Cross-Protection as a Control Strategy for *Pepino mosaic virus* (PepMV) in Greenhouse Tomato

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Abstract

***Pepino mosaic virus* (PepMV) is a highly infectious *Potexvirus*, which has been observed for the first time in tomato crops in The Netherlands about a decade ago and has presently become a major disease of tomato crops worldwide. Control measures consist of strict hygienic measures to prevent infection, albeit these measures often fail, especially in dense tomato production areas. However, previous studies indicate that cross-protection can be effective. The potential of three mild PepMV isolates, belonging to the CH2, EU and LP genotypes, to protect a tomato crop against an aggressive challenge isolate of the CH2 genotype was assessed in a summer crop in plastic tunnels. Enhanced symptom display was observed in plants that were pre-inoculated with a protector isolate belonging to a different genotype (EU, LP) as the challenge isolate (CH2), while effective cross-protection was obtained using the mild CH2 isolate. As the PepMV population in Belgium and neighbouring countries is dominated by the CH2 genotype, cross-protection using this mild CH2 isolate could be an effective strategy for controlling PepMV. Therefore, in this study we further assess the potential of this vaccination strategy. The efficiency of the mild CH2 isolate to protect a crop from another aggressive, mutant CH2 variant (CH2-pvu), which is currently spreading in the area, was assessed in a summer crop in plastic tunnels. In addition glasshouse trials in conditions similar to commercial tomato production are currently being carried out, in which the response of a wide range of tomato varieties to the mild CH2 isolate is studied, next to the cross-protection efficiency under two different climate strategies.**

INTRODUCTION

Pepino mosaic virus (PepMV) is a highly infectious *Potexvirus* that was first isolated from pepino (*Solanum muricatum*) in Peru in 1974 (Jones et al., 1980). The virus causes a wide range of symptoms, including the typical marbling of tomato fruits, which reduces the economical value of the crop (Roggero et al., 2001; Spence et al., 2006). Currently, four PepMV genotypes have been reported in commercial tomato production (Hanssen and Thomma, 2010): the Peruvian genotype (LP) which is similar to the original isolate from pepino (López et al., 2005; Pagan et al., 2006); the European tomato genotype (EU), which was first reported in European greenhouse tomato production in 1999 (Verhoeven et al., 2003; Pagan et al., 2006); the CH2 genotype, which was first found on tomato seeds from Chile (Ling, 2007) and the US1 genotype, which was first described in the United States (Maroon-Lango et al., 2007). Molecular methods such as sequencing, RT-qPCR or RT-PCR-RFLP are needed to distinguish these four genotypes, as biological differences between isolates from the same genotype can be larger than

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biological differences between the genotypes (Hásiow-Jaroszewska et al., 2009; Hanssen et al., 2009). The EU and LP genotypes are closely related as they share a nucleotide sequence homology of 95%. By contrast, the CH2 genotype shares only 78 to 80% sequence homology with the EU and LP genotypes. Also the US1 genotype is rather different, as it shares only 78% sequence homology with CH2 and 82% with EU/LP genotypes. Initially, the PepMV population in European tomato production was quite homogenous and consisted of isolates from the EU genotype. In recent years however, the CH2 genotype has largely replaced the EU genotype in commercial tomato production in several European countries, whereas in the United States and Canada the EU genotype is still predominant (Hanssen and Thomma, 2010).

