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Cross-protection or enhanced symptom display in greenhouse tomato co-infected with different *Pepino mosaic virus* isolates

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The potential of three mild *Pepino mosaic virus* (PepMV) isolates, belonging to the CH2, EU and LP genotypes, to protect a tomato (*Solanum lycopersicum*) crop against an aggressive challenge isolate of the CH2 genotype was assessed in greenhouse trials and PepMV symptoms were rated at regular time points. After challenge infection, enhanced symptom display was recorded in plants that were pre-inoculated with a protector isolate belonging to a different genotype (EU, LP) from the challenge isolate. A quantitative genotype-specific TaqMan assay revealed that in these plants, the accumulation of the challenge isolate only temporarily slowed down. By contrast, efficient cross-protection was obtained using the mild isolate of the CH2 genotype, and in this case the challenge isolate was barely detectable in the pre-inoculated plants. These results suggest that the interaction between PepMV isolates largely depends on RNA sequence homology and that post-transcriptional gene silencing plays an important role in cross-protection.

Keywords: *Solanum lycopersicum*, symptomatology

Introduction

Pepino mosaic virus (PepMV), a highly infectious *Potexvirus* that was first isolated from pepino (*Solanum muricatum*) in Peru in 1974 (Jones *et al.*, 1980), is a major viral disease in greenhouse tomato (*Solanum lycopersicum*) production worldwide (van der Vlugt *et al.*, 2000; French *et al.*, 2001; Mumford & Metcalfe, 2001; Cotillon *et al.*, 2002; Maroon-Lango *et al.*, 2005; Pagan *et al.*, 2006; Hanssen *et al.*, 2008; Hasiów *et al.*, 2008; Ling, 2008). The virus causes a wide range of symptoms, of which the typical fruit marbling is considered to be the most devastating, as it reduces the economic value of the fruit (Soler *et al.*, 2000; Roggero *et al.*, 2001; Spence *et al.*, 2006). Recently reported open fruits and sepal necrosis similarly contribute to fruit quality devaluation (Hanssen *et al.*, 2009a). Damage and economic losses caused by PepMV vary greatly, not only between different production areas, but also between different infected crops in the same area. This variation can, at least partially, be attributed to differential symptomatology caused by different PepMV isolates (Hanssen *et al.*, 2009a).

Currently, four PepMV genotypes are distinguished: the Peruvian PepMV genotype (LP), which was first isolated from *Lycopersicon peruvianum* and is similar to the original pepino (*S. muricatum*) isolate (López *et al.*, 2005; Pagan *et al.*, 2006); the European tomato genotype (EU), which was first reported in greenhouse tomato production in Europe (Mumford & Metcalfe, 2001; Aguilar *et al.*, 2002; Cotillon *et al.*, 2002; Verhoeven *et al.*, 2003; Pagan *et al.*, 2006); the CH2 genotype, which was first isolated from tomato seeds from Chile (Ling, 2007), and the US1 genotype, which was first described in the USA (Maroon-Lango *et al.*, 2005). The different genotypes cannot be distinguished based on biological characteristics, as biological differences between isolates from the same genotype can be considerable (Córdoba-Sellés *et al.*, 2008; Hanssen *et al.*, 2009a). The EU and LP genotypes share a nucleotide sequence homology of 95% and cluster phylogenetically. The CH2 genotype is rather different as it displays only 78–80% sequence homology with the EU and LP genotype groups. The US1 genotype shares 78% sequence homology with CH2 and 82% with EU/LP genotypes. An RT-PCR-RFLP-based method was developed to distinguish the different genotypes without

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Plant Pathology

RNA sequence determination (Hanssen *et al*., 2008). In recent years, the CH2 genotype has largely replaced the EU genotype in commercial tomato production in several European countries, whereas in the USA and Canada the EU genotype is predominant (French *et al*., 2008; Gómez *et al*., 2008; Hanssen *et al*., 2008 and unpublished; Ling, 2008).

Viral cross-protection was first described by McKinney (1929), who observed that tobacco plants that were systemically infected by a mild strain of *Tobacco mosaic virus* (TMV) were not affected by subsequent infection by a severe strain of TMV, which otherwise induced yellow mosaic symptoms. Since then, cross-protection has been applied both in research, to study relationships between viruses, and in commercial crop cultivation, to control viral diseases (Lecoq & Lemaire, 1991). The most successful examples of cross-protection in vegetable production are the control of TMV conferred by the mild MII-16 protector isolate, which was used in greenhouse tomato production worldwide until resistant plant varieties became available, and control of *Zucchini yellow mosaic virus* (ZYMV) in field squash production by the mild WK protector isolate of ZYMV (Rast, 1972; Lecoq & Lemaie, 1991; Lecoq, 1998). In perennial crops efficient cross-protection was obtained in papaya fields for control of *Papaya ringspot virus* (PRSV) and in citrus orchards against *Citrus tristeza virus* (CTV) (Muller, 1980; Yeh & Gonsalves, 1984). Cross-protection is of particular interest to control a narrow-host-range virus that is characterized by high incidence and substantial crop damage in a geographic region where it constitutes the major viral disease of the crop (Lecoq, 1998; Gal-On & Shiboleth, 2006). Since PepMV is the most prevalent virus in tomato production in north-western Europe (Belgium, the Netherlands and the UK), and taking into account the lack of alternative control strategies, cross-protection is potentially suitable to control PepMV. It has been speculated that early PepMV infections are less damaging than infections that occur later in the growing season and that economic losses may be reduced by inoculation of tomato plants with a mild isolate at an early stage (Spence *et al*., 2006; Hanssen *et al*., 2008). Isolates belonging to the LP genotype have been reported to cause only mild symptoms in tomato (López *et al*., 2005), indicating that isolates from this genotype could be interesting candidates for cross-protection. A mild LP isolate of PepMV is currently used in greenhouse tomato cultivation in the Netherlands for cross-protection (Brakeboer, 2007), but no efficiency data are available yet. In contrast, Belgian tomato growers have largely abandoned the PepMV cross-protection strategy after negative experiences with early inoculation during the 2005 growing season (Hanssen *et al*., 2009b). Greenhouse experiments showed enhanced symptom severity in tomato crops that were simultaneously infected by two different PepMV genotypes compared with crops infected by just one of the genotypes (Hanssen *et al*., 2008). These observations have raised questions on the interaction of different PepMV isolates and genotypes in mixed infections, as well as on risks associated with cross-protection.

