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Genetic characterization of *Pepino mosaic virus* isolates from Belgian greenhouse tomatoes reveals genetic recombination

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Abstract Over a period of a few years, *Pepino mosaic virus* (PepMV) has become one of the most important viral diseases in tomato production worldwide. Infection by PepMV can cause a broad range of symptoms on tomato plants, often leading to significant financial losses. At present, five PepMV genotypes (EU, LP, CH2, US1 and US2) have been described, three of which (EU, LP and US2) have been reported in Europe. Thus far, no correlation has been found between different PepMV genotypes and the symptoms expressed in infected plants. In this paper, the genetic diversity of the PepMV population in Belgian greenhouses is studied and related to symptom development in tomato crops. A novel assay based on restriction fragment length polymorphism (RFLP) was developed to discriminate between the different PepMV genotypes. Both RFLP and sequence analysis revealed the occurrence of two genotypes, the EU genotype and the CH2 genotype, within tomato production in Belgium. Whereas no differences were observed in symptom expression between plants infected by one of the two genotypes, co-infection with both genotypes resulted in more severe PepMV symptoms. Furthermore, our study revealed that PepMV recombinants frequently occur in mixed infections under natural conditions. This may possibly result in the generation of viral variants with increased aggressiveness.

Keywords Cross-protection · Genotyping · *Potexvirus* · PepMV · *Solanum lycopersicum* · Synergism

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Introduction

Worldwide, tomato (*Solanum lycopersicum*) is one of the most widely grown vegetable crops. In many

regions where tomato is cultivated, viral diseases have become one of the main limiting factors in tomato production over the last decades. A virus that has recently caused a large impact on tomato cultivation is *Pepino mosaic virus* (PepMV). PepMV is a positive-sense single-stranded RNA virus that belongs to the potexviruses, with *Potato virus X* as the type species. The genomic RNA of the virus is approximately 6,400 nt long and encodes five open reading frames (ORFs) encompassing an RNA-dependent RNA polymerase (RdRp), a triple gene block, a coat protein (CP), and two short untranslated regions that flank the coding regions, including a 3′ poly(A) tail (Aguilar et al. 2002; Cotillon et al. 2002). PepMV was first identified in Peru in 1974 from young leaves of pepino (*Solanum muricatum*) that displayed yellow mosaic symptoms (Jones et al. 1980). Presence of the virus in tomato crops was not reported until 1999 when it was found in Dutch tomatoes (Van der Vlugt et al. 2000), after which the virus quickly spread in tomato crops throughout Europe and North America (Soler et al. 2000; French et al. 2001; Jorda et al. 2001; Mumford and Metcalfe 2001; Roggero et al. 2001). A wide range of symptoms has been associated with PepMV infection in tomatoes including leaf mosaic, leaf distortions, nettle heads and stunting. Apart from these, fruit discoloration, mostly expressed as marbling or flaming, caused by irregular lycopene distribution, is considered as the most devastating result of PepMV infection because it reduces the commercial value of the fruit (Soler et al. 2000; Mumford and Metcalfe 2001; Roggero et al. 2001; Spence et al. 2006). In some cases, even complete plant collapse has been associated with PepMV infection (Soler-Aleixandre et al. 2005). In general environmental factors such as light and temperature are thought to play a crucial role in symptom development (Jorda et al. 2001). In some tomato cultivation areas up to 90% of the greenhouse tomato crops are infected with PepMV, leading to up to 40% production losses (Soler et al. 2000). Since no resistant varieties are available and no curative measures exist, prevention of PepMV infection by hygienic measures is important. To reduce economic losses caused by PepMV infection, some tomato growers deliberately inoculate their plants with PepMV at the start of the growing season since it has been speculated that early PepMV infections are less damaging than infections that occur later in the growing season (Spence et al. 2006). This immunization strategy is based on the principle of cross-protection, which was successfully used in the 1970s to protect tomato plants against *Tobacco mosaic virus* (Rast 1972). Recently, resistance sources have been identified within the *Solanum* genus that may be used for future resistance breeding against PepMV (Ling and Scott 2007; Soler-Aleixandre et al. 2007).

In addition to the complete nucleotide sequence of four European PepMV isolates, that of a Peruvian PepMV isolate from *Lycopersicon peruvianum* has also been determined (Aguilar et al. 2002; Cotillon et al. 2002; Lopez et al. 2005). Generally, PepMV isolates that have been identified in European tomato crops are highly similar (99% nucleotide identity) and differ from the Peruvian isolate (95% nucleotide identity). Therefore, these European PepMV isolates were grouped in the so-called tomato genotype, while the Peruvian isolate represents the so-called Peruvian (LP) genotype (Mumford and Metcalfe 2001; Aguilar et al. 2002; Cotillon et al. 2002; Verhoeven et al. 2003; Lopez et al. 2005; Pagan et al. 2006). In addition, three significantly different PepMV genotypes have recently been identified, two derived from isolates from diseased tomato plants in the USA, the so-called US1 and US2 genotypes, and one derived from an isolate from infected tomato seeds in Chile, the so-called CH2 genotype (Maroon-Lango et al. 2005; Ling 2007). To distinguish the original ‘tomato genotype’ from the novel PepMV genotypes identified on tomato, the original genotype is further referred to as the European (EU) genotype (Pagan et al. 2006). In Spain, members of the LP and US2 PepMV genotypes have been found in tomato crops, always occurring in mixed infections with the EU genotype (Martinez-Culebras et al. 2002; Pagan et al. 2006). Apart from the study of Pagan et al. (2006), the genetic structure of the tomato PepMV population has not been analyzed in a tomato growing area. Furthermore, until now no correlation has been found between different PepMV isolates or genotypes and the severity of symptom expression in infected tomato plants (Pagan et al. 2006). Here, the genetic diversity among PepMV isolates in Belgian greenhouses is studied and related to symptom development in tomato crops. In addition, it is shown that PepMV recombinants frequently occur in mixed infections with different PepMV genotypes.

Materials and methods

PepMV assessment in commercial greenhouses

From January until November 2006, a monthly survey for the occurrence of PepMV was conducted. In total, 48 commercial Belgian greenhouse tomato production facilities located in areas with a high PepMV infection pressure were used in this study, giving preference to greenhouses with a history of PepMV infections (Table 1). On a monthly basis, different plant parts (head, foliage and fruit) were examined for PepMV occurrence by horticultural experts according to a specific rating schedule from 1 (no symptoms) to 6 (dead plant part; Table 2). In each greenhouse, approximately 100 plants that belong to a single tomato variety located in a marked rating block of two plant rows were examined, and one average score was given for each type of symptom. Furthermore, samples were collected monthly to assess PepMV presence. The samples were composed of young leaves from the heads of 10 different, randomly chosen tomato plants from the marked rating block (one leaf per plant; 10 leaves per sample). As such, each sample represented the overall situation of a PepMV infection in a given greenhouse at a given point in time. Following homogenization, subsamples were used for further analysis.

