Pepino mosaic virus:
an endemic pathogen of tomato crops

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Thesis

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Chapter 1:
General introduction and outline

Emerging viral diseases of tomato crops

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Abstract

Viral diseases are an important limiting factor in many crop production systems. As antiviral products are not available, control strategies rely on genetic resistance or hygienic measures to prevent viral diseases, or on eradication of diseased crops to control such diseases. Increasing international travel and trade of plant materials enhances the risk of introducing new viruses and their vectors into production systems. In addition, changing climate conditions can contribute to a successful spread of newly introduced viruses or their vectors and establishment of these organisms in areas that were previously unfavorable.

Tomato is economically the most important vegetable crop worldwide and many viruses infecting tomato have been described, while new viral diseases keep emerging. *Pepino mosaic virus* (PepMV) is a rapidly emerging virus which has established itself as one of the most important viral diseases in tomato production worldwide over recent years. Begomoviruses and other whitefly transmitted viruses are invading into new areas, and several recently described new viruses such as Tomato torrado virus and new *Tospovirus* species are rapidly spreading over large geographic areas. In this manuscript emerging viruses of tomato crops are discussed.

Introduction

Despite tremendous efforts in human, animal and plant health management, viral diseases remain notoriously difficult to control or eradicate. Moreover, currently used control strategies are compromised by the continuous emergence of new strains of existing viruses or of completely new viruses. Owing to their large population size and short generation time, viruses have a great potential to quickly evolve and adapt under natural selection pressure. The high incidence of mutation, recombination and reassortment in viral genomes enhances the generation of new variants that, in case the mutation results in a biological advantage, quickly spread throughout the viral population (Moya et al., 2004). Especially RNA virus quasispecies, composed of large mutant swarms that contain major and minor mutations in their genome sequences, are highly effective in adapting to varying selection pressures as the pool of variants in the quasispecies "cloud" is a rich source of variation which can contain successful variants (Domingo, 2000).

There are several definitions of emerging viruses. The world health organisation (WHO) defines an emerging virus as 'one that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range'. Others consider an emerging virus as a virus that has recently

changed or appeared to occupy and spread within a new niche (Rojas and Gilbertson; 2008). Emerging viruses can be newly described viruses that were previously unknown. However, more frequently, emerging viruses are known viruses with an increased incidence in a certain niche due to changes in the environment, the vector, the host and/or in the viral genome.

Virtually no antiviral products are available for plant disease management, leaving eradication or prevention through hygiene measures as the only immediate control strategies, albeit that these practices have only met limited success. The best control strategy for a viral disease is the introduction of genetic resistance in the plant host. However, introgression of genetic resistance is time-consuming, and will in most cases become available only after the emerging virus has become well-established.

Increasing international travel and trade of seeds, seedlings, cuttings and fruits enhances the risk of introducing new viruses and their vectors into production areas. Changing climate conditions can contribute to a more successful spread of the virus or its vector and establishment of these organisms in areas that were previously unfavorable, thus enhancing viral spread.

With a worldwide production of 130 million metric tons and a value of over 30 billion international dollars in 2007, tomato (*Solanum lycopersicum*) is by far the most important vegetable crop (source: Food and Agricultural Organization (FAO), United Nations (UN); http://faostat.fao.org/site/339/default.aspx). In terms of economical value, tomato constitutes 72% of the value of fresh vegetables produced worldwide. The number of described viral species that infect tomato crops amounts to 136, while this number is notably lower for other vegetable crops with, for example, 49 viruses that infect pepper (*Capsicum annuum*), 53 that infect lettuce (*Lactuca sativa*), 46 that infect melon (*Cucumis melo*), 54 that infect potato (*Solanum tuberosum*) and 44 that infect eggplant (*Solanum melongena*). Only for cucumber (*Cucumis sativis*) even more viral pathogens (153) have been described (Brunt et al., 1996 onwards, Plant viruses online, VIDE database).

The high number of viral pathogens of tomato can partly be explained by the sensitivity of tomato to begomoviruses, a genus that harbors a large variety of species. In addition, years of intensive breeding for improved production may have narrowed the genetic basis for viral disease resistance in commercially grown tomato varieties. Furthermore, the intensification of tomato production with large areas of protected monocropping under controlled climate conditions may generate the conditions for efficient spread and survival of viruses and their vectors, increasing the potential for emerging viruses to become endemic. Finally, although it may seem trivial, also the

economical importance of tomato makes the study of pathogens and pests affecting this crop a major topic in phytopathological research. Consequently, pathogens infecting tomato are generally well studied and characterized. Over the recent years, several viral diseases including *Tomato yellow leaf curl virus* (TYLCV; genus *Begomovirus*), *Pepino mosaic virus* (PepMV; genus *Potexvirus*) and Tomato torrado virus (ToTV) have emerged in greenhouse tomato crops and are presently impacting the fresh market tomato production in diverse geographic areas worldwide.

Considering the past 20 years, in this manuscript the most important emerging viral pathogens of tomato are discussed, encompassing: (i) new viral species; (ii) known viral pathogens of other crops that are emerging in tomato; and (iii) tomato viruses with a rapidly increasing incidence. In addition, the re-emergence of old viruses, that largely disappeared with the introduction of resistance, due to 'specialty market'-driven culture of old tomato varieties, is discussed.

New viral species that infect tomato

Emerging species assembled in the novel 'Torradovirus' genus

In the past decade, two related viral species that are clearly distinct from previously described plant viruses simultaneously emerged in two distinct geographic regions, Spain and Mexico. Both viruses caused necrotic or burn-like disease symptoms in tomato crops. From 2001 onwards, tomato plants with severe necrotic leaf symptoms were observed in protected tomato crops in the Murcia area in the South-East of Spain (Verbeek et al., 2007; Alfaro-Fernández et al., 2007a). Because of the burn-like appearance of affected leaves, the disease was locally referred to as 'torrado' (roasted) disease. Initial disease symptoms consist of necrotic spots that are surrounded by a light green or yellow area at the base of the leaflets (Figure 1a). In a later stage, leaves and fruits display severe necrosis and plants suffer an overall growth reduction resulting in serious economical damage (Verbeek et al., 2007). Diagnostics revealed the presence of *Pepino mosaic virus* (PepMV) in infected plants, but as the symptoms were atypical for PepMV further studies were performed. In addition to the rod-shaped PepMV particles, isometric viral particles were observed by electron microscopy. Through inoculation of the indicator plants *Physalis floridana* and *Nicotiana glutinosa* that are not susceptible to PepMV, a virus with a bipartite positive stranded (ss) RNA genome with three open reading frames (ORFs) was purified. The virus shares virion characteristics and nucleotide sequence similarities with viruses from the genera *Sequivirus*, *Waikavirus*,

Sadwavirus and *Cheravirus*, but phylogenetic analyses on two different genome regions revealed a separate taxonomic position. The name 'Tomato torrado virus' (ToTV) was proposed, and the species was proposed to belong to a novel genus (Verbeek et al., 2007; Table 1).

Intriguingly, from 2003 onwards a highly similar disease (leaf, stem and fruit necrosis; Figure 1e) was observed in Mexican tomato crops, locally known as 'marchitez' (wilted) disease, which was initially thought to be caused by a *Sw5* resistance-breaking strain of *Tomato spotted wilt virus* (TSWV; genus Tospovirus) (Turina et al., 2007), although this virus could not be detected in symptomatic plants. Electron microscopy revealed the presence of isometric viral particles in symptomatic plant material (Turina et al., 2007). Virus characterization and partial sequencing revealed that the virus was distinct from previously described viruses and the name 'tomato apex necrosis virus' (ToANV) was proposed (Turina et al., 2007). In a parallel study on the same disease, analyses of morphology and genome structure showed that the virus causing 'marchitez' disease was highly similar to ToTV (Verbeek et al., 2008). However, subsequent nucleotide sequence analyses revealed that the disease was caused by a new viral species that is clearly distinct from, albeit related to, ToTV with nucleotide sequence identities of 85%, 63 and 66% for the three ORFs, and the name 'Tomato marchitez virus' (ToMarV) was proposed (Verbeek et al., 2008; Table 1). Phylogenetic analyses showed that ToTV, ToMarV and ToANV are related and these new viruses are thought to belong to the same novel genus for which the name 'Torradovirus' was proposed, with ToTV as the type species (Verbeek et al., 2008; Sanfaçon et al., 2009). ToMarV is considered a distinct species of the same genus, and because partial sequence comparison revealed 95% sequence homology, ToANV is considered to be a ToMarV isolate (Verbeek et al., 2008).

The generation of a new family within the order of Picornavirales, which currently contains mainly animal viruses that belong to the families Picornaviridae, Comoviridae, Dicistroviridae, Marnaviridae, Sequiviridae and the unassigned plant virus genera Cheravirus and Sadwavirus, was recently proposed (Le Gall et al., 2008). The name 'secoviridae' was proposed for the new family, which includes all plant viruses within the order and comprise the genera *Comovirus*, *Fabavirus*, *Nepovirus*, *Sequivirus*, *Waikavirus*, *Cheravirus*, *Sadwavirus* and the new genus Torradovirus (Sanfaçon et al., 2009).

Rapidly after the initial characterisation of ToTV, the virus was reported in greenhouse tomato crops in the Canary Islands (Alfaro-Fernández et al., 2007b), where the typical symptoms were already observed in 2003. Similarly, typical symptoms were observed in tomato crops in Poland already in 2003, and based on nucleotide sequence

comparisons ToTV was identified afterwards as the causal agent (Pospieszny et al., 2007). While poor mechanical transmission was reported, efficient vectoring by the greenhouse whitefly *Trialeurodes vaporiarium* was demonstrated (Pospieszny et al., 2007). More recently, the whitefly *Bemisia tabaci* was shown to efficiently transmit the virus and other Solanaceous crops, including potato, eggplant, pepper and tobacco (*Nicotiana tabacum*), were reported as hosts (Amari et al., 2008). In addition, natural infection of weed hosts, possibly serving as alternative hosts in close proximity to Solanaceous crop production systems, was reported in Spain (Alfaro-Fernández et al., 2008a). In 2008, tomato plants exhibiting ToTV-like symptoms were observed in Panama and molecular analysis confirmed the presence of both ToTV and *Cucumber mosaic virus* (CMV). No difference in symptom expression was found between plants infected with both viruses or with ToTV alone (Herrera-Vasquez et al., 2009). Interestingly, out of 87 ToTV-infected samples with typical 'torrado' disease symptoms that were collected between 2003 and 2006 in Spanish greenhouse tomato crops, 83 were simultaneously infected with *Pepino mosaic virus* (PepMV) (Alfaro-Fernández et al., 2007a). Recently, the virus was also reported in tomato crops in Hungary (Alfaro-Fernández et al., 2009a) and in Australia (EPPO reporting service 2009), showing a rapid migration of ToTV over large geographical distances (Figure 2), possibly in association with whiteflies. By contrast, ToMarV is thus far only reported in Mexico and information regarding ToMarV transmission is currently not available.

New *Crini-* and *Tospovirus* species

The criniviruses represent a group of viruses that emerged over the last decades in association with the worldwide emergence of whiteflies (Wisler et al., 1998a; Jones, 2003; Wintermantal, 2004). Whitefly populations (mainly the *Bemisia tabaci* B biotype) have drastically increased worldwide since the 1970s, especially in tropical and subtropical areas (Wisler et al., 1998a). Even the greenhouse whitefly *Trialeurodes vaporariorum*, for which epidemics have long been restricted to greenhouse crops, have been emerging in open field vegetable production over the last 20 years with large populations in summer crops and weed reservoirs (Wintermantel, 2004). Increased insecticide resistance, global warming, changing of agricultural practices and increasing global trade of plant materials have been suggested as factors enhancing whitefly emergence (Wintermantel, 2004). Members of the genus *Crinivirus* within the *Closteroviridae* family have a bipartite ssRNA genome with the two segments separately encapsidated, and are transmitted by several species from *Bemisia* and *Trialeurodes* whiteflies in a semi-persistent manner (Wintermantel, 2004). In the last decade two

criniviruses emerged as a problem in tomato production, *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV).

TICV was first identified in field-grown tomato crops in 1993 in California, with an estimated yield loss of 2 million US dollars in that year alone (Duffus et al., 1996; Wisler et al., 1996). Tomato plants showing symptoms reminiscent of TICV infection have been reported in greenhouse tomato crops in Florida since 1989 (Wisler et al., 1998b). The disease was referred to as 'yellow leaf disorder' and had been attributed to nutritional disorders or pesticide phytotoxicity, as initial analyses could not detect viral presence. However, transmission experiments revealed that this 'yellow leaf disorder' was efficiently transmitted by whiteflies. A *Crinivirus* that was distinct from TICV, both in terms of RNA sequence, vector specificity and host range was isolated and named *Tomato chlorosis virus* (ToCV) (Wisler et al., 1998b).

TICV is transmitted solely by the greenhouse whitefly *Trialeurodes vaporariorum*, while ToCV is transmitted by a number of whitefly species which include the greenhouse whitefly, the banded wing whitefly *Trialeurodes abutilonea*, and the *Bemisa tabaci* biotypes A, B and Q (Navas-Castillo, 200; Wintermantal and Wisler, 2006). The *B. tabaci* A biotype is also known as the sweet potato whitefly, and the B biotype is also known as the silverleaf whitefly (Jones, 2003). *T. vaporariorum* is present in all temperate areas worldwide, wile *T. abutilonea* has only been described in Cuba and the US. *B. tabaci* was originally described in tropical and subtropical regions, but it has spread to temperate regions as well. The B biotype is considered highly invasive and with worldwide spread, has been shown to be the most efficient vector for ToCV transmission (Wintermantal and Wisler, 2006).

Both TICV and ToCV induce practically indistinguishable "yellowing disease" in tomato, which include interveinal yellowing and thickening of mature leaves, while the new growth at the plant apex appears normal. Disease symptoms usually appear 3-4 weeks following inoculation, and are readily mistaken for nutritional disorders or pesticide phytotoxicity. Although TICV and ToCV do not induce any symptoms on tomato fruit, fruits of infected plants are smaller, decreased in number, the ripening process is impeded, and the plants seems to go through early senescence. All of which results in yield and economical losses (Wintermental, 2004; Dalmon et al., 2009).

Although both TICV and ToCV have a wide host range, only TICV infects lettuce. While both TICV and ToCV induce very similar disease symptoms in tomato plants, they can be discriminated using the indicator plants *Nicotiana benthamiana* and *N. clevelandii* (Wisler et al., 1998b). As symptoms of both viruses can be confused with nutritional disorders or poor growing conditions, it has been speculated that the virus might have

been present in tomato crops long before being identified (Rojas and Gilbertson, 2008). Moreover, both viruses are phloem-limited and infected plants carry low viral titers, which complicates accurate diagnostics.

Over the last decade, criniviruses were reported to infect field and greenhouse tomato crops in Cuba, Turkey, Cyprus, Portugal, Spain, Italy, Israel, Jordan, La Réunion Island, Morocco, Greece, Puerto Rico and Taiwan (British Society for Plant Pathology, New Disease Reports; Louro et al., 2000; Navas-Castillo et al., 2000; Accotto et al., 2001; Wintermantel et al., 2001; Dovas et al., 2002; Varia et al., 2002; Tsai et al., 2004; Segev et al., 2004; Anfoka and Abhary, 2007; Martinez-Zubiaur et al., 2008). Interestingly, ToCV was identified in Israel in 2004, while in 2007 TICV was identified in tomato fields in the Jordan Valley in Jordan (Segev et al., 2004; Anfoka and Abhary, 2007). Although the distance between the tomato fields in Jordan and in Israel is only a few kilometers, ToCV has not yet been detected in Jordan and TICV has not yet been detected in Israel. This may be linked to different agricultural practices affecting whitefly populations, with most tomatoes in Jordan grown in open fields, while in Israel the majority of the tomatoes are grown in greenhouses.

In addition to whitefly emergence, also thrips populations have notably increased over the last decades, thus facilitating the emergence of thrips-transmitted viruses (Prins and Goldbach, 1998). More specifically, the proliferation of thrips-vectored Tospoviruses has been associated with increasing thrips populations, mainly attributed to the rapid expansion of the western flower thrips (*Frankliniella occidentalis*) combined with increasing insecticide resistance in thrips populations (Prins and Goldbach, 1998; Rojas and Gilbertson, 2008). Over recent years, several new species from the *Tospovirus* genus, infecting tomato crops in Australia, Asia and the Middle-East, have been reported (Hassani-Mehraban et al., 2005; Dong et al., 2008; Knierim et al., 2006). Tospoviruses are enveloped viruses with a tripartite ssRNA genome that belong to the *Bunyaviridae* family and that cause significant losses in tomato, pepper and other crops worldwide. Presence of tospoviral species, and more specifically of *Tomato spotted wilt virus* (TSWV; the *Tospovirus* type species) and members of the *Watermelon silver mottle virus* (WSMoV) serogroup, has been documented in Asian tomato production since the mid-1980s. Since then, an increasing number of tospoviruses has been found in different crops all over Asia (Whitfield et al., 2005; Dong et al., 2008). In 1998 a tospovirus-like disease was reported in tomato crops with necrotic lesions on the foliage and chlorotic ring spots on the fruits in Iran (Hassani-Mehraban et al., 2005). Serological and molecular characterisation revealed that this *Tospovirus* isolate represents a new species, which was tentatively named ‘tomato yellow ring virus’ (TYRV; Table 1).

In 2002, a new *Tospovirus* species infecting pepper and tomato in Queensland (Australia) was characterized and tentatively named 'Capsicum chlorosis virus' (CaCV; McMicheal at al., 2002; Table 1), which was later reported to infect tomato plants in Thailand (Knierim et al., 2006). The virus has also been reported to occur in Taiwan and China (Dong et al., 2008).

In 2005 a devastating new disease was observed in tomato and chili pepper crops in Yunnan, China. Based on electron microscopy, serological studies and complete nucleotide sequences of the three RNA fragments, it was concluded that the virus represents a new *Tospovirus* species, and the name 'Tomato zonate spot virus' (TZSV) was proposed (Dong et al., 2008; Table 1). Phylogenetic analyses show that TZSV clusters in an Asian group of tospoviruses, comprising *Calla lily chlorotic spot virus* (CCSV; Taiwan), *Watermelon silver mottle virus* (WSMoV; Taiwan and Japan), Capsicum chlorosis virus (CaCV; Taiwan, Australia, Thailand and China), watermelon bud necrosis virus (WBNV; India and Southeast Asia) and melon yellow spot virus (MYSV; Japan and Taiwan). All these tospoviruses seem to originate from, and are presently restricted to, Asia (Figure 2), suggesting that they share a common origin. The high incidence of new *Tospovirus* species in tropical Asian regions suggests a 'hot spot' of viral genetic diversity in reservoir hosts variants from where they are transmitted to commercial crops through increasing vector populations (Rojas and Gilbertson, 2008).

In addition to new *Tospovirus* species, recently another unknown, putatively thrips-transmitted virus has been reported in open field tomato crops in the Central Valley of California (Batuman et al., 2009). Necrotic symptoms were observed on tomato leaves, stems and petioles (Figure 1g), most similar to those induced by *Tobacco streak virus* (TSV). Although numerous fields were affected, disease incidence in the field was rather low (5 to 20%). The causal agent was identified as an *Ilarvirus* (*Bromoviridae*) with three RNA fragments, and nucleotide sequence analyses revealed 81 to 84% and 64 to 80% identity for the three RNAs of *Parietaria mottle virus* (PMoV) and TSV ilarviruses, respectively. The mode of transmission has not clearly been demonstrated but may involve thrips feeding on infected pollen, as was previously shown for TSV (Sdoodee & Taekle, 1987). It was proposed that this virus is representative of a new *Ilarvirus* species, tentatively named 'Tomato necrotic spot virus' (TNSV; Batuman et al., 2009; Table 1).

Table 1. Emerging viruses of tomato crops reviewed in this manuscript

Family	Genus	Species	Geographical spread	Reference[1]
Secoviridae	Torradovirus	Tomato torrado virus (ToTV)	Spain, Canary Islands, Panama, Poland, Hungary, Australia	Verbeek et al., 2007
		Tomato marchitez virus (ToMarV)	Mexico	Verbeek et al., 2008
Closteroviridae	*Crinivirus*	*Tomato infectious chlorosis virus* (TICV)	US (California), Europe (South), Middle-East	Duffus et al., 1996
		Tomato chlorosis virus (ToCV)	Europe (South), US, Middle-East	Wisler et al., 1998b
Bromoviridae	*Ilarvirus*	Tomato necrotic spot virus (TNSV)	US (Florida)	Batuman et al., 2009
	Anulavirus	*Pelargonium zonate spot virus* (PZSV)	Israel, Europe (South), US (California)	Gallitelli, 1982
Bunyaviridae	*Tospovirus*	Tomato yellow ring virus(TYRV)	Iran	Hassani-Mehraban et al., 2005
		Capsicum chlorosis virus (CaCV)	Australia, China, Thailand, Taiwan	McMicheal at al., 2002
		Tomato zonate spot virus (TZSV)	China	Dong et al., 2008
Flexiviridae	*Potexvirus*	*Pepino mosaic virus* (PepMV)	Worldwide (except Middle-East)	van der Vlugt et al., 2000
Geminiviridae	*Begomovirus*	*Tomato yellow leaf curl virus* (TYCLV) and related species	Tropical and subtropical areas	Cohen and Harpaz, 1964

[1]first description for new viruses; first report in tomato for known viruses that recently emerged in tomato

Known viral pathogens that emerged in tomato

Pepino mosaic virus, a *Potexvirus* that was initially isolated from pepino (*Solanum muricatum*) in 1974 in Peru (Jones et al., 1980), was observed for the first time in tomato crops only a decade ago (van der Vlugt, 2000; Table 1). In only a few years time, the virus became a major disease of glasshouse tomato crops worldwide (French et al., 2001, Mumford and Metcalfe 2001, Cotillon et al., 2002, Aguilar et al., 2002; Maroon-Lango et al., 2005; Ling 2006, Pagán et al., 2006; Figure 2). Potexviruses are mechanically transmitted viruses with flexuous rod-shaped particles and an ssRNA genome containing five ORFs. PepMV isolates originating from tomato crops in Europe between 2000 and 2003 shared over 99% nucleotide sequence identity among each other but were clearly distinct (96% nucleotide sequence identity) from the original pepino isolate (Verhoeven, 2003). In addition, whereas the original pepino isolate was

asymptomatic in tomato, the tomato isolates caused a wide variety of symptoms on fruits and vegetative plant parts (van der Vlugt et al., 2000, French et al., 2001, Mumford and Metcalfe 2001, Cotillon et al., 2002, Aguilar et al., 2002). These isolates were thus considered as distinct from the original pepino-infecting strain, and these were subsequently designated 'European tomato strain' of PepMV (Mumford and Metcalfe, 2001; Pagán et al., 2006; Verhoeven, 2003). Since 2005, new genotypes sharing only 80% nucleotide sequence identity with the European tomato strain have been identified, originating from tomato crops in the US (US1 and US2; Maroon-Lango et al., 2005) and from tomato seeds from Chile (CH1 and CH2; Ling, 2006).

The typical fruit marbling (Figure 1c) is generally considered as the most important PepMV symptom because it significantly impacts the economic value of the crop (Mumford and Metcalfe, 2001; Jones and Lammers, 2005; Spence et al., 2006). In addition, uneven or blotchy ripening of tomato fruit has been associated with PepMV infection (Roggero et al., 2001; Spence et al., 2006). Symptoms on the vegetative plant parts include yellow angular spots on the leaves, the so-called 'nettleheads' (upper young leaves that are distorted, serrated and upright with a reduced leaf surface), leaf mosaics, leaf scorching and leaf blistering or bubbling. The economic impact of PepMV on the tomato industry varies for different tomato producing areas, as it depends on the structure of the tomato market and more specifically on the local marketability of lower quality fruit (Jones and Lammers, 2005; Spence et al., 2006). In addition, large differences in symptom severity and damage have been observed between subsequent cropping seasons and between different geographic areas, suggesting an impact of climate conditions on symptom display (Spence et al., 2006; Jordá et al., 2001). Greenhouse trials conducted in the UK from 2001 to 2003 revealed considerable differences in damage between subsequent years, with the percentage of downgraded tomato fruit due to PepMV-induced quality loss ranging from 6 to 38%. In a questionnaire conducted among Belgian tomato growers, yield losses caused by PepMV were estimated negligible while losses due to fruit marbling were estimated around 5% in the 2005 growth season. However, large differences were noted between individual growers. By contrast, in 2006 quality losses were less pronounced but the overall yield loss was estimated between 5 and 10% (Hanssen et al., 2009a). Since economical margins are limited, losses of 5 to 10% can significantly affect the economical profitability of the crop, which is reflected by the fact that 50% of the Belgian tomato growers estimates the damage caused by PepMV as 'moderate to very severe' (Hanssen et al., unpublished data).

It is presently unclear what triggered the sudden emergence of PepMV in tomato, although long distance spread through infected seeds has been suggested (Rojas and Gilberston, 2008). In addition, as the virus is highly infectious, increasing global trade of tomato fruits and maybe also cuttings of ornamental pepino varieties may have contributed to long distance spread.

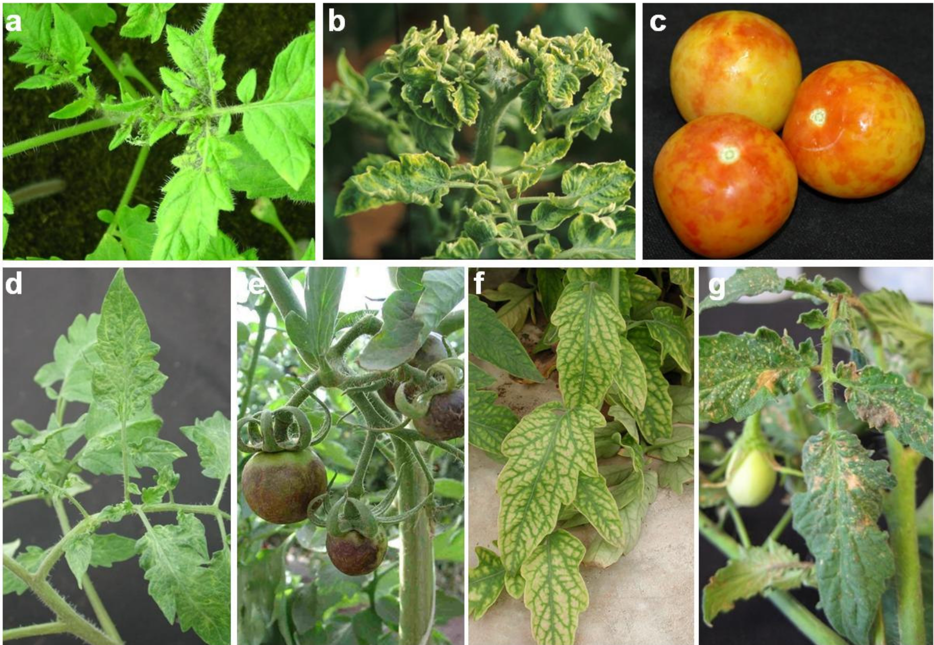

Figure 1. Symptoms of emerging viruses in tomato plants. (A) necrotic spots at the leaflet base induced by Tomato torrado virus (ToTV); (B) Leaf deformation, yellowing and stunting induced by *Tomato yellow leaf curl virus* (TYLCV); (C) Fruit marbling induced by *Pepino mosaic virus* (PepMV); (D) Chlorotic rings and line patterns on leaves induced by *Pelargonium zonate spot virus* (PZSV); (E) Fruit necrosis induced by Tomato marchitez virus (ToMarV); (F) Interveinal leaf chlorosis induced by *Tomato chlorosis virus* (ToCV); (G) Necrotic leaf spots induced by Tomato necrotic spot virus (TNSV). Pictures E and G are kindly provided by Dr. Paul Maris (De Ruiter Seeds, Bergschenhoek, the Netherlands) and Dr. Robert Gilbertson (University of California, USA), respectively.

Known tomato viruses with a rapidly increasing incidence

The worldwide emergence of whiteflies, especially of the *B. tabaci* B biotype, has contributed significantly to the rapid and successful emergence of the tomato yellow leaf curl disease (TYLCD), caused by a complex of viral species and constituting a serious threat to tomato production in many tropical and subtropical regions worldwide (Table 1; Figure 2). The causal agents are members of the *Begomovirus* genus within the family of the *Geminiviridae*, plant viruses with a circular ssDNA genome. Begomoviruses are transmitted by the whitefly *B. tabaci* in a persistent and circulative manner, and their genome generally consists of two DNA molecules. However, nearly all begomoviruses that cause TYLCD have only one genomic component. Many different species have been described within this group, most of which have been given the name *Tomato yellow leaf curl virus* (TYLCV), or derivatives thereof. TYLCD symptoms comprise upward curling of leaflet margins, reduction of leaf surface, yellowing of young leaves, severe stunting and flower abortion (Moriones and Navas-Castillo, 2000; Figure 1b). A disease with similar symptoms to TYLCD was already reported in tomato crops in Israel in the late 1930s, but the virus was first identified in 1961, and tomato production in the Middle East has been severely affected from the 1970s onwards (Cohen and Antignus, 1994; Czosnek and Laterrot, 1997; Cohen and Lapidot, 2007). However, due to a worldwide spread of the B biotype of the vector *B. tabaci*, which has a wider host range than other biotypes and is highly invasive, new TYLCV-like viruses emerged from weed and endemic plant reservoirs in the late 1980s. In addition, with the rapid emergence of whiteflies into new geographic regions, *Begomovirus* infections have been reported in new tomato production areas over the last decade. The disease was described in Japan, Mexico and the US in the late nineties (Polston et al., 1994; Polston and Anderson, 1997; Kato et al., 1998; Ascencio-Ibáñez et al., 1999).

TYLCV hosts that can serve as virus reservoirs might have a large effect on viral emergence to new tomato production regions. While TYLCV can produce severe symptoms in tomato, it is also able to establish symptomless infections in both wild and cultivated species. For instance, in many tomato-growing areas pepper is grown in close proximity. Since pepper was known as a non-host of TYLCV, whitefly management was not practiced rigorously in pepper plots (Polston and Lapidot, 2007). After former, conflicting reports regarding the susceptibility of peppers to TYLCV (Mansour and Al-Musa, 1992; Reina et al., 1999), it was recently demonstrated that some, albeit not all, cultivars of pepper are in fact symptomless hosts of TYLCV (Morilla et al., 2005; Polston et al., 2006). Moreover, despite the lack of TYLCV-induced disease symptoms whiteflies were able to acquire TYLCV from infected pepper plants and transmit the virus to tomato

(Polston et al., 2006). Hence, symptomless infected pepper plants can serve as virus reservoir for the acquisition and transmission of TYLCV. Other asymptomatic hosts of TYLCV may act as reservoirs in a similar manner.

Remarkably, in 2007 a TYLCV outbreak outside the climatic zone in which *B. tabaci* thrives was reported. TYLCV infection was reported in 19 neighboring greenhouse tomato crops in the Netherlands, but the virus has been successfully eradicated and no new outbreaks have been reported since then (Botermans et al., 2009). The source of infection could not be identified, but nucleotide sequence analyses revealed a high similarity between isolates from the different crops (> 99% identity to the Spanish TYLCV-Alm strain) and therefore infection was thought to be initiated from the same source, while local spread most likely occurred through *B. tabaci* vectoring (Botermans et al., 2009). As *Begomovirus* emergence in tomato crops has been extensively discussed in several recent reviews (Moriones & Navas-Castillo, 2000; Varma & Malathi, 2003; Seal et al., 2006; Rojas & Gilbertson, 2008), this topic is not further addressed in this manuscript.

Pelargonium zonate spot virus (PZSV) was first isolated from *Pelargonium zonale* (Quacqurelli and Gallitelli, 1979) and subsequently from tomato crops in Italy. PZSV-infected tomato displayed concentric chlorotic or necrotic rings and line patterns on leaf, stems and fruits (Gallitelli, 1982), and has been the causal agent of multiple disease outbreaks in commercial tomato crops in various geographic regions. PZSV was detected in the late 1990s in greenhouse tomato crops and weeds growing in the immediate vicinity in Spain (Luis-Arteaga and Cambra, 2000), and in 2000 in greenhouse-grown tomato plants in south-eastern France (Gebre-Selassie et al., 2002). More recently, the virus was isolated from open field tomatoes in California (Liu and Sears, 2007) and in Israel (Lapidot et al., 2009; Figure 2). It was reported that the virus is seedborne in the weed species *Diplotaxis erucoides* and transmitted to tomatoes in association with pollen grains carried by thrips, as was previously described for *Ilarvirus* species (Finetti-Sialer and Gallitelli, 2003). Also particle morphology and a number of physiochemical properties are shared with ilarviruses. As the genome comprises three ssRNA species with an organisation that is similar to viral species of the *Bromoviridae*, it was suggested that PZSV represents a new genus within this family (Finetti-Sialer and Gallitelli, 2003), for which the name 'Anulavirus' was proposed (Gallitelli et al., 2005). The virus is seedborne in several hosts and is efficiently transmitted mechanically (Gallitelli, 1982; Lapidot et al., submitted).

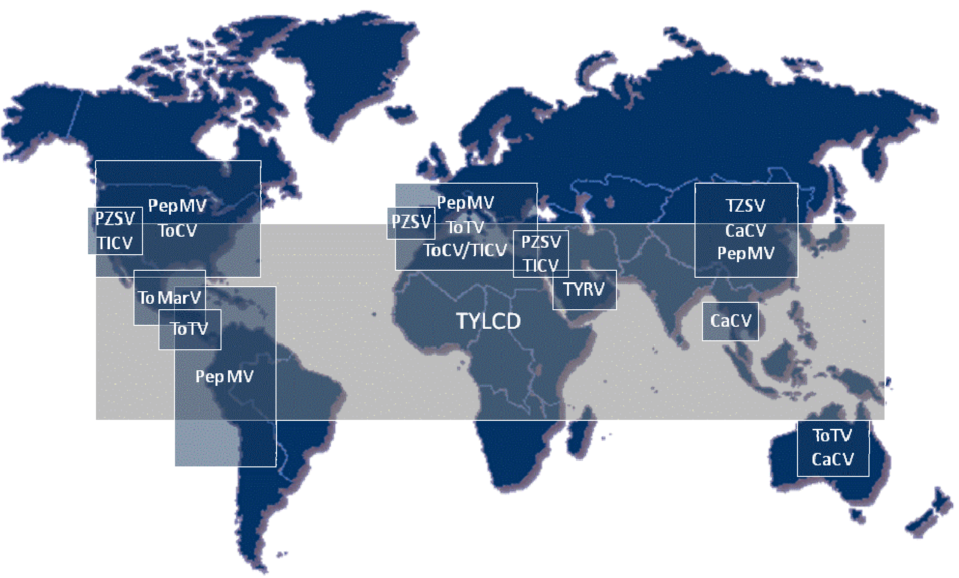

Figure 2. Geographic distribution of the emerging viruses in tomato crops reviewed in this manuscript.