The use of mild virus isolates to protect a crop against more severe isolates from the same or a closely related viral species is generally referred to as cross-protection. The mechanism was first described by McKinney (1929) in tobacco plants that were systemically infected by a mild strain of *Tobacco mosaic virus* (TMV). A subsequent infection with a severe strain of the virus, which normally induces yellow leaf mosaics, did not cause any symptoms. Since then, cross-protection has been used to study relationships between viruses and to control viral diseases in commercial crop production systems (Lecoq and Lemaire, 1991). An important role of the coat protein in cross-protection has indeed been evidenced for *Cucumber mosaic virus* (CMV) and for TMV (Dodds et al., 1985; Sherwood and Fulton, 1982). This type of resistance is not strain specific and can be broken under high infection pressure of the challenge strain (Lomonosoff, 1995). Experiments by Koo et al. (2004), who constructed transgenic plants expressing the coat protein of TMV under an inducible promoter, showed that expression of the TMV coat protein was sufficient to obtain resistance to related TMV strains. However, the observation that also coat protein defective viruses and even viroids can confer cross-protection to related strains (Niblett et al., 1978; Gerber and Sarkar, 1989) triggered further study of the mechanism behind cross-protection. Ratcliff et al. (1999) provided evidence for post-transcriptional gene silencing (PTGS) as an underlying mechanism of cross-protection. The authors proved the involvement of RNA recognition by performing co-infection experiments of two unrelated viruses, PVX and *Tobacco rattle virus* (TRV), sharing the nucleotide sequence of the same reporter gene GFP. Leaves that were systemically infected with PVX-GFP could not be infected with TMV-GFP. 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 and Shiboleth, 2006). This type of resistance is strain specific, as a high RNA sequence homology between protector and challenge isolate is required for efficient protection.

Some mild viral isolates have been used in commercial vegetable production for vaccination of the crop. The most classical example is the successful, worldwide application of the mild TMV isolate MII-16 in greenhouse tomato production to control TMV (Rast, 1972). The strategy was gradually abandoned when TMV resistant tomato cultivars became available. Also *Zucchini yellow mosaic virus* (ZYMV) was temporarily controlled using cross-protection. Watermelon and squash production fields in Israel and France were vaccinated with the mild isolate ZYMV-WK (Lecoq and Lemaie, 1991; Lecoq, 1998). However, outbreaks of the *Watermelon mosaic Potyvirus* (WMV), which interacts synergistically with ZYMV, dramatically reduced the economic potential of the vaccination strategy. A mild isolate of *Papaya ringspot virus* (PRSV) was used in papaya fields in Hawaii, Taiwan, Thailand and Mexico to control this devastating papaya disease (Gal-On and Shiboleth, 2006). As predominant isolates in Taiwan shared only 84-90% sequence homology with the predominant isolates in Hawaii, the mild isolate from Hawaii did not protect papaya crops in Taiwan, indicating that the mechanism was strain specific and thus, most probably, RNA mediated (Yeh et al., 2005). These examples demonstrate that cross-protection can be a promising strategy to control a damaging virus with a high incidence and a homogenous population in a certain region, where it

constitutes the major viral disease of the crop (Lecoq, 1998; Gal-On and Shiboleth, 2006). Since PepMV is the most prevalent virus in greenhouse tomato production in north-western Europe, and taking into account the lack of alternative control strategies, cross-protection could be a good strategy to control PepMV disease in tomato crops. In addition, it has been speculated that early PepMV infections cause less economic losses than infections that occur later in the production cycle (Spence et al., 2006; Hanssen et al., 2008). Therefore, we performed several greenhouse trials to assess the potential of cross-protection for the control of PepMV in greenhouse tomato production.