Determination of viral presence

Samples were analysed for PepMV presence using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) employing a commercially available antiserum (Agdia Inc., Elkhart, USA) according to the supplier's instructions. Samples were rated positive if the absorbance level exceeded the background level by three times. Background absorption was defined as the mean value of at least two wells containing all reagents except the sample. In case ELISA testing was inconclusive, PepMV presence was determined by reverse transcriptase PCR (RT-PCR).

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from the tomato samples (300 mg of homogenized leaf tissue) using a phenol-based extraction procedure described by Eggermont et al. (1996). Subsequently, cDNA was synthesized using the Qiagen Quantitect Reverse Transcription kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions using the PepMV-specific reverse primers Pep4 and PepMV UTR R (Mumford and Metcalfe 2001; Pagan et al. 2006), targeting a fragment of the RNA-dependent RNA polymerase (RdRp) gene and the Coat Protein (CP) gene, respectively. Prior to PCR amplification, the cDNA was treated with RNase to eliminate residual RNA. Amplification was performed in a 20 µl reaction volume containing 1 µl of cDNA, 1 µM of each primer and 1 unit Titanium Taq DNA polymerase (Clontech Laboratories, Inc., Palo Alto, CA, USA). Primers Pep3 and Pep4 generated a single, 625 bp RdRp amplicon and primers PepMV TGB F and PepMV UTR R generated a single CP amplicon of 845 bp (Mumford and Metcalfe 2001; Pagan et al. 2006). The PCR temperature profile consisted of denaturation at 94°C for 2 min, followed by 30 cycles of 45 s at 94°C, 45 s at 53°C, and 45 s at 72°C, with a final extension step at 72°C for 10 min. Amplified products (1 µl) were resolved electrophoretically in a 1.5% agarose gel. All reactions were performed at least twice.

Reverse transcriptase PCR restriction fragment length polymorphism (RT-PCR-RFLP)

A RT-PCR assay combined with RFLP analysis has previously been proposed as a rapid method for discrimination of the PepMV isolates known at that time (Martinez-Culebras et al. 2002). However, since this method was not developed to discriminate between the currently occurring five PepMV genotypes, a similar RT-PCR-RFLP assay was developed for these five genotypes. RdRp and CP sequences of reference PepMV genotypes available in GenBank (Table 3) were screened *in silico* for genotype-specific restriction enzyme recognition sites. According to theoretical predictions from sequence alignments, digestion of the RdRp fragment with *Eco*RI and *Bgl*II results in three different RFLP groups, one encompassing the EU and the LP genotype, one with the CH2 genotype and one consisting of the US1 and US2 genotypes (Table 3). Based on *in silico* prediction, no discrimination between the EU and the LP genotype or between the US1 and US2 genotype was possible with restriction analysis of the RdRp frag-

Table 1 PepMV occurrence in 48 commercial Belgian greenhouse tomato production facilities in the 2006 growing season assessed upon monthly monitoring

Greenhouse[a]	Location	First detection of PepMV	Genotype(s) detected[b]	Genotype(s) autumn 2006[b]
01	Melsele	February 2006	EU + CH2	EU + CH2
02	Merksplas	March 2006	EU	EU
03	Rijkevorsel	February 2006	CH2	CH2
04	Rijkevorsel	none	None	None
2005	Rijkevorsel	December '05	EU + CH2	EU + CH2
06	Rijkevorsel	None	None	None
07	Rijkevorsel	February 2006	EU	EU + CH2
08	Merksplas	June 2006	CH2	EU + CH2
09	Merksplas	May 2006	CH2	EU + CH2
11	Wuustwezel	June 2006	EU	EU
12	Ravels	None	None	None
13	Hoogstraten	May 2006	CH2	CH2
14	Meer	None	None	None
15	Meer	February 2006	CH2	CH2
16	Borsbeek	April 2006	CH2	CH2
17	Broechem	January 2006	EU + CH2	EU + CH2
18	Broechem	January 2006	EU	EU
19	Broechem	January 2006	CH2	CH2
20	Broechem	February 2006	EU	EU + CH2
21	Melsele	June 2006	CH2	CH2
22	Melsele	April 2006	CH2	CH2
23	Hoogstraten	January 2006	CH2	CH2
24	Rijkevorsel	None	None	None
31	Aartselaar	February 2006	EU	EU + CH2
32	Boechout	February 2006	EU + CH2	EU + CH2
33	Rumst	May 2006	CH2	CH2
34	Duffel	February 2006	CH2	CH2
35	Duffel	None	None	None
36	Lier	None	None	None
37	Putte	February 2006	EU	EU
38	Putte	January 2006	CH2	CH2
39	St.-Kat.-Waver	March 2006	CH2	CH2
40	St.-Kat.-Waver	February 2006	CH2	CH2
41	St.-Kat.-Waver	February 2006	EU + CH2	EU + CH2
42	St.-Kat.-Waver	August 2006	CH2	CH2
43	St.-Kat.-Waver	February 2006	CH2	CH2
44	St.-Kat.-Waver	July 2006	CH2	CH2
45	St.-Kat.-Waver	February 2006	CH2	CH2
46	Koningshooikt	March 2006	CH2	EU + CH2
47	Koningshooikt	February 2006	EU + CH2	EU + CH2
48	St.-Kat.-Waver	January 2006	CH2	CH2
49	St.-Kat.-Waver	February 2006	CH2	CH2
50	St.-Kat.-Waver	January 2006	CH2	CH2
51	St.-Kat.-Waver	January 2006	EU	EU + CH2
52	Boechout	June 2006	EU	EU + CH2
53	Duffel	October 2006	EU	EU
54	St.-Kat.-Waver	February 2006	CH2	CH2
55	St.-Kat.-Waver	February 2006	EU + CH2	EU + CH2

[a] Greenhouses in italics were selected for phylogenetic analyses.

[b] PepMV genotype determined by RT-PCR-RFLP (Reverse transcriptase PCR restriction fragment length polymorphism assay).

Table 2 Score table used for monthly assessment of PepMV symptoms[a] by horticultural experts

Plant part	Symptom type	Score
Head	Leaf bubbling (a)	Mean of a and b (A)
	Nettle head (b)	
Foliage	Leaf bubbling (c)	Mean of c, d, e and f (B)
	Yellow spots (d)	
	Stem necrosis (e)	
	Leaf necrosis (f)	
Fruit	Discoloration	Maximum of g and h (C1)
	Marbling (g)	
	Flaming (h)	
	Scars and open fruits	Maximum of i and j (C2)
	Scars (i)	
	Open fruits (j)	
	Rare symptoms	Maximum of k and l (C3)
	Sunken spots on the fruit surface (k)	
	Brown spots on the fruit surface (l)	
		Mean of C1, C2 and C3 (C)
General score		Mean of A, B and C

[a] PepMV symptoms were scored between 1 (symptoms not observed) and 6 (die-off of the respective plant part).

ment using these enzymes (Table 3). However, further discrimination between these genotypes was possible upon restriction of the CP fragment with a set of four restriction endonucleases (*Hind*III, *Nde*I, *Pvu*II and *Sac*I; Table 3). Actual digestion of RT-PCR fragments was performed according to the manufacturer's instructions (New England Biolabs Inc., Ipswish, MA, USA).The analyses were performed twice, each time on two different samples from the same greenhouse.