Re-emerging old viruses

In recent years, the tomato market in Europe has evolved towards a specialized, marketing-driven industry. So-called 'specialty' varieties with varying colors (brown or pink), sizes or shapes (cherry, plum or beef), tastes (sweet or rather salty) and health promoting properties (high lycopene content) are gaining importance (e.g. Flandria specialty street; www.specialtystreet.be). Interestingly, part of this evolution is the reintroduction of "old" tomato varieties such as Coeur-de-Boeuf onto the market. Especially in France, old tomato varieties are gaining popularity because of their atypical appearance and flavor (e.g. 'Saveurs Anciennes'; www.savéol.com). Remarkably, these market-driven changes have consequences for plant disease management as these old varieties usually lack the resistance genes that are commonly exploited in more recently developed commercial tomato varieties. As a consequence, *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV), two tobamoviruses that caused serious losses in tomato production worldwide before resistant varieties were introduced, are re-emerging in tomato production areas where these "old" varieties are grown. Grower organizations are considering reintroduction of the cross-protection strategy that was frequently used in the 1970s, consisting of the inoculation of young tomato plants with the mild TMV isolate MII-16 (Rast, 1972), to protect crops from infection with more aggressive TMV or

ToMV variants. However, complications can occur when TMV infects a susceptible variety that is grafted onto a TMV-resistant rootstock. As TMV resistance is generally conferred by the $Tm2^2$ resistance gene (Pelham, 1966) and based on a hypersensitive response (HR), infection of the susceptible scion will result in necrosis at the grafting point and subsequent plant collapse and death. In addition, the high TMV infection pressure in areas where both susceptible and resistant varieties are grown, results in a higher incidence of leaf and stem necrosis in resistant varieties due to the activation of the HR upon challenge with TMV. Finally, as these established viruses might occur in mixed infections with currently emerging viruses like PepMV or ToTV, there may be a risk of synergism, or even for recombination events, which might lead to new viral disease problems in the future.

Control strategies

Over the past 20 years, several viruses have emerged in tomato production worldwide, some of which pose a considerable threat to greenhouse and open field tomato production. Especially the devastating begomoviruses and the highly contagious PepMV are currently hampering the economic profitability of tomato production worldwide. Optimal control strategies are virus-specific and depend on the incidence, transmission and availability of resistant varieties. Within the European Union, control strategies for plant virus emergence depend on Pest Risk Analyses (PRAs) and the phytosanitary measures that are taken, which can include a quarantine status on seeds, seedlings and/or commercial crops (www.eppo.org/QUARANTINE/quarantine.htm). Begomoviruses and the criniviruses TICV and ToCV are listed in the EPPO A2 alert list. TYLCV and related species were assigned a quarantine status for tomato in EU member states (EU directive 2000/29/EC), while PepMV (Commission Decision 2001/536/EC and 2004/200/EC) currently has a quarantine status only on tomato seeds.

Control of whitefly-transmitted viruses depends mainly on the availability of efficient insecticides, insect-proof greenhouses and resistant varieties (Polston and Lapidot, 2007). Tomato varieties with resistance to TYLCV, ToTV and ToMarV are commercially available. Genes controlling resistance to TYLCV and other tomato-infecting begomoviruses (*Ty* genes) were introgressed from several wild Solanum species. The intermediate TYLCV resistance gene Ty1, introgressed from *Solanum chilense* LA1969 (Michelson et al., 1994; Zamir et al., 1994), is the most commonly used source of TYLCV resistance, while information on ToTV resistance has not been published. A recent F_1 diallele study (Vidavski et al., 2008) demonstrated that several of these *Ty* resistance genes may interact with each other, and in some cases result in hybrid plants displaying

higher TYLCV-resistance compared to their parental lines. This suggests that pyramiding resistance genes originating from different resistance sources can be effective in obtaining cultivars that are highly resistant to begomoviruses (Vidavski et al., 2008).

In contrast, varieties with resistance to criniviruses are not yet available. The Tospovirus TSWV-resistance gene *sw5* (Stevens, 1964) is widely used in commercial varieties, but resistance for the newly described *Tospovirus* species is not yet available in commercial tomato varieties. Resistance strategies for *Tospovirus* control are compromised by the continuous emergence of resistance-breaking strains and new species (Pappu et al., 2009). Control of tospoviruses is also hampered by the rapid development of insecticide resistance within thrips populations. Alternatives to chemical thrips control comprise the use of thrips predators or thrips-proof insect nets (Jones, 2004), although the small mesh size required for thrips-proof nets leads to reduced ventilation and thus to suboptimal climate conditions in the greenhouse. A promising future disease management strategy for whitefly and thrips transmitted viruses could consist of genetic resistance to the insect vectors rather than to the viruses they transmit.

For the highly contagious, mechanically transmitted PepMV and PZSV, control depends largely on hygienic measures, as resistant varieties are not yet available. In addition, cross-protection strategies might be developed in production areas with a high infection pressure, provided that only one virus is dominant and that the viral population is homogenous (Lecoq, 1998; Gal-On and Shiboleth, 2006).

Future control strategies for plant virus diseases may include transgenic resistance. Especially the introduction of an inverted repeat (IR) transgene, that is derived from viral sequences and is thus able to trigger gene silencing of the viral gene expression, into the plant genome is a promising strategy (Prins et al., 2008).

Concluding remarks

Viral diseases are an important limiting factor in many crop production systems. Owing to their large population size and short generation time, viruses have a great potential to quickly evolve and adapt under natural selection pressure. Tomato is by far the most important vegetable crop, and many viral species that infect tomato crops have been described. Over the recent years, several viral diseases have emerged in greenhouse tomato crops and are presently impacting the fresh market tomato production in diverse geographic areas worldwide. These are (i) new viral species, (ii) known viral pathogens of other crops that are emerging in tomato, (iii) tomato viruses with a rapidly increasing incidence, and (iv) old viruses that re-emerge due to the cultivation of old varieties that

lack the resistance genes that are commonly exploited in more recently developed commercial tomato varieties. Optimal strategies to reduce the impact of emerging viruses on current and future tomato production systems will most probably consist of an integrated pest management (IPM) approach, in which the most efficient control measures are combined depending on the biology of the virus and its vector (Jones, 2004). Consequently, a good insight in the biology and epidemiology of these viruses is critical for the development of suitable control strategies.

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Thesis Outline

In **Chapter 1**, the most important emerging viruses of tomato crops were reviewed. This includes Pepino mosaic virus (PepMV), a Potexvirus with a single stranded RNA genome which has rapidly become one of the most important viral pathogens of tomato crops worldwide over the recent years, and subject of this thesis research.

In **Chapter 2**, the genetic diversity of the PepMV population in Belgian greenhouses is studied and related to the symptom development in tomato crops. Presently, five PepMV genotypes (EU, LP, CH2, US1 and US2) have been described worldwide, three of which (EU, LP and US2) have previously been reported to occur in Europe. A novel assay based on restriction fragment length polymorphism (RFLP) was developed to discriminate the different PepMV genotypes. Both RFLP and sequence analysis revealed the occurrence of two genotypes, the EU genotype and the CH2 genotype, within Belgian tomato crops, with a clear dominance of the CH2 genotype. Whereas no differences were observed in symptom expression between plants infected by either of the two genotypes, co-infection with both genotypes resulted in more severe PepMV symptoms. Nevertheless, it remained unclear whether different PepMV isolates can cause differential symptom severity. Therefore, the symptomatology that is induced by different PepMV isolates was studied in **Chapter 3**. Based on the survey described in Chapter 2, four isolates that differed in symptom expression in the crop of origin were selected for greenhouse trials. PepMV symptom development was assessed regularly and extensive sampling followed by ELISA analyses, genotyping and nucleotide sequencing was performed to study viral presence and variation in PepMV sequences throughout the trial period. These trials demonstrated that PepMV isolates can cause differential symptomatology as two isolates that were selected based on mild symptom expression in the crop of origin caused only mild symptoms in the trial, while two other isolates that were selected for severe symptom display, caused considerably more severe symptoms.

In **Chapter 4**, seed transmission of PepMV in tomato is demonstrated. Fruit was harvested from the greenhouse trials described in Chapter 3 and over 100,000 seeds were collected. Subsequently, in the framework of a European project, seeds were distributed to 10 different laboratories in three separate batches and germinated for seedling analyses by ELISA. The results show that PepMV can be transmitted through seeds, albeit with low transmission rates.

In **Chapter 5**, the potential of three mild PepMV isolates, belonging to the CH2, EU and LP genotypes, to protect a tomato crop against an aggressive CH2 isolate, was assessed in greenhouse trials. The 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.

A custom-designed Affymetrix tomato GeneChip array that contains probe sets to interrogate over 22,000 tomato transcripts was used in **Chapter 6** to study transcriptional changes in response of tomato to inoculation with the highly similar (99.4% nucleotide sequence identity) mild and aggressive CH2 isolates that are characterized in Chapter 3.

Finally, **Chapter 7**, the general discussion, is a PepMV pathogen profile in which the results obtained in this work are discussed and integrated into a review on the current knowledge on this highly successful pathogen of tomato crops.

Chapter 2:
Genetic characterization of *Pepino mosaic virus* isolates from Belgian greenhouse tomatoes reveals genetic recombination

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Abstract

In just a few years time, *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 to occur 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 the different PepMV genotypes. Both RFLP and sequence analysis revealed the occurrence of two genotypes, the EU genotype, as well as the CH2 genotype, within the 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. Possibly, this may result in generation of viral variants with increased aggressiveness.

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 a 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; Jordá 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, possibly 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 (Jordá et al., 2001). In some tomato cultivation areas up to 90% of the greenhouse tomato plants 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 seventies 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 & 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 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; Pagán 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. 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; Pagán et al., 2006). Apart from the study of Pagán 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 (Pagán et al., 2006). Here, the genetic diversity among PepMV isolates in Belgian greenhouses is studied and related to the 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 untill 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 ten different, randomly chosen tomato plants from the marked rating block (one leave per plant; ten leaves per sample). As such, each sample was supposed to represent 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 2 wells containing all reagents except the sample. In case ELISA testing was inconclusive, PepMV presence was determined by reverse transcriptase PCR (RT-PCR).

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

[a]Greenhouses marked with an asterisk(*) 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 score fruit symptoms	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).

Reverse transcriptase 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; Pagán 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; Pagán 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 fragment 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.

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

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

[a]RdRp= RNA dependent RNA polymerase; CP = coat protein.

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

[c]Sizes (bp) of cDNA fragments are based on theoretical digests of reference sequences retrieved from GenBank (Table 3).

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 three to ten 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).

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, an 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 of 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 ten 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 more than 98% with the CH2 sequence present in GenBank (DQ000985). Altogether, these results illustrate that the CH2 genotype was dominant in the 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; Figure 1). As shown in Figure 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 that were assessed (head, foliage and fruit), the mean scores in the head of the plant show the most significant differences between the different groups (Figure 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 eight 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 also the CH2 genotype was detected, coinciding with a sudden increase in symptom severity in September, especially on the fruits. Scores for fruit marbling increased from one to four between August and October. A concurrent increase in symptom severity was not seen in plants only infected with the EU or the CH2 genotype (data not shown).

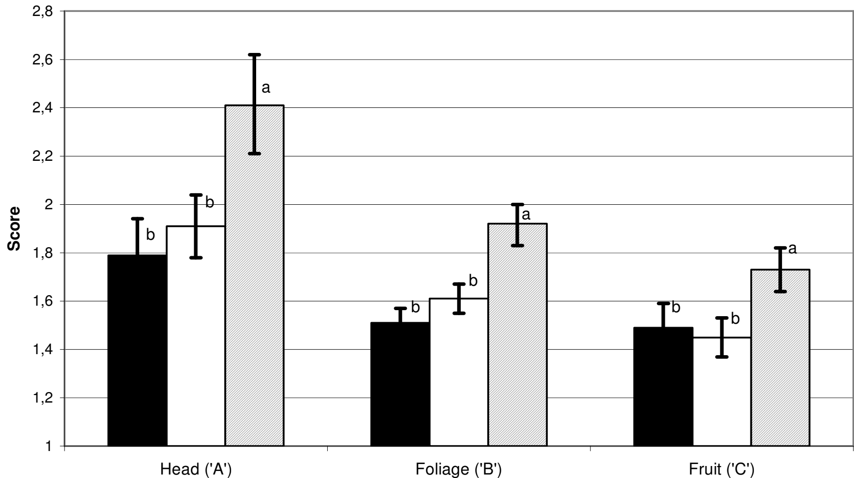


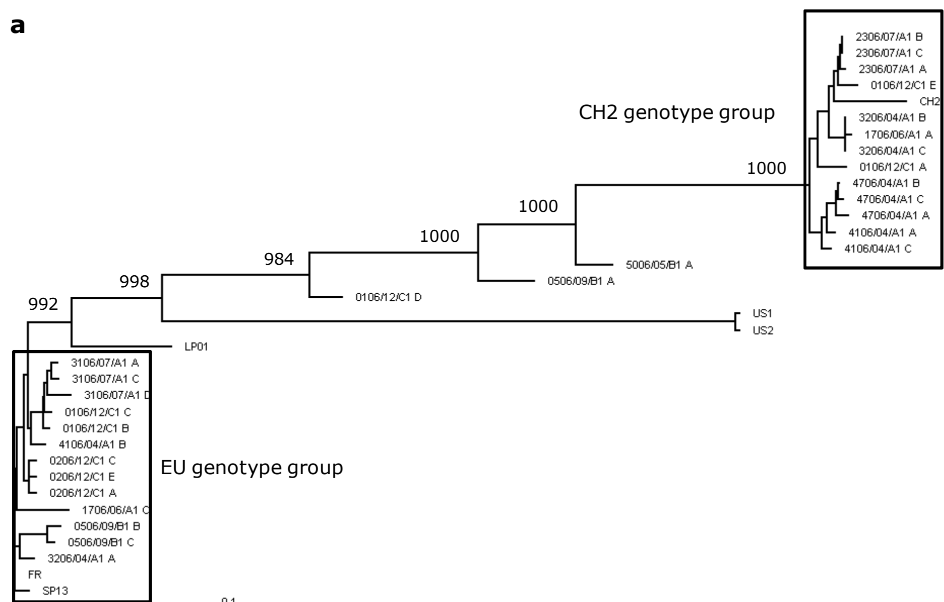
Figure 1. Relation between symptom expression and PepMV genotype. All greenhouses included in the survey were grouped based on the occurrence of a given PepMV genotype, generating three groups (EU genotype in black bars, CH2 genotype in white bars, and mixed infections of EU and CH2 genotypes in grey 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 posthoc *Bonferroni* tests.

Phylogenetic analyses of PepMV isolates from ten 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 ten 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 (Figure 2a), that each share 97% to 100% similarity, while the two groups share only about 80% homology between each other. 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 (Figure 2a). Thirteen sequences (originating from 7 samples) were assigned to the EU genotype, while 13 other sequences (originating from 6 samples) landed in the CH2 genotype group (Figure 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 (Figure 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 genotype was found (Figure 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 five of the ten 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 similar 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.


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
EU genotype group
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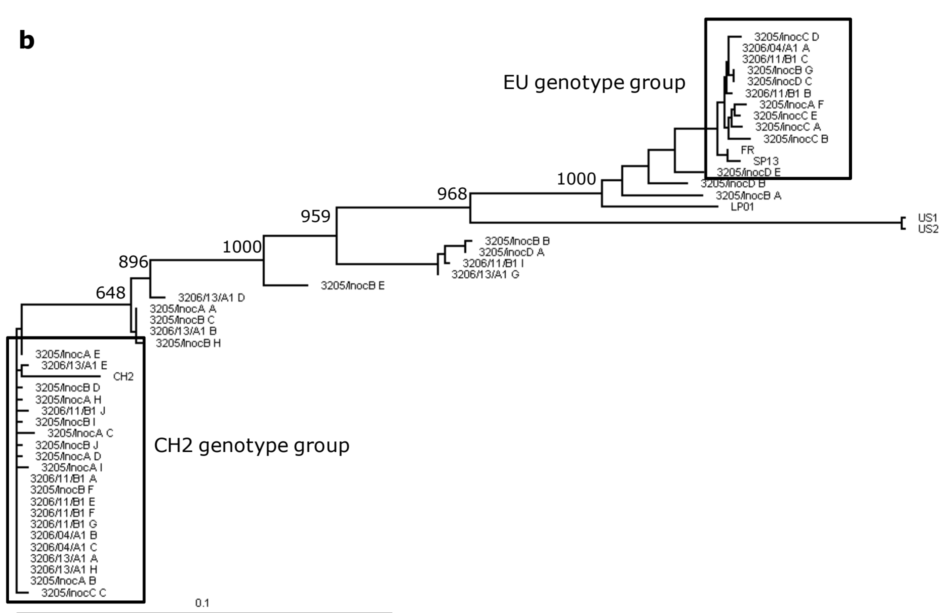
0506/09/B1 C
3206/04/A1 A
FR
SP13
0.1


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
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3206/11/B1 J
3205/InocB I
3205/InocA C
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3205/InocA I
3206/11/B1 A
3205/InocB F
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3205/InocC C
CH2 genotype group
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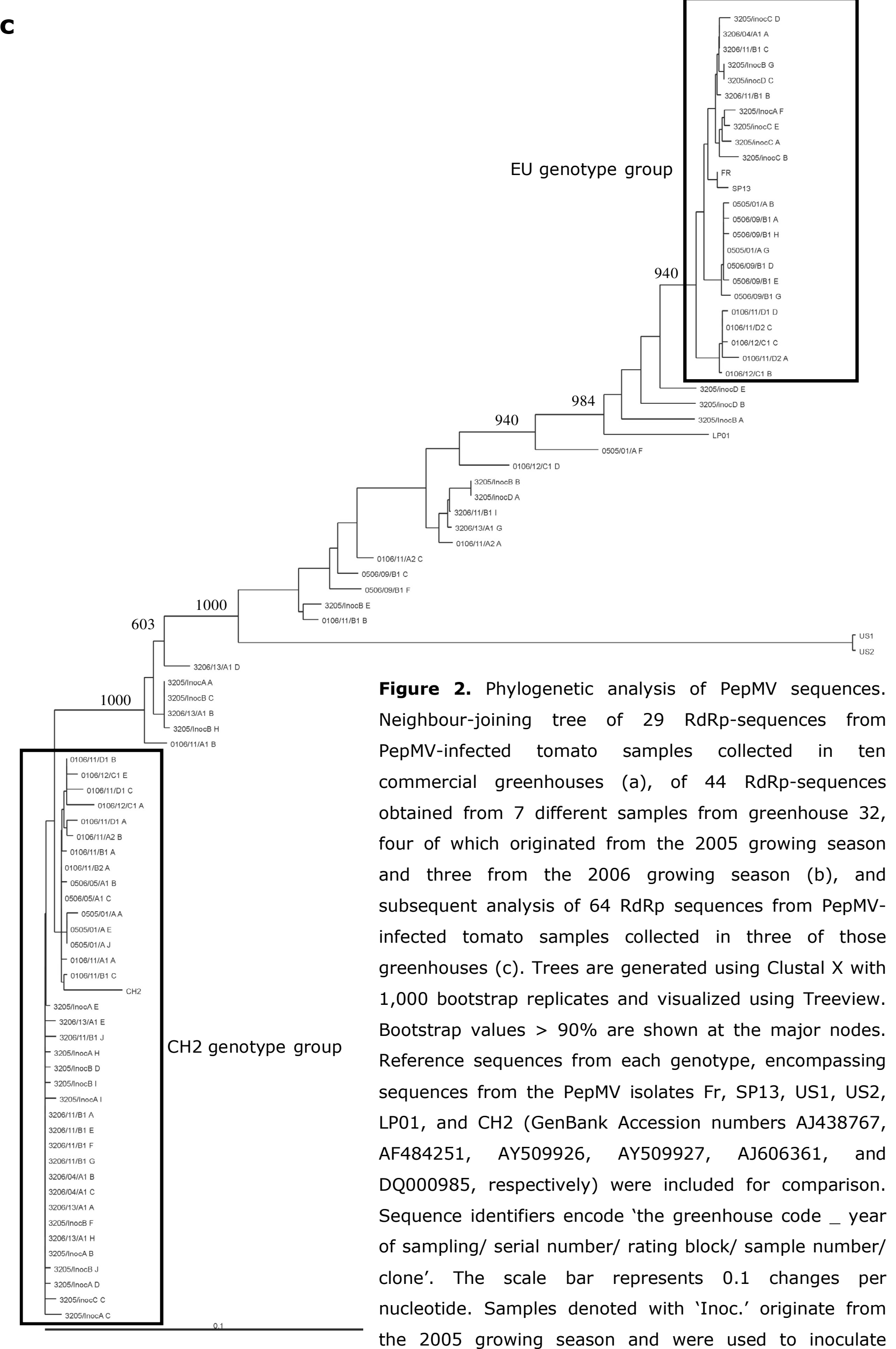

Figure 2. Phylogenetic analysis of PepMV sequences. Neighbour-joining tree of 29 RdRp-sequences from PepMV-infected tomato samples collected in ten commercial greenhouses (a), of 44 RdRp-sequences obtained from 7 different samples from greenhouse 32, four of which originated from the 2005 growing season 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.

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 genotype. For each greenhouse, two samples taken between April and June 2006 were analysed. For two greenhouses (05 and 32), also samples from the 2005 growing season were included. For each sample, RdRp fragments were cloned and eight to ten 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 (Figure 2c). Also in this case the majority of sequences fell within the 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 (Figure 2c).

Detailed sequence analysis of the RdRp fragments showed that 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 Figure 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 identified as well. For example, the recombinant RdRp sequences 3206/11/B1_I and 3206/13/A1_G that were obtained from samples taken at different time points in the same greenhouse were 100% identical. When aligned with other sequences that were 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 share 100% homology over the first 250 nucleotides with sequence 3206/13/A1_A, while the subsequent 375 nucleotides share 100% homology with the EU sequence (Figure 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 (Figure 3b).

