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High similarity between tomato isolates of *Pepino mosaic virus* suggests a common origin

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Abstract

The almost simultaneous outbreaks of *Pepino mosaic virus* in tomato crops in different European and non-European countries, was reason to have a closer look at the relationship between these isolates and the original isolate from pepino. Fifteen isolates from tomato from different locations and the original pepino isolate, were compared on the basis of their symptomatology on a series of plant species. In addition, PCR fragments derived from the viral polymerase gene were sequenced and aligned. Both studies showed that the isolates from tomato clearly differed from the pepino isolate. The different tomato isolates, however, exhibited only minor differences to each other, both in symptomatology and nucleotide sequence. These results support the conclusion that the tomato isolates should be considered as a distinct strain (Mumford and Metcalfe (2001) Archives of Virology 146: 2455–2460; Van der Vlugt et al. (2000) Plant Disease 84: 103; Van der Vlugt et al. (2002) Bulletin OEPP/EPPO Bulletin 32: 503–508). Moreover, the high similarity of the different tomato isolates suggests the existence of a common source of infection for the recent outbreaks.

Introduction

Until 1999, the known incidence of *Pepino mosaic virus* (PepMV) was limited to two pepino crops (*Solanum muricatum*) in the coastal region of Peru (Jones et al., 1980). In the beginning of that year, however, PepMV was identified as the cause of a new disease in protected tomato crops in the Netherlands (Van der Vlugt et al., 2000; 2002). Comparison of this tomato isolate with the original isolate from pepino revealed some clear differences, which was reason for considering the tomato isolate as a different strain (Van der Vlugt et al., 2002).

Soon after the first outbreak of PepMV in the Netherlands, the virus was also detected in tomato crops in other European and non-European countries. However, in the different countries, striking differences in symptomatology and yield losses were reported. In Germany (D-E Lesemann, pers. comm.) and the Netherlands hardly any yield losses were observed, while in the United Kingdom (D Wright, pers. comm.), losses up to 15% were reported. In addition, in these countries remarkable differences were observed in the reaction of *Nicotiana glutinosa* after mechanical inoculation with local tomato isolates. It was not clear, whether these differences could be ascribed to differences between the virus isolates, the accessions of *N. glutinosa*, or the environmental conditions. Therefore, one of the questions to be answered was how the tomato isolates occurring in different geographical regions relate to each other. Moreover, would it be possible to trace their origin on the basis of this information and relate them to the original pepino isolate.

This paper describes the results of a comparative study including fifteen tomato isolates from different European and non-European countries and the original pepino isolate. The isolates were compared with each other on the basis of their symptomatology on a range

of plant species and on sequence alignments of 547-nt PCR fragments of the viral polymerase gene.

Materials and methods

Virus isolates

Sixteen PepMV isolates from ten different countries (Table 1) were obtained, either as samples submitted to the Plant Protection Service in the Netherlands (PPS) or kindly supplied by D-E Lesemann (BBA1137 (pepino) and BBA99-550; Biologische Bundesanstalt für Land-und Forstwirtschaft (BBA), Braunschweig, Germany) and D Wright (PD99903244; Central Science Laboratory (CSL), York, United Kingdom). On tomato, all tomato isolates showed (part) of the symptoms reported before (Van der Vlugt et al., 2002), except for a Canadian isolate (PD21001936) causing additional necrotic lesions on both leaves and stems. The initial identification of the tomato isolates was based on DAS-ELISA, using the antiserum prepared against a Dutch isolate of the tomato strain (PD99901066; Van der Vlugt et al., 2002), and the reactions of *Chenopodium quinoa*, *Datura stramonium*, *N. glutinosa* (accession PPS) and *N. occidentalis*-P1 upon mechanical inoculation. The pepino isolate (BBA1137) was maintained and propagated by mechanical inoculation to *N. glutinosa*-PPS at intervals of about three weeks, while all tomato isolates were maintained and propagated in *D. stramonium*.

Table 1. Pepino mosaic virus isolates from tomato and pepino used for comparative studies

Isolate	Origin
Tomato isolate	
BBA99-550	Germany
PD99901066	The Netherlands
PD99903244	United Kingdom
PD99910242	The Netherlands
PD99912968	USA, Arizona
PD99913775	Austria
PD99913776	Spain, Murcia
PD20000790	France, Bretagne
PD20001003	Belgium
PD20001287	Spain, Canary Isles
PD20001809	Germany
PD20005574	USA, Texas
PD20006266	Ukraine
PD20007805	The Netherlands
PD21001936	Canada
Pepino isolate	
BBA1137	Peru

Comparative study on test plants

At least four plants of the species listed in Table 2 were inoculated with each isolate. The inoculated plants were inspected visually and symptoms recorded during the following three weeks. If no symptoms occurred during this period, DAS-ELISA was, in most cases, used to check for symptomless infections. Different accessions of *N. glutinosa* were kindly provided by D-E Lesemann (BBA) and D Wright (CSL).