Phylogenetic analyses

Genetic characterization of PepMV isolates was performed based on two genomic regions, a fragment of the RdRp gene and a fragment of the CP gene, obtained by RT-PCR as described earlier. Amplified products were directly cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced using the vector-specific primers M13-F and M13-R flanking the insert (Macrogen Inc., Seoul, South Korea). Depending on the experiment, for each sample 3 to 10 clones were sequenced. Multiple sequence alignments were performed using CLUSTAL X (Thompson et al. 1997), including six PepMV reference sequences available in GenBank (Table 3). Subsequently, a neighbour-joining tree (Saitou and Nei 1987) was constructed and displayed by Treeview (v. 1.6.6; Page 1996). Robustness of the generated phylogenetic relationships was assessed by subjecting the data set to 1,000 bootstrap replicates. All sequences determined in this study have been deposited in GenBank under Accession numbers EF599505–EF599604.

Statistical analyses

Analysis of variance (ANOVA) and post-hoc Bonferroni tests were used to determine the effects of PepMV genotype on symptom expression in different plant parts. All statistical analyses were performed with SPSS software (v. 10.0; SPSS Inc., Chicago, IL, USA).

Table 3 Rapid typing of PepMV genotypes by restriction endonuclease digestion of RT-PCR products (RT-PCR-RFLP)

PepMV fragment	Restriction enzyme	PepMV genotype				
		EU[a]	LP[a]	CH2[a]	US2[a]	US1[a]
RdRp	None	625[b]	625	625	625	625
	*Eco*RI	230	230	625	625	625
		395	395			
	*Bgl*II	625	625	338	625	625
				287		
CP	None	845	845	845	845	845
	*Hind*III	845	845	845	845	562
						283
	*Nde*I	367	367	845	845	845
		478	478			
	*Pvu*II	278	278	845	644	643
		122	122		201	202
		445	445			
	*Sac*I	845	386	845	845	845
			459			

RdRp RNA dependent RNA polymerase; *CP* coat protein.

[a] GenBank accession numbers of used reference sequences: EU (Fr, AJ438767; Sp13, AF484251), LP (LP2001, AJ606361), US1 (AY509926), US2 (AY509927) and CH2 (DQ000985).

[b] Sizes (bp) of cDNA fragments are based on theoretical digests of reference sequences retrieved from GenBank.

Results

Assessment of PepMV occurrence in commercial greenhouses

In the 2006 growing season, a monthly survey for the occurrence of PepMV was conducted in 48 commercial Belgian greenhouse tomato production facilities (Table 1). At the end of the growing season, tomato crops in 41 of the 48 greenhouses were infected with PepMV (Table 1). However, since areas with a high PepMV infection pressure and greenhouses with prior PepMV infections were selected, it should be noted that the high PepMV presence in this study is not representative of the PepMV incidence in the total Belgian tomato cultivation.

PepMV genotyping by RT-PCR-RFLP

In order to develop a rapid screening method to discriminate between the different PepMV genotypes, a RT-PCR-RFLP assay was designed. The robustness of the assay was verified by sequence analysis of all sequences obtained in this study as well as those retrieved from GenBank. For all sequences, a perfect correlation was obtained between the presence or absence of the RFLP restriction sites and the PepMV genotype (data not shown), demonstrating the reliability of the assay.

Once PepMV was detected in a given sample, the genotype was determined using the developed RT-PCR-RFLP method (Table 1). At the time of detection, the EU genotype was found in 10 greenhouses while in 24 greenhouses the CH2 genotype was detected. In seven greenhouses a mixed infection with these two genotypes was recorded. At the end of the growing season, genotyping by RT-PCR-RFLP was performed again to see whether the composition of the PepMV population had changed over the growing season. At that time, the CH2 genotype was detected solitary in 21 greenhouses, while infection with only the EU genotype was found in five greenhouses. Mixed infections with both genotypes were found in 15 greenhouses and no other PepMV genotypes were detected. In seven greenhouses, PepMV was not detected during the entire growing season and no symptoms were observed.

To assess the homogeneity of the PepMV population present in a greenhouse, RT-PCR-RFLP results obtained from mixed plant samples were compared to results obtained from individual plant samples. In total, 30 individual plant samples, originating from greenhouses 01, 31 and 32, were analysed together with the corresponding mixed plant sample. Individual plant samples from greenhouse 31 generated an EU genotype specific RT-PCR-RFLP pattern, consistent with the result obtained from the mixed plant sample, whereas individual plant samples from greenhouses 01 and 32 resulted in mixed infection patterns, with the exception of one plant that appeared to be infected only with the CH2 genotype. These results show that mixed plant samples are, in general, representative of individual plants in the rating block, implying that the PepMV population is homogenous within a greenhouse, and that co-infection with the EU genotype and the CH2 genotype occurs within individual plants.

Since the CH2 genotype was not previously reported in European tomato cultivation, the RT-PCR-RFLP identification of one isolate (2206/06/A1, obtained from greenhouse 22) that resulted in a CH2 specific RT-PCR-RFLP pattern was confirmed by determining the complete sequence (GenBank Accession number EF599605), resulting in an overall nucleotide identity of >98% with the CH2 sequence present in GenBank (DQ000985). Altogether, these results illustrate that the CH2 genotype was dominant in Belgian tomato production of 2006, as it was found in 36 of 41 greenhouses in which PepMV was detected.

PepMV genotype occurrence and symptom development

Each greenhouse was rated monthly for plant vigour and PepMV symptom expression in multiple plant parts according to a specific rating scale (Table 2). Subsequently, results were grouped based on the encountered PepMV genotypes (EU, CH2 or mixed; Fig. 1). As shown in Fig. 1, no differences were observed in symptom expression between plants infected by one of the two genotypes. Nevertheless, the obtained PepMV symptom scores were significantly higher ($P<0.05$) for greenhouses with mixed infections. When comparing results for the three different plant parts assessed (head, foliage and fruit), the mean scores for the head of the plant showed the most significant differences between the different