a

```
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_CH        ATGAGGTTGTCTGGTGAAGGTCCCACATTTGACGCTAACACTGAGTGTTCGATTGCATAC 60
                          *********************** ** ***** ** ***** ** ***** ** ***** 

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3206/11/B1_I              ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
3206/13/A1_G              ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
3205/InocB_E              ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
CH_3206/13/A1_A_CH        ACTGCCACAAGATTCCATATTGACAATACTATTAAGCAAGTGTATGCCGGTGACGACATG 120
                          ***** *******  *** ****   ***  * ***** ** ***** ** ** ** ***

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3206/11/B1_I              GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
3206/13/A1_G              GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
3205/InocB_E              GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
CH_3206/13/A1_A_CH        GCATTAGATGGAGTTGTGAGTGAAAAGAAATCATTCAGGAAGTTACAAAATCTACTAAAA 180
                          *****************    *****    ** ****  **  **** **    ** ***

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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_CH        CTCACTTCAAAAACGCTGTACCCAAAACAGGTTAAAGGGGATTACGCTGAATTTTGTGGT 240
                          ***** ***** ** ** *  ***************** ***** ******** ******

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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_CH        TGGACTTTCACACCAGGGGGTATAATTAAAAATCCACTTAAAATGCATGCCTCAATTATG 300
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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_CH        CTGCAAGAAGCCATTGGCAATCTGCACACAGCAGCCAGATCTTATGCAATTGACATGAAG 360
                           ******* ** ** ****** * ***** ** ******** ***** ************

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3205/inocD_A              CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
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3206/13/A1_G              CATTCATACCAAATGGGTGATGAGCTGCACAATTACTTAACACCAGATGAAGCTGAACAA 420
3205/InocB_E              CATTCATACCAAATGGGTGACCAACTGCATGACTACTTAACACCAGATGAAGCTGAACAA 420
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3206/13/A1_G              CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
3205/InocB_E              CACTTCCTTGCTGTTCGGAAGTTGCACAAGTTACACCAAGGAGAAGCAATGAGACTTGGT 480
CH_3206/13/A1_A_CH        CATTTCCTAGCTGTGAGAAAGCTTCACAAACTCCATCAAGGCGAGGCCATGCGTCTTGGT 480
                          ** ***** *****  * *** * *****  * ** ***** ** ** *** * ******

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3206/13/A1_G              GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGGTTAAGTTTTCCCCAGTTCGAAATGGA 540
3205/InocB_E              GAAAAGAGCCCTCCAAAAGCAACACATTGAGGGATTAAGTTTTCCCCAGCTCGAAATGGA 540
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                          ** ** ** ** **** * **** **** **** ************** * ********
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3206/13/A1_G           AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 599
3205/InocB_E           AAAATCAACTCTGATTAATTTACTTCAATTGCACCACTTCGAGCCAAAACTCAGTGTTGA 600
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                       ** ******* **** **********  ** *** * ** ** *  **  * *     **

EU_3206/04/A1_A        AGGAATCATAGTTGTGCACGGAATT 625
3205/inocD_A           AGGAATCATAGTTGTGCACGGAATT 624
3206/11/B1_I           AGGAATCATAGTTGTGCACGGAATT 625
3206/13/A1_G           AGGAATCATAGTTGTGCACGGAATT 624
3205/InocB_E           AGGAATCATAGTTGTGCACGGAATT 625
CH_3206/13/A1_A_CH     AGGAATCATAGTTGTGCACGGAATT 625
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b

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3206/13/A1_G         LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
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CH_3206/13/A1_A      LTSKTLYPKQVKGDYAEFCGWTFTPGGIIKNPLKMHASIMLQEAIGNLHTAARSYAIDMK 120
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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
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3206/13/A1_G         LHHFEPKLSVEGIIVVHGI 199
3205/InocB_E         LHHFEPKLSVEGIIVVHGI 199
CH_3206/13/A1_A      LHKFEHKINTEGIIVVHGI 199
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Figure 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). Figure 3a 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). Sequences of the primers to check for recombination are underlined. Figure 3b 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.

To eliminate the possibility that the recombinant sequences were generated by artefacts during the RT-PCR, an 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. In addition, primers were developed to specifically detect the obtained recombinant sequences, with a forward primer (5′ TGAAGGTCCCACATTTGACGC 3′) specific for the CH2 genotype and a reverse primer (5′ CTGAGTTTTGGCTCGAAGTGG 3′) specific for the EU genotype (Figure 3a). Under stringent PCR conditions, with an annealing temperature of 66°C, an amplicon of the expected size was obtained only in samples containing recombinant sequences, and not in samples containing only the CH2 or the EU genotype, nor in a mixture of two samples containing only the CH2 or the EU genotype. These results provide further evidence for recombination events to occur in tomato plants co-infected with both PepMV genotypes.

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 occurs 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 infected already at an early stage of the 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 three weeks after inoculation only the CH2 genotype could be detected, while only two months later also the presence of the EU genotype could 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 (Pagán 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 Pagán 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 that are 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 (Figure 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 recombination events within complete viral RNAs have not been described yet 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 (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 (Figure 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 by only 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 2000 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.

Acknowledgements

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Chapter 3:
Pepino mosaic virus isolates and differential symptomatology in tomato

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Abstract

After a first detection in 1999, *Pepino mosaic virus* (PepMV) swiftly spread through greenhouse tomato production worldwide. Currently, three different PepMV genotypes (the European tomato (EU), the CH2 and the Peruvian (LP) genotype) have been reported to occur in European tomato production. So far, it is unclear whether different viral isolates cause different symptom severity. Based on a survey conducted in commercial tomato production in Belgium in 2006, four PepMV isolates that differed in symptom expression in the crop of origin were selected for greenhouse trials. The selected isolates were inoculated onto tomato plants grown in four separate plastic tunnels. PepMV symptom development was assessed regularly and extensive sampling followed by ELISA analyses, genotyping and sequencing was performed to study viral presence and variation in PepMV sequences throughout the trial period. Two isolates (EU mild and CH2 mild) that were selected based on mild symptom expression in the crop of origin caused only mild symptoms in the trial, while two other isolates (CH2 aggressive and EU+CH2) that were selected for severe symptom display, caused considerably more severe symptoms. Sequence homology between CH2 mild and CH2 aggressive was as high as 99.4%. Results of this study show that differential symptom expression can, at least partially, be attributed to the PepMV isolate, which may be related to minor differences at the nucleotide level between isolates.

Introduction

Over recent years, tomato (*Solanum lycopersicum*) greenhouse crops are increasingly affected by the highly contagious *Potexvirus Pepino mosaic virus* (PepMV), which was first described in 1980 on pepino (*Solanum muricatum*) in Peru (Jones et al., 1980). Infection of tomato was not reported until 1999, when the virus was first detected in tomato greenhouse crops in the Netherlands (van der Vlugt et al., 2000). Since then, a fast spread of the virus occurred throughout tomato production worldwide, with official reports of PepMV incidence from Spain, France, Italy, the United Kingdom, Poland, Belgium, the United States and Canada (Soler et al., 2000; French et al., 2001; Jorda et al., 2001; Mumford & Metcalfe, 2001; Roggero et al., 2001; Cotillon et al., 2002; Pospieszny et al., 2006; Hanssen et al., 2008).

The PepMV viral RNA genome is approximately 6400 nucleotides long and, similar to other potexviruses, contains five open reading frames (ORFs) including a replicase gene, a triple gene block (TGB) and a coat protein gene (CP). Sequence data revealed a high genome identity (approximately 99%) between isolates from different European countries (Aguilar et al., 2002; Cotillon et al., 2002; Mumford & Metcalfe, 2001; Verhoeven et al., 2003). Since these European isolates showed only 96% sequence

homology to a Peruvian PepMV isolate (LP 2001), they are considered as a distinct genotype and further referred to as the EU tomato genotype (Lopez et al., 2005). Two other PepMV isolates, originating from diseased tomato plants in the United States (US1 and US2; Maroon-Lango et al,. 2005), displayed between 79% and 82% homology to the EU tomato genotype. A fifth genotype, the so-called CH2 genotype which showed more sequence homology with the US2 genotype (91%) than with the EU tomato genotype (about 80%), was found on contaminated tomato seeds originating from Chile (Ling, 2007).

A phylogenetic study in Spanish tomato production revealed the presence of the Peruvian and US2 genotypes in addition to the EU tomato genotype of PepMV (Pagan et al., 2006). Recently, the CH2 genotype has spread throughout Europe and was reported to occur in Belgium and Poland (Hanssen et al., 2008; Hasiów et al., 2008), but has also been identified in France, the Netherlands and Morocco (Hanssen et al., unpublished data). Genetic characterisation of PepMV isolates from Belgian greenhouse tomatoes demonstrated dominance of the CH2 genotype over the EU tomato genotype and the frequent occurrence of recombinants between both genotypes in mixed infections (Hanssen et al., 2008).

Initially, symptoms such as nettleheads, dwarfing, leaf distortions, leaf mosaics, yellow rectangular leaf spots and marbling or flaming of fruit were associated with PepMV infection in tomato. As leaf or head symptoms were usually mild and persisted only for a short period after infection, fruit discoloration was generally considered the most devastating consequence of PepMV infection (Soler et al., 2000; Roggero et al., 2001; Spence et al., 2006). Nevertheless, since the emergence of new PepMV genotypes, the symptom severity seems to be increasing as not only the common leaf and head symptoms are becoming more persistent and severe, but also new symptoms (e.g. leaf scorching or premature leaf senescence, open fruit and scars on the fruit surface) are observed (Spence et al., 2006; Hanssen et al., 2008). So far, no correlation has been observed between different PepMV genotypes and the severity of symptom expression in infected tomato plants (Pagan et al., 2006; Hanssen et al., 2008). However, co-infection with both genotypes resulted in enhanced PepMV symptoms (Hanssen et al., 2008). A detailed follow-up of symptom display in Belgian greenhouse tomatoes infected with PepMV in 2006 gave rise to the hypothesis that severity and nature of symptoms induced in tomato plants differ between isolates, and even within the same genotype. From this survey four PepMV-isolates, obtained from these naturally infected commercial tomato crops were selected for further study. Here, we compared PepMV symptom expression caused by those four different PepMV isolates in greenhouse trials. In addition, complete genome sequences were determined and sequence evolution during the infection was assessed.

Materials and Methods

Experimental design

From May to October 2007, a greenhouse trial was conducted in five separate plastic tunnels to assess PepMV symptom expression upon inoculation with different PepMV isolates. Tomato seeds (cultivar Tricia, De Ruiter Seeds, Bergschenhoek, the Netherlands) were disinfected with sodium hypochlorite (1° active chlorine for 30 min) and rinsed thoroughly with tap water. Subsequently, the seeds were germinated on rockwool trays in a lettuce greenhouse facility, isolated from tomato production facilities to prevent PepMV infection. Five weeks after sowing, 100 tomato plants were transferred to each of the tunnels. One month after planting, the tomato plants in four separate tunnels were individually inoculated with four different PepMV strains, while plants in a fifth tunnel were mock-inoculated with phosphate buffer.

Based on a survey conducted in 2006 in commercial tomato production greenhouse facilities in Belgium, four PepMV isolates ('1806', '1906', '0506' and 'PCH 06/104') obtained from different greenhouses with distinct PepMV symptom expression in tomato were selected (Hanssen et al., 2008). Here, a PepMV isolate is defined as the viral inoculum derived from PepMV infected plants from one specific tomato production site. Inoculation was performed by rubbing one lower leaf per plant with an extract of infected tomato leaf material, prepared by grinding 30 g of leaf material in 60 ml of phosphate buffer (pH 7.4). Viral concentration in the PepMV inoculum of the four isolates was standardized by using infected leaf material with a similar viral titre as determined by ELISA. This method was verified to result in 100% disease incidence.

Each tunnel with 100 plants was divided in ten sampling blocks, each containing ten adjacent plants. Until the fourth week post inoculation (WPI), a weekly sample consisting of a single leaf from the head of each of the 10 plants in the sampling block was taken from all 10 sampling blocks per tunnel. After four weeks, sampling was performed once in two weeks. An overview of the sampling schedule is given in Table 1.

Genetic characterization of PepMV isolates

The genotypes of PepMV isolates were determined using a previously described RT-PCR-RFLP method (Hanssen et al., 2008). Whole genome sequences of the three isolates containing a single genotype (EU mild, CH2 mild and CH2 aggressive) were determined by amplifying, cloning and sequencing seven partially overlapping regions of the PepMV genome (Table 2). 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 (Macrogen Inc., Seoul, South Korea). Alignment of the full genome sequences was performed using the CLUSTAL X algorithm (Thompson et al., 1997). In

addition, for the EU+CH2 isolate a 625bp fragment of the genome referred to as RdRp (Pagan et al., 2006; Hanssen et al., 2008), obtained using primers Pep3 and Pep4 (Table 2; Mumford & Metcalfe, 2001) was cloned and a total of ten clones was sequenced.

Table 1. Schedule of sampling and *Pepino mosaic virus* analyses on tomato

WPI[a]	Sampling date [b] ('07)	PepMV analyses		
		ELISA	Genotyping [c]	Sequencing [d]
1	20/06	10 samples per tunnel	/[e]	1 sample per tunnel, 7-10 clones
2	29/06	10 samples per tunnel	/	/
3	05/07	10 samples per tunnel	/	1 sample per tunnel, 7-10 clones
4	13/07	10 samples per tunnel	/	/
6	27/07	10 samples per tunnel	3 samples per tunnel (sampling block 1, 5 and 10)	/
8	10/08	10 samples per tunnel	/	/
10	24/08	10 samples per tunnel	/	/
12	07/09	10 samples per tunnel	/	1 sample per tunnel, 7-10 clones
14	20/09	10 samples per tunnel	/	/
16	01/10	10 samples per tunnel	3 samples per tunnel (sampling block 1, 5 and 10)	1 sample per tunnel, 7-10 clones

[a] WPI = weeks post inoculation

[b] A sample consisted of a mixture of 10 young leaves (one from each plant in the sampling block) from the heads of the plants. As the heads of the plants were pruned in the sixth WPI, leaves were taken from young shoots in the highest plant parts after six WPI.

[c] by RT-PCR-RFLP

[d] RdRp fragment, after cloning (analysis not performed for EU+CH2 isolate '0506')

[e] analysis not performed

Table 2. Primer sets used for amplification and sequence determination of the *Pepino mosaic virus* genome

Primer[a]	Target region	5'Position [b]	Sequence (5'-3')	T_{ann}[c]	Amplicon size
Apa15	Replicase gene	36	CTAACACAACATAACCACG	57°C	1172
Rep1-R1		1190	GTTGCATGGGTGCAACCA		
Rep2-F	Replicase gene	1075	GAATTGTATGACCCTGATG	54°C	1316
Rep2-R		2371	GGTTGAATCATTGCTTTCTC		
Rep3-F2	Replicase gene	2166	TCAAAATGCAACATGAAGAC	54°C	1105
Rep3-R		3252	GTTGATGTTGGAAAAGTTG		
Rep4-F1	Replicase gene	2951	ACACCATATCTCAAAGC	51°C	1160
Rep4-R		4094	CCTTTAACCTGTTTTGG		
Pep3-F [d]	Replicase gene	3893	ATGAGGTTGTCTGGTGAA	53°C	625
Pep4-R [d]		4500	AATTCCGTGCACAACTAT		
Apa23-F	Triple gene block	4411	GTTTTCCCCAGTTTGAAATGG	54°C	1147
Apa25-R		5537	CCAAGGGGAGAAGTTGATTGC		
Ker1	Coat protein gene	5379	CACCAATAAATTTAGTTTTAGC	56°C	996
FL-R		6359	AGAAAACCCACTCTGA		

[a] F is sense primer, R is antisense primer

[b] reference sequence CH2, GenBank accession number DQ000985

[c] Annealing temperatures

[d] Mumford & Metcalfe, 2001.

Evaluation of PepMV symptoms

During the trial period, at ten time points (0, 1, 2, 4, 5, 6, 8, 9, 10 and 14 WPI) 30 individual plants from each treatment were examined for PepMV symptoms. In addition, at three points in time (3, 7 and 12 WPI) all 100 plants were evaluated. In each case, the head of the plants, the foliage and stem, and the fruit were examined. Symptoms were rated using a scale from one (not present) to four (severe symptom display) (Table 3). In addition, at 9, 13 and 15 WPI all tomatoes in the mature clusters from each treatment were examined to determine the percentage of marbled and flamed tomatoes.

Determination of PepMV presence and infection level

All plant samples were analysed for PepMV presence using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using a commercial antiserum (Agdia Inc., Elkhart, USA), 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. Assay reproducibility was confirmed by including a duplicate sample of a standardized positive control of known viral concentration in each ELISA analysis. A mean OD value of 3.76 with a 95% confidence interval of 3.72 till 3.80 was obtained for the positive controls in each of the ELISA analyses, demonstrating the reproducibility of the analysis. The linear range of OD values was determined using a dilution series of the positive control of known viral concentration and ranged from 0.6 to 3.8. Since nearly all OD values for the samples from this trial fell within this range, the sample OD was considered as an indication for the PepMV viral titre. OD values per tunnel and per sampling point were determined by calculating the mean of the ten OD values obtained for each sampling block.

Viral genome variation

For the three single isolates used in this study, viral genome variation was studied by cloning and sequencing the 625bp RdRp fragment (Mumford & Metcalfe, 2001; Pagan et al., 2006; Hanssen et al., 2008). From each treatment, four samples obtained at different time points (1, 3, 12 and 16 WPI), were used. Amplified products were directly cloned and sequenced (seven to ten clones per sample) as described above. Multiple sequence alignments were performed using CLUSTAL X (Thompson et al., 1997) and neighbour-joining trees were constructed and displayed using TREEVIEW v. 1.6.6 (Page, 1996).

Table 3. *Pepino mosaic virus* symptom rating scale for tomato

Plant part	Symptom type	Score	Description	Picture
Head[a]	Nettle-head	1	Absent	
		2	Leaves are somewhat pointed and upright with a slightly reduced surface	
		3	Leaves are pointed, upright or curled, with a reduced surface	
		4	Leaves resemble nettle leaves, with a serrated leaf margin and a reduced surface	
	Leaf bubbling	1	Absent	
		2	One bubbled leaf[b]	
		3	Two to four bubbled leaves[b]	
		4	All leaves are bubbled[b]	
Foliage[c]	Premature leaf senescence	1	Absent	
		2	Scorching – leaflet margins	
		3	Scorching – entire leaflets of min. one leaf	
		4	Scorching – more than one leaf	
Fruit	Marbling	1	Absent	
		2	One marbled fruit[b]	
		3	Two marbled fruits[b]	
		4	More than two marbled fruits[b]	
	Flaming	1	Absent	
		2	One flamed fruit[b]	
		3	Two flamed fruits display flaming[b]	
		4	More than two fruits display flaming[b]	
	Open fruit	1	Absent	
		2	One open fruit[b]	
		3	Two open fruits[b]	
		4	More than two open fruits[b]	
	Necrosis of the sepals	1	Absent	
		2	One fruit with sepal necrosis[b]	
		3	Two fruits with sepal necrosis[b]	
		4	More than two fruits with sepal necrosis[b]	

[a]Upper youngest leaves (planttop)
[b]Per plant
[c]Lower leaves

Results

Differential PepMV infection levels

A greenhouse trial was conducted in five separate plastic tunnels that each contained 100 cluster tomato plants, to assess PepMV symptom expression upon inoculation with four different PepMV isolates that were obtained from commercial greenhouse tomato crops with distinct PepMV symptoms (Hanssen et al., 2008). PepMV isolate '1806' (EU mild) belonged to the EU tomato genotype and did not cause typical PepMV symptoms in the tomato crop of origin. Isolate '1906' (CH2 mild) belonged to the CH2 genotype and caused mild symptoms. Isolate 'PCH 06/104' (CH2 aggressive) also belonged to the CH2 genotype but caused severe PepMV symptoms. Finally, isolate '0506' (EU+CH2), containing both the EU tomato and the CH2 genotype, caused severe symptoms. To verify that only the inoculated genotype was present in the PepMV infected plants, genotyping analyses were performed using a previously described RT-PCR-RFLP genotyping method (Hanssen et al., 2008) on samples harvested at six and 16 WPI (Table 1), confirming that no cross-contamination occurred between treatments throughout the trial period (data not shown).

ELISA analyses on samples taken one day before inoculation confirmed that the plants were not infected with PepMV prior to inoculation. One week after inoculation (1 WPI), however, the majority of the samples tested positive for PepMV. For the EU-mild and CH2-aggressive inoculation, three out of ten sampling blocks tested negative, while in the CH2-mild and the EU+CH2 inoculation, only one sampling block tested negative. At 2 WPI, all sampling blocks tested positive, except for the same three sampling blocks of the EU-mild inoculation. These blocks tested positive from 3, 4 and 6 WPI onwards, respectively. These results suggest that the EU mild isolate slower colonizes the tomato crop as compared to the other isolates (Figure 1). The viral titres observed in the different samples appeared to be different for the various inoculations (Figure 1). The OD values obtained for the EU mild isolate were considerably lower at most of the sampling points, although plants in this tunnel displayed the largest variation in viral titres between subsequent sampling points (Figure 1). At the end of the trial period, all samples contained a similar viral load (OD ~ 2.5), except for the CH2+EU inoculated plants (OD ~ 3.8), which contained a minimum 10 times higher viral concentration. Samples from the mock-inoculated control tunnel remained negative in the ELISA assay throughout the entire trial period.

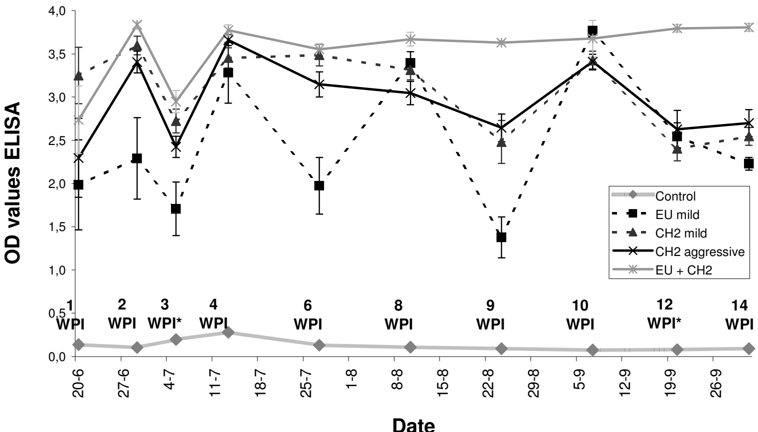


Figure 1. Viral accumulation in the different *Pepino mosaic virus* inoculations on tomato, displayed as mean optical density values obtained from 10 samples per tunnel (one sample from each sampling block) and per sampling point (indicated using the number of weeks post inoculation (WPI) and the actual date on the horizontal axis), throughout the trial period. Standard errors bars are indicated for each measurement. Each sample consisted of a mixture of 10 young leaves (one from each plant in the sampling block) from the heads of the plants. As the heads of the plants were pruned in the sixth WPI, leaves were taken from young shoots in the highest plant parts after six WPI.

Differential symptom expression upon PepMV inoculation

The typical PepMV symptoms were monitored at regular intervals during the entire trial period (Figure 2; Table 3). The same thirty plants were monitored weekly per treatment, except for three, seven and 12 WPI when all 100 plants were monitored.

Typical nettlehead symptoms were seen from two WPI onwards (Figure 2a). From two till five WPI, the most severe display of nettleheads was monitored in plants inoculated with the CH2 aggressive and the EU+CH2 isolate, with the most sever nettlehead scores at six WPI. The CH2 mild inoculation, in contrast, resulted in considerably less severe symptoms. No nettlehead-like symptoms were seen in the EU mild inoculated plants or in the mock-inoculated control (Figure 2a). A similar pattern of symptom expression was recorded for bubbling of the leaf surface in the head of the plants (Figure 2b). At six WPI, scoring of nettlehead symptoms and bubbling of the leaf surface was terminated because the heads of the plants were pruned. On the foliage and stems, the incidence of necrosis was assessed. Necrosis on the stem did not occur, but premature senescence of the leaves observed as chlorosis and necrosis (Table 3), was prevalent in the trial mainly between three and nine WPI. Again, plants inoculated with

the CH2 aggressive isolate and the EU+CH2 isolate exhibited the most severe symptoms with significantly ($p<0.05$ at most sampling points) more premature leaf senescence throughout the trial period, symptoms that were not observed in the EU mild inoculated plants or the mock-inoculated control (Figure 2c).

With respect to fruit quality, scores were given for fruit marbling, fruit flaming or blotchy ripening, incidence of scars and open fruits, and necrosis or browning of the sepals (Table 3). In weeks 9, 13 and 15 all ripe tomatoes were rated for fruit marbling (Figure 3a). Marbling was most prevalent in the EU+CH2 inoculated plants, with almost 10% of the ripe tomatoes displaying severe marbling at 13 WPI. While fruit marbling is a very specific virus related symptom, flaming of tomatoes is a problem that can also be induced by environmental conditions in an otherwise healthy tomato crop. In this trial, PepMV infection significantly increased the number of flamed or discoloured tomatoes (Figure 3b), especially for the CH2 aggressive isolate. Open fruit is defined as tomato fruit that splits shortly after fruit setting, such that the seeds are visible in the flesh of the mature tomato (Table 3). From four WPI onwards, the incidence of scars and open fruit was significantly higher ($p<0.05$ at all sampling points from 4 to 12 WPI) in the CH2 aggressive inoculated plants as compared to the other inoculations (Figure 2d). In these plants, the overall percentage of clusters with at least one open fruit was 10%, as compared to 4% and 3% in plants inoculated with EU+CH2 and EU mild, respectively.

In this trial it was clearly shown that PepMV can cause sepal necrosis (Table 3), a symptom that is not typically associated with PepMV infection, but that radically reduces the commercial value of cluster tomatoes. A high incidence was recorded in plants infected with the CH2 isolates and in the mixed infection, with the CH2 aggressive inoculation resulting in the highest score at five WPI, followed by the CH2 mild and EU+CH2 inoculations (Figure 2e). No sepal necrosis was seen in the control treatment or in the EU mild inoculation.

Plant vigour and yield were assessed by counting the number of tomato clusters, as well as the total number of tomatoes per plant. Plants in the mock-inoculated control produced significantly more tomatoes per cluster than plants inoculated with the four PepMV isolates (Figure 3c). No significant differences were seen between plants inoculated with the EU mild, CH2 mild and CH2 aggressive isolates. However, the number of clusters per plant was significantly lower for plants inoculated with the EU+CH2 isolate (Figure 3c).

Overall, it can be concluded that the isolates CH2 aggressive and EU+CH2 caused significantly more severe symptoms than the isolates EU mild and CH2 mild.

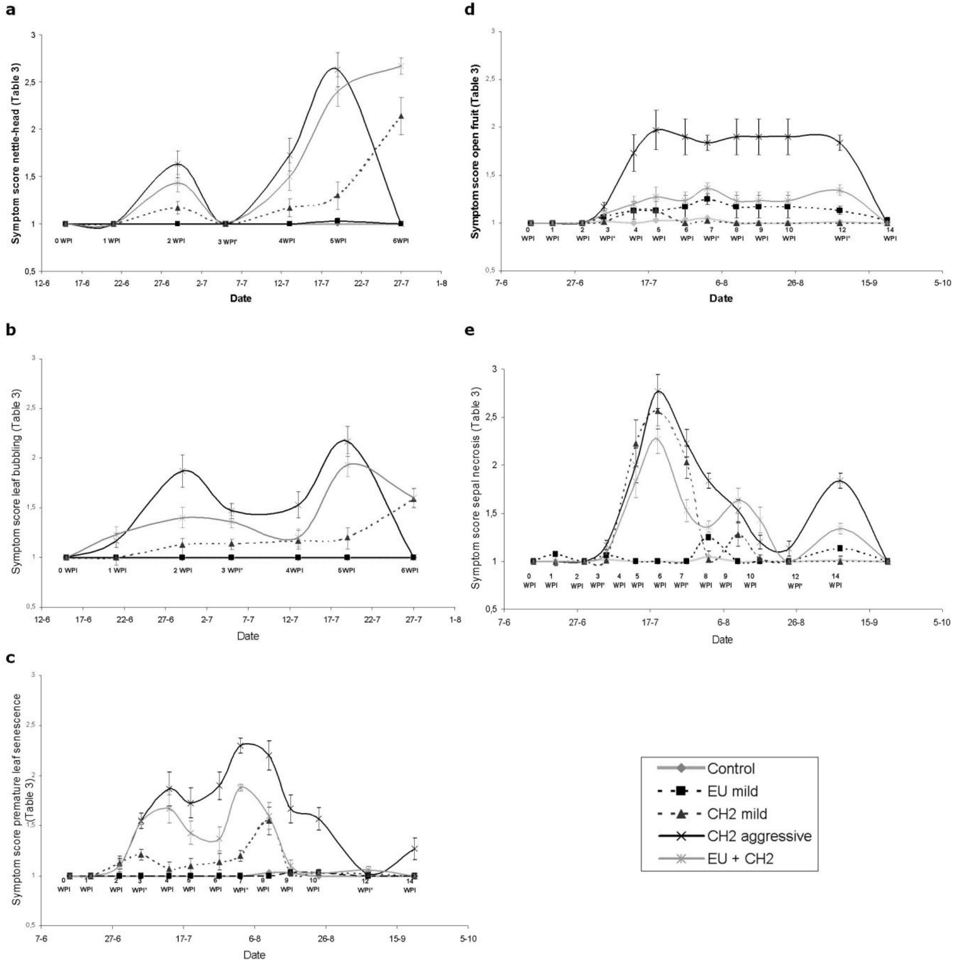
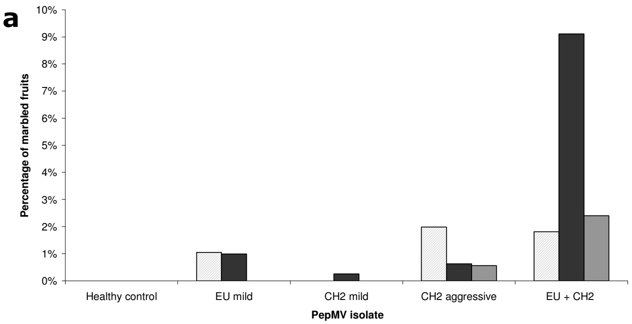
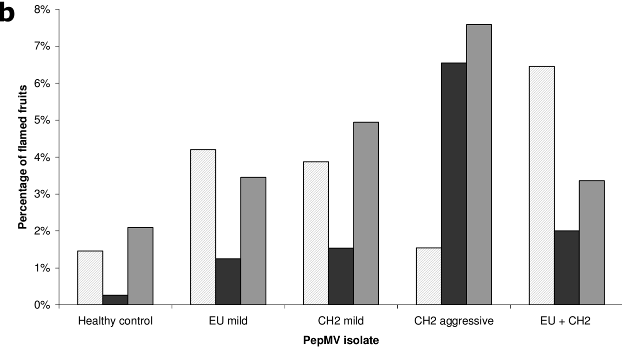


Figure 2. *Pepino mosaic virus* (PepMV) symptom expression on tomato, presented as the evolution of symptom scores (Table 3) in tomato plants inoculated with the different PepMV isolates, throughout the trial period. Plants were inoculated with PepMV on June 13th. Each point represents the mean of 30 scores obtained from 30 plants at a certain time point, indicated using the number of weeks post inoculation (WPI) and the actual date on the horizontal axis. Time points marked with * represent the means of 100 scores obtained from 100 plants. Standard errors bars are indicated for each measurement. (a) Nettlehead (scores were given until the end of July, when plants were topped); (b) leaf bubbling in the head of the plants (scores were given until the end of July, when plants were topped); (c) Premature leaf senescence (necrosis and/or chlorosis); (c) open fruit; (e) browning of sepals.





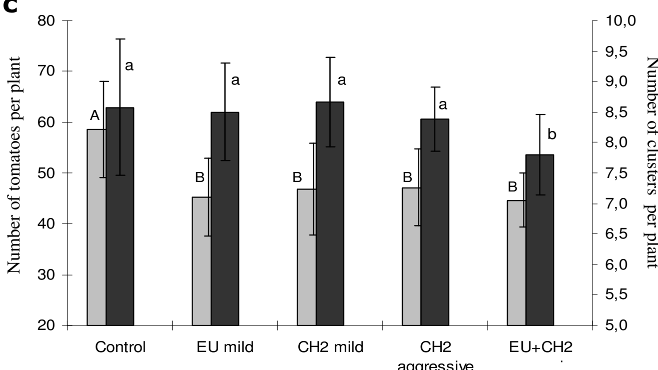


Figure 3. *Pepino mosaic virus* (PepMV) damage on tomato quality and fruit setting, caused by the different PepMV isolates. Percentage of tomatoes showing marbling (a) and flaming (discoloration and blotchy ripening) (b) at three different time points. Time point 9 weeks post inoculation (WPI) is shown in diagonally striped bars; time point 13 WPI is shown in dark grey bars and time point 15 WPI is shown in light grey bars; (c) Fruit setting: total number of tomatoes (left vertical axe – light grey bars) and clusters (right vertical axe – dark grey bars) per plant for the four different inoculations and the control, over the entire growing period. Statistical differences are indicated with a and b for the number of clusters and with A and B for the number of tomatoes (One-Way ANOVA, post-hoc Bonferroni, $p<0.05$). The error bars represent standard errors.

Comparison of PepMV whole genome sequences

The whole genome sequences, except for the 5' and 3' untranslated regions (UTR), were determined for the three single isolates EU mild, CH2 mild and CH2 aggressive. Several primer sets targeting seven partially overlapping regions of the PepMV genome were used for sequence determination of in total 6291 nt of the PepMV genome (Table 2). While the sequence homology between the EU mild isolate and both CH2 isolates (CH2 mild and CH2 aggressive) was only 79%, as could be expected (Ling, 2007; Hanssen et al., 2008), homology between both CH2 isolates was as high as 99.4% and the isolates differed only in 38 single nucleotide polymorphisms (SNPs). Collectively, these SNPs cause only nine amino acid differences in the respective predicted proteins between both CH2 isolates: three in ORF1; two in ORF2; one in ORF3; one in ORF4 and two in ORF5 (Table 4). The UTR between ORF1 and ORF2 (25 nt) was highly conserved between the PepMV isolates, with only one SNP between the EU and the two CH2 isolates and complete homology between the latter two, while the size of the UTR between ORF 4 and ORF5 varied between the EU mild isolate (38 nt) and both CH2 isolates (45 nt). A single SNP was found in this region when comparing the CH2 mild and the CH2 aggressive isolate. Sequencing of ten clones obtained from the 625bp RdRp fragment of the EU+CH2 isolate revealed the presence of three genotypes, including the EU tomato genotype, the CH2 genotype and a recombinant genotype.

Table 4. Amino acid polymorphisms in the predicted protein sequences of *Pepino mosaic virus* isolates CH2 mild and CH2 aggressive

	Position[a]	'1906' CH2 mild	'PCH 06/104' CH2 aggressive
ORF1	504	Glutamic acid	Alanine
	995	Valine	Isoleucine
	1051	Threonine	Serine
ORF2	154	Valine	Alanine
	192	Serine	Proline
ORF3	97	Serine	Asparagine
ORF4	24	Alanine	Threonine
ORF5	48	Threonine	Isoleucine
	244	Alanine	Threonine