RNA extraction, cDNA synthesis and PCR

Total RNA was isolated from leaf material (150 mg) from infected plants of *D. stramonium* or *N. glutinosa*, using the Rneasy Plant Mini Kit (Qiagen). RNA was eluted from the column with 100 µl sterile H_2O and precipitated with ethanol at $-20\,^{\circ}C$. After washing, the RNA was dissolved in 20 µl sterile H_2O, of which 3 µl was used for synthesis of cDNA with general potexvirus primer Potex 1RC. Five microlitres of the cDNA reaction was used for PCR with general potexvirus primers Potex 2RC and Potex 5, which amplify a 547-nt fragment from the potexvirus RNA-dependent RNA-polymerase region (Van der Vlugt and Berendsen, 2002). The annealing temperature was $51.5\,^{\circ}C$. PCR fragments were analysed on 1% TAE–agarose gels.

Cloning of PCR fragments and sequence analysis

PCR fragments were cloned into a pGEM-T easy vector (Promega) and transformed to *Escherichia coli* JM109 cells (Promega) according to the manufacturer's instructions. Purified recombinant plasmid DNA was sequenced on an ABI automatic sequencer using standard M13 forward and reverse primers. Sequence data, excluding the PCR primer sequences, were compiled and analysed using the Lasergene DNASTAR program package. Multiple nucleotide sequence alignments were performed with the ClustalX Package (Thompson et al., 1997). Phylogenetic trees were generated by the neighbour-joining method with 1000 bootstrap replications and visualised using Treeview (Page, 1996).

Table 2. Summary of the local and systemic reactions of fifteen tomato isolates and one pepino isolate of *Pepino mosaic virus* on various plant species

Isolate	*Capsicum annuum* 'W. G. Zoete'	*Chenopodium quinoa*	*Datura metel*	*Datura stramonium*	*Nicotiana glutinosa*-CSL	*Nicotiana glutinosa*-PPS	*Nicotiana occidentalis*-P1	*Nicotiana tabacum* *'White Burley'*	*Physalis floridana*	*Solanum melongena* *'B. Beauty'*
BBA1137	−/(m)[1]	−/−	nl/s	(nl)/nl	nl,n/cl,cr,vc	nl,n/cl,cr,vc	cl,nl/c,d,nl	−/(vc)	−/(cl,m,nl)	−/s
BBA99-550	−/−*	−/−*	−/m	−/c,m	(cl)/(cl,vc)	(cl)/(cl,vc)	cl,nl/c,d,nl	cl/cl,vc	−/−*	−/(m),s
PD99901066	−/−*	−/−	−/m	−/c,m	(cl)/(cl,vc)	−/(cl,vc)	cl,nl/c,d,nl	−/−*	−/−*	−/c,m
PD99903244	−/−*	−/−	−/m	−/c,m	−/−*	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/(m),s
PD99910242	−/−*	−/−	−/(m)	−/c,m	nt	−/−	cl,nl/c,d,nl	−/−*	−/−*	−/m
PD99912968	−/−*	−/−	−/cl	−/c,m	−/−*	−/−	cl,nl/c,d,nl	−/−*	−/−*	−/cl,m[2]
PD99913775	−/−*	−/−	−/m	−/c,m	−/−*	−/−	cl,nl/c,d,nl	−/−*	−/−*	−/m
PD99913776	−/−*	−/−	−/m	−/c,m	−/−*	−/−	cl,nl/c,d,nl	−/−*	−/−*	−/m
PD20000790	−/−*	−/−	−/m	−/c,m	−/−*	−/−	cl,nl/c,d,nl	−/−*	−/−*	−/s
PD20001003	−/−*	−/−	−/m	−/c,m	−/−*	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/m
PD20001287	−/−*	−/−	−/l,m	−/c,m	−/−*	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/s
PD20001809	−/−*	−/−	−/cl	−/c,m	−/−*	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/cl,m[2]
PD20005574	−/−*	−/−*	−/m	−/c,m	−/−*	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/m
PD20006266	nt	−/−*	nt	−/c,m	−/−	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/s
PD20007805	−/−*	−/−	nt	nl/c,l,m	−/−*	−/−	cl,nl/c,d,nl	−/−	−/−*	−/cl,m
PD21001936	−/−*	−/−	nl/n	−/c,m	nt	−/−*	cl,nl/c,d,nl	−/−*	−/−*	−/m,r

