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Multi-genotype cross-protection against *Pepino mosaic virus* in tomato

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ABSTRACT

The viral pathogen *Pepino mosaic virus* (PepMV) can cause serious problems in the crop tomato (*Solanum lycopersicum*). One strategy to control infections of this virus is the application of hygienic measures. Alternatively, tomato plants can be cross-protected by attenuated isolates of a PepMV genotype. After two weeks, these plants are cross-protected against virulent isolates of the same genotype. In this study, the effectivity of a multi-genotype cross-protection was assessed. In greenhouse experiments young tomato plants were inoculated with a mixture of attenuated isolates of the Peruvian (LP) and Chili-2 (CH2) genotype, named VX1 and VC1, respectively. Inoculated plants were challenged with virulent isolates of the EU and CH2 genotype. The mixture of attenuated isolates, named V10, prevented almost completely the appearance of viral symptoms like leaf and stem necrosis, stunting, loss of fruit quality and loss of yield, caused by the virulent isolates. In contrast, when only a single genotype (CH2) was used to cross-protect the plants, symptoms were not prevented after the challenge with the mixture of virulent isolates. This study offers perspectives for a large-scale application of a multi-genotype cross-protection against PepMV in tomato.

1. Introduction

Pepino mosaic virus (PepMV) is a major viral disease in tomato, *Solanum lycopersicum*, (Soler-Aleixandre et al., 2005; Van der Vlugt et al., 2000). PepMV is easily transmitted by plant sap through crop handling, like harvesting, pruning and other practices (Jones et al., 1980; Spence et al., 2006). Symptoms can vary from latent to severe. Marbling, blotchy ripening and malformation of the fruits, lead to economical loses for tomato growers. Additionally, symptoms on the leaves as yellow mosaic, chlorosis, necrosis, nettle heads, bubbling and stem necrosis can be caused by PepMV as well (Jorda et al., 2001; Soler et al., 2000; Spence et al., 2006; Van der Vlugt et al., 2000). The abnormalities in the leaves and stems result in distorted and stunted growth.

Two to three weeks after infection with PepMV, the first symptoms can be observed in tomato plants. In general, they slowly disappear in a couple of weeks afterwards (Van der Vlugt and Stijger, 2008). The display of symptoms may depend on light intensity and temperature (Jorda et al., 2001; Van der Vlugt and Stijger, 2008). Symptoms are more common during fall and winter months when temperatures and light levels are minimal.

The kind of PepMV symptoms is also dependent on intrinsic properties of the viral isolate (Hanssen and Thomma, 2010). Differences in the nucleotide sequences between isolates from the same genotype have been shown to be associated with enhanced virulence (Hasiów-Jaroszewska and Borodynko, 2012; Hasiów-Jaroszewska et al., 2011).

Preventing infections and limiting the spread of PepMV in greenhouses require stringent hygiene measures. In tomato cultivation areas, 90% of the greenhouses with tomatoes can be infected with PepMV (Soler-Aleixandre et al., 2005). To prevent virus infections by hygiene measures can be difficult under such circumstances, therefore cross-protection might be an alternative strategy to manage PepMV infections.

Crops can be cross-protected against infections of virulent isolates by a previous infection with closely related attenuated isolates of the same virus. This strategy has been applied for various viruses. Examples are the use of attenuated isolates of *Citrus tristeza virus* (Costa and Muller, 1980), *Tobacco mosaic virus* (Burgyán and Gáborjányi, 1984), *Papaya ringspot virus* (Yeh et al., 1988), *Cucumber mosaic virus* (Kosaka and Fukunishi, 1997), *Pepper mild mottle virus* (Yoon et al., 2006) and *Chinese yam necrotic mosaic virus* (Kondo et al., 2007). Attenuated isolates may originate from greenhouses or fields (Costa and Muller, 1980; Kondo et al., 2007) or can be developed in the laboratory by mutation techniques (Yeh et al., 1988). Cross-protection against PepMV in tomato has been

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described before (Schenk et al., 2010). Infection of tomato plants with attenuated PepMV isolates did not have negative effects on yield or fruit quality, contrary to infection with virulent isolates, but induced cross-protection. The underlying mechanism of cross-protection might be RNA silencing induced by the attenuated isolate (Ratcliff et al., 1999; Valkonen et al., 2002). In plants, RNA silencing has been shown to serve as a defence mechanism against virus infections. RNA silencing is based on targeting and degrading specific sequences of RNA. As a result replicated RNA of the invading virus is destroyed preventing further spread of the virus in the plant. Replicated RNA of almost identical virus isolates is also destroyed, preventing infection by these isolates as well (Nishiguchi and Kobayashi, 2011). Multiplication of any almost identical (attenuated or virulent) virus isolate (similarity between RNA of isolates $\geq$ 96%) that invades the plant, after RNA silencing is initiated, will therefore be prevented. This implies that the mechanism of cross-protection only works when tomato plants are inoculated with an attenuated isolate before being exposed to virulent isolates.