[a] Distance from the first amino acid of the protein

Variation of partial PepMV genome sequences

The variation of a 625 bp fragment of the replicase gene and the first ORF of the TGB was studied for the isolates EU mild, CH2 mild and CH2 aggressive throughout the trial period. The nucleic acid sequences obtained from samples taken at 1, 3, 12 and 16 WPI were compared (Table 1). For all three isolates, minor differences were observed between the sequences obtained from different clones and different time points. In isolate EU mild, only silent mutations were observed (data not shown). All sequences from this isolate obtained after three WPI differed in one base pair (position 348: A to C substitution) from the sequences obtained at one WPI, suggesting that a stable but silent

point mutation had occurred. Similarly, sequences obtained from both CH2 isolates displayed a number of SNPs at different time points, although most of the point mutations were again silent (Figure 4). For both CH2 isolates, also missense point mutations, leading to amino acid changes, were observed (Figure 4). None of these mutations in the studied genome region were stable as they had disappeared by sixteen WPI.

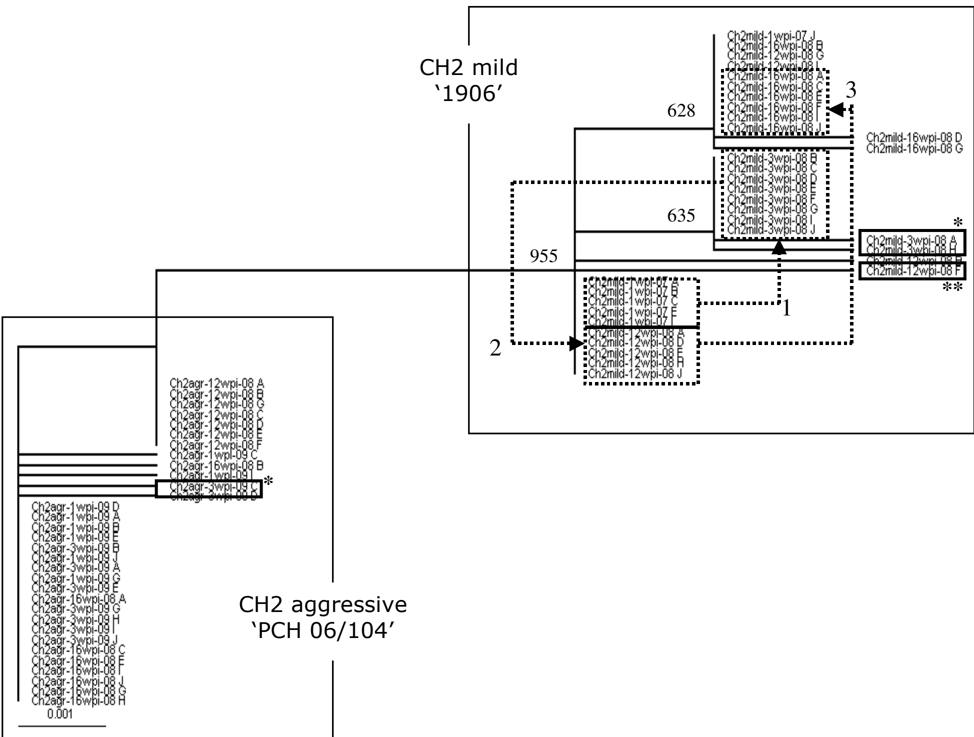


Figure 4. Sequence variation of the *Pepino mosaic virus* isolates CH2 mild and CH2 aggressive, presented as a neighbour joining tree of 67 sequences obtained from samples taken at 4 different time points. Sequence identifiers encode 'inoculation _ sampling time point - sampling block - clone'. The predicted translated sequences are identical for all sequences, except for the sequences marked with * (one amino acid substitution) or ** (two amino acid substitutions). Dotted arrows indicate reversible mutations in the CH2 mild isolate (1: one to three weeks post inoculation (WPI); 2: three to 12 WPI; 3: 12 to 16 WPI).

Discussion

Greenhouse trials were conducted to compare the symptoms caused by four different PepMV isolates, originally isolated from four different commercial Belgian tomato crops which differed considerably in PepMV symptom display. As the impact of environmental growth conditions and tomato genotype on PepMV symptom development is not yet fully understood, it was not clear whether the differences in symptom display in these commercial tomato greenhouses should be attributed to the viral isolate, or to environmental factors and cultural practices. In this study, the environmental conditions and plant genotype were standardized in order to study the impact of the viral isolate. Our analysis strongly suggests that the viral isolate largely determines symptom development. The EU isolate '1806' (EU mild) and the CH2 isolate '1906' (CH2 mild), which caused only mild symptoms in the crop of origin, caused rather mild symptoms in our analysis, while the CH2 isolate 'PCH06/104' (CH2 aggressive) and the mixed infection isolate '0506' (EU+CH2) that originated from two different tomato crops with severe PepMV symptom display, resulted in the most severe PepMV damage in our analysis. Generally, the CH2 aggressive isolate caused the most severe fruit and leaf symptoms throughout the trial period, followed by the EU+CH2 isolate. The latter caused considerably more fruit marbling than all the other isolates.

Interestingly, significant differences in symptom severity were recorded for isolates belonging to the same genotype. The occurrence of open fruit appeared to be associated with the CH2 aggressive isolate. This isolate was clearly more aggressive than the CH2 mild isolate, although only minor differences were found in the genome and amino acid sequences of both isolates. A total of 38 SNPs was found in the 6291 nt sequence that was determined when comparing both isolates, resulting in only nine differences at the predicted amino acid level. The SNPs were not concentrated in a specific region of the genome. These results confirm the hypothesis that minor differences at the nucleotide level can account for biological differences between isolates. A comparative study using test plants with fourteen EU tomato strain isolates, displaying 99.1 to 100% nucleic acid sequence homology in a 547 nt fragment of the replicase gene, revealed minor biological differences upon inoculation of test plants (Verhoeven et al., 2003). Comparison of the whole genome sequence of two EU tomato isolates differing slightly in symptom expression revealed 99% homology on the nucleotide level (López et al., 2005). Nevertheless, it remains unclear which regions of the PepMV genome are involved in the expression of symptoms.

ELISA analyses revealed a slower colonisation of the tomato crop by the EU mild isolate as compared to the other isolates. As plants were inoculated on the lower leaves and samples were taken from the upper, young leaves, the virus could only be detected after efficient systemic movement. Therefore, the slower colonisation could be due to

impeded phloem-dependent accumulation of this mild PepMV isolate as was also observed for the attenuated M strain of *Tobacco mosaic virus* (TMV) (Nelson et al., 1993). The mixed infection resulted in the highest and most stable titres throughout the entire period, while the viral titre in plants inoculated with the EU mild isolate was very unstable and generally the lowest. As viral synergism is often manifested by an increase in both symptom expression and viral accumulation (Hull, 2002), the higher titre observed in plants infected with the EU+CH2 isolate could indicate a synergistic interaction between the coinfecting EU and CH2 genotypes, but further research is needed to confirm this hypothesis.

Our results suggest a correlation between the aggressiveness of the PepMV isolate and the viral titre. Interestingly, a lower PepMV titre in symptomless tomato plants infected with a Peruvian genotype isolate (isolate LP 2001), when compared to tomato plants infected with EU tomato genotype isolates showing clear PepMV symptoms, has previously been reported (López et al., 2005). A lower viral titre may occur for viral isolates with reduced post-transcriptional gene silencing (PTGS) suppressor activity, as PTGS reduces viral accumulation (Ratcliff at al., 1999). PTGS has been associated with differential symptom expression, viral resistance and synergism of viruses in previous studies (Pruss et al., 1997; Ratcliff et al., 1999; Kubota et al., 2003).

It may be expected that the PepMV genome displays high mutation frequencies, as many different genotypes have emerged over a rather limited period of time. In general, RNA virus replication is characterised by high mutation rates, high yields and short replication times (Domingo & Holland, 1997). Therefore, RNA sequence stability of the PepMV isolates used in our greenhouse trails was studied by comparing sequences of a 625 nt fragment, containing the end of the replicase gene, an untranslated region and the start of the TGB, obtained from samples taken at four time points throughout the trial period. Comparison of sequences obtained from the beginning and the end of the trial period revealed that the number of mutations was rather limited, that most of the mutations that took place had no clear biological relevance as they were mostly silent and often reversible. Therefore, the RNA sequence in this part of the genome appeared to be relatively stable. These results are compatible with recent advances in plant virus evolution on random genetic drift and the existence of critical thresholds that limit viruses to a small portion of their potential sequence space (Domingo & Holland, 1997). It was shown that systemic movement of plant viruses through the vascular system results in population bottlenecks. A study on systemic movement of TMV revealed that the effective population numbers are a lot smaller than the census population numbers, indicating the importance of random genetic drift in virus evolution (Sacristan et al., 2003). Deleterious mutations can lead to average fitness losses, thus restricting the

types and numbers of mutations RNA viruses can tolerate (Domingo & Holland, 1997; Garcia-Arenal et al., 2001).

A high stability of the RNA sequence of the PepMV genome is also in line with the observation that sequence homology of different isolates from different origins but belonging to the same PepMV genotype groups, show high levels of sequence homology (>99%) (Verhoeven et al., 2002; Lopéz et al., 2005). However, it remains unclear how the variability in currently known PepMV genotypes was introduced in the viral genome.

A recent report on biological characterisation of several PepMV isolates on solanaceous test species in climate chambers revealed diverse pathogenic behaviours (Córdoba-Selles et al., 2008). However, to our knowledge this is the first study in which differential symptom display in greenhouse tomato production could unambiguously be attributed to the PepMV isolate. These results can at least partially explain the huge variation in the level of damage reported for PepMV in commercial tomato production. In addition, SNPs that may play a role in PepMV symptom expression were identified that might contribute to future identification of genome regions involved in the expression of PepMV symptoms in tomato.

Acknowledgements

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Chapter 4:

Seed transmission of *Pepino mosaic virus* in tomato

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Abstract

In this manuscript we provide evidence for seed transmission of *Pepino mosaic virus* (PepMV) in tomato. Fruit was harvested from a tomato crop artificially infected with both European and CH2 genotypes of PepMV and more than 100,000 seeds were extracted and cleaned using an enzymatic treatment without disinfection. Infection assays using indicator plants confirmed the presence of viable virus on the seeds. Seeds were distributed to 10 different laboratories in three separate batches, where they were germinated and the young plants tested with ELISA. In total over 87,000 plants were tested and 23 positives detected, indicating an overall transmission rate of 0.026%. However, the observed seed transmission rates varied from 0.005% to 0.057%, depending on the seed batch used. Results clearly showed that PepMV can be transmitted from seeds contaminated with virus to seedlings, highlighting the risk of using seeds from PepMV-infected plants and the potential for seed transmission to contribute to the further spread of PepMV.

Introduction

Since first appearing in tomato crops in 1999 *Pepino mosaic virus* (PepMV), a *Potexvirus* originally isolated from pepino (*Solanum muricatum*) in Peru in 1974 (Jones et al., 1980), has become a major disease of greenhouse tomato production worldwide (van der Vlugt et al., 2000; French et al., 2001; Mumford and Metcalfe, 2001; Cotillon et al., 2002; Maroon-Lango et al., 2005; Pagan et al., 2006; Hasiow et al., 2008; Hanssen et al., 2008; Ling, 2008a). The virus causes a wide range of symptoms both on fruits and on the vegetative plant parts, including fruit marbling and flaming, nettle-heads, leaf mosaics, dwarfing, leaf distortions and yellow leaf spots. Although the symptoms are often mild, an increase in symptom severity has been observed and more novel symptoms are now more common, including leaf scorching, sepal necrosis and open fruit (Spence et al., 2007; Hanssen et al., 2008; Hanssen et al., 2009b). Despite the wide range of symptoms, in terms of economic impact, those on fruit are generally regarded as the most damaging as they can lead to fruit being downgraded, reducing the economic value of a crop (Soler et al., 2000; Roggero et al., 2001; Spence et al., 2007).

Originally all PepMV isolates identified in European tomato production shared a high nucleotide sequence homology and differed in biological properties from the orginal pepino isolate (Mumford and Metcalfe, 2001; Aguilar et al., 2002; Cotillon et al., 2002; Verhoeven et al., 2003; Lopez et al., 2005; Pagan et al., 2006). For these reasons they were classified as a European tomato genotype (EU). Lopez et al. (2005) isolated a PepMV isolate (LP2001) from *Lycopersicon peruvianum*, which was similar to the original pepino strain in biological properties and shared a high nucleotide sequence identity

(96%) with the EU genotype. This isolate is now considered as the type-isolate for the Peruvian genotype of PepMV (LP). Since 2005, three divergent genotypes (US1/CH1, US2 and CH2) have been identified in tomato (Maroon-Lango et al., 2005; Ling, 2007). However, as no US2 isolates are available and nucleotide sequence alignment suggests that US2 might be a recombinant of US1 and CH2, we propose to distinguish four PepMV genotype groupings (LP, EU, US1/CH1 and CH2). In several European countries, the CH2 genotype has now become dominant and has largely replaced the EU genotype in commercial tomato production (Davino et al., 2008; Hanssen et al., 2008). However, in the United States, the EU genotype is still predominant (Ling, 2008a).

With the sudden appearance and rapid establishment of the different PepMV genotypes across large geographical areas, the question of how PepMV is spread over long distances remains unanswered. Along with the movement of germplasm and trade in infected fruit, the potential role of contaminated seed has also been suggested. Seed transmission has been reported for approximately 20% of plant viruses and can, even with low transmission rates, be an important means of viral dissemination (Yang et al., 1997). Although potexviruses are generally not considered to be seed-transmitted, the highly infectious nature of PepMV combined with an extremely rapid cross-continental spread has raised concerns with respect to seed transmission. However, despite the fact that it has previously been shown that seeds from PepMV-infected tomato plants contain high viral loads (Krinkels et al., 2001; Córdoba-Selles et al., 2007; Ling, 2008b), and that viral particles can be found on the seed coat, but not in the embryo (Ling, 2008a), the ability of PepMV to be transmitted via seed is still unclear. Previous studies have been ambiguous, as seed transmission rates obtained varied from zero to 1.84% (Krinkels et al., 2001; Salomone and Roggero 2002; Córdoba-Sellés et al., 2007; Ling, 2008b). In most previous studies the numbers of seeds tested were low; in others the seeds were not cleaned to ‘industry-standards’. Therefore, the work presented here was designed to obtain a statistically-sound estimation of the PepMV seed transmission rate in tomato.

MATERIALS AND METHODS

Seed harvest and cleaning

Seeds used in this study were harvested from tomatoes artificially infected with PepMV (cultivar Tricia; De Ruiter Seeds, Bergschenhoek, The Netherlands) and grown in plastic tunnels in Belgium during the 2007 growing season (Hanssen et al., 2009b). Plants were inoculated with a mixture of the EU and CH2 genotypes, and fruit was harvested at 8, 12 and 15 weeks post inoculation (WPI). Enzyme-linked immunosorbent assay (ELISA)

analyses on leaf samples confirmed similarly high viral concentrations in the mother plants at the three different harvesting points (Hanssen et al., 2009b).

In each harvest, all ripe tomatoes (500 to 800) were collected and cleaned to 'industry standards'. Seeds were manually separated from the tomato pulp and collected in containers to which an equal volume of tap water was added. Subsequently, citric acid pH 4 (6.7% v/v) and pectinase (Pectinex® Ultra SP-L, Novozymes A/S, Denmark; 0.25% v/v) were added and the pulp was incubated for three hours at 28°C, stirring every 30 minutes. Next, seeds were retrieved by sieving, thoroughly rinsed with tap water and dried for 24 hours in an oven at 26°C until the water content was below six percent.

Determination of viral presence and infectivity on seeds

PepMV contamination of the seeds was assessed using a commercially available ELISA assay according to the suppliers' instructions (Prime Diagnostics, Wageningen, The Netherlands). Twenty seeds were tested per seed batch, each sample containing one seed from the infected batch spiked into 250 healthy seeds according to a PepMV-specific seed testing protocol designed by the International Seed Health Initiative - section Vegetables (ISHI-Veg; Krinkels, 2001). All samples were tested in duplicate and were rated positive if the mean optical density at 405 nm (OD) of the sample was at least twice the mean OD of two wells containing extract from healthy tomato seeds. The PepMV genotypes present on the seed batches were determined by RT-PCR-RFLP (Hanssen et al., 2008).

Part of the seed batch harvested at 15 WPI was used for seed infectivity assays on *Nicotiana occidentalis* P1. Seeds were divided into subsamples, crushed in a mixture of sand and water and inoculated onto two plants using carborundum powder and cotton wool. Plant symptoms were evaluated two weeks after inoculation.

Seed transmission grow-out trials

The tomato seeds harvested from PepMV infected plants were distributed to 10 different partner laboratories, in batches of 4,000-5,000 seeds per laboratory. These were sown in stonewool blocks (10 cm square) within six weeks of the seed being harvested. Seedlings were irrigated individually with nutrient solution according to local practices and grown for at least four weeks in a glasshouse. Strict hygienic measures were taken to prevent external contamination of seeds and seedlings. Leaf samples were then taken from each plant, pooled and tested in groups of ten. In total, 8,778 pooled samples were obtained from 87,780 seedlings, across all 10 laboratories. All pooled samples were analysed for presence of PepMV by the ELISA assay as described above. Positive ELISA results were confirmed by an additional ELISA analysis or by RT-PCR.

Statistical analyses

To determine the number of seeds required for the grow-out trial, a binomial distribution of the probability of seed transmission (p) was assumed (Table 1). The seed transmission rate was calculated from grouped sample test results using the equation $P^* = 1 - (1 - R/N)^{1/i}$, where P^* is the maximum likelihood estimate of the seed transmission rate (0 to 1), N is the number of grouped samples, i is batch size, and R is the number of infected seedlings (adapted from Gibbs and Gower 1960). Transmission rates of seeds, harvested at 8, 12 and 15 WPI, were compared using an analysis of deviance (Generalised Linear Model with a binomial distribution error term and logit-link function). 95% lower and upper confidence intervals were calculated to indicate the errors associated with the transmission rates (Table 2). Generalised Linear Model analysis was carried out using Genstat Release 10.2 (Lawes Agricultural Trust). Confidence intervals were calculated using Seedcalc version 8.1 (International Seed Testing Association).

Table 1. Estimation of the number of PepMV infected seeds to be included in PepMV grow-out trials under different assumptions of transmission rates.

		X Value (Number of positive groups)					
		P=0.0001		P=0.001		P=0.01	
N=10000		X	CI	X	CI	X	CI
	K=50	1	0-0.0185	10	0-0.02	79	0.0012-0.0357
	K=25	1	0-0.0093	10	1.8E-7-0.011	89	0.0027-0.0252
	K=10	1	0-0.0039	10	2.5E-5-0.0055	96	0.0048-0.0182
N=50000							
	K=50	5	0-0.0035	49	3.5E-5-0.0066	395	0.0039-0.0191
	K=25	5	0-0.0019	49	0.0001-0.0035	444	0.0069-0.0146
	K=10	5	0-0.0009	50	0.0003-0.0023	478	0.0074-0.0131
N=100000							
	K=50	10	0-0.0020	98	0.0001-0.0036	790	0.0061-0.0154
	K=25	10	1.8E-8-0.0011	99	0.0003-0.0026	889	0.0072-0.0136
	K=10	10	2.5E-6-0.0006	100	0.0005-0.0018	960	0.0082-0.0121

N = Total number of seeds. K = Number of seeds per group. P = Probability of seed transmission. X = Number of positive groups. CI = Confidence interval**.** The confidence intervals were calculated using the binomial distribution $f(X) = [N!/(X!(N-X)!]\ P^X\ (1-P)^{N-X}$. If N seeds are analysed in batches of K, the frequency of positive batches, in which transmission has occurred, will be i and thus 1-i is the frequency of batches in which no seed transmission has occurred. Therefore, applying the above binomial probability distribution, $f(0) = 1-i = [K!/(0!(K-O)!]\ P^0\ (1-P)^K = (1-P)^K$. Therofore, $1-i = (1-P)^K$ and $p = 1 - (1-i)^{1/K}$.

Results

A total of more than 100,000 seeds was obtained by enzymatic treatment from fruit harvested from tomato plants artificially infected with PepMV. Three batches of seeds were produced at 8, 12 and 15 WPI. Seeds were tested for the presence and viability of PepMV particles and used in grow-out trials to determine the seed transmission rate. In total ten partner laboratories from different European countries participated in the grow-out trials.

Determination of viral presence and infectivity on the seeds

Before distribution of the seeds to the ten partners, the presence of PepMV on the seeds was determined by ELISA analyses on 20 samples per harvest, following the spiking method described above. The ELISA results revealed a high concentration of PepMV on or in seeds, as all samples were positive with mean OD values of 3.39 (st.dev. (±) 0.29), 3.35 (± 0.37) and 3.12 (± 0.57) obtained for the three subsequent harvests; mean OD values of 2.92 (± 0.60) and 0.07 (± 0.006) were obtained for the positive and negative control wells, respectively. Genotype analyses by RT-PCR-RFLP revealed that, like the mother plants, seeds from all three harvests were infected by both the EU and CH2 PepMV genotype.

Infectivity of PepMV particles present in seeds from the third harvest was determined by inoculation on *N. occidentalis*. To this end, 15 samples of 10 seeds each and 10 samples of 50 seeds each were inoculated on two *N. occidentalis* seedlings per sample. Five out of 30 plants inoculated with the 10-seed-samples developed PepMV symptoms, while 11 out of 20 plants inoculated with the 50-seed-samples developed PepMV symptoms, indicating that these seed batches contained infectious PepMV.

PepMV seed transmission

In order to determine the PepMV seed transmission rate in tomato, an extensive grow-out trial was performed. Based on previous studies, a seed transmission rate between 0 and 1% was anticipated (Krinkels et al., 2001; Salomone and Roggero, 2002; Córdoba-Sellés et al., 2007). Assuming that the probability of seed transmission is binomially distributed and that the rate is not lower than 0.01%, probability calculations resulted in the estimation that a total number of 100,000 seedlings in blocks of ten would be sufficient to obtain a reliable estimate (Table 1). The germination and grow-out of this many seedlings, arranged to avoid physical contact between the blocks, would require a huge amount of greenhouse space. Therefore the seed transmission grow-out trials were run in parallel in the greenhouse facilities of plant pathology laboratories of ten partners in Belgium, Bulgaria, Denmark, Greece, Italy, Norway, Poland, Portugal, Slovenia and the United Kingdom. To assess the influence of the time span between infection of the

mother crop and seed harvest on the transmission rate, seeds harvested at 8, 12 and 15 WPI were analysed in three subsequent grow-out trials.

Since the seeds were harvested from a commercial tomato hybrid, a heterogeneous germination and a suboptimal germination rate of 70-80% were expected. Therefore, seeds were sown in blocks of 16 to obtain at least ten homogenous seedlings per block to pool into one sample for ELISA analysis.

In total, 8,776 blocks consisting of 10 seedlings each were sampled four weeks after sowing, 23 of which tested positive for PepMV by ELISA (Table 2). Positive ELISA results were generally clearly above the threshold value (twice the negative control) (Table 3) and were in all 23 cases confirmed by additional ELISA analyses or by RT-PCR. In addition, typical PepMV symptoms such as nettlehead and leaf deformation were seen in infected plots. Thus, at least 23 out of 87,760 seeds resulted in an infected seedling, leading to a minimum PepMV transmission rate of 0.026% (confidence interval 0.0166 to 0.0359; Table 2). Interestingly, while only one and three infected blocks were obtained from the first and second harvest, respectively, the third harvest gave rise to 19 infected blocks, resulting in a significantly higher ($P<0.05$) transmission rate (0.0567%) as compared to the first and second harvest (0.0053% and 0.0085%, respectively). Positive samples obtained in Denmark, Italy, Norway Slovenia and the UK were analysed by a genotype specific TaqMan qRT-PCR (unpublished) or by RT-PCR-RFLP (Hanssen et al., 2008). Out of the 11 positive blocks obtained in those four countries, seven were infected only with the EU genotype, three with the CH2 genotype, and one with both the EU and CH2 genotype.

Table 2. Number of ELISA-positive plots out of the total number of plots[1] tested in grow-out trials

Partner	8 WPI harvest	12 WPI harvest	15 WPI harvest	Total
Bulgaria	0/382	0/395	0/338	0/1115
Denmark	0/240	0/360	2/320	2/920
Greece	0/270	0/366	2/416	2/1052
Italy	1/249	1/393	1/410	3/1052
Norway	0/346	0/350	2/411	2/1107
Slovenia	0/71	0/153	2/240	2/464
UK	0/329	0/365	2/348	2/1042
Poland		0/495	5/520	5/1015
Portugal		0/350	3/350	3/700
Belgium		2/311		2/311
Total	1/1887	3/3538	19/3353	23/8778
Transmission rate (%)[2]	0.0053[a]	0.0085[a]	0.0567[b]	0.026
Confidence interval (*P* =0.05)	0.0002 - 0.0295	0.0021 - 0.0248	0.0345 - 0.0885	0.0166 - 0.0395

[1] 8.760 plots consisting of 10 tomato seedlings each grown from infected seeds

[2] Different letter labels indicate significant differences (P=0.05).

Table 3. ELISA optical density (OD) values of controls and positive samples obtained from the grow-out trials by the different partner laboratories

Samples	Belgium	Denmark	Greece	Italy	Norway	Poland	Portugal	Slovenia	UK
Healthy controls[1]	0.111 ±0.004	0.090 ±0.008	-0.024 ±0.026	0.117 ±0.011	0.016 ± 0.013	0.024 ±0.005	0.020 ±0.008	0.082 ±0.015	0.064 ±0.003
Positive controls[1]	3.450 ±0.120	1.682 ±0.120	1.940 ±0.034	3.136 ±0.056	1.034 ±0.128	1.944 ±0.140	3.355 ±0.135	1.321 ±0.177	0.819 ±0.145
Positive sample 1	0.340	0.480	1.983	0.689	1.593	0.575	0.124	1.631	0.357
Positive sample 2	0.280	1.303	1.217	0.307	0.695	0.571	0.120	0.964	1.045
Positive sample 3	-	-	-	2.930	-	0.585	0.840	-	-
Positive sample 4	-	-	-	-	-	0.202	-	-	-
Positive sample 5	-	-	-	-	-	0.734	-	-	-
Negative samples[2]	0.116 ±0.006	0.087 ±0.006	0.014 ±0.020	0.102 ±0.022	0.020 ±0.007	0.029 ±0.006	0.018 ±0.005	0.076 ±0.003	0.066 ±0.008

[1] Mean OD value ± standard deviation obtained from all control wells

[2] Mean OD value ± standard deviation obtained from all negative sample wells

Discussion

The results presented in this extensive study, based upon a grow-out trial using almost 90,000 tomato seedlings, clearly demonstrated that PepMV can be transmitted to the next generation via contaiminated seed and provided a statistically sound estimation of 0.026% as the PepMV seed transmission rate in tomato.

In the past, the seed transmission of PepMV has proved a controversial subject, with previous studies on the subject giving conflicting results. For example, Salomone and Roggero (2002) did not find any seed transmission by testing 52 seedlings. Likewise, in a more recent study, none of 10,000 grow-out seedlings from an infected commercial seed lot were infected by PepMV as determined by symptom expression, ELISA tests or infectivity assays, although mechanical transmission demonstrated the virus on the seed was still viable (Ling, 2008b). In contrast, other studies have found seed transmission, including one conducted in collaboration with the seed industry, which revealed seed transmission rates between 0.06% and 0.03% for seeds that were cleaned by natural fermentation and dried (Krinkels et al., 2001). In another grow-out trial with 168 seedlings a seed transmission rate of 1.84% was found for PepMV infected tomato seeds that were only rinsed without fermentation or enzymatic treatment (Córdoba-Selles et al., 2007). The contrasting conditions used in these previous studies and the study presented in this paper does make direct comparison of results difficult. The fact that different PepMV genotypes, seed ages and seed treatments were used will potentially have influenced the final results obtained. Moreover, in contrast to our latest study, the numbers of seeds used in most of the previous studies were too low for a statistically sound estimation of the seed transmission rate. However it is interesting that

the results found in our study are in line with those from Krinkels et al. (2001), who obtained similar transmission rates and applied a comparable seed treatment. In contrast, the much higher transmission rate reported by Córdoba-Selles et al. (2007) can be explained by the fact that seeds were not cleaned to 'industry-standards'. A high viral titre on seeds harvested from PepMV infected tomato plants was previously reported (Córdoba-Selles et al., 2007; Ling, 2008b). Taking into account the highly efficient mechanical transmission of the virus, it is strongly suspected that seed transmission of PepMV occurs as a result of contact between the germinating seedling and the virus contaminated seed coat. For this reason, seed cleaning and treatment will have a large influence on the transmission rate. This was demonstrated by further seed treatment and disinfestation studies performed by Córdoba-Selles et al. (2007), who were able to significantly reduce transmission from their uncleaned seed using various chemical treatments.

Of course it should be noted that as the seeds used in this study were harvested from heavily infected plants, where all the fruit was infected, and where no post-cleaning disinfection treatment of the seeds was applied prior to sowing, the transmission rate obtained does represent a potential worst case scenario. In practice, procedures (both statutory and internal quality ones) are in place that should virtually eliminate the risk from commercial seed. For example, within the European Union, PepMV has had quarantine status on seeds since 2001 and regulations are in force to prevent the introduction and further spread of PepMV through infected tomato seeds (Commission Decision 2001/536/EC and 2004/200/EC). These include the seed producer having to provide proof of absence of PepMV, either in the production area, in the mother crop by monitoring, or through seed testing. In addition, acid extraction of tomato seeds is mandatory. In general, seed production companies combine the different criteria. Established seed production methods do exist and protocols such as the widely-used ISHI-Veg approved ELISA-based testing procedure (http://www.worldseed.org/en-us/international_seed/ishi_vegetable.html) can provide reliable detection of PepMV in the contaminated seed lots, as shown in this study.

However, this study does provide conclusive proof that PepMV can be transmitted from tomato seeds produced from infected fruit and for that reason the risk must be taken seriously. The continued imposition of strict regulations for seed harvest and reliable PepMV seed testing methods are necessary to prevent spread of PepMV by tomato seeds. While the efficiency of transmission is obviously low, the highly infectious nature of PepMV in tomato crops means that even one infected plant derived from contaminated seed is sufficient to start an outbreak in a commercial crop that might have tens if not hundreds of thousands of plants. So ultimately in risk matrix terms, while the risk of infection from seed is low, the probability of it causing an outbreak is

high. For example, transmission via seed provides the most likely explanation of how PepMV was able to spread so widely and so rapidly throughout worldwide greenhouse tomato production in the late 1990s and early 2000s, prior to the introduction of the current regulations.