The aims of this study were to provide a better understanding of the interaction between PepMV isolates. The cross-protection potential of a mild LP isolate to protect tomato against an aggressive PepMV isolate from the CH2 genotype, which is dominant in Europe, was evaluated in greenhouse trials. In addition, the cross-protection potentials of a mild EU isolate and a mild CH2 isolate against the aggressive CH2 isolate were evaluated.

Materials and methods

Experimental design

A greenhouse trial was conducted in four plastic tunnels to assess the potential of a mild PepMV isolate 5608, belonging to the LP genotype and further referred to as LP-mild (protector isolate) to protect tomato plants against the more aggressive CH2 isolate PCH 06/104 (challenge isolate), hereafter referred to as CH2-aggressive (GenBank Accession No. FJ457097; Hanssen *et al*., 2009a). Here, a PepMV isolate is defined as the viral inoculum derived from PepMV-infected plants from one specific tomato production site. The genotype of both isolates was determined using a previously described RT-PCR-RFLP method (Hanssen *et al*., 2008). In addition, isolate pureness was confirmed using a PepMV genotype-specific TaqMan RT-qPCR detection method (Gutiérrez-Aguirre *et al*., in press). Tomato seeds (cv. Tricia, De Ruiter Seeds) were disinfected, sown and germinated as previously described (Hanssen *et al*., 2009a). Five weeks after sowing, 100 tomato plants were transplanted to each of the tunnels in stonewool substrate. One week after planting, the absence of PepMV from all tunnels was demonstrated by ELISA analyses, and subsequently tomato plants of the first and second tunnels were inoculated individually with the LP mild isolate as previously described, while plants in the third and fourth tunnels were mock-inoculated with phosphate buffer (Hanssen *et al*., 2009a). Inoculations were performed on the third-youngest leaf of each plant. Three weeks later, after confirmation of systemic infection by ELISA analyses, all plants in the second tunnel were inoculated again, but this time with the CH2-aggressive challenge isolate. At the same time, plants in the third tunnel were inoculated (for the first time) with CH2-aggressive. This time point was defined as 0 weeks post-inoculation (WPI) and the period before this time point is referred to as weeks ante-inoculation (WAI). Plants in the fourth tunnel were kept as non-infected controls. The plants that were first inoculated with LP-mild and subsequently with CH2-aggressive are further referred to as the pre-inoculated plants. Plants that were inoculated only with LP-mild are further referred to as the LP-mild reference plants and those inoculated only with the challenge isolate as the CH2-aggressive reference plants. Each tunnel was

divided into 10 sampling blocks, each containing 10 adjacent plants. At regular intervals, samples were taken from the youngest leaves in the heads of the plants, as previously described (Hanssen *et al*., 2009a).

PepMV detection and relative quantification of viral titres

To confirm PepMV presence in inoculated plants and absence in non-infected plants, all plant samples were analysed for PepMV presence with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using a commercial antiserum (Prime Diagnostics), according to the supplier's instructions. The optical density (OD) was measured at 405 nm and samples were rated positive if the OD exceeded the mean value of two negative control wells by three times.

To relatively quantify the viral titres, a genotype-specific TaqMan RT-qPCR method was applied. For each tunnel, three samples (sampling blocks 1, 4 and 8) were analysed at eight time points (1 WAI and 1, 3, 5, 7, 9, 11 and 14 WPI). RNA was extracted from fresh plant material using the RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed using the high-capacity cDNA archive kit (Applied Biosystems). qPCR reactions were performed in 10- μL final reaction volumes, including TaqMan universal PCR master mix (Applied Biosystems), forward and reverse primers at 900 nM each, 200 nM TaqMan probe and 2 μL cDNA. Primers and TaqMan probes specific for EU/LP or CH2/US2 were used for specific quantification of PepMV genotypes (Table 1; Gutiérrez-Aguirre *et al*., in press). Plant cytochrome oxidase (COX)-specific primers and a Taqman probe (Weller *et al*., 2000) were used as internal controls to account for variations resulting from the RNA extraction. The qPCR was performed in 384-well plates (Applied Biosystems). Reactions were run in triplicate on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) using universal cycling conditions (2 min at 50°C, 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C). The threshold cycle (Ct) for each individual amplification was obtained using SDS 2·3 software (Applied Biosystems). Buffer-extraction controls were used to evaluate potential contamination within the RNA-extraction procedure. Non-template controls were used to monitor for potential contamination within the qPCR reagents. Based on previous observations, a Ct of 35 was considered the highest detectable Ct (Gutiérrez-Aguirre *et al*., in press).