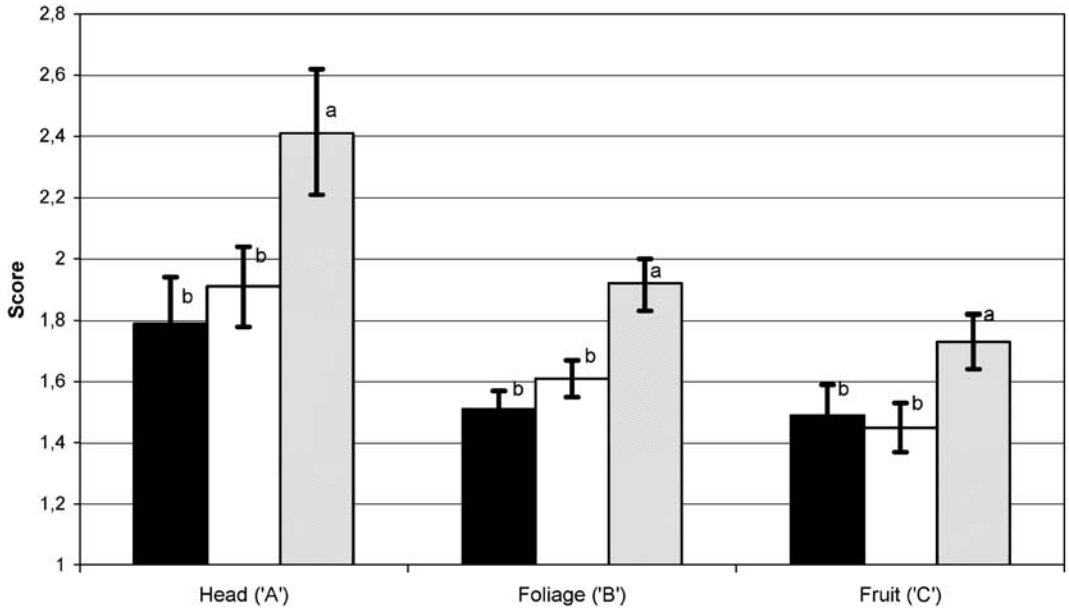


Fig. 1 Relation between symptom expression and PepMV genotype. All greenhouses included in the survey were grouped based on the occurrence of a given PepMV genotype, generating three groups (EU genotype in *black bars*, CH2 genotype in *white bars*, and mixed infections of EU and CH2 genotypes in *grey bars*). Symptoms were scored on a rating scale from 1 (no symptoms) to 6 (die-off) as shown in Table 2. Overall means of score 'A', 'B' and 'C' (Table 2) and a 95% confidence interval per group are shown in the graph, calculated for each factor using SPSS software. Differences between groups were studied using one-way ANOVA and post hoc Bonferroni tests

groups (Fig. 1). Nevertheless, it should be noted that in general, differences between the groups were rather small. This is mainly due to the fact that means were calculated based on monthly ratings throughout the entire growing season, while PepMV symptom expression typically occurs periodically. Long periods without symptoms are usually observed that level out extreme differences when monitoring a complete growing season. Compared with the situation when PepMV was detected for the first time in a greenhouse, in 8 out of 40 greenhouses with PepMV infected tomato plants, an additional PepMV genotype (five times the CH2 genotype and three times the EU genotype) invaded the crop during the growing season (Table 1). Upon invasion of this second genotype more severe symptoms were usually observed. In greenhouse 07, for example, PepMV infection with only the EU genotype was first detected in February. Upon monthly monitoring, from September onwards the CH2 genotype was also detected, coinciding with a sudden increase in symptom severity in September, especially on the fruits. Scores for fruit marbling increased from 1 to 4 between August and October. A concurrent increase in symptom severity was not seen in plants infected only with the EU or the CH2 genotype (data not shown).

Phylogenetic analyses of PepMV isolates from 10 greenhouses

To further assess the genetic diversity of the encountered PepMV isolates, the nucleotide sequences of a 625 bp fragment of the RdRp gene and a 845 bp fragment of the CP gene were determined in several samples taken in the beginning of the growing season, preferentially from greenhouses that were diagnosed with mixed infections of the EU and CH2 genotypes (Table 1). For the RdRp gene, two to three clones from samples from 10 greenhouses (01, 02, 05, 17, 23, 31, 32, 41, 47 and 50) were sequenced resulting in 29 nucleotide sequences. Subsequently, the sequences were compared with reference sequences from the different genotypes. Based on the sequence alignments, a phylogenetic tree was constructed that displays two main groups (Fig. 2a), that each share 97% to 100% similarity, while the two groups share between each other only about 80% homology. The

first group includes the European reference isolates Fr and SP13 and is further referred to as the EU genotype group. The second cluster encompassing the Chilean reference isolate CH2 is referred to as the CH2 genotype group (Fig. 2a). Thirteen sequences (originating from seven samples) were assigned to the EU genotype, while 13 other sequences (originating from six samples) landed in the CH2 genotype group (Fig. 2a). Sequence homology between both PepMV groups and the closest related viral species within the genus *Potexvirus* (*Scallion Virus X* and *Narcissus Mosaic Virus X*) ranged between 44 and 49%. Sequences that are homologous to the reference isolates US1 (US1 genotype), US2 (US2 genotype) and LP01 (LP genotype) were not detected in our study, confirming the results of the RT-PCR-RFLP analysis. However, within three greenhouses (01, 05 and 50), PepMV sequences were identified that clustered in between the two different groups (samples 0106/12/C1 clone D, 0506/09/B1 clone A and 5006/05/B1 clone A), suggesting the occurrence of PepMV recombinants (Fig. 2a).

The intra-specific sequence variance for the RdRp gene fragment within a greenhouse was studied for greenhouse 32, where a mixed infection with the EU and the CH2 genotypes was found (Fig. 2b). Sequence homology ranged from 79 to 100%, even when comparing different clones from a single sample. When studying sequences belonging to the same PepMV genotype but obtained from different clones from the same sample, a sequence homology of 98 to 100% was seen.

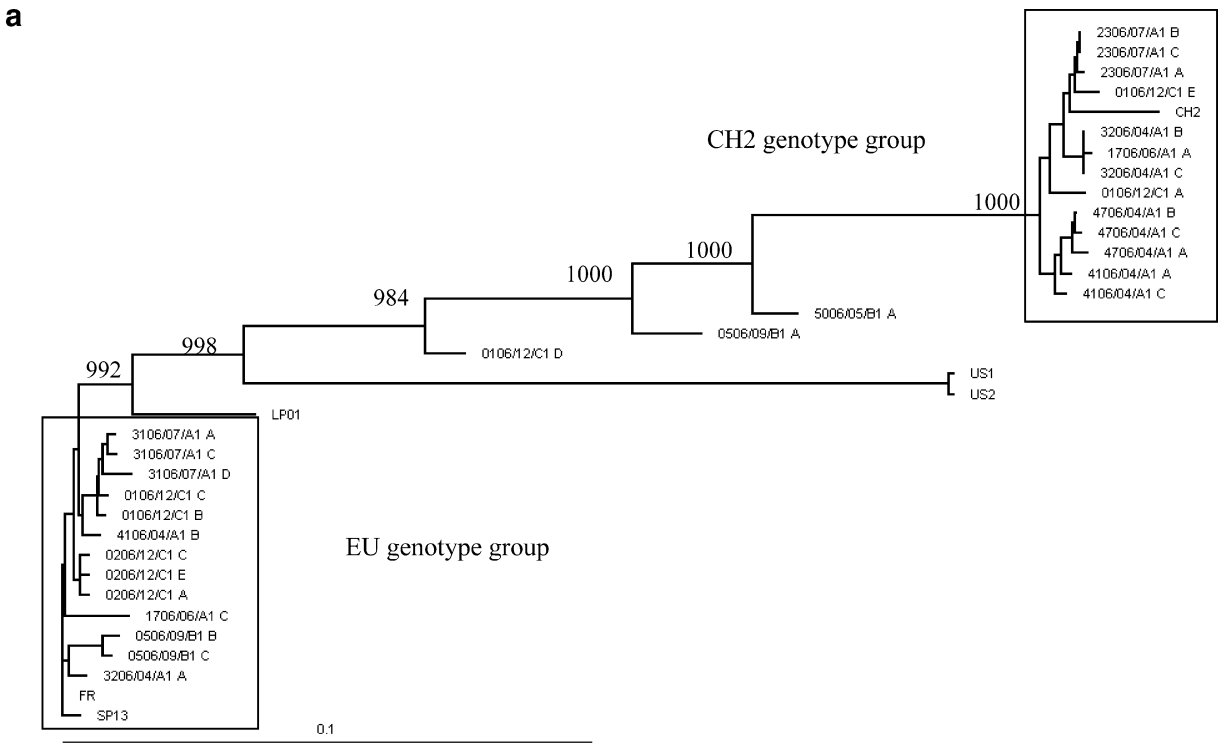
In addition, for 5 of the 10 selected greenhouses (01, 02, 23, 31 and 32) a 845 bp fragment of the CP gene was cloned and sequenced (four clones per sample). Sequence alignments of 20 sequences showed similar results for this part of the viral genome as for the RdRp gene (data not shown). Again, two distinct PepMV genotype groups were formed, and as for the RdRp gene fragment, both groups shared only 78% sequence homology. For the CP gene, no sequences were identified that clustered in between the two different groups.