While the occurrence of seed transmission is clear, the results do include some interesting observations. Firstly, they show that infected seedlings obtained from tomato seeds infected by two different genotypes of PepMV are not necessarily infected by both genotypes. Only one out of 11 infected plots from which the genotypes were determined was infected by both genotypes. These results strongly suggest that only a low number of viral particles initiated the infection and represent strong evidence of a population bottleneck during PepMV seed transmission. Population bottlenecks during horizontal transmission of plant viruses from host to host have thus far only been reported during aphid transmission (Ali et al., 2006). Very small numbers (between one and two) of effective founders have been reported for *Cucumber mosaic virus* (CMV) transmitted from one plant to another by the aphid vector *Aphis gossyppi* (Betancourt et al., 2008). Also the number of *Potato virus Y* (PVY) particles transmitted by the aphid vector *Myzus persicae* was found to be very limited, from 0.5 to 3.2 (Moury et al., 2007). However, to our knowledge, this is the first indication of a population bottleneck during seed transmission. The second interesting observation made during this study was the apparent increase in seed transmission risk seen as the interval between infection of the mother crop and seed harvest was extended. A ten times higher transmission rate was observed for seeds harvested at 15 WPI, as compared to seeds harvested at 8 and 12 WPI. The observed difference could not be explained by a higher viral titre in the mother plants at the time of harvest, nor by a higher concentration of virus in or on the seed batches. It has previously been reported that the time interval between infection of the mother plants and fruit harvest influences seed transmission rates and it was suggested that infection before initiation of florescence is required to obtain seed transmission (Blaszczak 1964). Taking into account a period of two weeks between inoculation and overall systemic spread of the virus (Hanssen et al., 2009b), and a period of nine to ten weeks between initial fruit set and tomato harvest, fruit set of tomatoes harvested at 15 WPI was initiated after systemic spread of the virus in the mother plants, in contrast to the 8 WPI harvest. If systemic spread of the virus at the time of fruit set is required for seed transmission to occur, this might explain both the absence of seed transmission in the 8 WPI harvest and the relatively high rate obtained from the 15 WPI harvest. Fruits harvested at 12 WPI were either set just before or just after systemic spread of the virus, which might account for the intermediate rate. While it is tempting to suggest a physiological explanation, further studies would undoubtedly be required to clarify the possible mechanisms behind this observation.

Acknowledgements

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Chapter 5:
Cross-protection or enhanced symptom display in greenhouse tomato co-infected with different *Pepino mosaic virus* isolates

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Abstract

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 that belonged to a different genotype (EU, LP) than 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.

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 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; Hasiow et al., 2008; Hanssen 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 economical value of the fruit (Soler et al., 2000; Roggero et al., 2001; Spence et al., 2007). Recently reported open fruits and sepal necrosis similarly contribute to fruit quality devaluation (Hanssen et al., 2009b). Damage and economical 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., 2009b).

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 (Lopez 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., 2007). 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., 2009; Hanssen et al., 2009b). The EU and LP genotypes share a nucleotide sequence homology of 96% and cluster phylogenetically. The CH2 genotype is rather different as it displays only 78 to 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 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; Hanssen et al., unpublished data; 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 et al., 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, 2005). 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 disease. 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 in 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 (Lopez 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., 2009a). Greenhouse experiments have shown enhanced symptom severity in tomato crops that were simultaneously infected by two different PepMV genotypes when compared to crops infected by 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 potential of a mild EU isolate and a mild CH2 isolate against the aggressive CH2 isolate was 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), further referred to as 'CH2 aggressive' (GenBank accession number FJ457097; Hanssen et al., 2009b). 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 the PepMV genotype-specific TaqMan RT-qPCR detection method. Tomato seeds (cultivar Tricia, De Ruiter Seeds, Bergschenhoek, the Netherlands) were disinfected, sown and germinated as previously described (Hanssen et al., 2009b). Five weeks after sowing, 100 tomato plants were transplanted to each of the tunnels in stonewool substrate. One week after planting, in all tunnels absence of PepMV was demonstrated by ELISA analyses, and subsequently tomato plants of the first and second tunnel were inoculated individually with the LP mild isolate as previously described, while plants in the third and fourth tunnel were mock-inoculated with phosphate buffer (Hanssen et al., 2009b). 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, however 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 is defined as 0 weeks post-inoculation (WPI), while 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 the LP mild isolate and subsequently with the CH2 aggressive isolate are further referred to as the pre-inoculated plants. Plants that were inoculated only with the LP mild isolate 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 ten sampling blocks, each containing ten adjacent plants. At regular intervals, samples were taken from the youngest leaves in the head of the plants, as previously described (Hanssen et al., 2009b).

PepMV detection and relative quantification of viral titres

To confirm PepMV presence in the inoculated plants and absence in the 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, Wageningen, the Netherlands), 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 8 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, Chatsworth, CA, USA) and reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems, Foster City, USA). qPCR reactions were performed in 10 µl of final reaction volume, including TaqMan universal PCR master mix (Applied Biosystems), forward and reverse primers at 900 nM each, 200 nM of the corresponding 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., 2009). Plant cytochrome oxidase (COX) specific primers and a Taqman probe (Weller et al., 2000) were used as internal control for accounting for variations due to 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 followed by 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 the 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, Ct of 35 was considered as the highest detectable Ct (Gutiérrez-Aguirre et al., 2009).

Table 1. PepMV genotype specific primers and Taqman probes used in this study (Gutiérrez-Aguirre et al., 2009)

Primer/Probe	Sequence	Position in genome
Forward primer EU/LP	5’ TGGAACATACTTCTCGACAGCAA 3’	6035-6057[1]
Reverse primer EU/LP	5’ TCCATCGAAGAAGTCAAATGCA 3’	6112-6133[1]
Probe EU/LP	5’FAM-ATTCCACCAGCAAATTGGGCCAAACTT-TAMRA 3’	6059-6085[1]
Forward primer CH2	5’ TGGGTTTAGCAGCCAATGAGA 3’	5832-5852[2]
Reverse primer CH2	5’ AACTTTGCACATCAGCATAAGCA 3’	5881-5903[2]
Probe CH2	5’FAM-CGGACCTGCCATGTGGGACCTC-TAMRA 3 ‘	5854-5875[2]

[1]SP13 reference sequence (GeneBank Accession number AF484251)

[2]CH2 reference sequence (GeneBank Accession number DQ000985)

Evaluation of PepMV symptoms

Symptoms were scored following a previously described assessment schedule (Hanssen et al., 2009b; 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 ($p<0.05$) 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., Chicago, IL, USA).

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 that is known to cause significant damage, and symptom display was recorded. As the LP genotype of PepMV was previously reported to cause symptomless infections in tomato (Jones et al., 1980; Lopéz et al., 2005), an LP isolate (LP mild) obtained in 2008 from a PepMV-infected symptomless commercial tomato crop in Belgium was selected as potential protector isolate. Tomato plants in one tunnel were first inoculated with the LP mild isolate, 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 (tunnel 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 one WPI onwards, and iii) that non-inoculated control plants remained free of PepMV (data not shown) . RT-PCR-RFLP analyses (Hanssen et al., 2008) 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 that were inoculated with the CH2 aggressive isolate, regardless of the pre-inoculation with the LP mild isolate (Figure 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 ($P<0.05$) 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 percentage of marbled and flamed fruits was 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 the LP mild isolate and subsequently with the CH2 aggressive isolate (Figure 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, while at the same time points only 2.5% and 2.0% marbled tomatoes were harvested from the CH2 aggressive reference plants. No or only few marbled tomatoes were seen in the control and the 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.

With regard to fruit flaming, symptoms were prevalent in both the LP mild pre-inoculated plants and the CH2 aggressive reference plants (Figure 2b). At 7 WPI, less flamed tomatoes were harvested from pre-inoculated plants (11%) when compared to the CH2 aggressive reference (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 the LP mild reference (17%) plants as compared to the

CH2 aggressive reference (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 (Figure 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 (Figure 3). The overall yield loss from these plants amounted to 13%. A minor yield loss (3%) was recorded in the 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 additional ratings of all 100 plants per 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. Nettlehead 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, while nettlehead symptoms did not occur in control plants (Figure 1b). Differences between control plants and inoculated plants were significant ($P<0.05$) at all time points from 1 WAI onwards. CH2 aggressive reference plants displayed significantly ($P<0.05$) more severe nettlehead symptoms then the other treatments at 1 and 2 WPI.

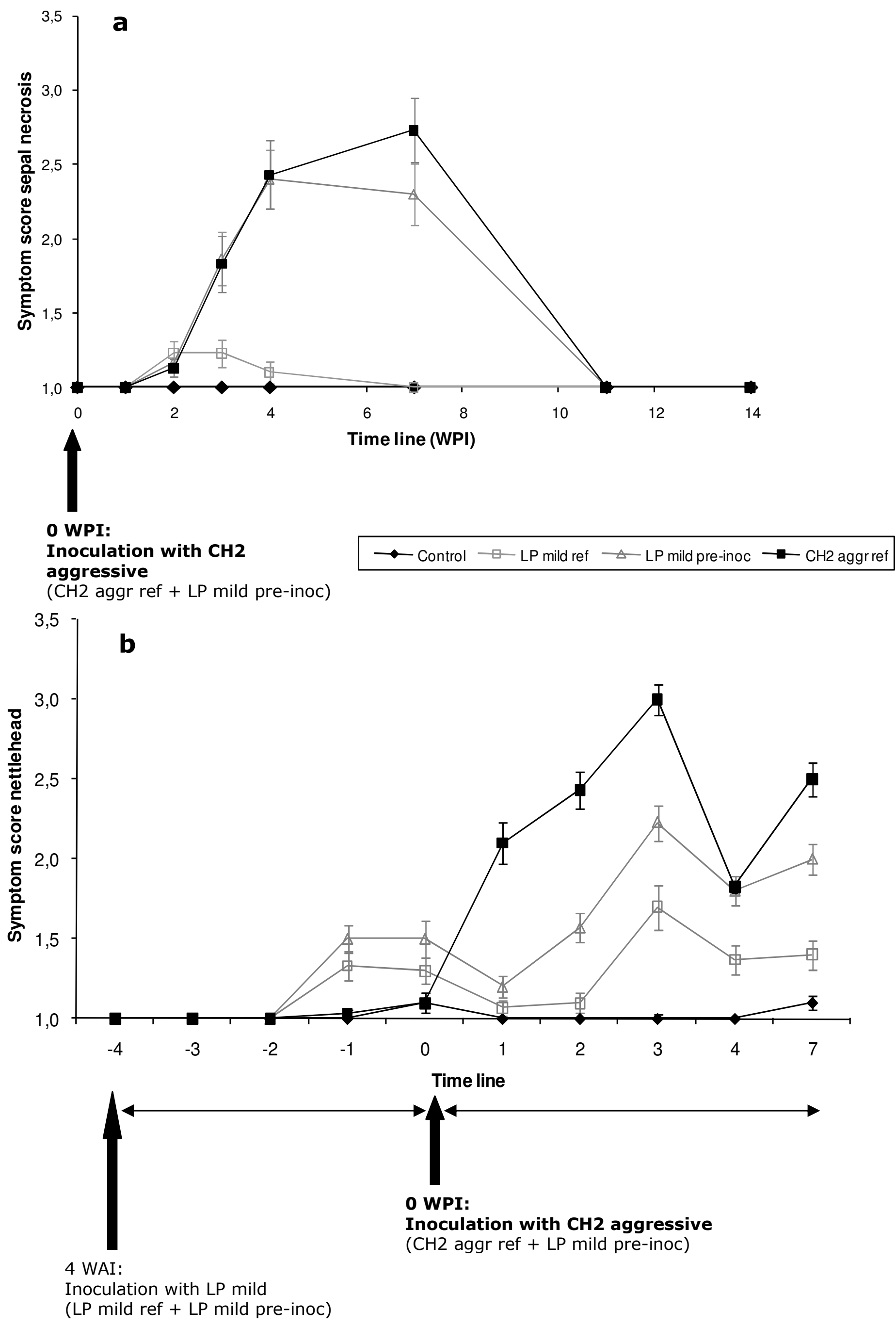

Figure 1. Symptom scores in LP mild pre-inoculation trial. Each point represents the mean score of 30 plants with standard errors. (a) Sepal necrosis on fruits of tomato plants inoculated with the various PepMV isolates. No sepal necrosis was observed before challenge inoculation at 0 WPI as fruit development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

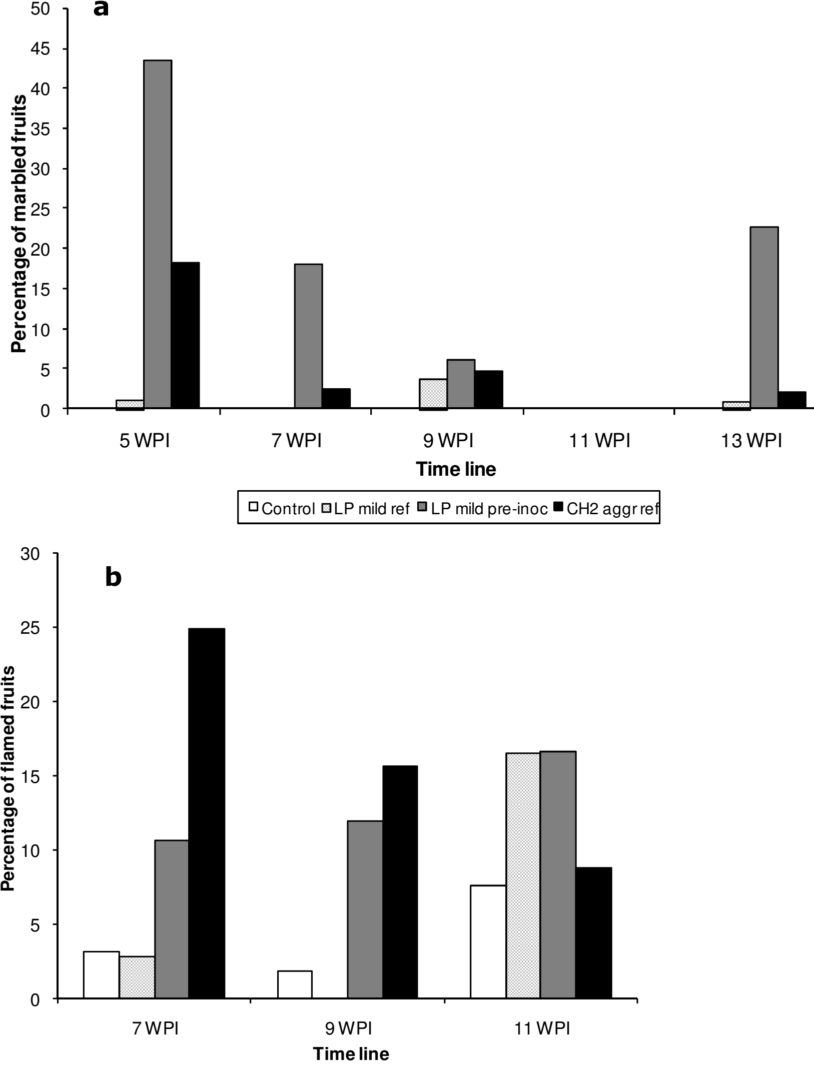

Figure 2. LP mild pre-inoculation trial: symptoms on tomato fruits caused by the different PepMV infections. Percentage of tomatoes showing (a) marbling and (b) flaming at various time points. The mean number of fruits harvested per week and per tunnel was 292, with the highest number of fruits harvested at 5 WPI.

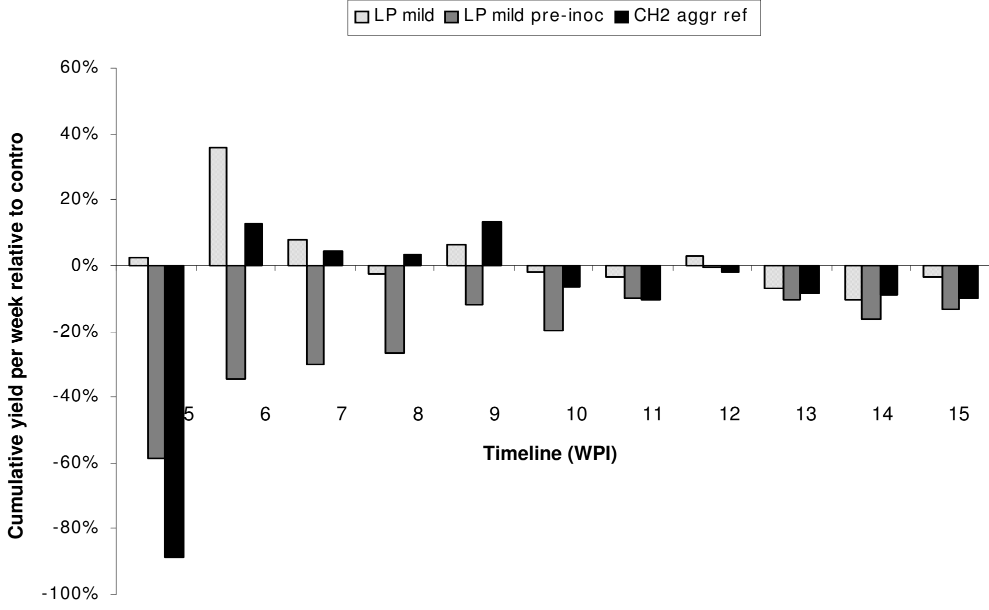

Figure 3. LP mild pre-inoculation trial: tomato production, determined as the cumulative weight of all ripe tomatoes (including fruit with typical PepMV symptoms) harvested once a week, from 5 until 15 WPI, in each tunnel and shown as the percentage relative to the weight obtained for the control tunnel. Total yield obtained from the 11 harvesting points amounted to 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).

Relative infection levels of the 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 standardisation. The index obtained (Ct(cox)-Ct(PepMVgenotype)) is proportional to the viral genome copy load in each analysed sample. The standardisation with COX excludes potential inter-sample variations due to 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 (Figure 4). The lower slope of the CH2 aggressive load curve is 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 the CH2 aggressive loads were comparable in the CH2

aggressive reference and the LP mild pre-inoculated plants (Figure 4). The viral load of the LP mild isolate was hardly influenced by challenge inoculation with the CH2 aggressive isolate.

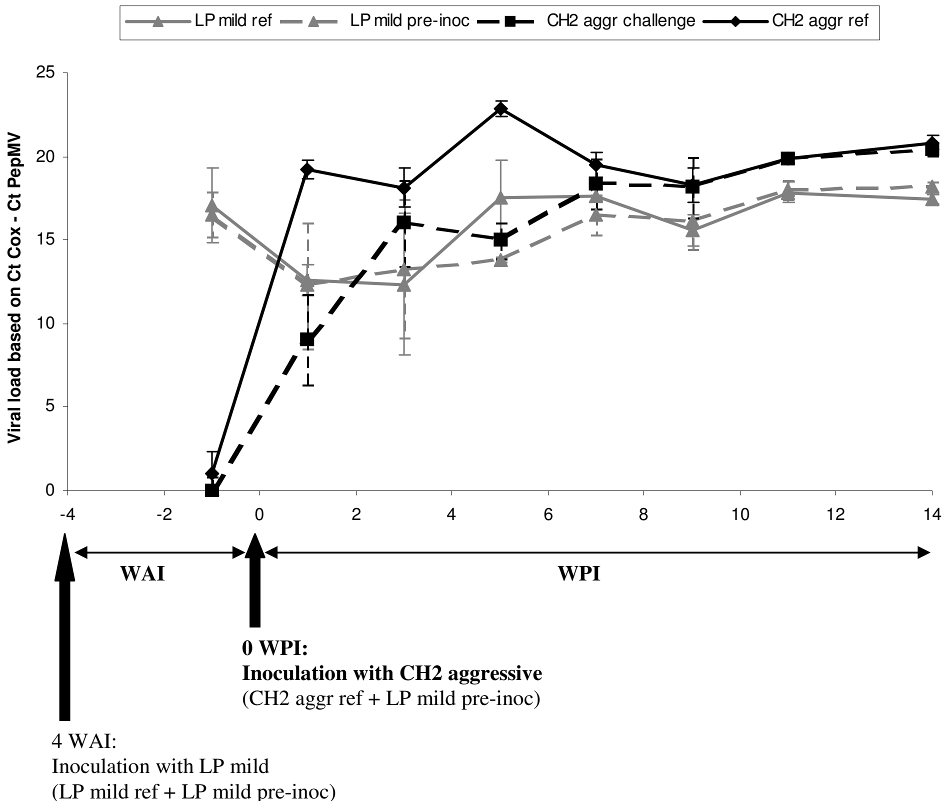

Figure 4. Relative quantification of viral loads visualised for the LP mild pre-inoculation trial. Values in the Y axis are based on a genotype specific TaqMan RT-PCR. The viral load in the reference plants is shown using closed symbols, while viral loads of both the protector (LP mild pre-inoc) and the challenge isolate (CH2 aggr challenge) in the pre-inoculated plants are shown using open symbols. Ct values obtained with LP/EU or CH2 genotype specific assays were subtracted from the Ct values obtained from the Cox (control) assay. Subsequently the lowest obtained value was 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 in the X axis.

Additional trials with mild CH2 and EU protector isolates

Next to the LP mild protector isolate, additional mild isolates belonging to different genotypes were tested for their cross-protection potential. Isolate '1806', further referred to as 'EU mild' (GenBank accession number FJ457098), and isolate '1906', further referred to as 'CH2 mild' (GenBank accession number FJ457096), were selected based on previous greenhouse trials (Hanssen et al., 2009b). 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 four 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 three weeks, a second 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 ($P<0.05$) as compared to 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 fruit in the pre-inoculated plants as compared to the CH2 aggressive reference plants at all time points (Figure 5a). Similar results were also obtained for fruit flaming (Figure 5b). By contrast, none of the fruits harvested from the plants that were pre-inoculated with the CH2 mild isolate showed marbling (Figure 5a). Only at 13 WPI few marbled fruits (2.7%) were seen. Also fruit flaming was clearly suppressed by pre-inoculation with the CH2 mild isolate (Figure 5b). In addition, plants in this tunnel were stronger and more vigorous as compared to plants that were pre-inoculated with the LP mild or the CH2 aggressive reference plants. No yield loss was recorded as the overall yield from both additional tunnels was comparable to 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 with LP mild pre-inoculated plants (Figure 6). A slower onset of viral accumulation of CH2 aggressive 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.

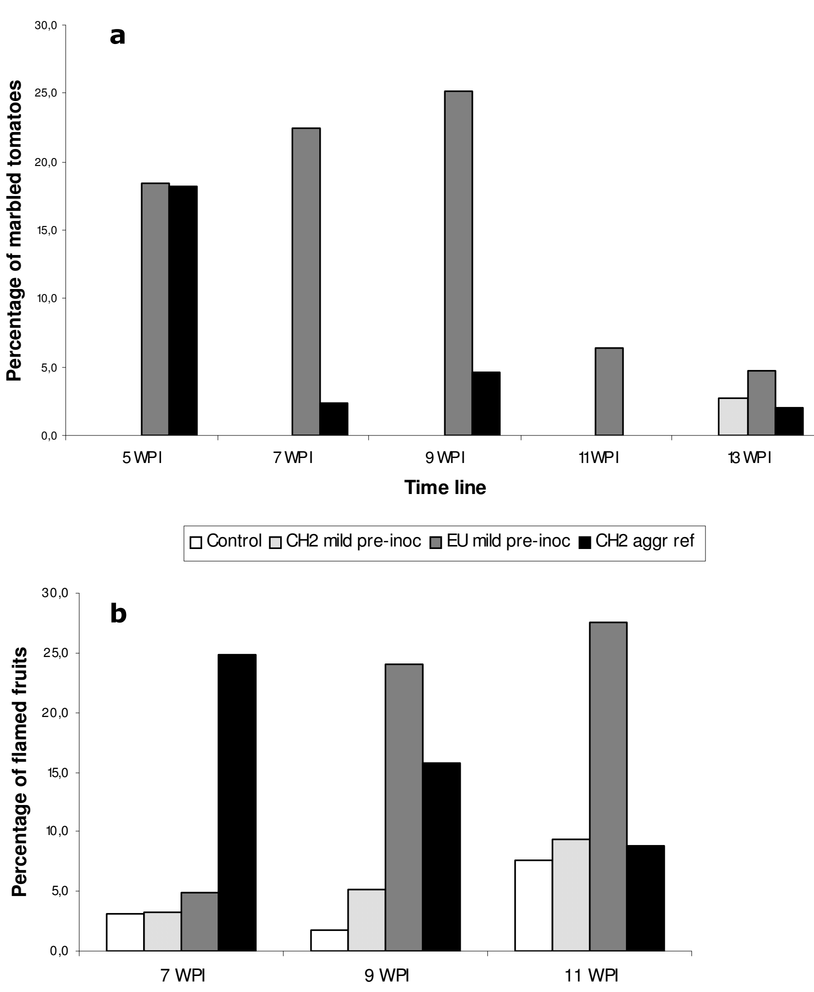

Figure 5. Additional trials: symptoms on tomato fruits caused by the different PepMV infections. Percentage of tomatoes showing (a) marbling and (b) flaming at various time points. The mean number of fruits harvested per week and per tunnel was 281, with the highest number of fruits harvested at 5WPI.

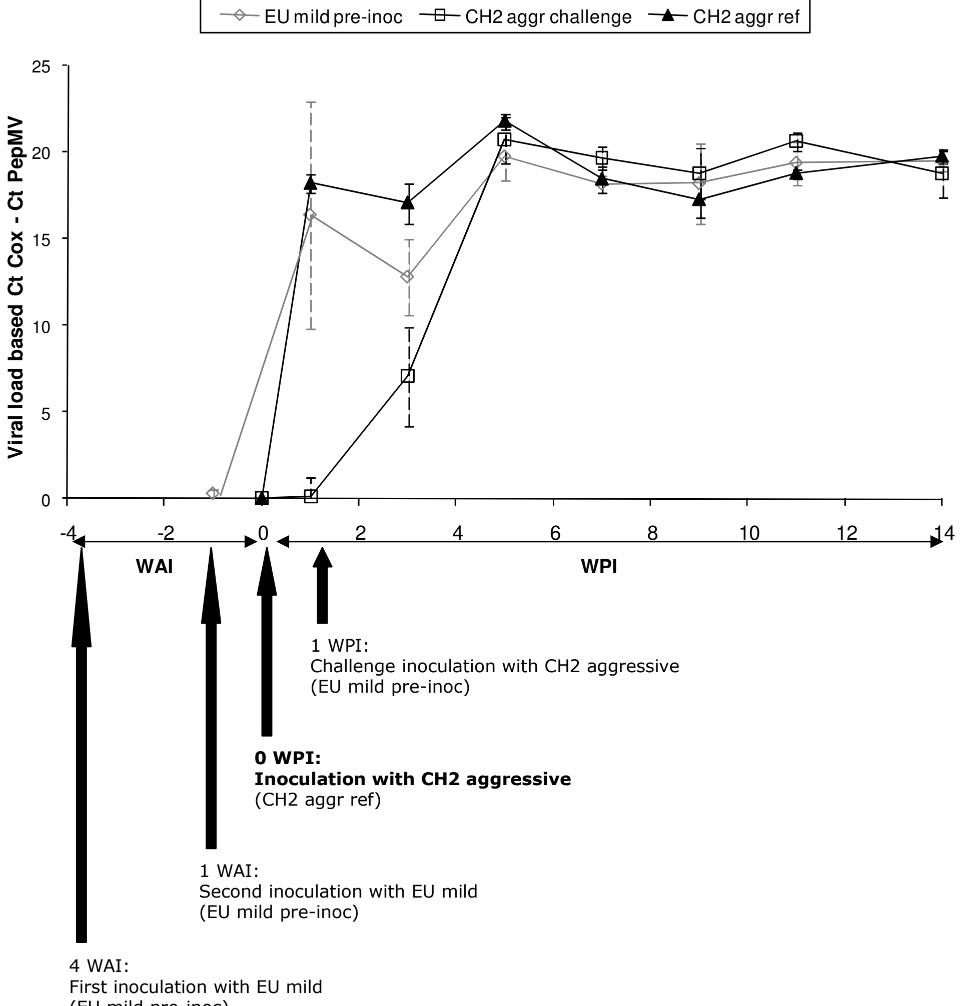

Figure 6. Additional trials: relative quantification of viral loads visualised for the EU mild pre-inoculation trial. As the first pre-inoculation with EU mild performed at 4WAI did not result in systemic infection, a second pre-inoculation was performed at 1 WAI and challenge inoculation was postponed with one week. Inoculation points of the various isolates are indicated using black arrows. The viral load in the reference plants is shown using closed symbols, while viral loads of both the protector (EU mild pre-inoc) and the challenge isolate (CH2 aggr challenge) in the pre-inoculated plants are shown using open symbols. Values in the Y axis are 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. Subsequently the lowest obtained value was equalized to zero for illustrative purposes. Each point represents the mean of three sampling blocks with standard errors.

To determine the relative concentration of the CH2 mild and CH2 aggressive isolates in CH2 mild pre-inoculated plants, nucleotide sequences of a total of 16 clones, each containing a 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., 2009b). 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' GTATTCTACTGTGTCGTCTTGT**G** 3'; mismatches with CH2 mild are indicated in bold). Due to the high sequence homology between both 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 down to which dilution pure CH2 aggressive cDNA could be detected in a background of CH2 mild cDNA. These tests revealed that the assay could detect 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 the CH2 mild pre-inoculated plants after challenge inoculation. Based on both the sequence analyses and the CH2 aggressive specific RT-PCR assay, it can be concluded that the concentration of CH2 aggressive is at least 10 times lower than the concentration of CH2 mild in CH2 mild pre-inoculated plants challenged with CH2 aggressive.

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 CH2 aggressive concentration was, at least, less than 50 times lower than the 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 an enhanced symptom severity rather than cross-protection in plants pre-inoculated with the LP mild isolate and subsequently challenged by an aggressive CH2 isolate. Especially the incidence of fruit marbling, the most typical and economically important PepMV symptom, increased considerably, while 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 an aggressive CH2 isolate had a serious impact on plant vigour and leaf development, and hence on yield, resulting in overall yield reduction of 13% as compared to the control plants. Fruit sepal necrosis was nor 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 to 7 WPI the viral load obtained for CH2 aggressive in the pre-inoculated plants was similar to the CH2 aggressive reference plants. This accumulation of the challenge isolate coincided with the start of the fruit ripening period and thus with the 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., 2009b). 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 the 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. Especially the incidence of PepMV typical fruit symptoms was notably reduced. Remarkably, the titre of the CH2 mild isolate was significantly higher when compared to the CH2 aggressive challenge isolate, which was barely detectable by cloning and by specific conventional RT-PCR.

Overall, our 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 results in more severe symptoms (Hanssen et al., 2008). Whether the enhanced symptom severity is due to synergism between different PepMV genotypes, or to 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* (PVY) (Stouffer and Ross, 1961) and for *Blackeye cowpea mosaic virus* (BICMV) in combination with *Cucumber mosaic virus* (CMV) (Andersson et al., 1996). Synergistic interactions with potyviruses are generally characterised 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* (RTBV) and *Rice tungro spherical virus* (RTSV) 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., 2009b). 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 to divergent strains of the virus (Wang et al., 1991; Desbiez and 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 to W-type isolates (Yeh et al., 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 wildtype strains, but in this case efficient cross-protection to a more divergent strain was also obtained (Ziebell et al., 2007).

Ratcliff et al. (1999) have 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* (TRV) and *Potato virus X* (PVX) constructs sharing a common sequence that one viral construct can 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).

To our knowledge, this is the first report on cross-protection between different PepMV isolates in tomato. Our findings that the interaction between PepMV isolates differs largely depending on nucleotide sequence homology between the isolates have 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 et al., 2008; Hanssen et al., 2008; Hasiow 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 hygienic measures are taken during cultivation and between different cropping cycles.

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Chapter 6:

Differential tomato transcriptomic responses induced by *Pepino mosaic virus* isolates with differential aggressiveness

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Submitted

Abstract

As plant viruses are obligate intracellular parasites that hijack host cellular functions and resources for their replication and movement, they generally induce a wide variety of alterations in host gene expression and cell physiology. *Pepino mosaic virus* (PepMV) is a highly infectious *Potexvirus* and a major disease of greenhouse tomato crops worldwide. Damage and economical losses caused by PepMV vary greatly, and can at least partially be attributed to differential symptomatology caused by different PepMV isolates. In this study, we used a custom-designed Affymetrix tomato GeneChip array that contains probe sets to interrogate over 22,000 tomato transcripts to study transcriptional changes in response to inoculation with a mild and an aggressive PepMV isolate that share 99.4% nucleotide sequence identity. Interestingly, our results show that both isolates induce differential transcriptomic responses in the tomato host despite similar viral titers. PepMV inoculation resulted in an extensive transient repression of host genes which clearly affected primary metabolism. Especially the defense response intensity was higher upon inoculation with the aggressive isolate and was mediated by salicylic acid signaling rather than by jasmonate signaling. Our results furthermore show that PepMV differentially regulates the RNA silencing pathway, suggesting a role for PepMV encoded silencing suppressors, and the ubiquitination pathway. Finally perturbation of pigment biosynthesis, as monitored by differential regulation of the flavonoid/anthocyanin and lycopene biosynthesis pathways, was monitored, which can be associated with the typical PepMV-induced marbling of tomato fruit.

Introduction

Global transcriptional profiling provides insight in the cellular biology of the host upon pathogen infection (Quirino and Bent, 2003; van Baarlen et al., 2008; Wise et al., 2007). As viruses are obligate intracellular parasites that hijack host cellular functions and resources for their replication and movement, plant viruses generally induce a wide variety of alterations in host gene expression and cell physiology (Whitham et al., 2003; Whitham et al., 2006; Alfenas-Zerbini et al., 2009). Over recent years, the use of transcriptomics approaches such as microarrays and subtractive libraries, has resulted in significant advances in identifying such responses (Whitham et al., 2003; Marathe et al., 2004; Senthil et al., 2005; Whitham et al., 2006; Dardick, 2007; Yang et al., 2007; Alfenas-Zerbini et al., 2009). In most compatible plant-virus interactions, transcriptomic analyses reveal a general virus-induced host gene repression shortly after infection (Wang and Maule, 1995; Maule et al., 2002; Marathe et al., 2004; Dardick, 2007; Baebler et al., 2009). Nevertheless, genes related to cell death, cell rescue, defense, ageing, stress and protein degradation are often induced in response to viral infection,

both in compatible and incompatible interactions (Whitham et al., 2003; Marathe et al., 2004 Senthil et al., 2005; Dardick, 2007). Viruses are also known to affect plant hormone signaling, as the abnormal growth of virus-infected plants has been related to alterations in auxin, abscisic acid, cytokinin, giberellin or ethylene levels, depending on the specific virus-host combination (Whitham et al., 2006).

Another prominent virus-induced host response is the induction of the post-transcriptional gene silencing (PTGS) machinery of the plant (Baulcombe, 2004). Antiviral PTGS involves the processing of viral double-stranded RNA (dsRNA) by Dicer-like enzymes (DCL) into small interfering RNAs (siRNAs), which are subsequently incorporated into the multi-component RNA-induced silencing complex (RISC). RISCs contain endonucleolytic Argonaute (AGO) enzymes that, upon activation by siRNAs, cleave RNA strands that have high sequence homology to the incorporated siRNAs (Xhie et al., 2004; Vaucheret et al., 2004; Ding and Voinnet, 2007). In addition to its implication in defense against viruses (Dalmay et al., 2001; Mourrain et al., 2000; Qu et al., 2005; Schwach et al., 2005), evidence accumulates for RNA silencing to play a role in interactions with other types of pathogens and pests, more specifically in bacterial defense (Padmanabhan et al., 2009; Ruiz-Ferrer and Voinnet, 2009), fungal defense (Ellendorff et al., 2009), nematode defense (Hewezi et al., 2008) and insect defense (Pandey et al., 2008). Furthermore, RNA silencing also plays an important role in the regulation of endogenous gene expression through microRNA (miRNA) function (Baulcombe, 2004). Host-adapted viruses have evolved strategies to counteract PTGS by encoding RNA silencing suppressors (Ding and Voinnet, 2007). As viruses are inducers, suppressors and targets of RNA silencing, virus-induced symptom development in infected plants can be influenced by the siRNA and miRNA pathways in many different ways (Baulcombe, 2004).

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 disease of greenhouse tomato crops worldwide (Hanssen and Thomma, 2010). The virus causes a wide range of symptoms, of which the typical fruit marbling and the occurrence of open fruits are particularly devastating as they reduce the economical value of the fruit (Soler et al., 2000; Roggero et al., 2001; Spence et al., 2007; Hanssen et al., 2009a; b; 2010a). Damage and economical losses caused by PepMV vary greatly, and can at least partially be attributed to differential symptomatology caused by different PepMV isolates (Hanssen et al., 2009b; 2010a). Currently, four PepMV genotypes are distinguished: the Peruvian genotype (LP) which was first isolated from *Lycopersicon peruvianum* and is similar to the original Pepino (*L. muricatum*) isolate (Lopez et al., 2005; Pagán et al., 2006); the European tomato genotype (EU), which was first reported in greenhouse tomato production in Europe (Mumford and Metcalfe, 2001; Aguilar et al.,

2002; Cotillon et al., 2002; Verhoeven et al., 2003; Pagán 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 United States (Maroon-Lango et al., 2007). Over recent years, the CH2 genotype has largely replaced the EU genotype in commercial tomato production in several European countries (Hanssen et al., 2008; Goméz et al., 2009). Two Belgian CH2 PepMV isolates have recently been characterized in detail and were designated mild (isolate 1906, further referred to as the mild CH2 isolate) and aggressive (isolate PCH 06/104, further referred to as the aggressive CH2 isolate), respectively, based on symptom expression in naturally infected commercial greenhouse tomatoes and in subsequent greenhouse trials (Hanssen et al., 2009b; 2010a). Inoculation of greenhouse tomatoes with the mild isolate resulted in rather mild PepMV symptomatology, while inoculation of greenhouse tomatoes with the aggressive isolate resulted in severe PepMV symptomatology with considerably more fruit marbling and a higher incidence of open fruits and premature leaf senescence. Intriguingly, the nucleotide sequence identity between both isolates was as high as 99.4% (Hanssen et al., 2009b). Because of the economical impact of PepMV symptoms, unraveling the molecular basis of (differential) symptom display is warranted.

In this study, we used a custom-designed Affymetrix tomato GeneChip array that contains probe sets to interrogate over 22,000 tomato transcripts (van Esse et al., 2007; 2008; 2009) to study transcriptional changes in response to inoculation with the mild and aggressive PepMV isolate. Interestingly, our results show that both isolates induce differential transcriptomic responses in the tomato host despite the accumulation of similar viral titers.

Materials and methods

Plant and virus materials, inoculation and disease evaluation

Tomato seedlings, cultivar Tricia (De Ruiter seeds, Bergschenhoek, the Netherlands) were grown in stonewool in a climate chamber (22 and 20°C during day and night periods of 10 and 14 hours, respectively, at 75% relative humidity). At 29 days after planting, plants were inoculated with a mild (‘1906’; GenBank accession number FJ457096) or an aggressive (‘PCH 06/104’; GenBank accession number FJ457097) PepMV isolate of the CH2 genotype (Hanssen et al., 2009b). Inoculation was performed on the second fully developed leaf as previously described (Hanssen et al., 2008).

The development of typical nettlehead-like PepMV symptoms was recorded at 4, 8 and 12 days post inoculation (DPI) on 20 plants per treatment. Symptoms were scored from 0 (no symptoms) to 3 (severe symptoms; Figure 1b; Hanssen et al., 2008) and

statistically analyzed by analysis of variance (one-way ANOVA) and post-hoc Bonferroni tests using SPSS software (v. 10.0; SPSS Inc., Chicago, IL, USA).

Viral accumulation was measured in the microarray samples using a PepMV-specific RT-qPCR assay with forward primer Pep5 (5' ATGAAGCATTCATACCAAAT 3') and reverse primer Pep4 (5' AATTCCGTGCACAACTAT 3'; Mumford and Metcalfe, 2001). The PCR program consisted of an initial denaturation step at 95°C for 15 min, 45 cycles of 15s at 94 ºC, 30 s at 50 °C and 30 s at 72 °C, followed by a final incubation step of 2 min at 72°C. Standard curves based on cDNA dilution series were generated to determine the relative concentrations of amplified viral RNA. Based on 4 replicates, run in two different analyses, a reaction efficiency of around 90% was obtained. Ct values obtained from the PepMV-specific RT-qPCR assay were standardized by subtraction from an internal control assay on the tomato actin gene (GenBank U60480.1) using forward primer Q-LeActinF 5' CTAGGCTGGGTTCGCAGGAGATGATGC 3' and reverse primer Q-LeActinR 5' GTCTTTTTGACCCATACCCACCATCACAC 3' (using the thermal profile mentioned above with an annealing temperature of 60°C).

Microarray sample preparation and data analysis

Per time point and treatment three biological replicates, each consisting of pooled RNA extracts obtained from the youngest fully developed leaves of two seedlings, were collected. Total RNA was extracted using the RiboPure RNA extraction kit (Ambion) and reverse transcribed with labeled oligo-dT primers for hybridization onto custom-designed Affymetrix tomato GeneChip arrays (Syngenta Biotechnology, Inc., Research Triangle Park, North Carolina, US).

Microarray quality control and data analysis were performed as described previously (van Baarlen et al., 2008; van Esse et al., 2009). Pathway reconstruction was performed using the BioNetBuilder plug-in (Avila-Campillo et al., 2007), an open-source tool that is able to generate biological networks by integration of information from several databases that include the Biomolecular Interaction Network Database (BIND) (Gilbert, 2005), Prolinks (Bowers et al., 2004), and the Kyoto Encyclopedia of Genes and Genomes database (KEGG) (Kanehisa et al., 2002).

Microarray data

Microarray data have been deposited in ArrayExpress as accession E-MEXP-2389.

Results

Differential disease development in tomato infected by different PepMV isolates

The phenotypic response of tomato seedlings to inoculation with a mild and an aggressive CH2 PepMV isolate was evaluated by recording the development of typical PepMV symptoms at 4, 8 and 12 DPI on 20 seedlings per treatment.

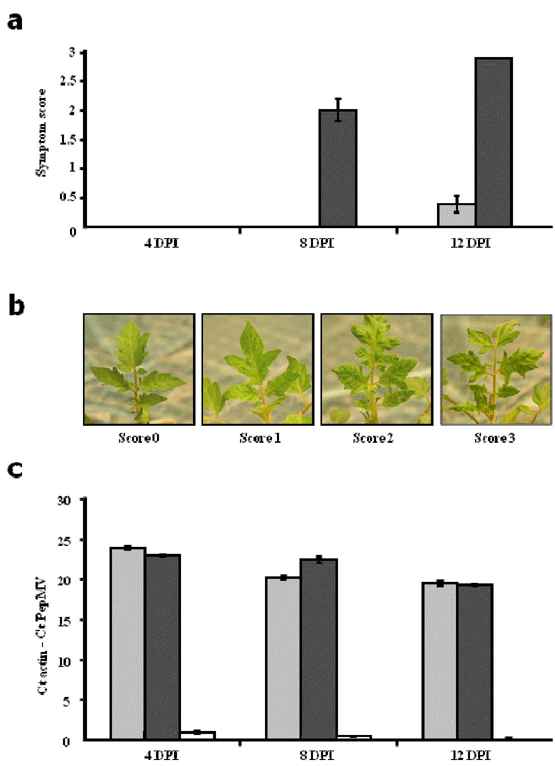