Evaluation of PepMV symptoms

Symptoms were scored following a previously described assessment schedule (Hanssen *et al*., 2009a; this manuscript also contains representative pictures of typical symptoms) with minor modifications; fruit marbling and flaming were not assessed on the plants, but by examining all ripe tomatoes after harvest. All harvested tomatoes were weighed to determine the yield per tunnel. Significant ($P < 0{\cdot}05$) differences in symptom scores were identified by analysis of variance (one-way ANOVA) and post-hoc Bonferroni tests using SPSS software (v. 10·0; SPSS Inc.).

Results

A PepMV cross-protection trial was conducted in four plastic tunnels, each containing 100 tomato plants. To this end, tomato plants were inoculated with a PepMV isolate known to cause few or no symptoms, subsequently challenged with an aggressive isolate known to cause significant damage, and symptom display recorded. As the LP genotype of PepMV was previously reported to cause symptomless infections in tomato (Jones *et al*., 1980; López *et al*., 2005), an LP isolate (LP-mild) obtained in 2008 from a PepMV-infected symptomless commercial tomato crop in Belgium was selected as a potential protector isolate. Tomato plants in one tunnel were first inoculated with LP-mild and, after systemic spread, a second inoculation with an aggressive challenge isolate belonging to the CH2 genotype was performed. As a reference, both isolates (LP-mild and CH2-aggressive) were inoculated separately in two distinct tunnels. Plants in the fourth tunnel were kept as non-infected controls.

ELISA analyses confirmed: (i) that all plants inoculated with the LP mild isolate (tunnels 1 and 2) were systemically infected prior to inoculation with the CH2 aggressive isolate, (ii) that plants inoculated solely with the CH2 aggressive isolate (tunnel 3) were systemically infected from 1 WPI onwards, and (iii) that non-inoculated control plants remained free of PepMV (data not shown) . RT-PCR-RFLP analyses (Hanssen *et al*., 2009a)

Table 1 *Pepino mosaic virus* genotype-specific primers and Taqman probes used in this study (Gutiérrez-Aguirre *et al*., in press)

Primer/Probe	Sequence	Position in genome
Forward primer EU/LP	5′-TGGAACATACTTCTCGACAGCAA-3′	6035–6057[a]
Reverse primer EU/LP	5′-TCCATCGAAGAAGTCAAATGCA-3′	6112–6133[a]
Probe EU/LP	5′-FAM-ATTCCACCAGCAAATTGGGCCAAACTT-TAMRA-3′	6059–6085[a]
Forward primer CH2	5′-TGGGTTTAGCAGCCAATGAGA-3′	5832–5852[b]
Reverse primer CH2	5′-AACTTTGCACATCAGCATAAGCA-3′′	5881–5903[b]
Probe CH2	5′-FAM-CGGACCTGCCATGTGGGACCTC-TAMRA-3′	5854–5875[b]

[a]SP13 reference sequence (GenBank Accession No. AF484251).
[b]CH2 reference sequence (GenBank Accession No. DQ000985).

at 4 and 13 WPI confirmed: (i) that plants were infected by the inoculated genotypes only, (ii) that plants inoculated with two different genotypes were systemically infected by both genotypes, and (iii) that no cross-contamination occurred between treatments throughout the trial period (data not shown).

Enhanced symptom severity and yield loss in pre-inoculated plants

Large differences in fruit symptom severity were obtained for the different treatments. A high incidence of sepal necrosis was observed between 2 and 11 WPI on all plants inoculated with CH2-aggressive, regardless of pre-inoculation with LP-mild (Fig. 1a). In the LP-mild reference and control plants, sepal necrosis did not occur. Differences between LP-mild pre-inoculated plants and LP-mild reference plants were significant ($P < 0{\cdot}05$) at 3, 4 and 7 WPI. The incidence of open fruits was rather low in this trial. The percentage of tomato clusters with at least one open fruit was 0·51% in the control plants, 0·45% in the LP-mild reference plants, 1·60% in the pre-inoculated plants and 4·32% in the CH2-aggressive reference plants. The percentages of marbled and flamed fruits were determined by examining all ripe tomatoes at harvest. Interestingly, throughout the entire trial period, the highest percentages of marbled tomatoes were obtained from the plants that were first inoculated with LP-mild and subsequently with CH2-aggressive (Fig. 2a). At 5 WPI, the percentage of marbled tomatoes obtained from these pre-inoculated plants was as high as 43%, whilst only 18% marbled tomatoes were harvested from the CH2 aggressive reference plants. Similarly, 18 and 23% marbled tomatoes were harvested from these plants at 7 and 13 WPI respectively, whilst at the same time points only 2·5 and 2·0% marbled tomatoes were harvested from the CH2-aggressive reference plants. No or only a few marbled tomatoes were seen in the control and LP-mild reference plants. Overall percentages of marbled tomatoes harvested at the five time points were 0·1% for the control plants, 1·3% for the LP-mild reference plants, 4·2% for the CH2-aggressive reference plants and 17·9% for the LP-mild pre-inoculated plants.