PEPMV CROSS-PROTECTION TRIALS AND VALIDATION IN SEMI-COMMERCIAL PRODUCTION

We have identified several PepMV isolates that differ significantly in aggressiveness: a mild EU isolate ('EU mild', isolate 1806; Hanssen et al., 2009); a mild LP isolate ('LP mild'; Hanssen et al., 2010); a mild CH2 isolate ('CH2 mild', isolate '1906'; Hanssen et al., 2009) and two aggressive CH2 isolates, designated 'CH2 aggressive' (isolate 'PCH 06/104'; Hanssen et al., 2009) and 'CH2 pvu'. In 2008, greenhouse trials in plastic tunnels were conducted to assess the cross-protection potential of the three mild isolates 'LP mild', 'EU mild' and 'CH2 mild' against an aggressive isolate from the dominant CH2 genotype, 'CH2 aggressive' (Hanssen et al., 2010). Interestingly, pre-inoculation of the tomato plants with a mild EU or LP isolate and subsequent challenge inoculation with the aggressive CH2 isolate resulted in more severe PepMV symptoms as compared to a single infection with the aggressive CH2 isolate (Hanssen et al., 2010). Mainly the incidence of fruit marbling increased dramatically at certain harvest points, e.g., from 18% in the reference plants only infected with 'CH2 aggressive' to 43% in the plants pre-inoculated with 'LP mild' and later on challenged with 'CH2 aggressive', and from 4% in the reference plants only infected with 'CH2 aggressive' to 24% in the plants pre-inoculated with 'EU mild' and later on challenged with 'CH2 aggressive'. Also the overall yield was negatively affected, especially by pre-inoculation with the 'LP mild' isolate. However, efficient cross-protection was obtained when plants were first inoculated with 'CH2 mild' and later on challenged with 'CH2 aggressive'. In this case, tomato fruits displayed no fruit marbling and the yield was not affected (Hanssen et al., 2010). These results clearly show that PepMV cross-protection can be efficient to control the disease, but that the mechanism is genotype specific, indicating that the obtained resistance is mediated by RNA silencing.

To further asses the protective potential of the 'CH2 mild' isolate, a second greenhouse trial in plastic tunnels was conducted in 2009. In this trial a different, aggressive challenge isolate from the dominant CH2 genotype, 'CH2 pvu', was used. This isolate is characterized by an atypical mutation in the coat protein gene and can cause severe fruit marbling and a high incidence of open fruits (fruit that splits shortly after fruit setting, such that the seeds are visible in the flesh of the mature fruit), especially in beef tomatoes. In four different plastic tunnels, 100 tomato plants (cultivar 'Tricia', De Ruiter Seeds, Bergschenhoek, The Netherlands) were planted for a summer cropping cycle (May to October). In the first tunnel, the plants were mock-inoculated (control). In the second (CH2 mild reference) and third tunnel, plants were inoculated with 'CH2 mild'. Two weeks later, plants in the third tunnel were subjected to a secondary challenge inoculation with the aggressive isolate 'CH2 pvu' (CH2 mild + CH2 pvu). At the same time, plants in the fourth tunnel, that were still healthy at that point in time, were also inoculated with this aggressive isolate (CH2 pvu reference). The first fruits were harvested eight weeks after challenge inoculation. For six subsequent weeks, all harvested tomato fruits from each tunnel were weighed to determine the yield and evaluated for PepMV symptoms such as fruit marbling, open fruits, discoloration or flaming of the fruits and necrosis of the fruit sepals. In addition, every two weeks the plants were evaluated for typical PepMV plant symptoms like nettlehead, bubbling and deformation of the leaves, yellow leaf spots and leaf scorching. Interestingly, marbling of the tomato fruits did not occur in the 'CH2 mild + CH2 pvu' tunnel, while quite high percentages of marbled fruit were seen in the

'CH2 pvu reference' tunnel at certain harvest points (37% at 8DP and 13% at 9 DPI; Fig. 1A). The plants in the 'CH2 mild reference' and 'control' tunnels did not display fruit marbling (Fig. 1A). Similar results were seen for the typical PepMV induced necrosis of the fruit sepals, with a rather high incidence in the 'CH2 pvu reference' tunnel and a very low incidence in the other tunnels (Fig. 1B). With regards to leaf symptoms, the differences were less pronounced, with a rather low incidence of nettleheads, leaf scorching and yellow leaf spots in all four tunnels (data not shown). Total yield was reduced with 9% in the 'CH2 pvu reference' tunnel as compared to the control tunnel, while no significant effect on yield was observed in the 'CH2 mild + CH2 pvu' or in the 'CH2 mild reference' tunnel. These results confirm that isolate 'CH2 mild' can be used efficiently to prevent damage caused by more aggressive isolates belonging to the same genotype, and that the protector isolate by itself is very mild.