Evidence for recombination between the European and Chilean PepMV genotypes

A detailed study was performed of the RdRp sequences obtained from the samples of three greenhouses (01, 05 and 32) that displayed a mixed infection with both the EU and CH2 genotypes. For each greenhouse, two samples taken between April and June 2006 were analysed. For two greenhouses (05 and 32), samples from the 2005 growing season were also included. For each sample, RdRp fragments were cloned and 8 to 10 colonies were sequenced. Multiple sequence alignments and subsequent phylogenetic analyses were performed on a dataset of in total 70 RdRp sequences including those of the six reference PepMV isolates (Fig. 2c). Also in this case the majority of sequences fell within two clusters that represent the EU and the CH2 genotypes. In addition, again a considerable subset of sequences fell in between both clusters, with the different sequences in a gradual transition from one cluster to the other (Fig. 2c).

Detailed sequence analysis of the RdRp fragments showed all sequences that landed in between both clusters were partially identical to the CH2 genotype and partially identical to the EU genotype. This is exemplified by Fig. 3a showing a sequence alignment of a small subset of sequences. In general, the transition site between the two sequences differed from sequence to sequence, even for sequences derived from a single sample. Nevertheless, some sequences with identical recombination sites were also identified. For example, the recombinant RdRp sequences 3206/11/B1_I and 3206/13/A1_G obtained from samples taken at different time points in the

Fig. 2 Phylogenetic analysis of PepMV sequences. Neighbour-joining tree of 29 RdRp-sequences from PepMV-infected tomato samples collected in 10 commercial greenhouses (**a**), of 44 RdRp-sequences obtained from seven different samples from greenhouse 32, four of which originated from the 2005 growing season and three from the 2006 growing season (**b**), and subsequent analysis of 64 RdRp sequences from PepMV-infected tomato samples collected in three of those greenhouses (**c**). Trees are generated using Clustal X with 1,000 bootstrap replicates and visualized using Treeview. Bootstrap values >90% are shown at the major nodes. Reference sequences from each genotype, encompassing sequences from the PepMV isolates Fr, SP13, US1, US2, LP01, and CH2 (GenBank Accession numbers AJ438767, AF484251, AY509926, AY509927, AJ606361, and DQ000985, respectively) were included for comparison. Sequence identifiers encode 'the greenhouse code _ year of sampling/ serial number/ rating block/sample number/clone'. The scale bar represents 0.1 changes per nucleotide. Samples denoted with 'Inoc.' originate from the 2005 growing season and were used to inoculate plants in 2006


a
CH2 genotype group
2306/07/A1 B
2306/07/A1 C
2306/07/A1 A
0106/12/C1 E
CH2
3206/04/A1 B
1706/06/A1 A
3206/04/A1 C
0106/12/C1 A
4706/04/A1 B
4706/04/A1 C
4706/04/A1 A
4106/04/A1 A
4106/04/A1 C
1000
1000
1000
984
998
992
5006/05/B1 A
0506/09/B1 A
0106/12/C1 D
US1
US2
LP01
3106/07/A1 A
3106/07/A1 C
3106/07/A1 D
0106/12/C1 C
0106/12/C1 B
4106/04/A1 B
0206/12/C1 C
0206/12/C1 E
0206/12/C1 A
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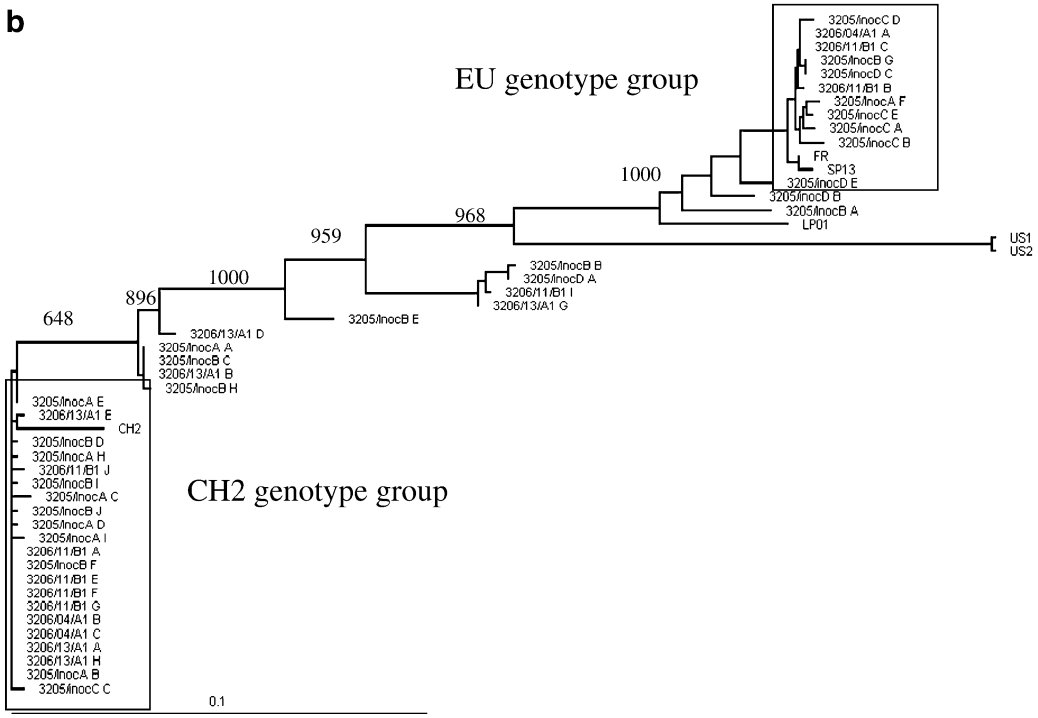
EU genotype group
0.1