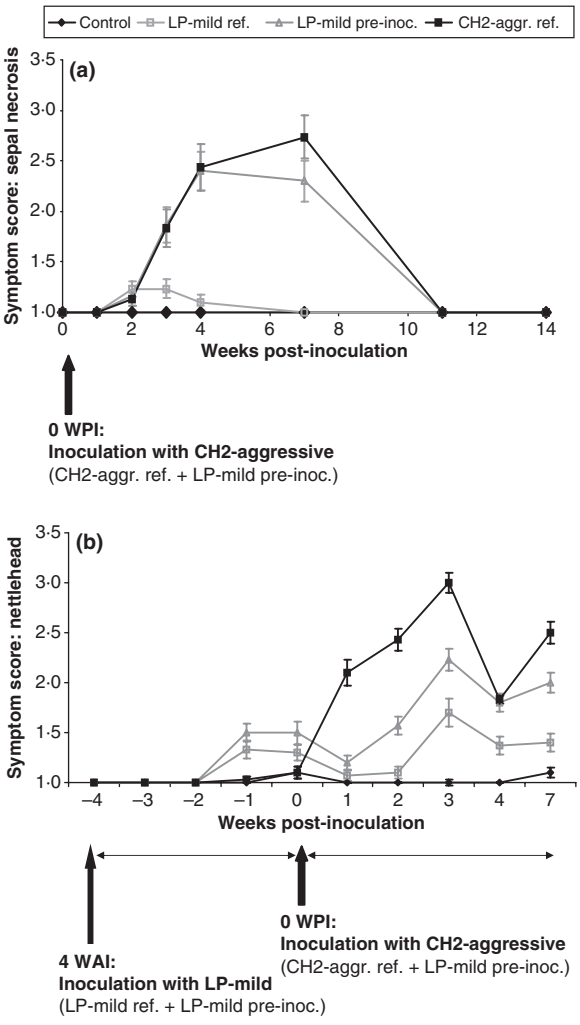

Figure 1 Symptom scores in LP-mild *Pepino mosaic virus* (PepMV) pre-inoculation trial. Each point represents the mean score of 30 tomato plants with standard errors at weeks ante-inoculation (WAI) and post-inoculation (WPI). (a) Sepal necrosis on fruits. No sepal necrosis was observed before challenge inoculation at 0 WPI as fruit development had not yet started. (b) Nettlehead symptoms (scores given until 7 WPI, when the plants were topped).

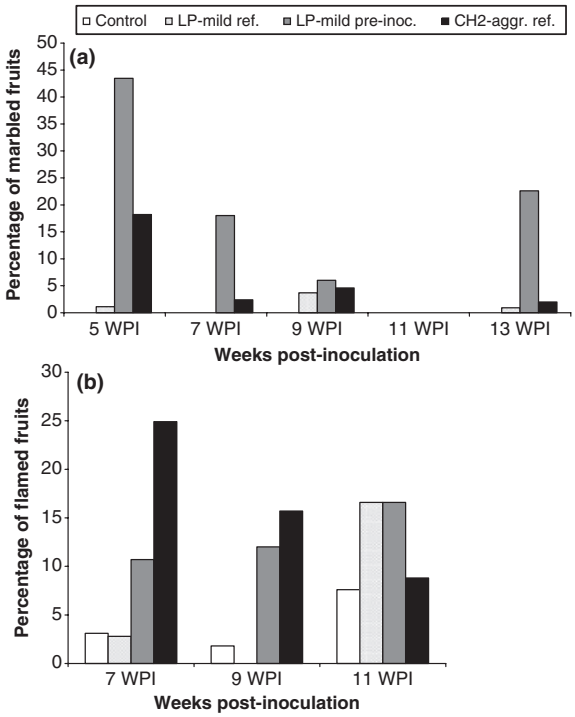

Figure 2 LP-mild pre-inoculation trial: symptoms on tomato fruits of plants inoculated with various *Pepino mosaic virus* isolates. Percentage of fruits showing (a) marbling and (b) flaming at different weeks post-inoculation. The mean number of fruits harvested per week and per tunnel was 292, with the highest number of fruits harvested at 5 WPI.

With regard to fruit flaming, symptoms were prevalent in both the LP-mild pre-inoculated plants and the CH2-aggressive reference plants (Fig. 2b). At 7 WPI, less flamed tomatoes were harvested from pre-inoculated plants (11%) than from CH2-aggressive reference plants (25%), whilst at 9 WPI the difference was less pronounced (12 and 16%, respectively). By 11 WPI, more flamed tomatoes were harvested from the pre-inoculated (17%) and LP-mild reference (17%) plants than the CH2-aggressive reference plants (9%). Overall, percentages of flamed tomatoes harvested at the three time points were 3·8% for the control plants, 5·0% for the LP-mild reference plants, 16·5% for the CH2-aggressive reference plants and 12·6% for the LP–mild pre-inoculated plants.

