaf bulging, c) *C. chacoense* "CGN21477": necrotic lesions, leaf abscission, d) *C. chinense* "CGN21557": necrotic
454 lesions, e) *C. frutescens* "CGN21546": chlorotic and necrotic lesions.

455 **S3 Appendix. Raw DAS-ELISA values of Table 2-4.**