As the results obtained with this mild CH2 isolate in plastic tunnels were promising, trials in semi-commercial tomato production conditions are currently being conducted. The effect of the 'CH2 mild' isolate on tomato cultivars with diverse genetic backgrounds is being assessed on two different locations, in year-round glasshouse tomato crops with plants from the 13 most commonly grown tomato cultivars in Belgium. In addition, the cross-protection efficiency is being tested under two different climate regimes, one similar to the standard regime used by commercial tomato growers and one with colder night temperatures.

CONCLUSIONS AND PERSPECTIVES

PepMV is an important viral disease of tomato. As resistant cultivars are not yet available, the only control strategy for tomato growers is the application of very strict hygiene measures to prevent infection. However, in dense tomato production areas, these measures often fail. Especially the highly prevalent and infectious CH2 genotype of PepMV often infects the crops, despite all precautions. Therefore, we assessed the potential of several mild PepMV isolates ('EU mild', 'LP mild' and 'CH2 mild') to provide protection against severe damage caused by an aggressive challenge isolate from the CH2 genotype. Our study revealed that efficient cross-protection against the prevalent CH2 genotype of PepMV can be obtained by pre-inoculation with a mild CH2 isolate, but that enhanced symptom severity can occur when the protector and challenge isolates belong to different genotypes (Hanssen et al., 2010). A second greenhouse trial in plastic tunnels was conducted in 2009 to verify the cross-protection efficiency of the mild CH2 isolate against a second, more recently identified, aggressive CH2 isolate designated 'CH2 pvu'. Again efficient cross-protection was obtained, indicating that cross-protection between isolates from the same genotype is not isolate specific. Moreover, as this aggressive 'CH2 pvu' isolate can cause significant damage in commercial greenhouse tomato production, the obtained results imply that the mild CH2 protector isolate could efficiently be used in commercial production to control PepMV. Therefore, trials in semi-commercial, glasshouse tomato crops are currently being conducted to assess the impact of the mild CH2 protector isolate on a wide range of tomato cultivars, and to verify the cross-protection efficiency under different climate regimes. Altogether, the results of these trials should provide sufficient foundations to decide whether application of this mild protector isolate could be an efficient and safe strategy to control PepMV in greenhouse tomato production. However, the strategy can only be used under certain premises. Firstly, our results clearly show that a major premise is the homogeneity of the PepMV population. Only one PepMV genotype should dominate the population, with no or only limited infection pressure of other genotypes. Therefore, the viral population should be monitored continuously. Secondly, taking into account the experience with the mild ZYMV strain, which reacted synergistically with WMV, cross-protection to control PepMV should only be considered in areas where PepMV constitutes the major viral disease of tomato. Thirdly, as our mild CH2 protector isolate only provides complete protection against the dominant CH2 genotype of PepMV, growers should continue to apply very strict hygiene measures to prevent mixed infections with different PepMV

genotypes.

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Literature Cited

Dodds, J.A., Lee, S.Q. and Tiffany, M. 1985. Cross protection between strains of *Cucumber mosaic virus*: effect of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. Virology 144:301-309.

Gal-On, A. and Shiboleth, Y.M. 2006. Cross protection. p.261-268. In: G. Loebenstein and J.P. Carr (eds.), Natural Resistance mechanisms of plants to viruses. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Gerber, M. and Sarkar, S. 1989. The coat protein of *Tobacco mosaic virus* does not play a significant role for cross-protection. J. Phytopath. 124:1-4.

Hanssen, I.M., Paeleman, A., Vandewoestijne, E., Van Bergen, L., Bragard, C., Lievens, B., Vanachter, A.C.R.C. and Thomma, B.P.H.J. 2009. *Pepino mosaic virus* isolates and differential symptomatology in tomato. Plant Pathol. 58:450-460.

Hanssen, I.M., Paeleman, A., Wittemans, L., Goen, K., Lievens, B., Bragard, C., Vanachter, A.C.R.C. and Thomma, B.P.H.J. 2008. Genetic characterization of *Pepino mosaic virus* isolates from Belgian greenhouse tomatoes reveals genetic recombination. Eur. J. Plant Pathol. 121:131-46.

Hanssen, I.M. and Thomma, B.P.H.J. 2010. *Pepino mosaic virus*: a successful pathogen that rapidly emerged from emerging to endemic in tomato crops. Mol. Plant Pathol. 11:179-189.