b
EU genotype group
3205/InocC D
3206/04/A1 A
3206/11/B1 C
3205/InocB G
3205/InocD C
3206/11/B1 B
3205/InocA F
3205/InocC E
3205/InocC A
3205/InocC B
FR
SP13
3205/InocD E
3205/InocD B
3205/InocB A
LP01
US1
US2
1000
968
959
1000
896
648
3205/InocB B
3205/InocD A
3206/11/B1 I
3206/13/A1 G
3205/InocB E
3206/13/A1 D
3205/InocA A
3205/InocB C
3206/13/A1 B
3205/InocB H
3205/InocA E
3206/13/A1 E
CH2
3205/InocB D
3205/InocA H
3206/11/B1 J
3205/InocB I
3205/InocA C
3205/InocB J
3205/InocA D
3205/InocA I
3206/11/B1 A
3205/InocB F
3206/11/B1 E
3206/11/B1 F
3206/11/B1 G
3206/04/A1 B
3206/04/A1 C
3206/13/A1 A
3206/13/A1 H
3205/InocA B
3205/InocC C
CH2 genotype group
0.1

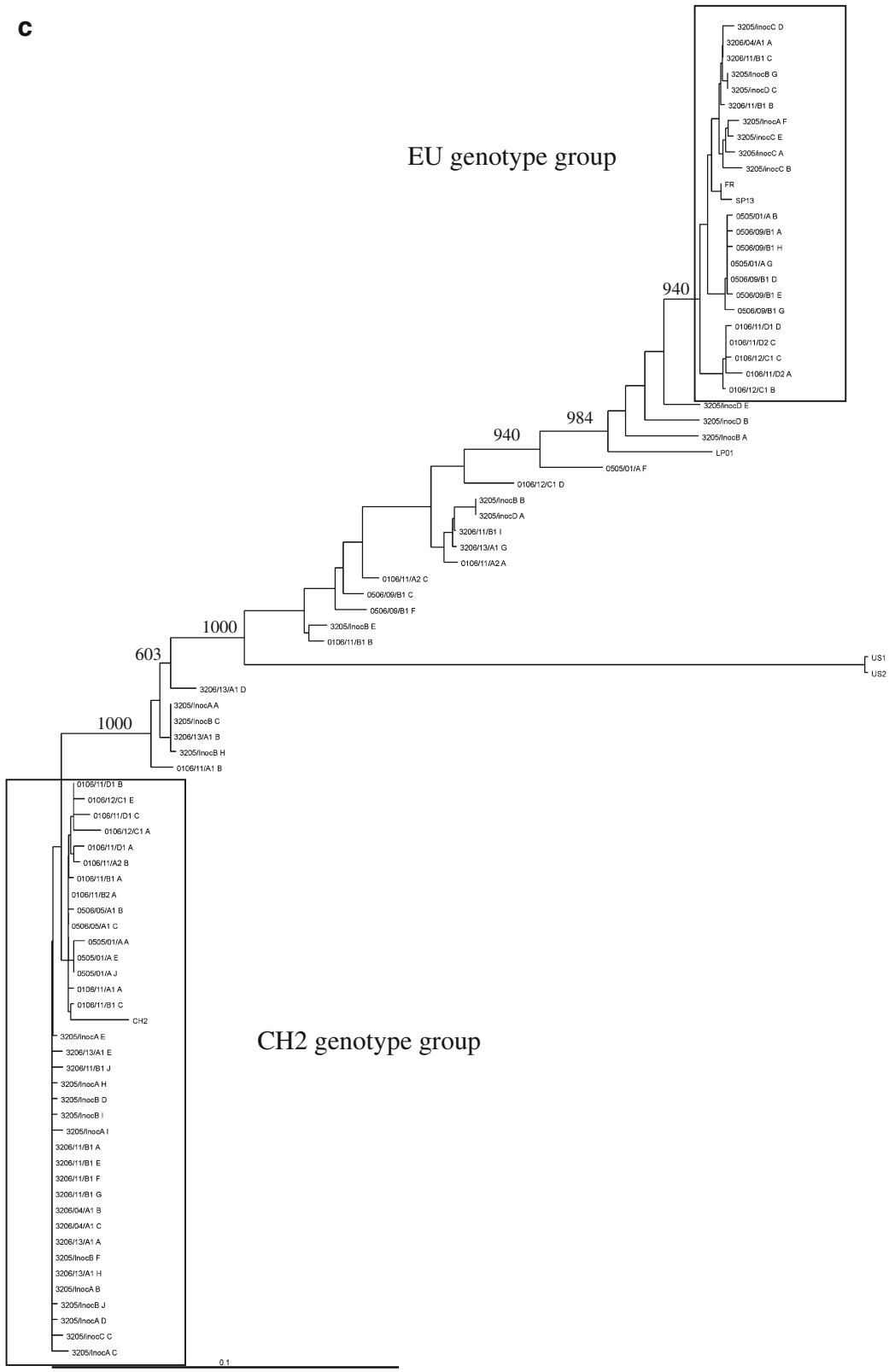


Fig. 2 (continued)

same greenhouse were 100% identical. When aligned with other sequences also obtained from this greenhouse, namely one belonging to the CH2 genotype (sequence 3206/13/A1_A) and one belonging to the EU genotype (sequence 3206/04/A1_A), both sequences shared 100% homology over the first 250 nt with sequence 3206/13/A1_A, while the subsequent 375 nt shared 100% homology with the EU sequence (Fig. 3a). The predicted translated sequences of the respective clones show that the original codons have been preserved and that all sequences encode amino acid sequences that are partially identical to the CH2 genotype and partially identical to the EU genotype (Fig. 3b).

To eliminate the possibility that the recombinant sequences were generated by artefacts during the RT-PCR, a RNA extract derived from PepMV infected plants from greenhouse 11 (infected with the EU genotype only) and one from plants from greenhouse 19 (infected with the CH2 genotype only) were mixed before RT-PCR analysis. Subsequently, the generated cDNA products were amplified, cloned and sequenced. In total 16 clones were sequenced, all of which were found to be derived from either the EU genotype or the CH2 genotype and no recombinant sequences were obtained.