Four major genotypes or strain groups can be distinguished: European (EU), Peruvian (LP), Chilean (CH2) and North-American (US1). These genotypes share genome nucleotide sequence identities between 78% and 96%. The EU-genotype, which is very similar to the LP-genotype (95–96% identity), was the predominant genotype in Europe. After 2004, the CH2-genotype became predominant. Infections of CH2-genotype occurred as a single infection or as a mixed infection with the EU-genotype. The CH2-genotype is genetically very distinct from the EU-genotype (79% nucleotide identity). Isolates of the US1-genotype, which are genetically clearly different from the EU-, LP- and CH2-genotypes (identities of 78–82%), are found only occasionally in Europe (Hanssen and Thomma, 2010).

Cross-protection is supposed to work only when the attenuated and virulent isolates are closely related, hence belonging to one genotype. In this study, we have investigated whether an inoculation with a mixture of attenuated isolates of the LP and CH2 genotype, effectively protects the plants against virulent isolates of the EU and CH2 genotype.

2. Material and methods

2.1. Virus isolates

Two attenuated isolates and two virulent isolates were isolated from greenhouses in The Netherlands. VX1 belongs to the LP genotype and VC1 to the CH2 genotype. The virulent isolates were named by their genotype, virEU and virCH. V10 is a mixture of VX1 and VC1.

2.2. Inoculation experiments (greenhouse compartments)

2.2.1. Trial sites

A trial was laid out, consisting of six treatments (Table 1). A mixture of the virulent isolates virEU&virCH was used as a virulent virus control. The trial was repeated six times in two greenhouse testing facilities. Three trials were conducted in Delfgauw, The Netherlands and three in Bleiswijk, The Netherlands, both during spring 2012, fall 2012 and spring 2013. Another trial consisted of five treatments (Table 2). This trial was performed once during the fall of 2012 in Bleiswijk.

The plots were laid out with four replicates. Each replicate consisted of a different tomato cultivar; Brioso (cocktail tomato), Endeavour, Komeett and Levanzo (all truss tomato). Each plot consisted of 20 plants. Each block of four replicates (one treatment) was separated from other treatments by sweet pepper plants (non-host for PepMV).

2.2.2. Treatments

The first application with attenuated viruses was conducted when the crop was 10–30 cm high. The application with the virulent virus was conducted three or seven weeks later. The sequence of the different virus applications is shown in Tables 1 and 2

2.2.3. Application details

The concentrations of undiluted VX1 and VC1 were 10–50 mg/L PepMV. Undiluted V10 contains 5–25 mg/L VX1 and 5–25 mg/L VC1. The attenuated viruses were applied as a foliar spray with a dosage of 2%. The equipment used to carry out the first application with attenuated viruses was a high-pressure spraying arm carrying nozzles of type XRTEEJET 11003VK. The amount of spray liquid used was 0.5 L/m^2 plant bed, sprayed at pressures ranging from 12 to 15 bar. The spray arm height was approximately 10–15 cm above the crop. Carborundum (0.8%; Silicon carbide from Saint-Gobain, particle size 17 μm) was added to the spray solution to provide enough abrasion to ensure infection. During the applications the plots were separated by plastic screens to avoid spray drift. The second application with the virulent isolates was carried out by dipping fingers (covered with latex or nitril gloves) in the virus suspension, derived from sap from infected tomato plants (dosage of 10% in combination with 1–2% carborundum) and rubbing two leaves on each plant. Two-three weeks after the first and second application, leaf samples (one sample per plant) were taken for ELISA to assess the absence of virus in the negative control treatment and 100% presence of the attenuated and the virulent virus isolates in the treated plants.

2.2.4. Growing conditions

The target temperatures were set during daytime at 20 °C and during nighttime at 18 °C. The realized mean 24 h-day temperatures were between 18 and 24 °C. Relative humidity varied between

Table 1
Treatments, virus isolates and application timings for six trials.

Treatment	A1	A2
1	untreated (water)	virEU + virCH
2	untreated (water)	–
3	V10 (VX1 + VC1)	virEU + virCH
4	V10 (VX1 + VC1)	–
5	VC1	virCH
6	VX1	virEU

–, untreated; VX1, attenuated Peruvian genotype; VC1, attenuated Chile-2 genotype; virEU, virulent European genotype; virCH, virulent Chile-2 genotype; A1, application time point 1 and A2, application time point 2.

Table 2
Treatments, virus isolates and application timings for one trial.

Treatment	A1	A2
1	untreated (water)	virEU + virCH
2	untreated (water)	–
3	V10 (VX1 + VC1)	virEU + virCH
4	V10 (VX1 + VC1)	–
5	VC1	virEU + virCH

–, untreated; VX1, attenuated Peruvian genotype; VC1, attenuated Chile-2 genotype; virEU, virulent European genotype; virCH, virulent Chile-2 genotype; A1, application time point 1 and A2, application time point 2.