Figure 1. Characterization of tomato infection by a mild and aggressive Pepino mosaic virus (PepMV) isolate. (a) Scores for symptomatology on mock-inoculated plants (white bars), plants inoculated with the mild CH2 isolate (light grey bars) and the aggressive CH2 isolate (dark grey bars) of PepMV. Twenty plants were evaluated per treatment and error bars represent standard errors. (b) Representative pictures corresponding to symptom scores presented in (a). Symptoms were scored on a scale from 0 to 3 with 0 = no symptoms, 1 = reduced leaf surface, slight leaf bubbling, 2 = moderate leaf deformation, and 3 = severe leaf deformation. (c) Relative viral loads for mock-inoculated plants (white bars), plants inoculated with the mild CH2 isolate (light grey bars) and the aggressive CH2 isolate (dark grey bars) of PepMV obtained using a PepMV-specific RT-qPCR assay. PCR was performed pooled total RNA from the youngest fully developed leaves from two plants per sample (3 replicates). Ct values obtained from PepMV positive samples were subtracted from Ct values obtained from a actin control RT-qPCR assay. Subsequently the lowest value (obtained for mock-inoculated plants at 12 DPI) was set at zero. Error bars represent standard errors.

While at 4 DPI no symptoms were recorded yet, at 8 DPI tomato seedlings inoculated with the aggressive isolate displayed severe nettlehead symptoms, while plants inoculated with the mild isolate remained symptomless (Figure 1a, b). By 12 DPI, mild symptoms had developed in the seedlings inoculated with the mild isolate, while symptoms in seedlings inoculated with the aggressive isolate had aggravated (Figure 1a, b). The significant difference ($P<0.05$) in symptom severity between the mild and the aggressive CH2 isolate is consistent with previous observations (Hanssen et al., 2009b; 2010a). Mock-inoculated plants remained free of viral symptoms throughout the trial period (Figure 1a, b).

In addition to characterization of symptomatology, viral accumulation was measured in the various samples using a PepMV-specific RT-qPCR assay on pooled RNA extracts obtained from the youngest fully developed leaves. While no virus was detected in the mock-inoculated plants throughout the trial, rather equal amounts of virus were detected in the plants inoculated with the two PepMV strains. At 4 DPI the viral load was at the maximum level, and decreased slightly throughout the trial period (Figure 1c). Interestingly, the titer of the mild isolate already decreased at 8 DPI, while the titer of the aggressive isolate decreased to a similar level at 12 DPI. Most importantly, overall the viral titers were similar for both isolates and are thus no reason to expect significant differences in symptom severity and global transcription profiles between the two isolates.

PepMV infection results in host gene repression

Differentially regulated tomato genes (≥2-fold change; P-value $\leq$0,001) upon inoculation with the two PepMV isolates were identified at 4, 8 and 12 DPI when compared with mock-inoculated control plants. Following the dynamics of the viral accumulation, the number of differentially regulated genes was maximal at 4 DPI (3880 and 4020 for the aggressive and the mild isolate, respectively), and subsequently decreased (1315 and 744 genes for the aggressive and the mild isolate, respectively, at 8 DPI) to less than 10% of the maximum response at 12 DPI (381 and 317 for the aggressive and mild isolate, respectively). Despite the observation that by far the largest amount of differentials was identified at 4 DPI, also at 8 and 12 DPI novel differentially regulated genes emerged (Figure 2a, b). When comparing the total amount of differentially regulated genes between the two isolates, roughly three quarters of the differentials overlapped (Figure 2c). Interestingly, at 8 DPI when the titer of the mild isolate but not the aggressive isolate had already decreased, almost double the amount of genes were differentially expressed in the plants that were infected by the aggressive isolate (Figure 2e). This difference in response intensity between both isolates is also reflected by the number of highly-induced genes (≥4-fold change), which amounted to 55 and 11 genes

upon infection by the aggressive isolate and 18 and 3 genes upon infection by the mild isolate at 8 and 12 DPI, respectively.

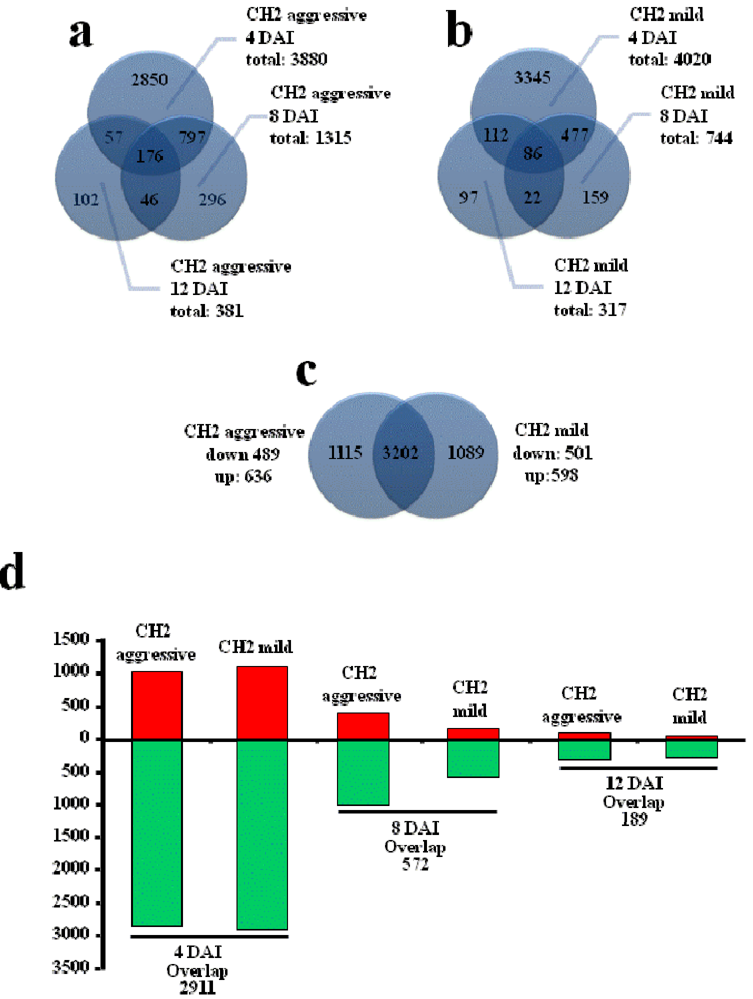


Figure 2. Differentially regulated tomato genes upon infection by a mild and aggressive *Pepino mosaic virus* (PepMV) isolate. (a, b) Venn diagram displaying the overlap in differentially regulated tomato gene sets upon inoculation with an aggressive (a) or a mild (b) isolate of the PepMV CH2 genotype at 4, 8 and 12 days post inoculation (DPI). (c) Venn diagram displaying the overlap in differentially regulated tomato gene sets upon inoculation with an aggressive or a mild isolate of the PepMV CH2 genotype. (d) Induced and repressed tomato genes in response to inoculation with an aggressive and a mild isolate of the PepMV CH2 genotype at 4, 8 and 12 DPI.

As has previously been noted for other viruses, host gene repression was more pronounced than induction at each of the time points, as at least 70% of the differentially regulated genes appeared to be repressed for both isolates (Figure 2e). Remarkably, when comparing the total amount of differentially regulated genes between the two isolates, relatively more repressed than induced genes overlapped between the two isolates. In the set of repressed genes up to 67% is commonly repressed by both isolates, compared to only 42% overlap in the set of induced genes. This suggests that, despite the observation that the majority of differentials is repressed, an important difference between the isolates is biased towards the induced differentials.

Over-representation analysis on repressed genes: primary metabolism

To identify differentially regulated biological processes, we performed over-representation analysis (ORA), a method by which over- and under-represented functional categories in a given gene set are identified using the web-based platform GeneTrail that can use *Arabidopsis* Reference Sequence (RefSeq) identifiers (Backes et al., 2007; van Baarlen et al., 2008). For 15,835 of the 22,721 genes that are monitored with the microarray, *Arabidopsis* homologues with significant similarity (E value $\leq 10^{-6}$) were identified (van Esse et al., 2009). ORA on the set of genes that are commonly repressed by both isolates (overlap; Figure 2c) identified as many as 267 functional categories involved in basic cell homeostasis and primary metabolism with a prominent occurrence of photosynthesis-related categories. ORA on the isolate-specific repressed gene sets revealed a few remarkable differences. Transcription and translation processes were more severely affected by the aggressive isolate, while photosynthesis was more severely suppressed by the mild isolate (Table 1). In addition to the photosynthesis-related categories identified in the commonly repressed genes, ORA identified another 17 photosynthesis-related categories in differentials that are down-regulated by the mild isolate exclusively.

Since ORA ignores induction or suppression amplitudes, hierarchical clustering (HCL; Eisen et al., 1998) on all photosynthesis-related genes that can be monitored with the array was used to cluster genes and treatments within ORA categories based on gene expression profiles to further study the behavior of these genes. HCL of these genes revealed that the expression profiles cluster by time point rather than by isolate (Figure 3a). This demonstrates that photosynthesis is similarly affected by both isolates, although the extent of the differential regulation clearly differs. Overall, the repression of photosynthesis-related genes is stronger in plants infected by the mild isolate, with the strongest repression at 4 DPI (Figure 3a). By 12 DPI, several photosynthesis-related genes, mainly involved in chlorophyll binding and light harvesting, were induced in both interactions, indicative of the recovery of photosynthetic activity. Interestingly, also other

energy processes appeared to be similarly repressed by PepMV infection. For instance, nearly all genes encoding citrate cycle enzymes showed significant repression (P<0.05) upon inoculation with both isolates at 4 DPI, which decreased by 8 DPI and was restored by 12 DPI (Figure 4). Thus, primary metabolism is temporarily affected by the PepMV-induced host gene repression.

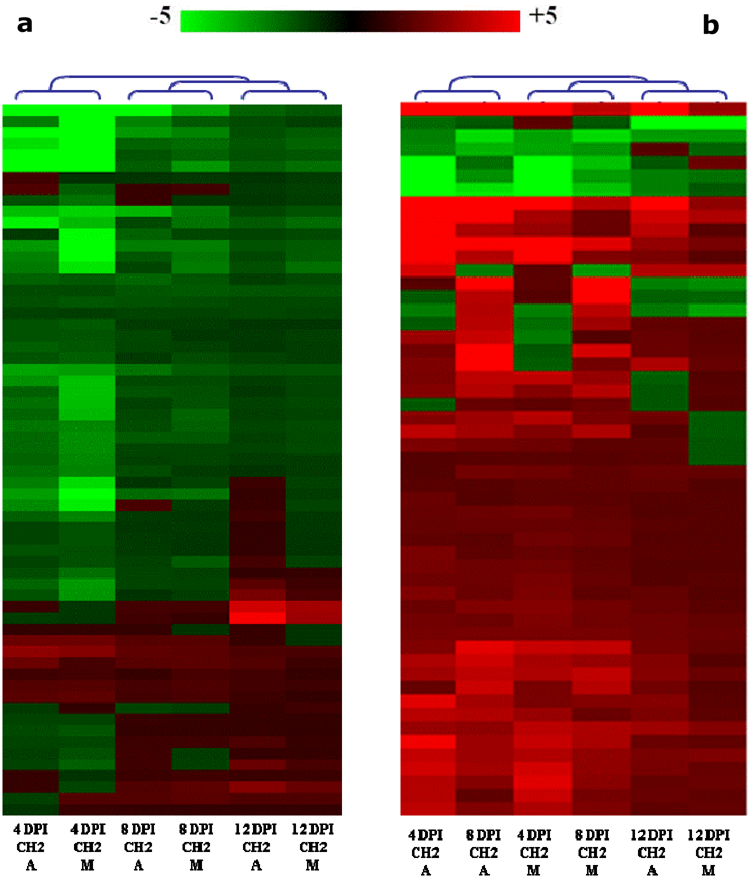


Figure 3. *Pepino mosaic virus* (PepMV) infection of tomato affects photosynthesis and host defense**;** (a) Transcriptional regulation of the 64 photosynthesis- and light harvesting-related genes that are represented on the tomato GeneChip in the interactions of tomato with the mild (CH2 M) and aggressive (CH2 A) CH2 isolate of PepMV at 4, 8 and 12 days post inoculation (DPI). (b) Transcriptional regulation of 53 defense-related genes that are identified in the over-representation analysis in the interactions of tomato with the mild (CH2 M) and aggressive (CH2 A) CH2 isolate of PepMV at 4, 8 and 12 days post inoculation (DPI).

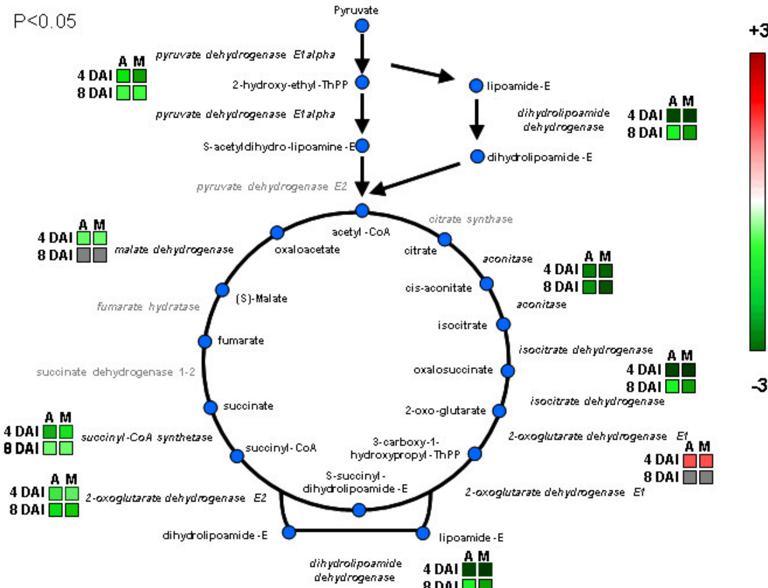


Figure 4. *Pepino mosaic virus* (PepMV) infection of tomato affects the citrate cycle. Graphic representation of the citrate cycle with intermediate products indicated as a blue dot and enzymes in italic (black = represented, grey = not represented on the tomato GeneChip). Expression data at 4 and 8 days post inoculation (DPI; P<0,05) are plotted using the indicated fold-change color scale with A = the aggressive CH2 isolate and M = the mild CH2 isolate. Expression levels similar to the control are indicated in grey.

Table 1. Over-representation analysis of genes that are specifically suppressed on at least one time point in tomato plants inoculated with the mild or aggressive CH2 isolate, respectively.

Gene Ontology subcategory	P-value[a]
CH2 mild	
Intracellular	1.41E-08
Cytoplasm	1.41E-08
Intracellular part	1.78E-08
Organelle	4.87E-08
Intracellular organelle	4.87E-08
Cytoplasmic part	4.87E-08
Organelle part	1.95E-06
Chloroplast part	1.95E-06
Plastid part	1.95E-06
Intracellular organelle part	1.95E-06
Intracellular membrane-bounded organelle	1.92E-05
Membrane-bounded organelle	2.10E-05
Photosynthetic membrane	7.95E-05
Thylakoid	8.90E-05
Thylakoid membrane	1.25E-04
Thylakoid part	1.50E-04
Chloroplast thylakoid membrane	1.69E-04
Plastid thylakoid membrane	1.69E-04
Organelle membrane	1.71E-04
Translation	1.74E-04

Cell	3.32E-04
Cell part	3.32E-04
Chloroplast thylakoid	4.41E-04
Chloroplast	4.41E-04
Plastid	4.51E-04
Plastid thylakoid	4.51E-04
Organelle subcompartment	5.19E-04
Ribosome	1.47E-03
Chlorophyll binding	1.47E-03
Cellular biosynthetic process	1.49E-03
Ribonucleoprotein complex	1.56E-03
Tetrapyrrole binding	1.66E-03
Oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	3.65E-03
Oxidoreductase activity, acting on heme group of donors	3.65E-03
Heme-copper terminal oxidase activity	3.65E-03
Cytochrome-c oxidase activity	3.65E-03
Biosynthetic process	5.47E-03
Structural molecule activity	6.03E-03
Monovalent inorganic cation transmembrane transporter activity	7.65E-03
Cellular metabolic process	9.26E-03
Structural constituent of ribosome	1.01E-02
Hydrogen ion transmembrane transporter activity	1.30E-02
Plastoglobule	1.61E-02
Intracellular non-membrane-bounded organelle	1.75E-02
Non-membrane-bounded organelle	1.75E-02
Metabolic process	1.83E-02
Organic acid metabolic process	2.15E-02
Cellular process	2.15E-02
Carboxylic acid metabolic process	2.15E-02
Macromolecular complex	2.35E-02
Stomatal movement	3.40E-02
Pigment metabolic process	3.71E-02
Cellular macromolecule biosynthetic process	3.71E-02
Tetrapyrrole metabolic process	3.71E-02
Oxidoreductase activity, acting on single donors with incorporation od molecular oxygen	3.71E-02
Plastid stroma	3.71E-02
Photosystem	3.71E-02
Macromolecule biosynthetic process	3.71E-02
Porphyrin metabolic process	3.71E-02
Catabolic process	3.87E-02
Response to radiation	4.03E-02
Porphyrin biosynthetic process	4.24E-02
Photosynthesis	4.24E-02
Tetrapyrrole biosynthetic process	4.24E-02
Cellular biopolymer biosynthetic process	4.56E-02
Biopolymer biosynthetic process	4.89E-02
CH2 aggressive	
Non-membrane-bounded organelle	7.70E-14
Intracellular non-membrane-bounded organelle	7.70E-14
Structural molecule activity	2.49E-12
Structural constituent of ribosome	3.76E-10
Ribonucleoprotein complex	3.76E-10
Translation	3.93E-10
Ribosome	1.33E-09
Chromatin	1.82E-08
Macromolecular complex	1.96E-08
Intracellular part	7.93E-08
Intracellular	1.56E-07

Chromosomal part	1.79E-07
Nucleosome	2.54E-07
Organelle	3.31E-07
Intracellular organelle	3.31E-07
Protein-DNA complex	4.52E-07
Chromosome	6.41E-07
Organelle organization	1.51E-06
Cytosolic ribosome	7.46E-06
Cytoplasmic part	1.12E-05
Chromatin assembly or disassembly	1.16E-05
Cell	1.55E-05
Cell part	1.55E-05
Cytosolic part	1.65E-05
Cytoplasm	1.83E-05
Nucleosome assembly	5.95E-05
Biosynthetic process	5.95E-05
Nucleosome organization	5.95E-05
Cellular biosynthetic process	5.95E-05
Establishment or maintenance of chromatin architecture	1.07E-04
Cellular biopolymer biosynthetic process	1.21E-04
Cellular biopolymer metabolic process	1.31E-04
Biopolymer biosynthetic process	1.31E-04
Chromatin assembly	1.36E-04
Chromosome organization	1.70E-04
DNA packaging	1.85E-04
Gene expression	1.90E-04
Intracellular organelle part	2.93E-04
Organelle part	2.97E-04
Anatomical structure organization	3.90E-04
Acyl carrier activity	4.00E-04
Cellular macromolecule biosynthetic process	4.22E-04
Macromolecule biosynthetic process	4.65E-04
Ribosomal subunit	4.90E-04
Protein-DNA complex assembly	5.25E-04
Cellular component organization	5.71E-04
Small ribosomal subunit	8.70E-04
Cytosol	1.10E-03
Anatomical structure morphogenesis	1.60E-03
Cellular process	1.78E-03
Structural constituent of cytoskeleton	2.08E-03
Cytosolic small ribosomal subunit	3.27E-03
Cellular metabolic process	4.50E-03
Anatomical structure development	6.86E-03
Protein metabolic process	7.91E-03
Primary metabolic process	8.80E-03
Cellular protein metabolic process	8.88E-03
Cellular macromolecule metabolic process	1.93E-02
Succinate-coa ligase activity	2.44E-02
Succinate-coa ligase (GDP-forming) activity	2.44E-02
Macromolecule metabolic process	2.62E-02
Nucleic acid binding	2.76E-02
Metabolic process	2.76E-02
Plastid organization and biogenesis	2.76E-02
Membrane-bounded organelle	3.09E-02
Intracellular membrane bounded organelle	3.80E-02
Fatty acid biosynthetic process	3.97E-02
Developmental process	4.65E-02

[a]False discovery rate

Over-representation analysis on induced genes: defense

Similar as for the repressed genes, ORA was used to compare the sets of induced genes (Figure 2c) between isolates and over time, showing significant differences in the induction of defense responses by the mild and the aggressive isolate. Although viral titers were similar (Figure 1c), defense responses were notably more pronounced upon inoculation with the aggressive isolate. The gene category “Pepsin A” activity was identified in both interactions at 4 DPI, which indicates a prominent role for protease activity at this time point (Table 2, 3). In addition, subtilase activity was induced by the mild isolate (Table 2, 3). The relevance of protease activity is further exemplified by the expression pattern of the cathepsin B-like cysteine protease, which at 4 DPI was induced 146-fold by the aggressive isolate and 100-fold by the mild isolate and remains highly induced throughout the PepMV-tomato interaction. Protease activity has been implicated in pathogen defense in many plant species including tomato (van Esse et al., 2008; van der Hoorn, 2008).

Table 2. Over-representation analysis of genes that are induced in tomato plants upon inoculation with the mild CH2 PepMV isolate.

Gene Ontology subcategory	P-value[a]
4 days post inoculation	
Pepsin A activity	2.53E-03
Subtilase activity	1.85E-02
8 days post inoculation	
Transcription factor activity	2.73E-02
Response to stress	2.73E-02
O-methyltransferase activity	2.73E-02
Response to wounding	2.73E-02
Response to light intensity	2.73E-02
Response to high light intensity	2.73E-02
Transcription regulator activity	2.73E-02
Response to stimulus	2.73E-02
Defense response	2.85E-02
Acid phosphatase activity	3.24E-02
Defense response to bacterium	3.46E-02
Response to temperature stimulus	3.60E-02
Defense response to bacterium, incompatible interaction	4.56E-02
12 days post inoculation	
chlorophyll binding	2.37E-06
tetrapyrrole binding	1.01E-05
photosynthesis, light harvesting in photosystem II	3.78E-03
photosynthesis, light harvesting	5.89E-03
posttranscriptional gene silencing	3.35E-02
gene silencing	4.57E-02

[a]False discovery rate

Table 3. Over-representation analysis of genes that are induced in tomato plants upon inoculation with the aggressive CH2 PepMV isolate.

Gene Ontology subcategory	P-value[a]
4 days post inoculation	
Pepsin A activity	2.51E-02
Anchored to membrane	4.59E-02
External encapsulating structure	4.59E-02
Carbon-oxygen lyase activity	4.59E-02
8 days post inoculation	
Defense response	4.30E-05
Response to biotic stimulus	4.79E-03
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	7.16E-03
Response to stress	1.00E-02
Response to wounding	1.00E-02
Defense response to bacterium	1.91E-02
Transcription regulator activity	2.40E-02
Response to other organism	2.67E-02
Response to light intensity	2.67E-02
Transcription factor activity	2.67E-02
Multi-organism process	2.74E-02
MAPKKK cascade	3.04E-02
Response to external stimulus	3.42E-02
Immune response	3.59E-02
Immune system process	3.59E-02
MAP kinase kinase activity	3.76E-02
Protein kinase cascade	3.76E-02
Response to high light intensity	4.82E-02
Response to stimulus	4.82E-02
12 days post inoculation	
Chlorophyll binding	7.34E-06
Tetrapyrrole binding	3.12E-05
Photosynthesis, light harvesting in photosystem II	6.79E-04
Photosynthesis, light harvesting	9.74E-03
Response to other organism	4.24E-02
Response to biotic stimulus	4.24E-02
Immune response	4.24E-02
Defense response	4.24E-02
Protein serinethreoninetyrosine kinase activity	4.24E-02
Immune system process	4.24E-02
Multi-organism process	4.69E-02
Response to chemical stimulus	4.69E-02
Photosynthesis, light reaction	4.69E-02
Response to stress	4.69E-02

[a]False discovery rate

At 8 DPI, many defense and stress-related categories were over-represented (Table 2, 3). Several defense-related categories are only over-represented upon inoculation with the aggressive isolate (Table 2, 3). Moreover, some defense-related categories that appear upon inoculation with both isolates (response to stress, response to wounding and defense response) are ranked higher upon inoculation with the aggressive isolate, indicating that more genes that belong to these categories are differentially regulated by this isolate. This more pronounced defense response induced by the aggressive isolate is also reflected by the set of highly induced genes (≥4-fold change) containing many defense-related genes at this time point. Similar to 8 DPI, at 12 DPI several defense-related categories were still over-represented and several defense genes were still highly induced in the plants inoculated with the aggressive but not with the mild isolate (Table 2, 3). This altogether demonstrates that, although plant defenses are activated in both

interactions, they are more pronounced, of higher magnitude and maintained longer in the aggressive isolate.

HCL of all defense-related genes from the ORA-identified categories showed that the expression profiles cluster by isolate at 4 and 8 DPI and not by sampling time point (Figure 3b). The 12 DPI time point of the aggressive isolate clusters with the three time points of the mild isolate, which is not surprising because the defense response is largely dampened by that point in time (Figure 3b). In addition, HCL reveals that most of the defense-related genes that were significantly induced at 8 DPI were already induced at 4 DPI, showing a rapid activation of host defense upon PepMV infection. The fact that the ORA did not reveal defense-related categories in addition to protease activity at 4 DPI is due to the extensive transcriptome change at this time point, which affects many processes and in which defense responses are not detected as over-represented.

Host signaling upon pathogen and herbivore attack has established the primacy of two pathways in plants, one involving salicylic acid (SA) and the other involving jasmonate (JA) hormones (Thomma et al., 1998; 2001; Stout et al., 2006). Homologues of the Arabidopsis pathogenesis-related proteins PR-1 and PR-5, known to be controlled by salicylic acid (SA; Ryals et al., 1996) were induced by PepMV infection while the homologue of JAR1, a JA-response protein that converts JA into the biologically active jasmonoyl-isoleucine (Staswick and Tiryaki, 2004), the jasmonate receptor CORONATINE INSENSITIVE 1 (COI1) (Xie et al., 1998; Yan et al., 2009), and the JA-inducible marker gene *PDF1.2* (Penninckx et al., 1998) were suppressed upon PepMV inoculation. This suggests that the tomato response to PepMV infection is mediated by SA rather than by JA, which is in line with the general observation that viruses are controlled by SA signaling (Huang et al., 2005; Vlot et al., 2009). Interestingly, the tomato homologue of Arabidopsis *Npr1*, which encodes a critical SA signal transducer, is not induced by PepMV. Similarly, in compatible Arabidopsis interactions with *Cucumber mosaic virus* (CMV) and *Oilseed rape mosaic virus* (ORMV) the majority of defense-related genes was induced by an SA-dependent, NPR1-independent signaling pathway (Huang et al., 2005). Nevertheless, a clear induction of the SA pathway upstream of the PR proteins was not detectable in our dataset.

Induction of post-transcriptional gene silencing

The key characteristic of RNA silencing is the formation of siRNAs that are produced by RNaseIII-like Dicer enzymes that are incorporated into a so-called RNA-induced silencing complex (RISC), which contains an Argonaute (AGO) protein with an siRNA-binding domain and endonucleolytic activity to cleave target RNAs. The PTGS pathway genes that have been implicated in antiviral defense and could be monitored with the GeneChip array ($P<0.05$) comprised the genes encoding the Dicer-like (DCL) enzymes DCL2 and

DCL4 (Bouché et al., 2006; Deleris et al., 2006), AGO proteins (Baumberger and Baulcombe, 2005), and the RNA-dependent RNA polymerase RDR6. A significant induction of the DCL2-encoding gene upon PepMV infection was observed, with a more pronounced induction upon inoculation with the aggressive isolate when compared with the mild isolate (>12-fold and 2,5-fold, respectively; Figure 5).

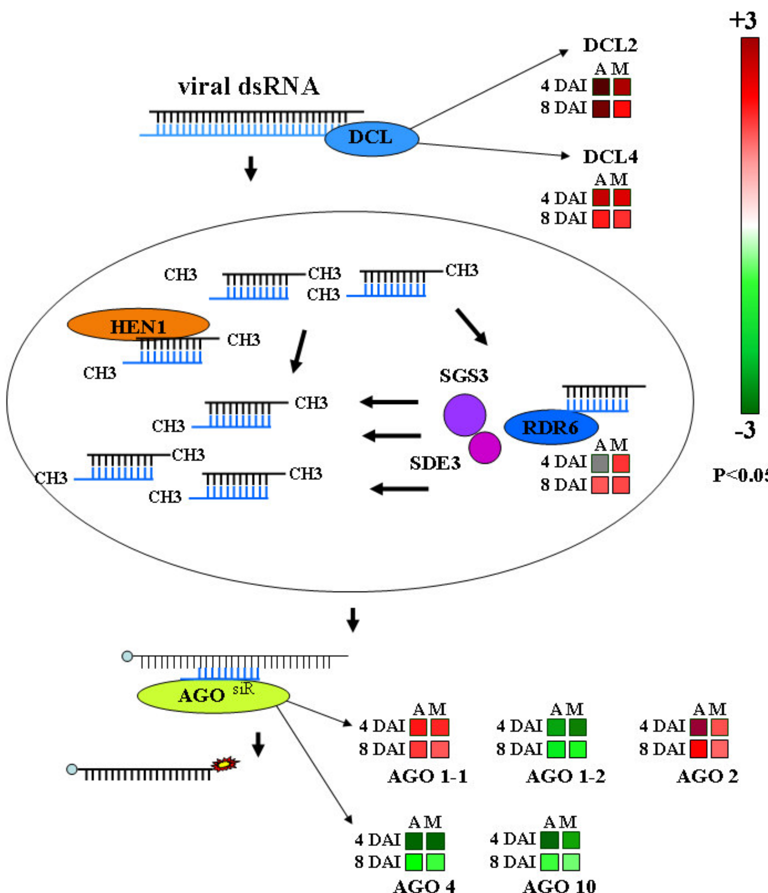


Figure 5. *Pepino mosaic virus* (PepMV) infection of tomato affects post-transcriptional gene silencing (PTGS). Expression data at 4 and 8 days post inoculation (DPI; P<0.05) are plotted using the indicated fold-change color scale with A = aggressive CH2 isolate and M = mild CH2 isolate. Expression levels similar to the control are indicated in grey.

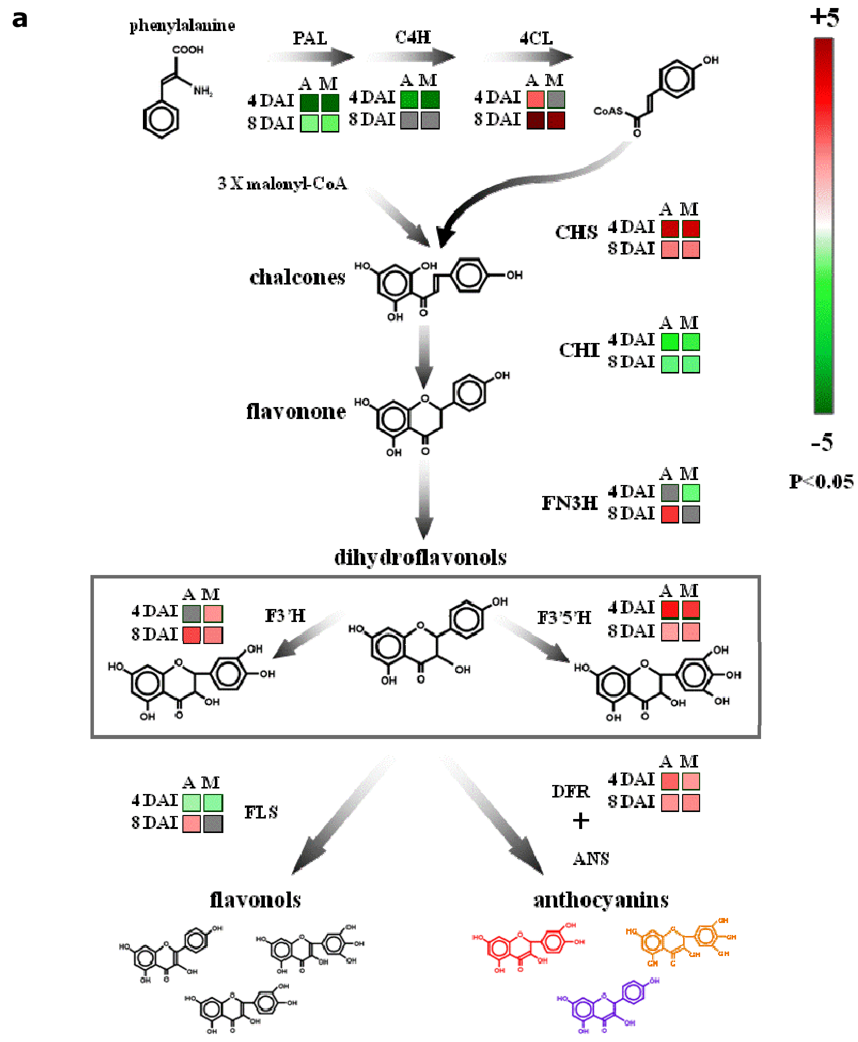
Also the gene encoding DCL4 was induced, although with a smaller amplitude (2.2-fold and 2.3-fold for the aggressive and the mild isolate, respectively). A rather mild up-regulation could be observed for RDR6 by both PepMV isolates. Furthermore, some AGO family proteins were differentially expressed. Probes corresponding to Arabidopsis AGO1, 2, 4, 6 and 10 were monitored. Interestingly, two AGO1 orthologues, *Ago1-1* and *Ago1-2* that were shown to play a role in RNA silencing in *Nicotiana benthamiana* (Jones et al., 2006), were differentially regulated. *Ago 1-2* was repressed, whereas *Ago1-1* was slightly induced by PepMV. AGO4 and AGO10 were repressed, while two AGO6 orthologues did not show differential expression. Interestingly, at 4 DPI AGO2 was induced 3.1-fold by the aggressive isolate and only 1.4-fold by the mild isolate. These data demonstrate that the antiviral PTGS pathway is differentially regulated upon PepMV infection (Figure 5).

Flavonoid and carotenoid biosynthesis

One of the most damaging effects of PepMV is the so-called "fruit marbling" which is likely due to irregular distribution of lycopene, the major red pigment in tomato fruits (Hanssen et al., 2008). To investigate whether PepMV has an effect on pigment production we studied the expression of genes that encode enzymes of the flavonoid/anthocyanin and carotenoid (lycopene) biosynthetic pathways ($P < 0.05$) (Figure 6). Interestingly, both the flavonoid/anthocyanin and lycopene biosynthetic pathways were severely affected by PepMV infection. In the flavonoid/anthocyanin biosynthetic pathway, the gene encoding 4-coumarate: CoA ligase (4CL) is induced upon infection with both CH2 isolates, although the induction is more pronounced in the aggressive isolate. At 8 DPI this gene is highly induced by both isolates (7,7-fold and 4,8-fold induction by the aggressive and the mild isolate, respectively; Figure 6a). Also a homologue of the gene encoding chalcone synthase (CHS; Arabidopsis RefSeq NP_196897, similar to Petunia hybrida Chalcone synthase B), the enzyme that initiates flavonoid biosynthesis (Holton and Cornish, 1995), is induced upon infection by both PepMV isolates at 4 DPI (amplitude 3.7) and 8 DPI (amplitude 1.9; Figure 6A). Interestingly, all homologues of the gene that encodes the next enzyme in the biosynthesis route, chalcone isomerase (CHI), that could be monitored with the array are slightly repressed by both isolates (Figure 6A). Also other genes encoding enzymes that act further downstream in the flavonoid/anthocyanin biosynthesis pathway are differently expressed (Figure 6a). In conclusion, the flavonoid biosynthetic pathway does not follow the trend of general host gene repression.

Interestingly, all genes encoding enzymes involved in the biosynthesis of lycopene, including GGPP-synthase, pythoene synthase, phytoeene desaturase and ζ-carotene desaturase, were suppressed upon PepMV infection (Figure 6b). The gene encoding phytoene synthase, also known as the tomato fruit ripening specific protein

(pTOM5; Bramley et al., 2005), was severely suppressed at all time points, although the level of repression gradually decreased from 7 and 13 times at 4DPI for the aggressive and mild CH2 isolate, respectively, to approximately 5 times for both isolates at 8 DPI and approximately 3 times for both isolates at 12 DPI. Thus, in contrast to the flavonoid/anthocyanin biosynthesis, the complete lycopene biosynthetic pathway is severely affected by the PepMV-induced host gene repression.



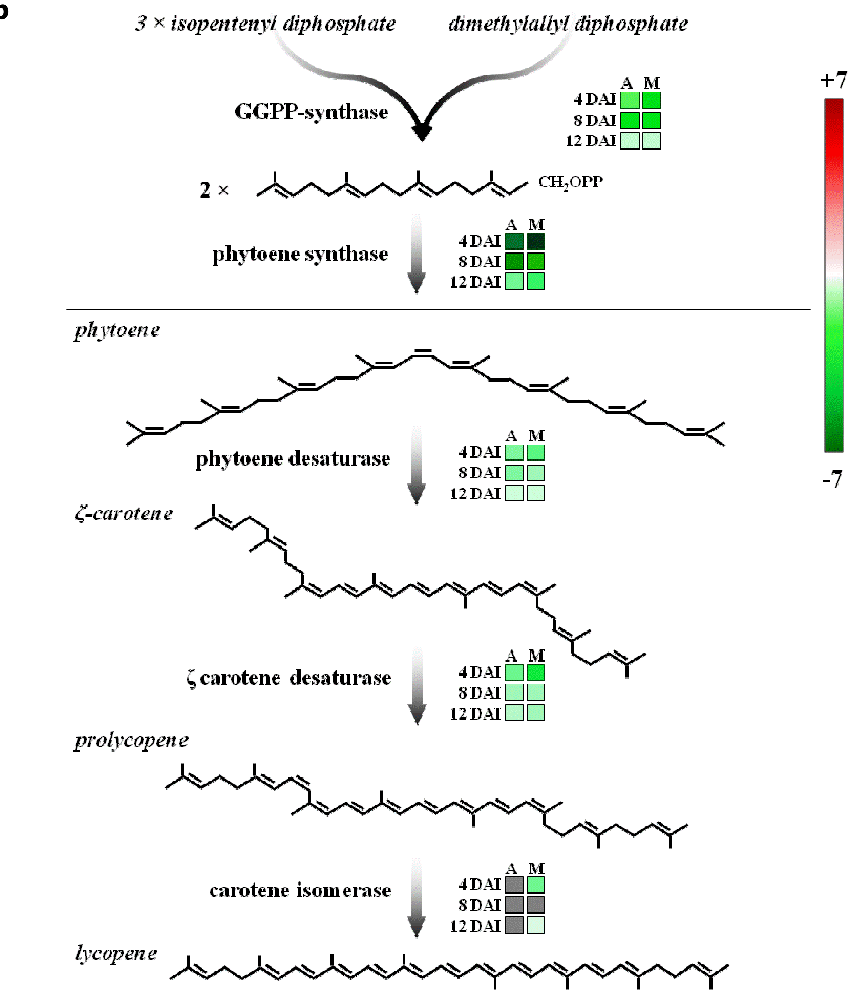


Figure 6. *Pepino mosaic virus* (PepMV) infection of tomato affects pigment biosynthesis pathways. (A) Transcriptional regulation of flavonoid/anthocyanin biosynthesis. Expression data at 4 and 8 days post inoculation (DPI; P<0,05) are plotted using the indicated fold-change color scale with A = the aggressive CH2 isolate and M = the mild CH2 isolate. Expression levels similar to the control are indicated in grey. (B) Transcriptional regulation of lycopene biosynthesis. Expression data at 4, 8 and 12 DPI (P<0,05) are plotted using the indicated fold-change color scale with A = the aggressive CH2 isolate and M = the mild CH2 isolate.

Pathway analysis reveals a role for the proteolytic machinery

Pathway reconstruction is a tool to identify cellular processes from transcriptome data, which has been widely used to analyze human and murine data, but has hardly been used to analyze plant data (van Baarlen et al., 2008; van Esse et al., 2009). Although the number of tools to facilitate cellular pathway reconstruction from Arabidopsis data is

increasing, pathway reconstruction in a non-sequenced plant such as tomato remains challenging (van Baarlen et al., 2008; van Esse et al., 2009). We performed pathway analyses using BioNetBuilder plug-in on all 15,835 genes with *Arabidopsis* homologues (E value $\leq 10^{-6}$; van Esse et al., 2009) that were differentially regulated (≥2-fold; P<0.01) upon infection by either PepMV isolate using the BioNetBuilder option to expand gene sets with neighboring nodes to find new interactions with molecular components identified in the previous step (Avila-Campillo et al., 2007). This resulted in the identification of 54 interacting factors that are involved in ubiquitination (Figure 7). Subsequently, the expression data of the differentially regulated genes (P<0.05) were grafted onto the pathway.

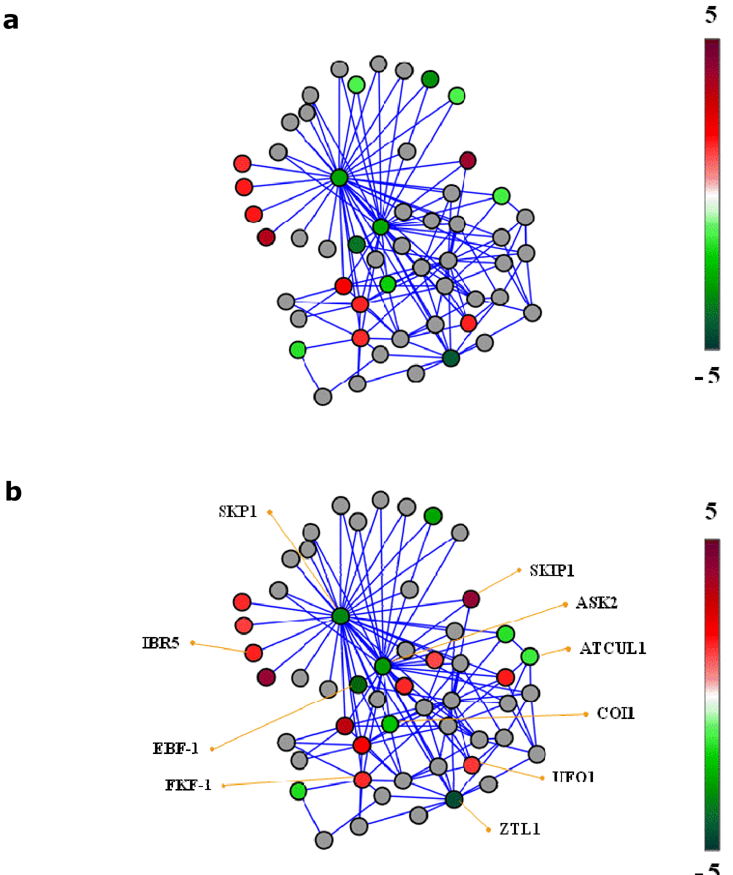


Figure 7 Pathway reconstruction reveals differential regulation of an ubiquitination pathway. Responses of tomato upon inculation with the aggressive (a) and mild (b) CH2 isolates of PepMV using the Bionetbuilder plug-in. A ubiquitination network implied in both interactions was retrieved and visualized in Cytoscape. Subsequently, expression data monitored with the tomato GeneChip were grafted onto the network.

The most extensive regulation of the network occurred at 4 DPI in both interactions, although differential regulation of the pathway was also observed at 8 and 12 DPI. Interestingly, in contrast to the general host gene repression, the identified pathway also contained genes that were induced in both interactions (Figure 7), suggesting that this ubiquitin-pathway plays an active role in the PepMV-tomato interaction.

An mild EU isolate induces similar host gene alterations

To determine whether transcriptome changes in response to PepMV inoculation are genotype-specific, transcriptome changes upon infection with a mild isolate belonging to the European tomato PepMV genotype (EU; isolate 1806; Hanssen et al., 2009b) were determined using a similar experimental design as with the CH2 isolates. Up to 12 DPI, plants inoculated with the EU isolate did not display any viral symptoms, although a slight reduction in plant vigor was observed. At all three time points, the number of induced genes was lower than for both CH2 isolates (Figure 8)HowhHH, further suggesting a correlation between isolate aggressiveness and the extent of host transcriptional reprogramming. However, surprisingly, the ratio of suppressed host genes was higher for the EU isolate when compared to the CH2 isolates. At 12 DPI, double the amount of genes was suppressed by the EU isolate (621) than by either of the CH2 isolates (257 and 302 for the mild and aggressive isolate, respectively). Thus, the intensity of the host gene repression, marked by the number of down-regulated genes, does not correlate with isolate aggressiveness.

In general, a similar pattern was seen for the EU isolate as with the CH2 isolates. At 4 DPI an early protease response (pepsin A, caspase and subtilase activity) was activated, coinciding with a down-regulation of general cellular processes related to photosynthesis, energy processes and translation activity. Interestingly, the gene encoding cathepsin B was not induced by the EU isolate, in contrast to its induction by the CH2 isolates. In general, the amplitude of protease induction was lower upon infection with the EU isolate. At 8 DPI the prevalent ORA categories comprised defense and stress responses (Table 4), while the general suppression of host genes became less severe. By 12 DPI, many photosynthesis-related genes were still down-regulated, but light harvesting and chlorophyll binding were induced (Table 4), similar to the situation as described for both CH2 isolates (Table 2, 3). Nevertheless, many general cellular processes, such as transcription, translation, metabolism and energy production were still suppressed. This is remarkable since, although a slight reduction in plant vigor was noted, the plants did not display obvious disease symptoms. Interestingly, the gene encoding DCL2 was not induced by the mild EU isolate, implying that the role of DCL2 is less important in tomato defense against this specific, mild PepMV isolate.

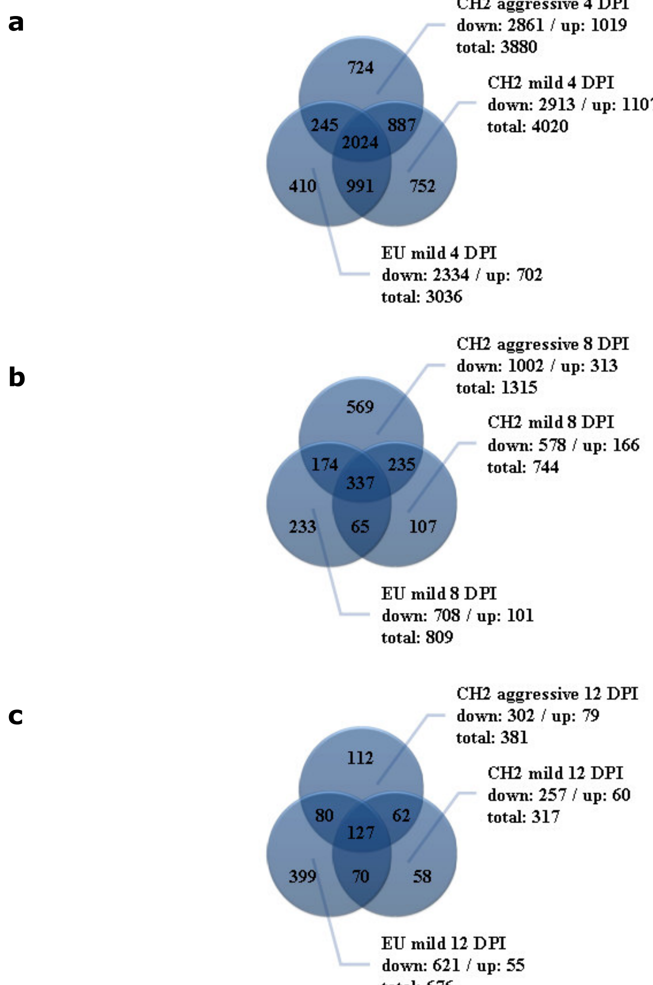


Figure 8. Differentially regulated tomato genes upon infection by three *Pepino mosaic virus* (PepMV) isolates. Venn diagrams displaying the overlap in differentially regulated gene sets between tomato responses upon inoculation with an aggressive and a mild isolate of the PepMV CH2 genotype and a mild isolate of the EU genotype at 4 (a), 8 (b) and 12 (c) days post inoculation (DPI).

Table 4. Over-representation analysis of genes induced in tomato plants inoculated with the mild EU PepMV isolate.

Gene Ontology subcategory	P-value[a]
4 days post inoculation[b]	
8 days post inoculation	
Steroid metabolic process	4.21E-04
Steroid biosynthetic process	4.67E-03
Defense response to bacterium	6.54E-03
Brassinosteroid metabolic process	6.54E-03