Hanssen, I.M., Gutiérrez-Aguirre, I., Paeleman, A., Goen, K., Wittemans, L., Lievens, B., Vanachter, A.C.R.C., Ravnikar, M. and Thomma, B.P.H.J. 2010. Cross-protection or enhanced symptom display in greenhouse tomato co-infected with different *Pepino mosaic virus* isolates. Plant Pathol. 59:13-21.

Hasiów-Jaroszewska, B., Pospieszny, H. and Borodynko, N. 2009. New necrotic isolates of *Pepino mosaic virus* representing the CH2 genotype. J. Phytopathol. 157:494-496.

Jones, R.A.C., Koenig, R. and Lesemann, D.E. 1980. *Pepino mosaic virus*, a new *Potexvirus* from pepino (*Solanum muricatum*). Ann. Appl. Biol. 94:61-68.

Koo, J.C., Asurmendi, S., Bick, J., Woodford-Thomas, T. and Beachy, R.N. 2004. Ecdysone agonist-inducible expression of a coat protein gene from *tobacco mosaic virus* confers viral resistance in transgenic Arabidopsis. Plant J. 37:439-448.

Lecoq, H. and Lemaire, J.M. 1991. Control of *Zucchini yellow mosaic virus* in squash by cross protection. Plant Dis. 75:208-211.

Lecoq, H. 1998. Control of plant virus diseases by cross protection. p.33-40. In: A. Hadidi, R.K. Khetarpal and H. Koganezawa (eds.), Plant Virus Disease Control. St Paul, MN, USA: APS Press.

Ling, K. 2007. Molecular characterization of two *Pepino mosaic virus* variants from imported tomato seed reveals high levels of sequence identity between Chilean and US isolates. Virus Genes 34:1-8.

Lomonossoff, G.P. 1995. Pathogen-derived resistance to plant viruses. Annu. Rev. Phytopathol. 33:323-343.

López, C., Soler, S. and Nuez, F. 2005. Comparison of the complete sequences of three different isolates of *Pepino mosaic virus*: size variability of the TGBp3 protein between tomato and *L. peruvianum* isolates. Arch. Virol. 150:619-627.

Maroon-Lango, C.J., Guaragna, M.A., Jordan, R.L., Hammond, J., Bandla, M. and Marquardt, S.K. 2005. Two unique US isolates of *Pepino mosaic virus* from a limited

source of pooled tomato tissue are distinct from a third (EU like) US isolate. Arch. Virol. 150:1187-1201.
McKinney, H.H. 1929. Mosaic diseases in the Canary islands, West Africa and Gibraltar. J. Agric. Res. 39:557-578.
Niblett, C.L., Dickson, E., Fernow, K.H., Horst, R.K. and Zaitlin, M. 1978. Cross protection among four viroids. Virology 91:198-203.
Pagán, I., Córdoba-Sellés, M.C., Martinez-Priego, L., Fraile, A., Malpica, J.M., Jordá, C. and García-Arenal, F. 2006. Genetic structure of the population of *Pepino mosaic virus* infecting tomato crops in Spain. Phytopathology 96:274-279.
Rast, A.T.B. 1972. MII_16, an artificial symptomless mutant of *Tobacco mosaic virus* for seedling inoculation of tomato crops. Neth. J. Plant Pathol. 78:110-112.
Ratcliff, F.G., MacFarlane, S. and Baulcombe, D.C. 1999. Gene silencing without DNA: RNA-mediated cross-protection between viruses. Plant Cell 11:1207-1215.
Roggero, P., Masenga, V., Lenzi, R., Coghe, F., Ena, S. and Winter, S. 2001. First report of *Pepino mosaic virus* in tomato in Italy. Plant Pathol. 50:798.
Sherwood, J.L. and Fulton, R.W. 1982. The specific involvement of coat protein in *Tobacco mosaic virus* cross protection. Virology 119:150-158.
Spence, N.J., Basham, J., Mumford, R.A., Hayman, G., Edmondson, R. and Jones, D.R. 2006. Effect of *Pepino mosaic virus* on the yield and quality of glasshouse-grown tomatoes in the UK. Plant Pathol. 55:595-606.
Verhoeven, J.T.J., van der Vlugt, R. and Roenhorst, J.W. 2003. High similarity between tomato isolates of *Pepino mosaic virus* suggests a common origin. Eur. J. Plant Pathol. 109:419-425.
Yeh, S.D. and Gonsalves, D. 1984. Evaluation of induced mutants of *Papaya ringspot virus* for control by cross protection. Phytopathology 74:1086-1091.