[1]c = chlorosis, cl = chlorotic lesions, cr = chlorotic rings, d = dwarfing, m = mosaic or mottle, n = necrosis, nl = necrotic lesions, nr = necrotic rings, nt = not tested, r = rugosity, s = symptomless infection, vc = veinal chlorosis, − = no symptoms, but not tested for symptomless infection, −* = no infection, () = symptoms occasionally observed.

[2]+ severe mosaic.

Results

Comparative study on test plants

Table 2 shows that on most inoculated plant species the pepino isolate of PepMV (BBA1137) reacted differently from the tomato isolates. The differences appeared most pronounced in *D. metel*, *D. stramonium* (Figure 1A and B) and *N. glutinosa* (Figure 1C). In *Capsicum annuum* 'Westlandse Grote Zoete', *N. tabacum* 'White Burley' and *Physalis floridana*, the pepino isolate occasionally caused systemic infections, while the tomato isolates did not. The symptomatology of the German tomato isolate BBA99-550 and the Dutch isolate PD99901066 most resembled the pepino isolate, especially on *N. glutinosa* and *N. tabacum* 'White Burley'.

Comparing the reactions of the different tomato isolates to each other revealed only minor differences. The American isolate PD99912968, the Canadian isolate PD21001936 and the German isolate PD20001809 reacted different in *D. metel*. While the majority of isolates showed a systemic mosaic, the American and German isolates both evoked chlorotic lesions at systemically infected leaves. Plants inoculated with the Canadian isolate reacted with necrotic local lesions and subsequently died. The American and German isolates also reacted different in *S. melongena* 'Black Beauty'. These isolates caused a distinct mosaic on systemically infected leaves, while all other tomato isolates showed a very mild mosaic or did not show symptoms at all.

Comparative study on N. glutinosa

Table 3 shows that the three accessions of *N. glutinosa* from the BBA (Germany), CSL (United Kingdom) and PPS (Netherlands), respectively, reacted similar upon inoculation with a German (BBA99-550), British (PD99903244) and Dutch (PD99901066) isolate. Considering the virus isolates, however, some differences were observed. The pepino isolate of PepMV infected all plants of all accessions systemically, symptoms appeared earlier and were more pronounced in comparison to those caused by the tomato isolates. Between the tomato isolates, however, a gradual difference was observed. The German isolate infected most plants, the Dutch isolate about half and the British isolate none of the inoculated plants. All plants that did not show symptoms reacted negative in DAS-ELISA with an antiserum to PepMV.

Figure 1. Pepino mosaic virus. A: mosaic on a leaf of *D. stramonium* infected by the tomato isolate PD99901066. B: necrotic spots on a leaf of *D. stramonium* infected by the pepino isolate BBA1137. C: chlorotic rings and spots on a leaf of *N. glutinosa* infected by the pepino isolate BBA1137; most tomato isolates do not infect this plant species.

RT-PCR and sequence analysis

In RT-PCR all sixteen PepMV isolates yielded fragments of approximately 600 nucleotides (nt). Sequence analysis of the cloned fragments, excluding the

Table 3. Number of *Pepino mosaic virus* infected plants/inoculated plants for each of the three *N. glutinosa* accessions from BBA, CSL and PPS, 21 days after mechanical inoculation with three isolates from the tomato strain and the original pepino strain.

Isolate	*N. glutinosa*-BBA	*N. glutinosa*-CSL	*N. glutinosa*-PPS
BBA1137 (pepino)	4/4; 4/4; 4/4	4/4; 4/4; 4/4	4/4; 4/4; 4/4
BBA99-550 (German)	2/4; 4/4	2/4; 4/4	2/4; 4/4
PD99901066 (Dutch)	2/4; 4/4; 1/4; 2/4	2/4; 2/4; 2/4; 4/4	2/4; 2/4; 0/4; 2/4
PD99903244 (Britsh)	0/4; 0/4	0/4; 0/4	0/4; 0/4

primer sequences, revealed a length of 547-nt for all isolates, and confirmed their identity as fragments from the PepMV RNA-dependent RNA polymerase. Nucleotide-sequence alignments of the pepino isolate with the tomato isolates showed a homology of 94.1–94.9%, with 20 nt unique for the pepino isolate. Nucleotide sequence alignments between the respective tomato isolates showed homologies from 99.1% to 100%. Homologies between the PepMV isolates and the corresponding sequence of *Potato virus X* (PVX; Acc. no. D00344, another potexvirus infecting tomato) ranged from 61.3% to 61.9% (results not shown).