All ripe tomatoes, including those displaying typical PepMV symptoms, were harvested and weighed weekly to determine the yield per tunnel. The cumulative weight relative to the non-infected control was determined for all harvesting points from 5 to 15 WPI (Fig. 3). Interestingly, yields of the LP-mild pre-inoculated plants were consistently lower than those of controls over the entire harvesting period, and were generally also lower than those of the CH2-aggressive reference plants (Fig. 3). The overall yield loss from these plants amounted to 13%. A minor yield loss (3%) was recorded in plants infected only with the LP-mild isolate, while the yield loss from the CH2-aggressive reference plants was 6%.

When monitoring the plants for PepMV symptoms, a large difference in general crop appearance and plant vigour was observed between the various tunnels. From 3 WPI onwards, the CH2-aggressive reference plants and especially the LP-mild pre-inoculated plants were clearly weaker, with a visibly reduced leaf surface and lower vigour than control plants.

PepMV symptom development was monitored at regular intervals by rating the same 30 plants at 12 time points and by performing additional ratings of all 100 plants per

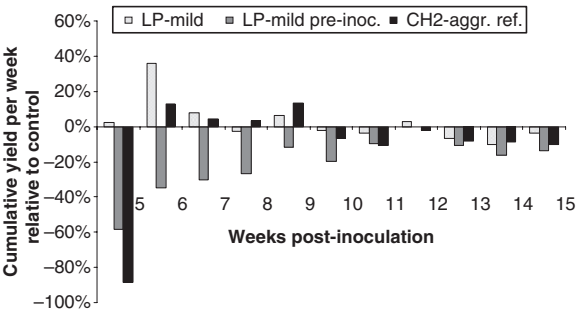

Figure 3 LP-mild *Pepino mosaic virus* (PepMV) pre-inoculation trial: tomato production, determined as the cumulative weight of all ripe tomatoes (including fruits with typical PepMV symptoms) harvested once a week, from 5 to 15 weeks post-inoculation (WPI), in each tunnel and shown as the percentage relative to the weight obtained for the control tunnel. Total yield obtained from the 11 harvesting points was 742 kg in the control tunnel, 721 kg in the LP-mild reference tunnel (3% yield loss), 641 kg in the LP-mild pre-inoculated tunnel (13% yield loss) and 695 kg in the CH2-aggressive reference tunnel (6% yield loss).

tunnel at two additional time points. Premature leaf senescence was seen from 3 WPI onwards in all tunnels, including the control tunnel, albeit to a lesser extent than in the treatments. Leaf bubbling was only rarely observed. Nettle-head scores obtained from pre-inoculated plants were between those from the LP-mild and CH2-aggressive reference plants, which were consistently the lowest and the highest, respectively, whilst nettlehead symptoms did not occur in control plants (Fig. 1b). Differences between control plants and inoculated plants were significant ($P < 0{\cdot}05$) at all time points from 1 WAI onwards. CH2-aggressive reference plants displayed significantly ($P < 0{\cdot}05$) more severe nettlehead symptoms then the other treatments at 1 and 2 WPI.

Relative infection levels of different PepMV isolates

To determine the relative infection level of the different PepMV isolates, genotype-specific TaqMan RT-qPCR assays were performed using a EU/LP-specific and a CH2/US2-specific assay. Mean Ct values obtained using the PepMV genotype-specific probes were subtracted from the mean Ct values of the internal control, COX, for standardization. The index obtained [Ct(COX) – Ct(PepMVgenotype)] was proportional to the viral genome copy load in each analysed sample. The standardization with COX excluded potential inter-sample variations caused by differences in the efficiency of the RNA-extraction procedure. Initially, replication of the CH2-aggressive isolate in LP-mild pre-inoculated plants was notably slower than in the CH2-reference plants (Fig. 4). The lower slope of the CH2-aggressive load curve was indicative of a somewhat slower onset after challenge inoculation, which was followed by a consistently lower load of the challenge isolate until 5 WPI. From 7 WPI onwards, loads of the CH2-aggressive isolate were comparable in CH2-aggressive reference and LP-mild pre-inoculated plants (Fig. 4). The viral load of the LP-mild isolate was hardly influenced by challenge inoculation with CH2-aggressive.

Additional trials with mild CH2 and EU protector isolates

As well as the LP-mild protector isolate, additional mild isolates belonging to different genotypes were tested for their cross-protection potential. Isolate 1806, hereafter referred to as EU-mild (GenBank Accession No. FJ457098), and isolate 1906, hereafter referred to as CH2-mild (GenBank Accession No. FJ457096), were selected based on previous greenhouse trials (Hanssen *et al*., 2009a). These isolates were tested in two additional tunnels following the same experimental design. In the first additional tunnel, plants were inoculated with the CH2-mild isolate and 4 weeks later, after confirmation of systemic spread of the virus, challenge inoculation was performed using the CH2-aggressive isolate. In the second additional tunnel, plants were inoculated with the EU-mild isolate. As ELISA analyses showed that the plants were not systemically infected after 3 weeks, a sec-