Phytosteroid metabolic process	6.54E-03
Sterol biosynthetic process	6.54E-03
Sterol metabolic process	6.54E-03
Response to light stimulus	6.54E-03
Response to radiation	6.54E-03
Defense response	6.54E-03
Response to stress	6.54E-03
Response to wounding	1.02E-02
Response to stimulus	1.07E-02
Response to bacterium	1.11E-02
Cellular lipid metabolic process	1.17E-02
Response to high light intensity	2.00E-02
Oxidoreductase activity, acting on CH-OH group of donors	2.00E-02
Response to abiotic stimulus	2.61E-02
Lipid metabolic process	2.88E-02
Response to biotic stimulus	2.97E-02
Response to light intensity	3.41E-02
Defense response to fungus	3.97E-02
Response to external stimulus	4.75E-02
12 days post inoculation	
Photosynthesis, light harvesting in photosystem II	2.09E-04
Photosynthesis, light harvesting	2.18E-03
Chlorophyll binding	3.11E-03
Tetrapyrrole binding	6.18E-03
Photosynthesis, light reaction	1.77E-02
Thylakoid	3.67E-02
Photosynthesis	3.67E-02
Cytosolic ribosome	3.67E-02
Cytosolic part	4.94E-02

[a]False discovery rate
[b]No categories identified with *P* value < 0.05.

Discussion

Tomato response to PepMV is transient: recovery?

In this study we monitored global transcriptional responses of tomato upon inoculation with two PepMV isolates of the CH2 genotype that differ considerably in aggressiveness although they share 99.4% nucleotide sequence identity (Hanssen et al., 2009b; 2010a). Inoculation with both isolates resulted in a very fast and extensive transcriptome change (4000 differentially regulated genes at 4DPI; amounting to ~20% of the total amount of genes that are monitored with the array) which, by 12 DPI, decreased to ~10% of the initial amount of differentially expressed genes. This dynamics is completely different than the dynamics observed upon inoculation with the foliar pathogen *Cladosporium fulvum* (Thomma et al., 2005) or the vascular fungus *Verticillium dahliae* (Fradin and Thomma, 2006) monitored over a ten-day time-frame using the same tomato array (van Esse et al., 2009). The number of differentially regulated genes gradually increased to

amount to over 3,000 differentials for *C. fulvum*, and over 500 and 1,000 genes for *V. dahliae* in foliage and roots, respectively. Interestingly, the pattern seems to follow the amount of pathogen propagules which is increasing upon inoculation with the fungal pathogens, while it is decreasing upon PepMV inoculation (van Esse et al., 2009; this study). Moreover, in contrast to progressive fungal disease development, viral symptoms are often transient as plants can recover from the initial infection shock, and can re-appear later in the infection process in response to environmental changes (Hull, 2002; Dardick, 2007). Recovery from PepMV symptoms after initial infection is indeed common in PepMV-infected tomato crops (Hanssen and Thomma, 2010), suggesting that the transient transcriptional response to PepMV is related to the recovery phenomenon.

Interestingly, the vast majority of the differentially expressed genes upon PepMV inoculation were repressed, with the strongest repression by the aggressive isolate, a phenomenon which is similarly observed in other plant-virus interactions (Aranda and Maule, 1998; Havelda et al., 2008). Remarkably, however, even more down-regulated genes were monitored upon inoculation with an asymptomatic EU isolate of PepMV.

Host gene repression – 'fuel for the fire' or viral infection strategy?

The majority (67%) of the repressed genes overlapped between both isolates, indicating that PepMV-induced host gene repression is not isolate-specific. ORA identified a general repression of photosynthesis and primary metabolism. Although increased photosynthesis to supply the energy required for plant defense may be anticipated, repression of photosynthesis and plastid function is a common host response not only to viruses (Aranda and Maule, 1998; Dardick, 2007; Havelda et al., 2008), but also to fungal and bacterial plant pathogens (Bolton, 2008). Apparently, as production of defense-related compounds becomes first priority, photosynthetic metabolism is reduced until pathogenic growth has been terminated. Apart from prioritizing resources towards the defense response, decreasing photosynthesis may also protect the photosynthetic machinery against oxidative damage, or, alternatively, be a consequence thereof (Bolton, 2008). Plant respiration, comprising glycolysis, the citrate cycle and mitochondrial electron transport, is generally induced upon pathogen attack to generate energy for the defense response (Bolton, 2008). Remarkably, while PepMV induces a broad spectrum of defense responses, a strong repression of the citrate cycle was observed. Thus, as suggested previously (Wang and Maule, 1995), our results suggest that, rather than host liberation of resources for defense responses, the virus modulates the host by repressing all processes that are not required for viral replication as a strategy for optimal and fast viral replication.

Intensity of defense responses correlates with viral aggressiveness

Host gene induction upon PepMV infection comprised protease activity specifically at 4 DPI and activation of defense responses at 4 and 8 DPI, which persisted up to 12 DPI in plants infected with the aggressive isolate. Interestingly, a stronger defense response was observed in plants inoculated with the aggressive CH2 isolate, which was not caused by higher viral accumulation as viral loads were similar for both isolates. Moreover, the stronger defense response was not sufficient to contain viral accumulation, nor to prevent symptom development. This indicates that PepMV aggressiveness is not correlated with the capacity to suppress basal plant defense responses, as was observed in *Arabidopsis thaliana* where an aggressive *Tobacco etch virus* (TEV) strain induced notably less defense-related genes when compared with a mildly aggressive strain (Agudelo-Romero et al., 2008). By contrast, enhanced symptom severity upon inoculation with the aggressive PepMV isolate may be related to a more severe perturbation of host metabolism leading to more severe developmental defects (Whitham et al., 2006).

PepMV differentially regulates antiviral PTGS

A major plant defense response to virus infection is the induction of the antiviral PTGS machinery. The response of genes encoding key PTGS components was studied in detail, and the genes encoding tomato homologues of the Arabidopsis dicer-like enzymes DCL2 and DCL4 were induced by both CH2 isolates. Interestingly, the induction of DCL2 was much stronger when compared to the induction of DCL4, which usually is the major contributor to antiviral PTGS (Bouché et al., 2006). However, DCL2 can substitute for DCL4 in antiviral defense when DCL4 is inhibited by viruses (Bouché et al., 2006; Deleris et al., 2006). Therefore, the high induction of DCL2 in our dataset may be indicative of a PepMV-encoded silencing suppressor that interferes with DCL4 activity. Alternatively, the induction of both DCL genes might be a result of a PepMV-encoded suppressor protein that acts upstream of the dicer-like enzymes. The *Potato virus X* silencing suppressor P25 has similarly been suggested to interfere with RDR6, to block an AGO protein, or interfere in another way with assembly of siRNA-containing effector complexes (Verchot-Lubicz et al., 2007; Bayne et al., 2007). Two AGO1 ortologues (*Ago1-1* and *Ago1-2*) that were previously identified in *N. benthamiana*, as well as AGO2 and AGO4 were differentially expressed. *Ago1-1* was slightly induced while *Ago1-2* was repressed. Also AGO4 was repressed, but AGO2 was strongly induced by the aggressive isolate (>3-fold at 4 DPI) and only slightly by the mild isolate. Although AGO1 is presented as a major antiviral slicer in Arabidopsis, the same situation is not necessarily true in tomato, and other AGO paralogues may display a similar activity as has previously been suggested (Ding and Voinnet, 2007). Interestingly, the induction amplitudes of the genes encoding

the tomato homologues of DLC2 and AGO2 correspond to PepMV isolate aggressiveness, with a strong induction by the aggressive CH2 isolate, a moderate induction by the mild CH2 isolate and no induction by the symptomless EU isolate. Possibly, this reflects the differential effectiveness of a silencing suppressor encoded by the various isolates. However, currently no PepMV-encoded silencing suppressor has been characterized.

Perturbation of flavonoid/anthocyanin and carotenoid biosynthesis

One of the most damaging effects of PepMV is the so-called "fruit marbling", which is likely to be caused by aberrant production of tomato pigments such as flavonoids, anthocyanins and carotenoids. The stronger induction of 4CL, involved in the flavonoid/anthocyanin biosynthetic pathway, is probably related to defense responses as this last enzyme in the general phenylpropanoid pathway leads to precursors for both flavonoids, but also wall-bound phenolics and lignins (Hahlbrock and Scheel, 1989; Whetten and Sederoff, 1995). However, induction of the 4CL gene was not associated with induction of PAL and C4H, as is often observed upon pathogen attack, elicitor treatment, and wounding (Fritzemeier et al., 1987; Schmelzer et al., 1989; Lee et al., 1995; Thulke and Conrath, 1998). A homologue of the gene encoding CHS, which synthesizes chalcones, was induced at both 4 DPI and 8 DPI. Chalcones are usually processed further by CHI to generate flavonones (Holton and Cornish, 1995; Schijlen et al., 2004). However, all homologues for the gene encoding CHI were repressed by both PepMV isolates, suggesting that chalcones might accumulate in PepMV-infected tomato. This yellow pigment is the most prevalent flavonoid compound in tomato fruits and it constitutes the major pigment of the yellow fruit skin. Although the effect of chalcone accumulation in tomato is not well known, it might change the color of flowers and fruits. CHI-silencing in tobacco resulted in changes in flower color and a natural CHI mutant in onion resulted in a gold colored variant (Nishihara et al., 2005; Kim et al., 2004). Furthermore, over-expression of petunia CHI in tomato fruits was shown to alter the color of tomato fruits, most likely caused by reduced levels of chalcone (Muir et al., 2001), demonstrating that differential chalcone levels can influence fruit color.

Also the lycopene biosynthetic pathway was severely affected by PepMV infection. Interestingly, all genes encoding enzymes involved in this pathway were repressed up to 12 DPI, suggesting that PepMV infection suppresses lycopene biosynthesis. As lycopene is the major red color pigment in tomato fruit, this finding might be involved in the typical fruit discoloration observed in PepMV infected tomato fruits. Especially the severe suppression of pTOM5 (phytoene synthase) is interesting in this respect, as inverted repeat-mediated knock-down of this gene was shown to result in yellow tomato fruits (Bramley et al., 1992).

Our study was performed on young plants and many transcriptome responses appeared to be transient over the observed time period. Also PepMV symptoms are mostly transient and disappear after the initial infection phase, but tend to reappear with changing environmental conditions later in the growth season. Similar fluctuations are likely to occur in the transcriptome, possibly related to viral replication in newly developing plant tissues. Local differences in PepMV replication and correlated host responses during fruit ripening might lead to local impairment of lycopene biosynthesis and/or local accumulation of chalcone in the fruit skin, leading to marbled of flamed fruits.

Induction of the ubiquitination machinery

The induction of the ubiquitination/proteasome machinery upon PepMV infection has similarly been observed in CMV-infected Arabidopsis (Marathe et al., 2004), and was shown to be important for resistance to TMV in tobacco (Liu et al., 2002). Furthermore, viral hijacking of the host proteolytic machinery to enhance viral replication has been reported in several cases. The *Lettuce mosaic virus* (LMV) helper component proteinase (HCPro) is a PTGS suppressor that interacts with the 20S proteasome (Ballut et al., 2005). Furthermore, the *Faba bean necrotic yellows virus* (FBNYV) protein Clink is able to bind to SKP1 for targeted degradation of host proteins (Aronson et al., 2000; Timchenko et al., 2006), while the P0 silencing suppressor of poleroviruses interacts with SKP1 via a minimal F-box motif to target and degrade AGO1 (Pazhouhandeh et al., 2006; Bortolamiol et al., 2007).

Interestingly, several components of the PepMV-induced ubiquitination pathway are involved in defense signaling, such as the jasmonate receptor COI1 that forms a complex with Skp and cullin proteins to degrades JAZ1 proteins that otherwise repress transcription of jasmonate-responsive genes (Thines et al., 2007). Repression of COI1 is in agreement with the observation that PepMV-induced defense responses are mediated by SA rather than by JA.

Another defense-related component identified in the PepMV-induced ubiquitination pathway is the EIN3-BINDING F BOX PROTEIN 1 (EBF1) that represses ethylene responses by degradation of the ethylene-responsive transcription factors EIN3 and EIL1 (Gagne et al., 2004; Binder et al., 2007). The gene encoding EBF1 was repressed in response to PepMV, suggesting that ethylene responses are induced. Furthermore, the gene encoding INDOLE-3-BUTYRIC ACID RESPONSE 5 (IBR5), which is able to promote auxin responses, is induced in our dataset which suggests that auxin is involved in the PepMV response (Monroe-Augustus et al., 2003; Binder et al., 2007). Auxin has been recognized as a regulator of plant defense against various pathogens (Navarro et al., 2006; van Esse et al., 2009; Kazan and Manners, 2009), and a role for auxin has been

demonstrated in the TMV-tomato interaction were the replicase protein of TMV actively disrupts auxin signaling by interacting with AUX/IAA protein LeIAA26, thus enhancing viral accumulation and symptom development (Padmanabhan et al., 2008).

In conclusion, infection with an aggressive and a mild PepMV isolate resulted in significant transcriptional changes. Furthermore, with novel strategies that allowed the use of state-of-the-art tools we identified several processes that are involved in the interaction between PepMV and tomato which provide new insight into the biology of PepMV.

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Chapter 7:
General discussion

Pepino mosaic virus: a successful pathogen that rapidly evolved from emerging to endemic in tomato crops

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Abstract

Taxonomy: Pepino mosaic virus (PepMV) belongs to the *Potexvirus* genus of in the *Flexiviridae* family.

Physical properties: PepMV virions are non-enveloped flexuous rods that contain a monopartite, positive sense, single stranded RNA genome of 6.4 kb with a 3' poly-A tail. The genome contains five major open reading frames (ORFs) encoding a 164 kDa RNA-dependent RNA polymerase (RdRp), three triple gene block proteins of 26, 14 and 9 kDa, and a 25kDa coat protein.

Genome diversity: four PepMV genotypes, with an inter-genotype RNA sequence identity ranging from 78 to 95%, can be distinguished: the original Peruvian genotype (LP); the European (tomato) genotype (EU), the American genotype US1 and the Chilean genotype CH2.

Transmission: PepMV is very efficiently transmitted mechanically, and a low seed transmission rate has been demonstrated. In addition, bumblebees have been associated with viral transmission.

Host range: Similar to other Potexviruses, PepMV has a rather narrow host range that is thought to be largely restricted to species of the *Solanaceae* family. After originally being isolated from pepino (*Solanum muricatum*), PepMV has been identified in natural infections of the wild tomato species *S. chilense*, *S. chmielewskii*, *S. parviflorum* and *S. peruvianum*. PepMV is causing significant problems in the cultivation of greenhouse tomato (*Solanum lycopersicum*), and has been identified in weeds belonging to various plant families in the vicinity of tomato greenhouses.

Symptomatology: PepMV symptoms can be very diverse. Fruit marbling is the most typical and economically devastating symptom. In addition, fruit discoloration, open fruit, nettle-heads, leaf blistering or bubbling, leaf chlorosis and yellow angular leaf spots, leaf mosaic and leaf or stem necrosis have been associated with PepMV. Severity of PepMV symptoms is thought to depend on environmental conditions as well as on properties of the viral isolate. Minor nucleotide sequence differences between isolates from the same genotype have been shown to lead to enhanced aggressiveness and symptomatology.

Control: Prevention of infection through strict hygiene measures is currently the major strategy for controlling PepMV in tomato production. Cross-protection can be effective, but only under well-defined and well-controlled conditions and the effectiveness heavily depends on the PepMV genotype.

Introduction

Pepino mosaic virus (PepMV) has been observed for the first time in tomato (*Solanum lycopersicum*) crops in the Netherlands only a decade ago (EPPO, 2000), and has presently become a major disease of greenhouse tomato crops worldwide (van der Vlugt et al., 2000; French et al., 2001; Mumford and Metcalfe 2001; Cotillon et al., 2002; Maroon-Lango et al., 2005; Pagán et al., 2006; Hasiów et al., 2008; Hanssen et al., 2008, Chapter 2; Ling 2006; Ling et al., 2008). The economic impact of PepMV on tomato industry is highly debated, as the impact largely depends on the structure of the tomato market, more specifically on the marketability and economic value of lower-quality fruits, which differs considerably between growing areas (Jones and Lammers, 2005; Spence et al., 2006). Furthermore, the high variability in nature and severity of symptom display complicates a reliable determination of the economic impact of PepMV on the tomato industry. In a questionnaire conducted among Belgian tomato growers, yield losses caused by PepMV were estimated between 5 and 10% in the 2006 growth season and negligible in 2005, while fruit quality losses were more pronounced in 2005 (Hanssen et al., 2009a). Greenhouse trials conducted in the UK from 2001 to 2003 revealed considerable differences in damage between subsequent years, with the percentage of downgraded tomato fruit due to PepMV-induced quality loss ranging from 6 to 38%.

In this pathogen profile we review the current knowledge on PepMV biology, genome diversity, population dynamics, symptomatology, transmission and control, which includes the results that have been obtained during this thesis research.

Host range and symptomatology

As indicated by its name, PepMV was originally isolated from pepino (*Solanum muricatum*) that showed yellow leaf mosaic symptoms in Peru (Jones et al., 1980). Its host range is thought to be mainly restricted to *Solanaceae* species (Salomone and Roggero, 2002; Soler et al., 2002; Verhoeven et al., 2003). In a survey in central and southern Peru the virus has been identified in natural infections of the wild tomato species *S. chilense*, *S. chmielewskii*, *S. parviflorum* and *S. peruvianum* (Soler et al., 2002). Furthermore, by performing mechanical inoculations, the host range of PepMV was shown to contain eggplant (*Solanum melongena*), potato (*Solanum tuberosum*) and species from the genera *Nicotiana* (e.g. *N. benthamiana*), *Datura* (e.g. *D. stramonium*), *Capsicum* (*C. annuum*) and *Physalis* (*P. floridana*) (Salomone and Roggero, 2002; Verhoeven et al., 2003; Jones et al., 1980; Martin and Mousserion, 2002). So far, Basil (*Ocimum basilicum*; Lamiaceae) is the only reported natural host that does not belong to the *Solanaceae* with plants displaying interveinal chlorosis (Davino et al., 2009).

Furthermore, in a survey of 42 native weed species growing in or around tomato production sites in Spain, PepMV infection was found in 18 weed species, including those belonging to the Amaranthaceae (e.g. *Chenopodium murale*), Convolvulaceae (e.g. *Calystegia sepium*), Brassicaceae (e.g. *Diplotaxis erucoides*), Boraginaceae (e.g. *Heliotropium europaeum*), Asteraceae (e.g. *Sonchus tenerrimus*), Plantaginaceae (*Plantago afra*), and Polygonaceae (*Rumex* sp.) (Córdoba et al., 2004). Interestingly, a recent study revealed that co-inoculation with a EU and a CH2 isolate extended the host range beyond the host range of the single isolates (Gómez et al. 2009). More specifically, neither the EU isolate Sp13, nor the CH2 isolate PS5, could establish infection in *N. glutinosa* or *N. tabacum*, while both host plants appeared to be susceptible upon inoculation with the mix of the two isolates (Gómez et al., 2009).

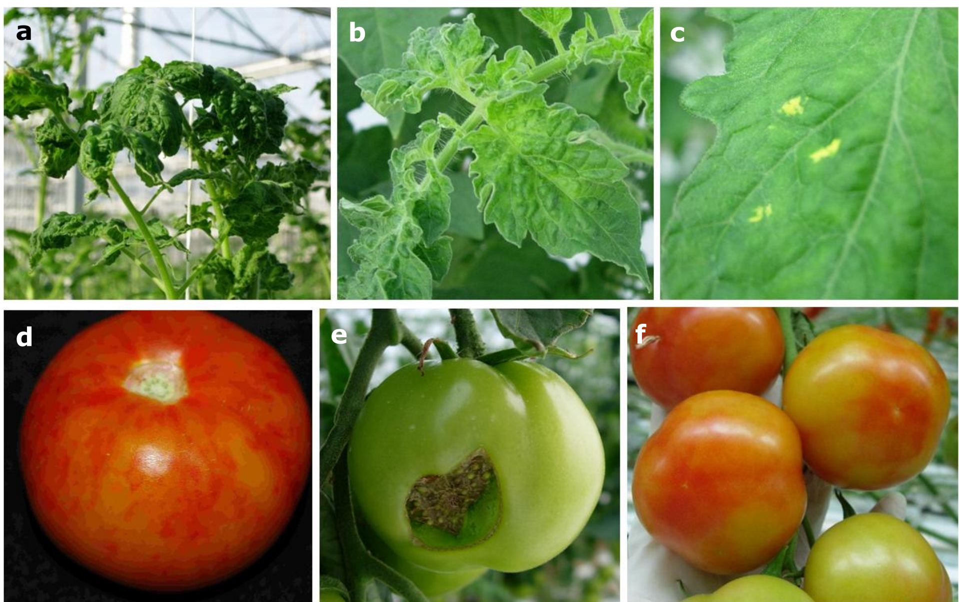

Figure 1. Typical PepMV symptoms on tomato. (a) nettlehead of young top leaves; (b) leaf bubbling; (c) yellow spots; (d) fruit marbling; (e) open fruit; (f) fruit discoloration (flaming).

PepMV symptomatology has been most extensively studied in cultivated tomato. A wide range of symptoms has been associated with PepMV infection. Fruit marbling is generally considered the most devastating symptom of PepMV infection as it diminishes the economical value of the crop, but other fruit symptoms like discoloration (botchy ripening or flaming), and the occurrence of 'open fruit' (fruit that splits shortly after setting such that the seeds become visible) can be similarly devastating (Figure 1; Spence et al., 2006; Hanssen et al., 2008, Chapter 2). Symptoms on vegetative plant

parts may comprise nettle-heads (upper young leaves distorted, serrated and upright with a reduced surface), leaf blistering or bubbling, chlorosis and yellow angular leaf spots, but also severe leaf mosaics and even leaf or stem necrosis (Figure 1; van der Vlugt et al., 2000; Roggero et al., 2001; Spence et al., 2006; Hasiów et al., 2008; Hanssen et al., 2009b, Chapter 3; Hasiów-Jaroszewska et al., 2009a). In addition, it has been suggested that the so-called 'tomato collapse' disease, a sudden and progressive wilt of tomato which eventually leads to plant death, is caused by necrosis of the vascular system due to PepMV accumulation (Soler-Aleixandre et al., 2005).

Transmission

PepMV is efficiently transmitted mechanically (Jones et al., 1980). The virus is highly contagious in tomato, as it easily spreads by the standard crop handling procedures in a greenhouse through contaminated tools, hands and clothing and by direct plant-to-plant contact (Wright and Mumford, 1999; Spence et al., 2006). Therefore, once the virus enters a tomato production facility, containment of further spread is virtually impossible and usually all plants will be infected eventually. It has been shown that bumblebees, often used for pollination in commercial tomato production, contribute to the spread of the virus (Lacasa et al., 2003; Shipp et al., 2008). In infected greenhouse tomato crops, nearly all the bumblebees were shown to carry PepMV and vectoring of the virus to non-infected plants was demonstrated (Shipp et al., 2008). Based on the infection levels in flowers, fruits and leaves, it was suggested that the infection occurred first in the pollinated flowers and then spread to other parts of the plant. Whether infection occurred through direct injury to the flowers or through fertilization with infected pollen could not be determined (Shipp et al., 2008).

Recently also the root-infecting parasitic fungus *Olpidium virulentus*, that has been implicated in the transmission of several plant viruses, was shown to be able to enhance PepMV spread (Alfaro-Fernández et al., 2009b). In addition, it was reported that PepMV can be efficiently transmitted by nutrient solution in a closed recirculation system leading to the infection of healthy tomato plants, although the virus itself was not detectable directly in nutrient solution (Schwarz et al., 2009).

Seed transmission of PepMV has been demonstrated in several studies, with rates up to ~2% depending on the time of seed harvest, the tomato variety and the seed cleaning or disinfection methods applied (Krinkels, 2001; Córdoba-Selles et al., 2007; Ling, 2008; Hanssen et al., 2010b, Chapter 4). In a recently performed grow-out trial with over 87,000 seedlings a seed transmission rate of 0.026% was found for seeds cleaned according to industry standards without disinfection (Hanssen et al., 2010b, Chapter 4). Interestingly, the rate of transmission increased as the interval between

infection of the mother crop and seed harvest increase. Disinfection treatments have been shown to efficiently reduce the seed transmission rate (Córdoba-Selles et al., 2007). Although the efficiency of seed transmission is low, the highly infectious nature of PepMV implies a substantial risk associated with tomato seeds harvested from an infected crop. Therefore, strict regulations aimed at eliminating the risk of viral spread through seeds are in place in the European Union (Commission Decision 2001/536/EC and 2004/200/EC). In addition, sensitive quantitative TaqMan RT-PCR detection methods to screen tomato seed lots for PepMV presence have been developed (Ling et al., 2007; Gutiérrez-Aguirre et al., 2009). As a consequence of these measures, long-distance dissemination of PepMV most likely occurs by the transfer of young infected plants from the nursery to the grower, through infected grafts, cuttings or fruits and even through seed-to-seedling transmission (Córdoba-Sellés et al., 2007).

Genome organization and diversity

PepMV belongs to the Potexvirus genus of the *Flexiviridae* family. Virions are non-enveloped flexuous rods of 508 nm (Jones et al., 1980). The positive single stranded RNA genome is 6.4 kb long and consist of 5 open reading frames (ORFs), a 5' and 3' untranslated region and a 3' poly-A tail. The ORFs encode a 164 kDa RNA-dependent RNA polymerase (RdRp) which contains the characteristic methyltransferase, nucleoside triphosphate (NTP)-binding and polymerase motifs, three triple gene block (TGB) proteins of 26, 14 and 9 kDa (assigned TGBp1, -2 and -3, respectively) and a 25kDa coat protein (Figure 2; Aguilar et al., 2002; Cotillon et al., 2002).

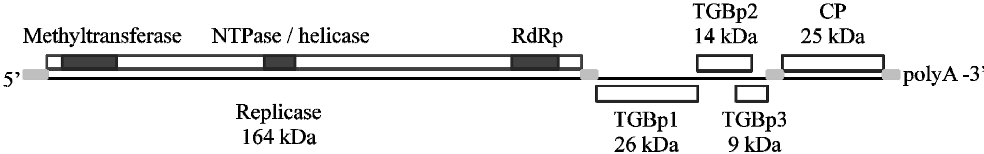

Figure 2. Schematic overview of the PepMV genome organisation, displaying the encoded gene products (adapted from Cottilon et al., 2002). Untranslated regions are shown in light grey bars; NTPase: nucleosidetriphosphatase; RdRp: RNA dependent RNA polymerase; TGBp: triple gene block protein; CP: coat protein. The size and overlap of the proteins is proportional to the actual sizes.

Phylogenetic analyses of replicase, TGBp1 and coat protein amino acid sequences revealed that PepMV is closely related to *Narcissus mosaic virus* (NMV), *Scallion virus X* (SVX), *Cymbidium mosaic virus* (CymMV) and *Potato aucuba mosaic virus* (PAMV) (Cottilon et al., 2002). The highest overall nucleotide identities are with *Narcissus*

mosaic virus (NMV) and *Cymbidium mosaic virus* (CymMV) (Aguilar et al., 2002). Initially, genetic characteristics, symptomatology and host range of different European PepMV isolates showed high similarity, suggesting a common origin of these isolates (Mumford and Metcalfe, 2001; Verhoeven et al., 2003). Nucleotide sequence comparisons of coat protein genes of 15 isolates originating from different European countries revealed 99% identity among the isolates, while these isolates shared only 96-97% identity with the original Peruvian pepino isolate (BBA1137; Mumford and Metcalfe, 2001). Therefore, and because the Peruvian pepino isolate does not cause symptoms in tomato, European isolates were considered of a distinct PepMV type (van der Vlugt et al., 2000; Mumford and Metcalfe, 2001). A comparative symptomatology and host range study of 15 PepMV tomato isolates and the original pepino isolate BBA1137 confirmed that the pepino isolate differed from the tomato isolates, as only the pepino isolate (occasionally) caused symptoms in *Nicotiana tabacum*, *Capsicum annuum* and *Physalis floridana* (Verhoeven et al., 2003). The comparison of the complete nucleotide sequences of two tomato isolates with an isolate from *L. peruvianum* (LP-2001) which was symptomless in tomato demonstrated that the tomato isolates shared over 99% identity while they shared approximately 96% identity with the LP-2001 genome (Soler et al., 2002). In addition, a two nucleotide deletion and some polymorphisms were identified in the 5’ UTR and the TGBp3 had two extra amino acids. These differences were suggested to play a role in the differential biological characteristics (López et al., 2005). At that time, only part of the sequence of the original PepMV isolate from pepino, BBA1137, was determined. As partial sequence comparison revealed a high identity between LP-2001 and BBA1137, the sequence of LP-2001 was considered a reference for the original pepino strain. Complete sequence determination of BBA1137 confirmed that both isolates share nucleotide sequence homologies of over 99% and can thus be considered as isolates from the distinct pepino or Peruvian type of PepMV, which is further referred to as LP (Pagán et al., 2006).

In 2005, two distinct isolates originating from US tomato production, designated US1 and US2, were described that shared only 86% sequence identity (Table 1; Maroon-Lango et al., 2005). Moreover, they shared only 78 and 81% sequence identity with the so-called European tomato isolates (Table 1; Maroon-Lango et al., 2005). As both US isolates caused disease in tomato, the designation ‘tomato strain’ which was used for the European isolates till then was no longer appropriate and most authors started referring to this group of isolates as the ‘European (tomato) genotype (EU)’ (Maroon-Lango et al., 2005; Pagán et al., 2006; Pospieszny and Borodynko, 2006; Hanssen et al., 2008, Chapter 2). In addition to EU, US1 and US2 genotypes, a divergent genotype was isolated from tomato seeds originating from Chile and designated CH2 (Ling, 2006). This CH2 isolate shares 78 to 80% nucleotide sequence identity with the LP and EU

genotypes and 78% with US1 (Table 1). Phylogenetic analyses revealed two main clusters, one containing the EU and LP genotype and the other consisting of the more recently described US and CH2 genotypes, suggesting two distinct evolutionary routes (Figure 3). As nucleotide sequence comparisons suggest that US2 is a recombinant of US1 and CH2, it was recently proposed to distinguish four PepMV genotypes: the original Peruvian genotype (LP); the European (tomato) genotype (EU), the American genotype US1 and the Chilean genotype CH2 (Hanssen et al., 2010b, Chapter 4).

Table 1. Nucleotide sequence identities between type isolates of the different PepMV genotypes

PepMV genotype	GenBank Accession	LP (LP2001)				CH2				US1				US2			
		G[1]	R[2]	T[3]	C[4]	G	R	T	C	G	R	T	C	G	R	T	C
EU[5]	AJ438767	95	95	96	97	79	78	79	77	82	81	85	83	79	79	80	77
LP[6]	AJ606361	*	*	*	*	79	78	80	77	82	81	85	84	79	78	80	77
CH2[7]	DQ000985					*	*	*	*	78	77	80	80	90	88	92	99
US1[8]	AY509926									*	*	*	*	86	87	86	80
US2[8]	AY509927													*	*	*	*

[1]Genome (complete sequence)
[2]Replicase gene
[3]Triple gene block
[4]Coat protein gene
[5]Cottilon et al., 2002
[6]López et al., 2005
[7]Ling, 2006
[8]Maroon-Lango et al., 2005

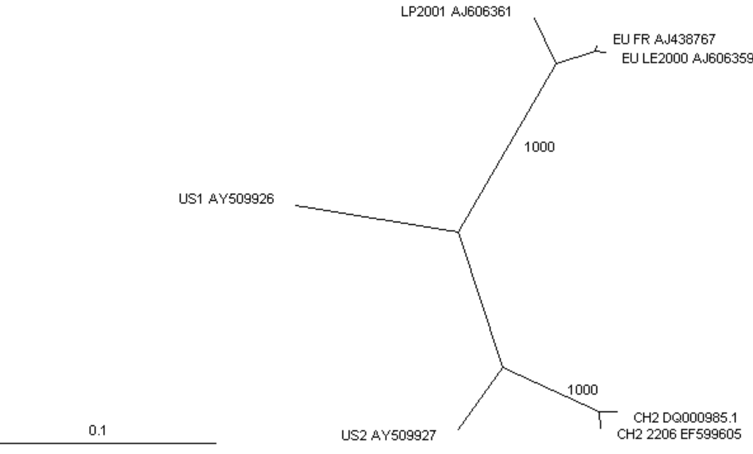

Figure 3. Unrooted distance tree of complete nucleotide sequences from various PepMV genotypes, including sequences from isolates FR (Cottilon et al., 2002) and LE2000 (Lopéz et al., 2005) of the EU genotype; isolates CH2 (Ling, 2006) and the Belgian '2206/06/A1' (Hanssen et al., 2008) of the CH2 genotype, LP2001 (Lopéz et al., 2005) of the LP genotype, and US1 and US2 (Maroon-Lango, 2005). Genbank accession numbers are indicated in the figure. The tree is generated using Clustal X with 1000 bootstrap values and visualized using Treeview. The scale bar represents 0.1 changes per nucleotide.

PepMV population dynamics