At the amino-acid level the homology between the tomato isolates ranged from 99.5% to 100%. Eleven isolates were found identical in the concerned region, and five isolates deviated one amino acid from the consensus. The pepino isolate showed two differences to the consensus, of which one was shared with the Canadian tomato isolate PD21001936.

Phylogenetic analyses of multiple nucleotide-sequence alignments of the PepMV isolates and PVX using ClustalX generated a phylogenetic tree (see Figure 2) in which PVX as an outgroup clearly branches apart from the PepMV isolates. Within the PepMV isolates the original pepino isolate BBA1137 clearly separated from the 15 tomato isolates.

Discussion

The results show that the tomato isolates of PepMV can be distinguished from the original isolate from pepino on the basis of symptomatology and sequence data. The different tomato isolates, however, appear highly similar to each other, as only minor differences in symptomatology and nucleotide sequence of a part of the polymerase gene were observed.

Considering the symptomatology, the pepino isolate clearly differed from the tomato isolates in most of the plant species tested. The tomato isolates BBA99-550 and PD99901066 showed some resemblance to the pepino isolate, as they occasionally infected *N. glutinosa* in contrast to the other tomato isolates. Based on the reactions of the other test plants and former results (Van der Vlugt et al., 2002), however, these two isolates clearly belonged to the group of tomato isolates. Moreover, analysis of another 60 isolates from tomato on *C. quinoa, D. stramonium, N. glutinosa*-PPS and *N. occidentalis*-P1, revealed reactions similar to the majority of the tomato isolates in this study (results not shown).

The remarkable differences observed in the reaction of *N. glutinosa* upon inoculation with local tomato isolates in Germany, the Netherlands and United Kingdom, was reason for a closer look. Both in Germany and the United Kingdom, systemic symptoms had been observed in this species (D-E Lesemann and D. Wright, pers. comm.), while in the Netherlands a systemic reaction had not been found (Van der Vlugt et al., 2002). The present results indicated that the different reactions of *N. glutinosa* could not be ascribed to the genotype of the accessions used. Moreover, they suggest a difference in the ability of the different virus isolates to infect this species. Differences in environmental conditions may account for the variation within repeats of the experiments.

The relationships between the tomato isolates and the pepino isolate of PepMV, indicated by the symptomatology, were confirmed by the sequence alignments of the 547-bp fragment of the viral polymerase gene. As expected from the lower level of nucleotide-sequence homology (94.1–94.9%), pepino isolate BBA1137 grouped separately from the tomato isolates in phylogenetic analyses. Both the pepino and the tomato isolates of PepMV, however, grouped separately from PVX, which was included in this analysis as a different potexvirus (Figure 2). The high levels of homology (>99.1%) between the 15 tomato isolates observed in a relative short sequence hamper the calculation of any reliable sub-clustering within these isolates. Bootstrap values varied from 59/1000 to 758/1000 (data not shown) indicating low reliability of the branching presented in the figure. PVX was always represented in the tree as an outgroup with a bootstrap probability of 100% (results not shown). Bootstrap values did not change significantly when