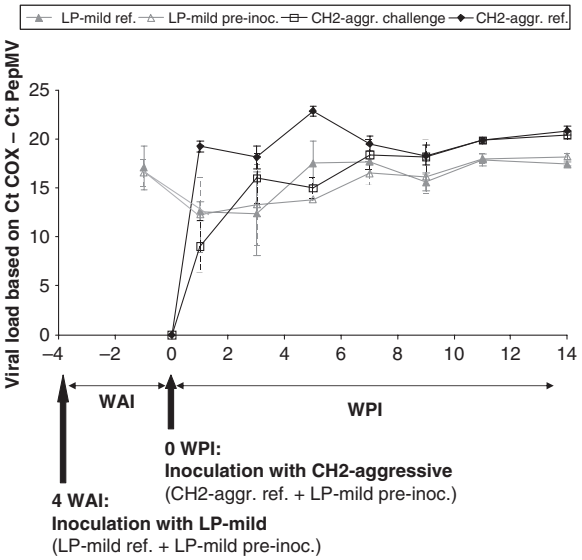

Figure 4 Relative quantification of viral loads for the LP-mild *Pepino mosaic virus* pre-inoculation trial on tomato, based on a genotype-specific TaqMan RT-PCR. Ct values obtained with LP/EU or CH2 genotype-specific assays were subtracted from the Ct values obtained from the COX (control) assay and the lowest obtained value equalized to zero for illustrative purposes. Each point represents the mean of three sampling blocks with standard errors. Inoculation time points are indicated by arrows. WAI, weeks ante-inoculation; WPI, weeks post-inoculation.

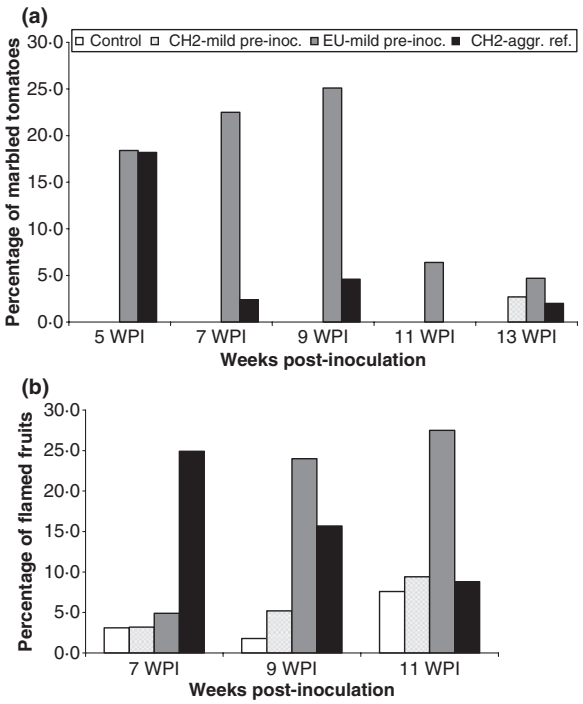

Figure 5 Symptoms on tomato fruits caused by the different *Pepino mosaic virus* infection treatments. Percentage of fruits showing (a) marbling and (b) flaming at various time points. The mean number of fruits harvested per week and per tunnel was 281, with the highest number of fruits harvested at 5 weeks post-inoculation (WPI).

ond inoculation with the same isolate was performed. Two weeks later, ELISA analyses confirmed systemic spread of the virus and the challenge inoculation with the CH2-aggressive isolate was performed.

In both tunnels, nettlehead scores were significantly lower ($P < 0{\cdot}05$) than the scores obtained in the CH2-aggressive reference plants at 1 and 2 WPI (data not shown). With regard to fruit marbling, results obtained with the EU-mild protector isolate were similar to those obtained with the LP-mild protector isolate, with a higher percentage of marbled fruits in the pre-inoculated plants than in the CH2-aggressive reference plants at all time points (Fig. 5a). Similar results were obtained for fruit flaming (Fig. 5b). By contrast, none of the fruits harvested from plants pre-inoculated with the CH2-mild isolate showed marbling (Fig. 5a). Only at 13 WPI were a few marbled fruits (2·7%) seen. Also, fruit flaming was clearly suppressed by pre-inoculation with the CH2-mild isolate (Fig. 5b). In addition, plants in this tunnel were stronger and more vigorous than the pre-inoculated LP-mild or CH2-aggressive reference plants. No yield loss was recorded as the overall yield from both additional tunnels was comparable to that of the control (data not shown). Genotype-specific TaqMan RT-qPCR assays were applied to quantify relative viral loads of the EU-mild and CH2-aggressive isolates in the EU-mild pre-inoculated plants. Similar results were obtained as for LP-mild pre-inoculated plants (Fig. 6). A slower onset of viral accumulation of the CH2-aggressive isolate was recorded in EU-mild pre-inoculated plants, but from 5 WPI onwards viral loads of CH2-aggressive in pre-inoculated and reference plants were comparable.