Discussion

The data provided in this study show that two PepMV genotypes occur in Belgian tomato production greenhouses, the EU genotype and the CH2 genotype. Until now, the EU genotype was considered the most prevalent PepMV genotype in European tomato production greenhouses (Aguilar et al. 2002; Cotillon et al. 2002; Ling 2007). Remarkably, while the CH2 genotype has not previously been detected in European tomato production facilities, we found that this genotype was present in 85% of the surveyed greenhouses with PepMV infected tomato crops (Table 1). This raises the question of how this genotype was introduced and why it is so widespread. Since the CH2 genotype has previously been shown to occur on tomato seeds (Ling 2007) and since many plants were found to be already infected at an early stage of cultivation (Table 1), an infection at the nurseries that grow young plantlets for delivery to greenhouses was considered as a potential cause of the widespread occurrence of the CH2 genotype. However, PepMV presence in these nurseries could not be demonstrated by ELISA testing. Alternatively, the widespread occurrence of the CH2 genotype suggests that this genotype may have a biological advantage over the EU genotype. Our data suggest that the CH2 genotype spreads faster than the EU genotype in greenhouses with mixed infections, as an individual plant only infected with the CH2 genotype could be identified in greenhouse 32, while infection only with the EU genotype was not observed. A similar situation was found in a Dutch greenhouse, where one out of six analyzed plants was infected with the CH2 genotype only, while the other five showed mixed infections upon RT-PCR-RFLP analysis. In addition, a mixed infection inoculation experiment showed that 3 weeks after inoculation only the CH2 genotype could be detected, while only 2 months later the presence of the EU genotype could also be demonstrated (data not shown).

It should be noted that primers Pep3 and Pep4 used in this study were originally designed based on the EU genotype (Pagan et al. 2006). As a result, a bias may be introduced in our PCR results by using these primers, perverting the dynamic range of the detected targets. Nevertheless, using this primer set Pagan et al. (2006) were able to obtain US2-like sequences. In addition, our results show that these primers can perfectly amplify the CH2 genotype, since the CH2 genotype was identified in almost 85% of the samples while the EU genotype was found in <50% of the samples.

A remarkable observation made in this study was the occurrence of recombination between the EU and CH2 genotype in plants infected with isolates of both genotypes. Identical recombinants were detected at different time points in different plants, suggesting that the recombinant genotype was sufficiently viable to be transmitted from one plant to another. The viability of the recombinants is further supported by the predicted translated sequences of the recombinant clones that in all cases represented perfect hybrid sequences between the EU and CH2 genotype (Fig. 3b). Recombination is known to play an important role in the evolution of RNA viruses (Nagy and Simon 1997; Garcia-Arenal et al. 2001; Moya et al. 2004). Nevertheless, information on recombination and its frequency in the absence of selection pressure is scarce, and to our knowledge recombina-

```
EU_3206/04/A1_A          ATGAGGTTGTCTGGTGAAGGTCCAACTTTTGATGCCAACACAGAATGTTCAATAGCATAT 60
3205/inocD_A             ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
3206/11/B1_I             ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
3206/13/A1_G             ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
3205/InocB_E             ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
CH_3206/13/A1_A          ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
                         ********************** ** ***** ** ***** ** ***** ** *****

EU_3206/04/A1_A          ACTGCTACAAGATATCATCTTGATTCTACAGTCAAGCAGGTTTATGCTGGAGATGATATG 120
3205/inocD_A             ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
3206/11/B1_I             ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
3206/13/A1_G             ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
3205/InocB_E             ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
CH_3206/13/A1_A          ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
                         ***** *******  *** ****   ***  * ***** ** ***** ** ** ** ***

EU_3206/04/A1_A          GCATTAGATGGAGTTGTCCAAGAAAAACCCTCTTTCAAAAAACTACAGAACAAGCTTAAA 180
3205/inocD_A             GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
3206/11/B1_I             GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
3206/13/A1_G             GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
3205/InocB_E             GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
CH_3206/13/A1_A          GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
                         ******************    *****    ** ****  **  **** **    ** ***

EU_3206/04/A1_A          CTCACCTCAAAGACACTATTTCCAAAACAGGTTAAAGGTGATTATGCTGAATTCTGTGGT 240
3205/inocD_A             CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGTGATTATGCTGAATTCTGTGGT 240
3206/11/B1_I             CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGGGATTACGCTGAATTTTGTGGT 240
3206/13/A1_G             CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGGGATTACGCTGAATTTTGTGGT 240
3205/InocB_E             CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGGGATTACGCTGAATTTTGTGGT 240
CH_3206/13/A1_A          CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGGGATTACGCTGAATTTTGTGGT 240
                         ***** ***** ** ** *  ****************** ***** ******** ******

EU_3206/04/A1_A          TGGACTTTCACTCCTGGTGGTATCATTAAAAACCCTTTGAAAATGCATGCTTCCATTATG 300
3205/inocD_A             TGGACTTTCACTCCTGGTGGTATCATTAAAAACCCTTTGAAAATGCATGCTTCCATTATG 300
3206/11/B1_I             TGGACTTTCACTCCTGGTGGTATCATTAAAAACCCTTTGAAAATGCATGCTTCCATTATG 300
3206/13/A1_G             TGGACTTTCACTCCTGGAGGTATCATTAAAAACCCTTTGAAAATGCATGCTTCCATTATG 300
3205/InocB_E             TGGACTTTCACACCAGGGGGTATAATTAAAAATCCACTTAAAATGCATGCCTCAATTATG 300
CH_3206/13/A1_A          TGGACTTTCACACCAGGGGGTATAATTAAAAATCCACTTAAAATGCATGCCTCAATTATG 300
                         *********** ** ** ***** ******** **  * *********** ** ******

EU_3206/04/A1_A          TTGCAAGAGGCAATCGGCAATTTACACACTGCTGCCAGATCATATGCCATTGACATGAAG 360
3205/inocD_A             TTGCAAGAGGCAATCGGCAATTTACACACTGCTGCCAGATCATATGCCATTGACATGAAG 360
3206/11/B1_I             TTGCAAGAGGCAATCGGCAATTTACACACTGCTGCCAGATCATATGCCATTGACATGAAG 360
3206/13/A1_G             TTGCAAGAGGCAATCGGCAATTTACACACTGCTGCCAGATCATATGCCATTGACATGAAG 360
3205/InocB_E             CTGCAAGAAGCCATTGGCAATCTGCACACAGCAGCCAGATCTTATGCAATTGACATGAAG 360
CH_3206/13/A1_A          CTGCAAGAAGCCATTGGCAATCTGCACACAGCAGCCAGATCTTATGCAATTGACATGAAG 360
                          ******* ** ** ****** * ***** ** ******** ***** *************

EU_3206/04/A1_A          CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
3205/inocD_A             CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
3206/11/B1_I             CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
```

◀ **Fig. 3** Alignment of recombinant nucleotide sequences (**a**) nt 1 to 510: part of RNA dependent RNA polymerase gene, nt 510 to 536: untranscribed region, nt 536 to 625: part of coat protein gene) and corresponding amino acid sequences (**b**) originating from different samples from one greenhouse (greenhouse 32), with a sequence belonging to the CH2 genotype (CH3206/13/A1_1) and a sequence belonging to the EU genotype (EU3206/04/A1_A). **a** Shows identical transition sites for 3206/11/B1_I and 3206/13/A1_G (nt 251) and different transition sites for 3205/inocD_A (nt 218) and 3205/InocB_E (nt 406). **b** Shows that recombination occurs in such way that codons stay intact and translation results in amino acid sequences that are partly identical to one of the parental genotypes

tion events within complete viral RNAs have not yet been described for potexviruses. Recombination most frequently occurs through the so-called copy-choice model, in which the viral RdRp enzyme switches templates during replication. Since each nucleotide may serve as a target for switching, recombination may occur randomly (Lai 1992; Shapka and Nagy 2004). However, some regions, so-called recombination hot spots, appear to display a higher recombination frequency which is generally explained by specific secondary structures such as stem loops