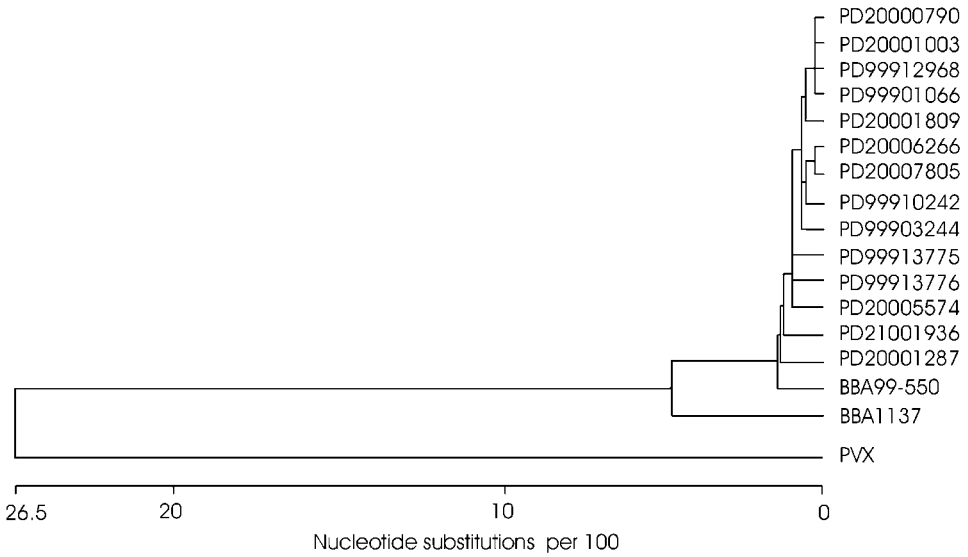

Figure 2. Neighbour-joining tree representing phylogenetic relationships between 15 isolates of the tomato strain, one isolate of the pepino strain (BBA1137) of *Pepino mosaic virus* and PVX as an outgroup. The relationships are based on nucleotide sequence identity in a 547-nt RT-PCR fragment derived from the viral RNA-dependent RNA polymerase. The scale bar indicates substitutions per 100 nt.

PVX was left out as an outgroup in the analysis. The phylogenetic analyses support the proposed grouping of the PepMV isolates into two strains.

Within the tomato isolates, no particular subclustering was derived from the limited sequence data. In addition, these data do not provide evidence for the deviation of the tomato isolates BBA99-550 and PD99901066 from the majority of the tomato isolates in their occasional infection of *N. glutinosa*. Also, different reactions of the isolates PD99912968 and PD20001809 on *D. metel* and *S. melongena* 'Black Beauty' were not related to differences in the nucleotide sequence of the PCR fragment. Moreover, the Canadian isolate PD21001936, the only isolate causing necrotic symptoms on tomato and systemic necrosis on *D. metel*, was not discriminated on the basis of the sequence alignments. Therefore, it had to be concluded that the sequence variation in the selected region of the polymerase gene, although suitable for discriminating the tomato isolates from the pepino isolate, could not be used for analysis of the relationship within the tomato isolates. This conclusion was in line with the study of Mumford and Metcalfe (2001), who compared the sequence of the coat-protein gene of fifteen PepMV isolates, including fourteen European tomato isolates and the original pepino isolate from Peru. Also in the coat-protein gene, the tomato isolates show homologies over 99%, while the homologies with the pepino isolate range from 96% to 97%. Together, the comparison of the sequences of part of the polymerase and coat-protein genes indicate that the tomato isolates are highly similar and clearly different from the pepino isolate. Comparison of other parts of the viral genome, therefore, seems needed to explain the differences in symptomatology.

Both the biological and molecular results support the separation of PepMV in a pepino and tomato strain (Mumford and Metcalfe, 2001; Van der Vlugt et al., 2000; 2002). Within the group of tomato isolates a high similarity was identified, both in host range and symptomatology and in the polymerase and coat-protein genes. Based on the high similarity of the tomato isolates, a common origin seems likely. However, where this origin is located and how it relates to the original infections in Peru remained unclear, as PepMV has not been reported since its first finding in 1974 (Jones et al., 1980). To locate the origin was complicated by the fact that the recent findings of the virus took place almost simultaneously in many European and non-European countries over the last three years, i.e. Austria (this report), Belgium (this report), Canada (French et al., 2001), France (this report), Finland (Tegel, pers. comm.), Germany (Pfeilstetter, et al., 2000), Italy (Roggero et al., 2001), the Netherlands (Van der Vlugt et al., 2000) Norway (Blystad, pers. comm.), Peru (Soler et al., 2002),

Spain (Jordá et al., 2000; Prohens et al., 2000), Ukraine, (this report), United Kingdom (Mumford and Metcalfe, 2001) and United States of America (French et al., 2001). Because of the often very weak symptomatology in wild and cultivated *Lycopersicon* spp., PepMV had probably spread before its first identification in the Netherlands. Within this context the recent paper of Soler et al. (2002) provides some interesting features, as they reported the natural occurrence of PepMV in Peru in 2000. Their identification of often symptomless infections of PepMV, in both wild *Lycopersicon* species and cultivated tomato crops, suggests that the virus might still have been present since its first finding. This could have lead to unnoticed spread of the virus from South America to other parts of the world. Sequencing of these recently collected isolates of PepMV from Peru might shed a new light on the origin of the tomato isolates and their relation to the original pepino isolate.

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