To determine the relative concentrations of the CH2-mild and CH2-aggressive isolates in CH2-mild pre-inoculated plants, nucleotide sequences of a total of 16 clones, each containing an 840-bp fragment of the PepMV coat protein gene, obtained from three samples taken at 3, 5 and 7 WPI in sampling block 8, were determined as described previously (Hanssen *et al.*, 2009a). Interestingly, each of these 16 clones contained a fragment derived from the CH2-mild genotype, suggesting that the concentration of the CH2-aggressive isolate in CH2-mild pre-inoculated plants was at least 10 times lower than the concentration of the CH2-mild isolate. The dominance of the CH2-mild isolate in these plants was further confirmed by a specifically developed RT-PCR assay by which a CH2-aggressive-specific amplicon was obtained (forward primer 5′-ATTAACACTGAAGG**C**ATCAT**A**-3′, reverse primer 5′-GTATTCTACTGTGTCGTCTT GTG-3′; mismatches with CH2-mild shown in bold). Because of the high sequence homology between the two isolates, the assay was only specific at a high annealing temperature (63°C), thus decreasing the sensitivity of the assay. Spiking experiments were performed to determine the greatest dilution at which pure CH2-aggressive cDNA could be detected in a background of CH2-mild cDNA. These tests revealed that the assay could detect

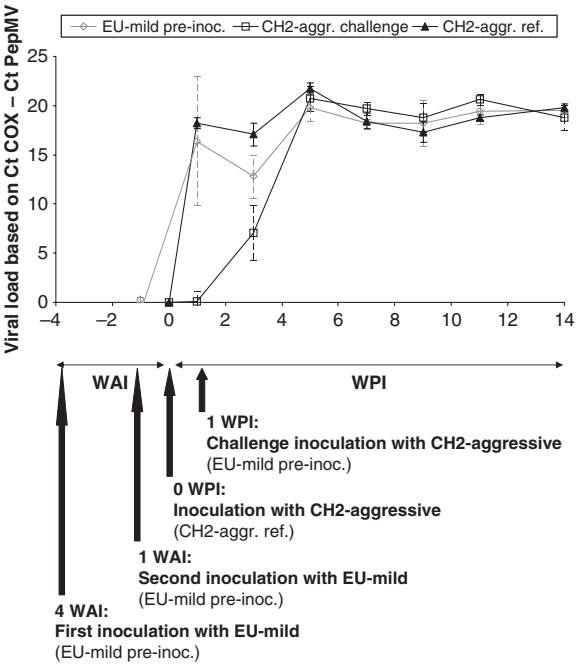

Figure 6 Relative quantification of viral loads of *Pepino mosaic virus* for the EU-mild pre-inoculation trial, based on a genotype specific TaqMan RT-PCR. As the first pre-inoculation with EU-mild performed at 4 weeks post-inoculation (WAI) did not result in systemic infection, a second pre-inoculation was performed at 1 WAI and challenge inoculation was postponed by 1 week. Inoculation points are indicated by black arrows. Ct values obtained with LP/EU or CH2 genotype specific assays were subtracted from the Ct values obtained from the COX (control) assay and the lowest obtained value equalized to zero for illustrative purposes. Each point represents the mean of three sampling blocks with standard errors. WAI, weeks ante-inoculation; WPI, weeks post-inoculation.

CH2-aggressive in a CH2-mild background down to a ratio of 1/50. Using this assay, the challenge isolate CH2-aggressive could not be detected in samples obtained from CH2-mild pre-inoculated plants after challenge inoculation.

Samples obtained from sampling blocks 2, 4 and 8 at 3, 5, 7 and 13 WPI (12 samples in total) were analysed using this assay and all tested negative, meaning that the concentration of CH2-aggressive was at least 50 times lower than that of CH2-mild in the pre-inoculated plants.

Discussion

In this work the potential of a mild LP isolate to provide cross-protection in a tomato crop against severe damage caused by an aggressive CH2 isolate was assessed in greenhouse trials. The study revealed enhanced symptom severity rather than cross-protection in plants pre-inoculated with the mild LP isolate and subsequently challenged by an aggressive CH2 isolate. In particular, the incidence of fruit marbling, the most typical and economically important PepMV symptom, increased considerably, whilst the incidence of fruit flaming, a less typical symptom which sometimes occurs as a physiological disorder, was less influenced. In addition, pre-inoculation with LP-mild and subsequent challenge inoculation with CH2-aggressive had a serious impact on plant vigour and leaf development, and hence on yield, resulting in overall yield reduction of 13% compared to control plants. Fruit sepal necrosis was neither reduced nor enhanced. Interestingly, additional trials with two other mild protector isolates resulted in similar results for plants pre-inoculated with a mild EU isolate and subsequently challenged by the aggressive CH2 isolate, while efficient cross-protection against the challenge isolate was obtained in plants pre-inoculated with a mild CH2 isolate.

In the LP-mild and EU-mild pre-inoculated plants, RT-PCR-RFLP demonstrated the presence of both the protector and the challenge genotype after challenge inoculation. Quantitative genotype-specific RT-qPCR assays revealed that viral accumulation of the CH2 challenge isolate was initially somewhat suppressed by the mild isolates, but that after 5–7 WPI the viral load obtained for CH2-aggressive in the pre-inoculated plants was similar to that in the CH2-aggressive reference plants. This accumulation of the challenge isolate coincided with the start of the fruit ripening period and thus with high incidence of fruit marbling. Nettlehead symptoms (nettle-like leaf deformations in the head of the plants) are usually more pronounced in the first weeks after inoculation and disappear later on, as previously observed in greenhouse trials (Spence *et al.*, 2006; Hanssen *et al.*, 2009a). In this trial, the incidence of nettlehead symptoms in the first weeks after challenge inoculation was lower in LP-mild and EU-mild pre-inoculated plants than in the reference plants only inoculated with the challenge isolate. This could be related to the initial suppression of challenge-isolate accumulation in the pre-inoculated plants. Overall, the EU-mild and LP-mild isolates did not induce durable cross-protection against an aggressive CH2 isolate.