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3206/13/A1_G          CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
3205/InocB_E          CATTCATACCAAATGGGTGACCAACTGCATGACTACTTAACACCAGATGAAGCTGAACAA 420
CH_3206/13/A1_A       CATTCATACCAAATGGGTGACCAACTGCATGACTACCTAACCCCTGATGAAGCTGAACAA 420
                      ********************  * *****  * *** **** ** ***************

EU_3206/04/A1_A       CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
3205/inocD_A          CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
3206/11/B1_I          CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
3206/13/A1_G          CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
3205/InocB_E          CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
CH_3206/13/A1_A       CATTTCCTAGCTGTGAGAAAGCTTCACAAACTCCATCAAGGCGAGGCCATGCGTCTTGGT 480
                      ** ***** *****  * *** * *****  * ** ***** ** ** *** * ******

EU_3206/04/A1_A       GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGGTTAAGTTTTCCCCAGTTCGAAATGGA 540
3205/inocD_A          GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGATTAAGTTTTCCCCAGTTCGAAATGGA 540
3206/11/B1_I          GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGGTTAAGTTTTCCCCAGTTCGAAATGGA 540
3206/13/A1_G          GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGGTTAAGTTTTCCCCAGTTCGAAATGGA 540
3205/InocB_E          GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGATTAAGTTTTCCCCAGCTCGAAATGGA 540
CH_3206/13/A1_A       GAGAAAAGTCCACCAAGATCAACCCATTAAGGGGTTAAGTTTTCCCCAGTTTGAAATGGA 540
                      ** ** ** ** **** * **** **** **** *************** * ********

EU_3206/04/A1_A       AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
3205/inocD_A          AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
3206/11/B1_I          AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
3206/13/A1_G          AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
3205/InocB_E          AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
CH_3206/13/A1_A       AAGATCAACTTTGATCAATTTACTTCTGTTACACAAATTTGAACACAAGATTAACACTGA 600
                      ** ******* **** **********  ** *** * ** ** * **  **  * *     **

EU_3206/04/A1_A       AGGAATCATAGTTGTGCACGGAATT 625
3205/inocD_A          AGGAATCATAGTTGTGCACGGAATT 625
3206/11/B1_I          AGGAATCATAGTTGTGCACGGAATT 625
3206/13/A1_G          AGGAATCATAGTTGTGCACGGAATT 625
3205/InocB_E          AGGAATCATAGTTGTGCACGGAATT 625
CH_3206/13/A1_A       AGGAATCATAGTTGTGCACGGAATT 625
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Fig. 3 (continued)

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EU_3206/04/A1_A      MRLSGEGPTFDANTECSIAYTATRYHLDSTVKQVYAGDDMALDGVVQEKPSFKKLQNKLK 60
3205/inocD_A         MRLSGEGPTFDANTECSIAYTATRFHIDNTIKQVYAGDDMALDGVVSEKKSFRKLQNLLK 60
3206/11/B1_I         MRLSGEGPTFDANTECSIAYTATRFHIDNTIKQVYAGDDMALDGVVSEKKSFRKLQNLLK 60
3206/13/A1_G         MRLSGEGPTFDANTECSIAYTATRFHIDNTIKQVYAGDDMALDGVVSEKKSFRKLQNLLK 60
3205/InocB_E         MRLSGEGPTFDANTECSIAYTATRFHIDNTIKQVYAGDDMALDGVVSEKKSFRKLQNLLK 60
CH_3206/13/A1_A      MRLSGEGPTFDANTECSIAYTATRFHIDNTIKQVYAGDDMALDGVVSEKKSFRKLQNLLK 60
                     ************************:*:*.*:***************.** **:**** **

EU_3206/04/A1_A      LTSKTLFPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
3205/inocD_A         LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
3206/11/B1_I         LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
3206/13/A1_G         LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
3205/InocB_E         LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
CH_3206/13/A1_A      LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
                     ******:**************************************************

EU_3206/04/A1_A      HSYQMGDELHNYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPKATHMEKSTLINLLQ 180
3205/inocD_A         HSYQMGDELHNYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPKATHMEKSTLINLLQ 180
3206/11/B1_I         HSYQMGDELHNYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPKATHMEKSTLINLLQ 180
3206/13/A1_G         HSYQMGDELHNYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPKATHMEKSTLINLLQ 180
3205/InocB_E         HSYQMGDQLHDYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPKATHMEKSTLINLLQ 180
CH_3206/13/A1_A      HSYQMGDQLHDYLTPDEAEQHFLAVRKLHKLHQGEAMRLGEKSPPRSTHMERSTLINLLL 180
                     *******:**:***********************************::****:*******

EU_3206/04/A1_A      LHHFEPKLSVEGIIVVHGI 199
3205/inocD_A         LHHFEPKLSVEGIIVVHGI 199
3206/11/B1_I         LHHFEPKLSVEGIIVVHGI 199
3206/13/A1_G         LHHFEPKLSVEGIIVVHGI 199
3205/InocB_E         LHHFEPKLSVEGIIVVHGI 199
CH_3206/13/A1_A      LHKFEHKINTEGIIVVHGI 199
                     **:** *:..*********
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Fig. 3 (continued)

(Olsthoorn et al. 2002). The occurrence of such recombination hot spots could not be demonstrated based on our data, as multiple recombination regions were identified for the sequences obtained in our study. However, sequences of all recombinants identified in this study were unidirectional as the 5′ end was consistently composed of a sequence of the CH2 genotype while the 3′ end contained a sequence of the EU genotype.

Significantly increased symptom severity was observed in tomato production greenhouses with a mixed infection when compared to greenhouses where plants were infected with only a single genotype (Fig. 1). In addition, when a second PepMV-genotype invaded a PepMV-infected tomato crop, more severe symptoms were observed. It is currently not known whether the increased symptom severity is due to synergistic activities between the different PepMV genotypes or whether recombinant genotypes account for more severe symptoms.

Obviously, scoring of symptoms can be subjective due to a lack of sufficient standardization. Therefore, all observations were performed only by two horticultural experts that have carried out scoring of tomato varieties and disease symptoms for many years. Scoring criteria were tweaked regularly in order to minimize bias. Eventually, a large dataset of over 900 and 2,000 scores for vegetative tissues and fruits, respectively, was analyzed. However, it should be stressed that our analysis concerns observations in different greenhouses with different cultivation practices. Controlled inoculation experiments using purified single or mixed virus isolates are required to substantiate our findings and clarify the role of different genotypes and mixed infections in symptom severity.

Importantly, however, our results show that plants infected with the EU PepMV genotype do not express cross-protection towards the CH2 genotype or vice versa, suggesting that the immunization carried out by some tomato growers in an attempt to protect their crops is not effective and may increase, rather than minimize, PepMV damage.

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