Figures

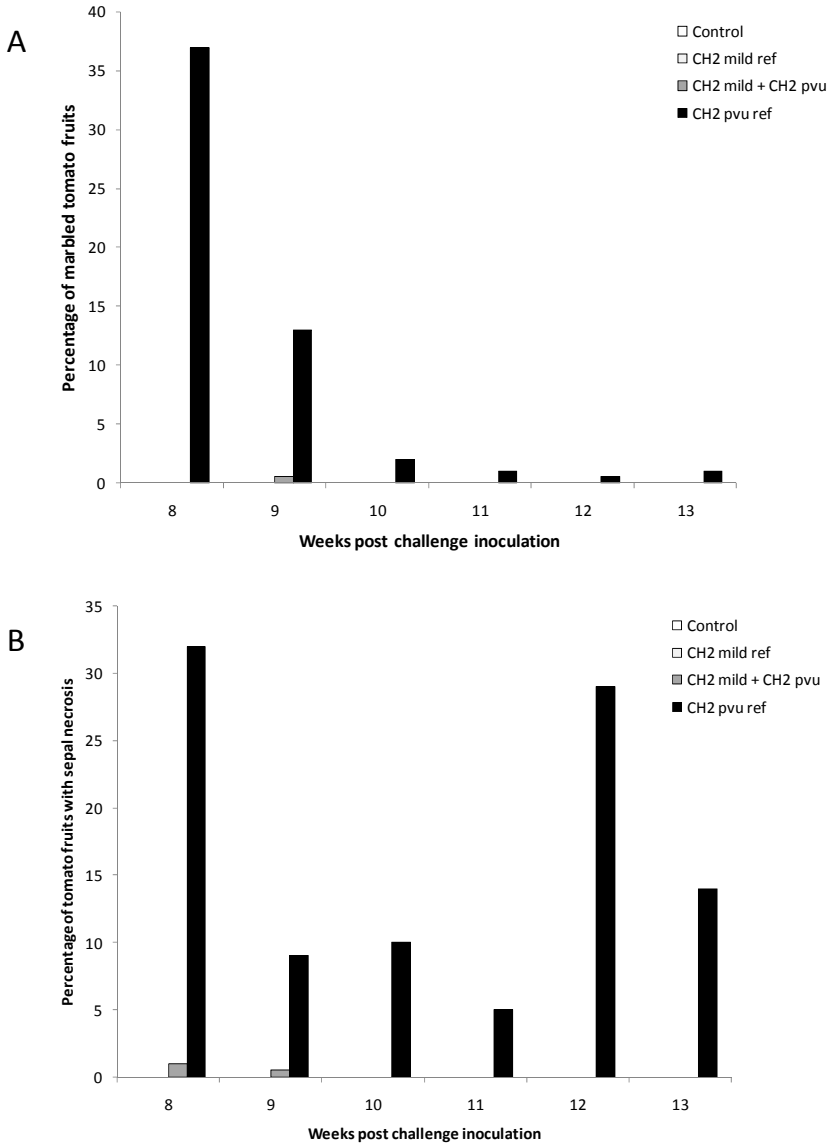

Fig. 1. Symptoms on tomato fruits caused by the different PepMV infections. (A) Percentage of tomatoes displaying (A) fruit marbling and (B) necrosis of fruit sepals at various harvest points from the different tunnels. The number of fruits harvested per week and per tunnel ranged from 250 to 500.