By contrast, efficient cross-protection against the CH2 challenge isolate was obtained by pre-inoculation with a mild CH2 isolate. In particular, the incidence of PepMV typical fruit symptoms was notably reduced. Remarkably, the titre of the CH2-mild isolate was significantly higher than that of the CH2-aggressive challenge isolate, which was not detectable by cloning and by specific conventional RT-PCR.

Overall, the results indicate that co-infection with different PepMV genotypes in the same plant can lead to enhanced symptom severity, and that pre-inoculation of a tomato crop with a mild isolate results in protection against an aggressive isolate only if it belongs to the same genotype. These observations are in line with previous observations that co-infection with two PepMV genotypes (EU and CH2) in commercial tomato crops resulted in more severe symptoms (Hanssen *et al.*, 2008). Whether the enhanced symptom severity is the result of synergism between different PepMV genotypes, or of PepMV recombinants, which were previously reported to occur in mixed infections (Hanssen *et al.*, 2008), is currently not

clear. Synergism between different viruses usually coincides with a drastic increase in viral titre of at least one of the two viruses, as previously shown for *Potato virus X* (PVX) and *Potato virus Y* (Stouffer & Ross, 1961) and for *Blackeye cowpea mosaic virus* in combination with *Cucumber mosaic virus* (CMV) (Anderson *et al*., 1996). Synergistic interactions with potyviruses are generally characterized by an unchanged concentration of the potyvirus and an increased concentration of the other virus (Hull, 2002). Relative quantification of viral titres of the co-infecting PepMV genotypes in this study did not reveal such an increase. However, a positive correlation between viral titre and symptom severity has not yet been unambiguously proven for PepMV in tomato. A synergistic interaction resulting in enhanced symptom severity without significant increase of either of the two interacting viruses was previously reported for *Rice tungro bacilliform virus* and *Rice tungro spherical virus* in rice tungro disease (Hull, 2002).

Interestingly, nucleotide sequence homology of the EU and LP genotypes with the CH2 genotype is as low as 79%, while the sequence homology between the mild and aggressive CH2 isolates used in this study is 99·4% (Hanssen *et al*., 2009a). These results suggest that RNA sequence homology is a determining factor in PepMV cross-protection efficiency in tomato, as was previously shown for other plant–virus interactions. It was demonstrated that the mild ZYMV-WK strain conferred efficient cross-protection against related but not divergent strains of the virus (Wang *et al*., 1991; Desbiez & Lecoq, 1997). Similar results were obtained for PRSV types P and W, which are serologically indistinguishable but differ in host range. A mild P-type isolate of PRSV confers efficient cross-protection against severe P-type isolates, but not W-type isolates (Yeh & Gonsalves, 1984). Albiach-Marti et al. (2000) demonstrated that mild strains of CTV conferring efficient cross-protection in Florida and Spain displayed high sequence homology with a diverse range of isolates. A CMV mutant lacking the 2b counter-defence protein gene was shown to provide protection against wild-type strains, but in this case efficient cross-protection against a more divergent strain was also obtained (Ziebell *et al*., 2007).

Ratcliff *et al*. (1999) provided convincing evidence that post-transcriptional gene silencing (PTGS) can be the underlying mechanism for cross-protection. The authors showed that for *Tobacco rattle virus* and PVX constructs sharing a common sequence, one viral construct could suppress the other through RNA-mediated cross-protection in co-infected plants. Based on this study, it was suggested that cross-protection is mediated by pre-activation of the RNA-induced silencing complex (RISC) with small interfering RNA (siRNA) derived from the protector virus RNA, thus inhibiting replication of the challenge isolate (Ratcliff *et al*., 1999; Gal-On & Shiboleth, 2006). By contrast, co-infection of two viruses with limited sequence homology could lead to synergism, mediated by inhibition of the PTGS defence mechanism by viral silencing suppressors (Gal-On & Shiboleth, 2006).

This is apparently the first report on cross-protection between different PepMV isolates in tomato. The finding that the interaction between PepMV isolates largely depends on nucleotide sequence homology between the isolates has important implications for PepMV disease management in practice. The risk of enhanced symptom severity in mixed infections caused by different PepMV genotypes undermines the potential of cross-protection and implies that the emergence of new PepMV genotypes in various tomato production areas (Alfaro-Fernández *et al*., 2008; Hanssen *et al*., 2008; Hasiów *et al*., 2008; Ling, 2008) poses a threat to the tomato industry worldwide. A management strategy based on cross-protection can only be successful in areas where one PepMV genotype is dominant, provided that the PepMV population is monitored intensively and that very strict hygiene measures are taken during cultivation and between different cropping cycles.

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