Since first appearing in greenhouse tomato crops in the Netherlands in 1999, PepMV rapidly established itself in tomato producing countries. An unprecedented, worldwide series of PepMV outbreaks within a just a few years time was reported, with disease reports from the UK, France, Italy and Spain, but also China, Canada and the US (Cotillon et al., 2002; French et al., 2001; Roggero et al., 2001; Jordá et al., 2001; Mumford and Metcalfe, 2001; Aguilar et al., 2002; Yaoliang and Zhongjian, 2003). Initially, all reported outbreaks were caused by the EU genotype of PepMV and the reported symptoms were rather mild. However, a study on the genetic structure of the PepMV population in Spain in 2005 revealed that the population was more diverse than assumed (Pagán et al., 2006). Although the EU genotype was dominant in Spanish tomato production, the LP genotype appeared to be present on the Canary Islands already in 2000, and US2-like isolates were present in peninsular Spain in 2004 (Pagán et al., 2006). In addition, the occurrence of mixed infections with two different genotypes (combinations of EU and US2-like, and of LP and US2-like) and with inter-genotype recombinants, was revealed. In 2006, the genetic diversity of the PepMV population in Belgian greenhouses was studied, revealing the occurrence of isolates belonging to the EU and CH2 genotypes, often in mixed infections, and the presence of recombinants (Hanssen et al., 2008, Chapter 2). A remarkable finding was the dominance of the CH2 genotype, which had not yet been reported in Europe until then, occurring in 85% of the infected crops while the EU genotype occurred in less than 50% of those crops (Hanssen et al., 2008, Chapter 2). Also the majority of the recent PepMV isolates from Dutch and French tomato crops that were genotyped belonged to the CH2 genotype (Hanssen et al., unpublished). In 2002 and 2005, two distinct Polish PepMV isolates were obtained from tomato and were shown to belong to the EU genotype and the CH2 genotype, respectively (Pospieszny et al., 2002; Pospieszny and Borodynko, 2006). Two additional Polish PepMV strains, isolated in 2007, that differed from the previously identified PepMV isolates in host range and symptomatology appeared to belong to the CH2 genotype (Hasiów-Jaroszewska et al., 2009a). Altogether these results are indicative a shift in the PepMV population, with the EU genotype gradually being overtaken by the CH2 genotype. It was suggested that the CH2 genotype has a biological advantage over the EU genotype, as it seemed to spread faster within a crop (Hanssen et al., 2008, Chapter 2). This was confirmed by a recent study on evolutionary dynamics of the PepMV population in Spain, in which RT-qPCR analyses in inoculated tomato plants showed that a CH2 isolate (PS5) accumulated faster and to higher viral loads than a EU isolate (Sp13) (Gómez et al., 2009). This study further revealed that PepMV populations in Spain are composed of isolates belonging to the EU and CH2 genotypes and that the CH2 type is predominant. Interestingly, EU isolates were shown

to persist in mixed infections, and it was suggested that mixed infections contribute to shaping of the population structure (Gómez et al., 2009). In addition, mutation analyses revealed very few non-synonymous substitutions, reflecting strong purifying selection. These results are in line with the observation that the number of mutations in the RNA sequence of PepMV isolates throughout a greenhouse trial period was rather limited and that most of the mutations that took place had no clear biological relevance (Hanssen et al., 2009b, Chapter 3).

Recent data on the Belgian PepMV population show a further dominance of the CH2 genotype, as in the 2008 growth season the EU genotype was only detected in 7% of the infected crops while the CH2 genotype occurred in 90% of the infected crops. Interestingly also the LP genotype was detected, with an incidence of 10% (Hanssen et al., unpublished data). The sudden occurrence of the LP genotype in Belgian tomato production may perhaps be linked to the use of a mild LP isolate in the Netherlands for cross-protection in commercial tomato greenhouses (Hanssen et al., 2010a, Chapter 5).

In retrospect, the US2-like sequences reported from Spain (Pagán et al., 2006) displayed high sequence identity to the later described CH2 genotype (Ling, 2006), suggesting that CH2 rather than US2 was present in Spain in 2004, indicating that the current PepMV CH2 epidemic in Europe was already initiated in or before 2004. Intriguingly, a recent population study on PepMV isolates in 31 infected North American greenhouse tomato crops revealed the occurrence of the EU, US1, US2 and CH2 genotypes, with a clear dominance of the EU genotype which was identified in all 31 crops (Ling et al., 2008). The remaining genotypes were only found rarely and exclusively in mixed infections. The low incidence of the CH2 genotype in North America is remarkable and might reflect different PepMV dissemination pathways linked to a different, less intensive structure of tomato growth facilities in North America as compared to Europe, where PepMV is mainly prevalent in dense greenhouse tomato cultivation areas. In the American situation mechanical transmission through workers or bumblebees may be subordinate to the long-distance transmission through young plants and seeds.

Factors contributing to PepMV population dynamics are currently not known. However, recently the existence of a population bottleneck during seed transmission was reported, with an apparent advantage of the EU genotype in transmission through seeds harvested from a mother crop co-infected by the EU and CH2 genotypes (Hanssen et al., 2010b, Chapter 4). As seed transmission was suggested as a major dissemination route of PepMV in 1999 and 2000, before strict sanitary regulations were in place (Córdoba-Sellés et al., 2007), this putative population bottleneck might be related to the original dominance of the EU genotype in European countries.

Recently, the US1 genotype was isolated in the Canary Islands from greenhouse tomato crops displaying leaf blistering and mosaic (Alfaro-Fernández et al., 2008b), the first time that this genotype is isolated in a different location than originally reported (North America; Maroon-Lango et al., 2005).

The diversity of symptom severity

The symptom intensity in PepMV infected tomato crops is highly variable, ranging from asymptomatic infections to very severe symptomatology (Jordá et al., 2001; Soler et al., 2000; Soler-Aleixandre et al., 2005; Hanssen et al., 2008). Observations in commercial tomato production have led to the hypothesis that environmental conditions play an important role in symptom severity. Low environmental temperatures and low light conditions are thought to result in more severe damage (Jordá et al., 2001). Furthermore, the PepMV-associated 'tomato collapse' is thought to be enhanced by temperature fluctuations throughout the growth season (Soler-Aleixandre et al., 2005). PepMV-induced leaf scorching was attributed to a period of high light intensity followed by a period of dull weather (Spence at al., 2006). However, a PepMV trial conducted under high-light conditions in 2003 resulted in considerably more damage than a trial conducted under lower-light conditions in 2001-2002 (Spence at al., 2006). Although growers in Belgium and the Netherlands confirm the importance of light and temperature, the interplay of environmental factors contributing to PepMV damage appears to be complex and remains to be elucidated.

As the impact of environmental growth conditions and tomato genotype on PepMV symptom development is not yet fully understood, it was not clear whether the differences in symptom display in commercial tomato greenhouses should also be attributed to the viral isolate that invaded the crop. In a population study conducted in Spanish tomato crops, no correlation between PepMV genotypes and symptomatology were found (Pagán et al., 2006). In a similar study of Belgian tomato crops no significant differences in symptom severity were detected between EU and CH2 isolates (Hanssen et al., 2008, Chapter 2). However, tomato crops that were simultaneously infected with isolates of both genotypes showed significantly enhanced symptom display on all plant parts when compared to crops infected with a single isolate (Hanssen et al., 2008, Chapter 2). Nevertheless, more and more evidence is accumulating showing a clear role of the viral isolate in PepMV symptomatology. Studies on Polish PepMV isolates revealed clear differences in host range and symptomatology of different isolates belonging to the CH2 genotype (Pospieszny et al., 2008; Hasiów-Jaroszewska et al., 2009a). Three necrotric CH2 isolates sharing over 99% sequence identity with non-necrotic isolates from the CH2 genotype were recently identified (Hasiów-Jaroszewska et al., 2009a).

Evidence for a role of the viral isolate in PepMV symptomatology is also found in recent greenhouse inoculation experiments (Hanssen et al., 2009b, Chapter 3). A CH2 isolate that was selected based on mild symptom expression in the crop of origin caused only mild symptoms in the trial, while another isolate with a sequence identity of 99.4% that was selected based on severe symptom display in the crop of origin caused significantly more severe symptoms in the same trial, including nettle-head and a high incidence of premature leaf senescence, open fruit and fruit flaming. These results demonstrate that minor differences at the nucleotide level can account for considerable differences in symptomatology between isolates that infect their crops under the same conditions. Nevertheless, presently it remains unclear which regions of the PepMV genome are important for symptomatology. The recent development of an infectious clone derived from a necrotic CH2 isolate is an important step forward to elucidate the role of certain regions and residues in PepMV symptomatology (Hasiów-Jaroszewska et al., 2009b).

Host responses to PepMV

Global transcriptional profiling, for instance with the use of micro-arrays, can provide insight in the cellular biology of the host upon pathogen infection (Quirino and Bent 2003; van Baarlen et al., 2008; Wise et al., 2007). As viruses establish infection in plants by exploiting the cellular components of the host, viruses can induce a wide range of alterations in host gene expression (Whitham et al., 2003). Presently, micro-array studies have been undertaken for a limited number of viral interactions with their hosts (Whitham et al., 2006; Wise et al., 2007). In most compatible plant virus interactions, a general virus-induced host gene repression occurs shortly after infection (Maule et al., 2002). However, genes related to cell death, cell rescue, defence, ageing and stress are often induced in response to viral infection (Whitham et al., 2003; Marathe et al., 2004; Senthil et al., 2005). Another important virus-induced host response is the induction of the RNA silencing machinery of the plant, which degrades or modifies viral RNAs to block translation of viral proteins (Baulcombe, 2004). This virus-induced post-transcriptional gene silencing (PTGS) mechanism involves the processing of viral double-stranded RNA (dsRNA) by Dicer-like enzymes (DCL) into small interfering RNAs (siRNAs) which are subsequently incorporated into protein complexes containing endonucleolytic Argonaute enzymes (Ding and Voinnet, 2007). PTGS is thought to be the mechanism behind the long known 'recovery' phenomenon, first described by Wingard (1928) who observed that upper leaves of tobacco plants infected with tobacco ringspot virus were asymptomatic and resistant to secondary infection (Ratcliff et al., 1999; Baulcombe, 2004). Host-adapted viruses have evolved strategies to counteract PTGS in their hosts by encoding viral suppressors of RNA silencing (Ding and Voinnet, 2007). As viruses are

inducers, suppressors and targets of the RNA silencing mechanism, virus-induced symptom development in infected plants can be influenced by the siRNA pathways in many different ways (Baulcombe, 2004), for example by perturbation of the endogenous microRNA (miRNA) function (Whitham, 2006). Moreover, it was recently shown that virus resistance induced by an NB-LRR-type disease resistance gene is mediated by Argonaute4-dependent inhibition of translation of virus-encoded proteins (Bhattacharjee et al., 20009). Therefore, PTGS components appear to be key factors in both compatible and non-compatible plant-virus interactions.

A custom-designed Affymetrix tomato GeneChip array (Syngenta Biotechnology, Inc., Research Triangle Park, NC, U.S.A.) that contains probe sets to interrogate over 22,000 tomato transcripts (van Esse et al., 2007) was used to study changes in the tomato transcriptome in response to inoculation with a mild and an aggressive PepMV isolate of the CH2 genotype (Hanssen et al., unpublished, Chapter 6). Over-representation analysis demonstrated a severe down-regulation of host genes involved in photosynthesis and energetic processes upon PepMV infection, while defence and stress responses were clearly induced. This reinforces the notion that, like bacteria and fungi, compatible viruses induce basal plant defense, although the mechanism to recognize the pathogen is likely to be different (Whitham et al., 2006; Ascencio-Ibáñez et al., 2008). Intriguingly, the induction of defence and stress responses was stronger and more persistent in plants that were inoculated with the aggressive CH2 PepMV isolate as compared with plants that were inoculated with the mild CH2 isolate, although viral loads were similar (Hanssen et al, unpublished, Chapter 6). Interestingly, dicer-like enzyme 2 (DCL2), a key factor in antiviral PTGS, was strongly induced by the aggressive isolate and only moderately by the mild isolate. In addition, several Argonautes were differentially regulated, suggesting that PTGS plays an important role in the interaction between PepMV and its host tomato. Nevertheless, these defense responses did not result in PepMV containment. Moreover, these results suggest that some of the symptoms provoked by the aggressive isolate may be caused by a more elaborate host defense response or perhaps a more severe perturbation of the plant miRNA function through PepMV-encoded silencing suppressors that have not yet been identified.

Another interesting observation is that PepMV infection results in the differential regulation of genes that code for several key enzymes in the flavanoid and lycopene biosynthesis pathway (Hanssen et al., unpublished, Chapter 6). This may possibly explain the impact of the virus on fruit symptoms such as fruit marbling and flaming. Although the use of microarrays has made it possible to profile changes in transcriptional activity of thousands of genes simultaneously, to link expression profiles to biological pathways as they occur in the cell remains a challenge (van Baarlen et al., 2008).

Therefore, functional analysis of candidate genes will have to reveal their role in viral defense and symptomatology.

Control strategies

Sources varying from moderate to full resistance have been identified in specific wild Solanum accessions, including *S. pseudocapsicum, S. chilense, S. peruvianum*, and *S. habrochaites* (Ling and Scott, 2007; Soler-Aleixandre et al., 2007). Especially the resistance that is segregating in accession LA1731 from *S. habrochaites* is thought to be promising because segregants of this accession displayed resistance against the CH1, CH2 and EU PepMV genotypes (Ling and Scott, 2007). As introduction of the identified resistance into cultivated tomato by breeding is a time-consuming process, commercial resistant varieties are not yet available. Therefore, prevention through hygiene currently remains the most important strategy for controlling PepMV in commercial tomato production. However, due to the high infectivity of the virus, prevention of infection through hygiene measures is a challenge, especially in dense tomato growing areas.

Many tomato growers, especially in the Netherlands, have chosen to inoculate their crops with a mild PepMV isolate in an attempt to protect their crops from severe damage upon natural infection by an aggressive isolate based on cross-protection (Spence et al., 2006; Hanssen et al., 2009a). In addition to the cross-protection effect which is aimed for, many growers feel that an infection early in the growing season is less harmful than an infection that occurs later in the growing season. In support of this, greenhouse trials conducted in the UK from 2001 to 2003 showed that the time of infection has an impact on PepMV-associated damage, as inoculations in May were more damaging than inoculations in February (Spence et al., 2006). Also from a questionnaire conducted among Belgian tomato growers it seems that early infections result in less damage than late infections (Hanssen et al., 2009a). Whether this is due to plant age, to activation of PTGS based plant defenses resulting in (partial) recovery, or to climate conditions has not been clarified. Although disease symptoms in infected crop sometimes decrease or disappear after a certain period, the virus remains detectable, also in the asymptomatic plant parts. However, a recent cross-protection study based on greenhouse trials revealed that efficient cross-protection against an aggressive isolate belonging to the CH2 genotype could only be achieved by pre-inoculation with a mild isolate from the same genotype. By contrast, enhanced symptom severity was observed when plants were pre-inoculated with a mild isolate belonging to the EU or to the LP genotype (Hanssen et al., 2010a, Chapter 5). These results suggest that the PepMV cross-protection efficacy largely depends on RNA sequence identity, as was similarly shown for other plantvirus interactions (Wang et al., 1991; Desbiez and Lecoq, 1997; Yeh and

Gonsalves, 1984). The role of PTGS in cross-protection was demonstrated by the observation that two viral constructs derived from different viruses but sharing a common sequence could suppress each other when co-inoculated in plants (Ratcliff et al., 1999). It has been suggested that cross-protection is mediated by pre-activation of the siRNA-induced silencing complex, thus inhibiting replication of the challenge isolate (Gal-On and Shiboleth, 2006).

Thus, although cross-protection can be efficient, the enhanced symptom severity in the case of limited nucleotide sequence identity between protector and challenge isolate undermines the potential of cross-protection as a general PepMV control strategy. Cross-protection can only be used successfully in areas where one single PepMV genotype is dominant, provided that a continuous monitoring of the PepMV population is performed and that strict hygienic measures are taken.

Future strategies to combat PepMV epidemics in tomato production might also include transgenic approaches. Coat protein-mediated resistance (CPMR), by which the expression of the viral coat protein confers resistance, could be an efficient strategy. However, the obtained protection efficiency ranges from immunity to delay or attenuation of symptoms and the mechanisms are not fully understood (Prins et al., 2008). Also, expression of replicase or dysfunctional movement proteins in transgenic plants can sometimes lead to resistance or symptom attenuation (Golemboski et al., 1990; Lapidot et al., 1993). A more promising strategy could be the introduction of an inverted repeat (IR) transgene, derived from a viral sequence, into the plant genome. Generation of long dsRNA precursors from those IR fragments will induce the siRNAs and the PTGS machinery, thus conferring sequence-specific antiviral resistance (Prins et al., 2008).

Conclusion

Since first appearing in protected tomato crops in Europe in 1999, PepMV has displayed a high potential to adapt to diverse environmental conditions. In only a few years time, not only the original EU genotype but also the more recently described CH2 genotype have established in tomato producing regions worldwide, and a recent shift in the PepMV population reveals a dynamic interplay between the different PepMV genotypes and their host. Symptoms can be very diverse, both in severity and nature. Although recent studies show that small differences in nucleotide sequence can account for large differences in biological properties and host responses, host and viral factors playing in a role in symptom severity remain unknown. Functional studies using host and viral mutants could identify viral factors that impact biological characteristics and increase our understanding of host responses to PepMV infection. Unraveling the role of PTGS and

viral encoded silencing suppressors in differential symptom severity might shed light on the interplay between different genotypes in mixed infections and could thus contribute to the further development of a sound cross-protection strategy. Although resistance sources have been identified in wild tomato species, commercial resistant varieties are not yet available and PepMV control is largely restricted to hygienic measures. However, currently applied prevention strategies often fail, demonstrating that our understanding of PepMV dissemination pathways is still too limited to contain spread of the virus.

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Summary

Owing to their large population size and short generation time, viruses generally have a huge potential to evolve and adapt under natural selection pressure. Despite tremendous efforts in human, animal and plant health management, viral diseases remain difficult to control and eradicate. Moreover, existing control strategies are compromised by the continuous emergence of new viruses. In **Chapter 1** emerging viruses of tomato crops are reviewed. This includes *Pepino mosaic virus* (PepMV), a Potexvirus with a single stranded RNA genome, a rapidly emerging virus which has become one of the most important viral diseases in tomato production worldwide over the recent years.

Infection by PepMV can cause a broad range of symptoms on tomato plants, of which especially the typical fruit marbling can lead to significant economical losses. Presently, five PepMV genotypes (EU, LP, CH2, US1 and US2) have been described worldwide, three of which (EU, LP and US2) have previously been reported to occur in Europe. As nature and severity of PepMV symptoms are highly variable, economical damage caused by PepMV is difficult to assess and the identification of factors contributing to symptom severity is warranted. In **Chapter 2** the genetic diversity of the PepMV population in Belgian greenhouses is studied and related to the symptom development in tomato crops. Previously, no correlation has been found between different PepMV genotypes and the symptomatology of infected plants. A novel assay based on restriction fragment length polymorphism (RFLP) was developed to discriminate the different PepMV genotypes. Both RFLP and sequence analysis revealed the occurrence of two genotypes, the EU genotype as well as the CH2 genotype, within the tomato production in Belgium. Surprisingly, a clear dominance of the CH2 genotype in the Belgian PepMV population was found, although this genotype has previously not been found in commercial tomato production. 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 such mixed infections.

So far, it remained unclear whether different PepMV isolates can cause differential symptom severity. Therefore, PepMV symptomatology of different isolates was studied in **Chapter 3**. Based on the survey described in Chapter 2, four isolates that differed in symptom expression in the crop of origin were selected for greenhouse trials. The selected isolates were inoculated onto tomato plants grown in separate plastic tunnels. PepMV symptom development was assessed regularly and extensive sampling followed by ELISA analyses, genotyping and nucleotide sequencing was performed to study viral presence and variation in PepMV sequences throughout the trial period. Two isolates (EU

mild and CH2 mild) that were selected based on mild symptom expression in the crop of origin caused only mild symptoms in the trial, while two other isolates (CH2 aggressive and EU+CH2) that were selected for severe symptom display, caused considerably more severe symptoms. Sequence identity between the mild and the aggressive CH2 isolates was as high as 99.4%. Results of this study show that differential symptom expression can, at least partially, be attributed to the PepMV isolate, which may be related to minor differences at the nucleotide level between isolates.

In **Chapter 4**, seed transmission of PepMV in tomato is demonstrated. Fruit was harvested from the greenhouse trials described in Chapter 3 and more than 100,000 seeds were extracted and cleaned using an enzymatic treatment without disinfection. Infection assays using indicator plants confirmed the presence of infectious virus particles on the seeds. In the framework of a European project, seeds were distributed to 10 different laboratories in three separate batches, and germinated for seedling analyses by ELISA. In total over 87,000 plants were tested, and 23 PepMV-infected plants were detected, indicating an overall transmission rate of 0.026 %. Our results clearly show that PepMV can be transmitted from contaminated seeds to seedlings, highlighting the risk of using seeds from PepMV-infected plants, and revealed the potential for seed transmission to contribute to spread of PepMV.

In **Chapter 5**, the potential of three mild PepMV isolates, belonging to the CH2, EU and LP genotypes, to protect a tomato crop against the aggressive CH2 isolate (Chapter 3) as the challenge isolate, was assessed in greenhouse trials. After challenge infection, enhanced symptom display was recorded in plants that were pre-inoculated with a protector isolate that belonged to a different genotype (EU, LP) than 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.

As plant viruses are obligate intracellular parasites that hijack host cellular functions and resources for their replication and movement, they generally induce a wide variety of alterations in host gene expression and cell physiology. In **Chapter 6**, we used a custom-designed Affymetrix tomato GeneChip array that contains probe sets to interrogate over 22,000 tomato transcripts to study transcriptional changes in response to inoculation with the highly similar (99.4% nucleotide sequence identity) mild and aggressive CH2 isolates that are characterized in Chapter 3. Interestingly, our results

show that both isolates induce differential transcriptomic responses in the tomato host despite accumulation to similar viral titers. PepMV inoculation resulted in an extensive transient repression of host genes which clearly affected primary metabolism. Especially the defense response intensity was higher upon inoculation with the aggressive isolate, and defense was mediated by salicylic acid signaling rather than by jasmonate signaling. Our results furthermore show that PepMV differentially regulates the RNA silencing pathway, suggesting a role for PepMV-encoded silencing suppressors, and the ubiquitination pathway. In addition, perturbation of pigment biosynthesis, as monitored by differential regulation of the flavonoid/anthocyanin and lycopene biosynthesis pathways, was monitored, which can be associated with the typical PepMV-induced marbling of tomato fruit.

Finally, **Chapter 7**, the general discussion, is a PepMV pathogen profile in which the results obtained in this work are discussed and integrated into a review on the current knowledge on this highly successful pathogen of tomato crops.

Samenvatting

Virussen hebben een enorm evolutie- en aanpassingsvermogen onder natuurlijke selectiedruk, onder meer dankzij de grootte van hun populaties en hun korte generatietijd. Ondanks aanzienlijke inspanningen in mens, dier en plant gezondheidsmanagement zijn virusziekten nog steeds moeilijk te beheersen of uit te roeien. Daarenboven worden bestaande beheersingsstrategieën bemoeilijkt door de continue opkomst van nieuwe virussen. In **hoofdstuk 1** wordt een overzicht gegeven van opkomende virussen in de tomatenteelt, waaronder het Pepinomozaïekvirus (PepMV). PepMV is een Potexvirus met een enkelstrengig RNA genoom, dat in de voorbije jaren wereldwijd uitgroeide tot een van de belangrijkste virusziekten in de tomatenteelt.

Infectie door PepMV kan een brede waaier van symptomen veroorzaken in tomatenplanten, waarvan het meest typische symptoom de vruchtmarmering is die tot significante economische verliezen kan leiden. Momenteel zijn er wereldwijd vijf genotypen van PepMV beschreven (EU, LP, CH2, US1 en US2), waarvan er drie zijn gevonden in Europa. Omdat de aard en ernst van de PepMV-symptomen in de tomatenteelt zeer variabel zijn, is de economische schade die het virus veroorzaakt moeilijk in te schatten. Identificatie van de factoren die de ernst van de symptomen bepalen is dan ook van groot belang. In **hoofdstuk 2** wordt de genetische diversiteit van de PepMV populatie in Belgische tomatenteelten bestudeerd en gerelateerd aan symptoomontwikkeling in het gewas. Eerdere studies vonden geen correlatie tussen verschillende PepMV genotypen en de symptomatologie in de plant. Een nieuwe toets, gebaseerd op 'Restriction Fragment Length Polymorphism' (RFLP), werd ontwikkeld om de verschillende PepMV genotypen te onderscheiden. Zowel RFLP als sequentieanalyse onthulden het voorkomen van twee genotypen, het EU en het CH2 genotype, in de Belgische tomatenproductie. Verassend was de uitgesproken dominantie van het CH2 genotype in de Belgische PepMV populatie, terwijl dit genotype eerder niet werd gedetecteerd in de commerciële tomatenproductie. Hoewel er geen verschillen in symptoomexpressie werden geobserveerd tussen planten geïnfecteerd met één van beide genotypen, resulteerde co-infectie met beide genotypen in sterkere PepMV symptomen. Daarnaast wees onze studie uit dat PepMV recombinanten frequent voorkomen in dergelijke menginfecties.

Tot nu toe was het onduidelijk of verschillende PepMV isolaten verantwoordelijk zijn voor verschillen in symptoomontwikkeling. Daarom wordt de symptomatologie van verschillende isolaten bestudeerd in **Hoofdstuk 3**. Op basis van de studie beschreven in Hoofdstuk 2 werden vier isolaten geselecteerd die verschilden in symptoomexpressie in

het gewas waaruit ze geïsoleerd werden. De geselecteerde isolaten werden geïnoculeerd op tomatenplanten in afzonderlijke plastic tunnels. PepMV symptoomontwikkeling werd op regelmatige tijdstippen beoordeeld en aan de hand uitgebreide bemonstering en ELISA analysen, genotypering en nucleotide-sequenering, werden de virustiter en de variatie in PepMV sequenties opgevolgd over de gehele proefperiode. Twee isolaten (EU mild en CH2 mild), die geselecteerd werden op basis van de milde symptoomexpressie in het gewas van oorsprong, veroorzaakten enkel milde symptomen, terwijl twee andere isolaten (CH2 agressief en EU + CH2), die geselecteerd werden omwille van de ernstige symptomen, aanzienlijk meer symptomen veroorzaakten. De nucleotidensequentie van het milde en agressieve CH2 isolaat waren voor 99.4% identiek. De resultaten van deze studie tonen aan dat differentiële symptoomexpressie op zijn minst gedeeltelijk toegeschreven kan worden aan het PepMV isolaat, mogelijk geassocieerd met zeer kleine verschillen op RNA niveau.

In **Hoofdstuk 4** wordt aangetoond dat PepMV zaadoverdraagbaar is in tomaat. Er werden vruchten geoogst van de praktijkproef beschreven in Hoofdstuk 3, waaruit meer dan 100,000 zaden werden geëxtraheerd die gereinigd werden aan de hand van een enzymatische behandeling zonder disinfectie. Infectietesten met indicatorplanten bevestigden de aanwezigheid van infectieuze viruspartikels op het zaad. In het kader van een Europees project werden de zaden in drie afzonderlijke batches verdeeld over 10 verschillende laboratoria, waar ze werden opgekweekt voor analyse van de zaailingen m.b.v. ELISA. In totaal werden meer dan 87,000 zaailingen getest, waarvan er 23 besmet bleken met PepMV, wat resulteert in een zaadtransmissie percentage van 0.026. Deze resultaten demonstreren dat PepMV overgedragen kan worden van besmette zaden naar zaailingen en dat het gebruik van PepMV-besmette planten een risico inhoudt, zeker omdat het virus zeer infectieus is. Zaadtransmissie speelt dan ook mogelijk een belangrijke rol spelen in de verspreiding van PepMV.

In **hoofdstuk 5** werd voor drie milde PepMV isolaten, behorende tot het CH2, EU en LP genotype, aan de hand van praktijkproeven nagegaan of ze een tomatengewas konden beschermen (crossprotectie) tegen het agressieve CH2 isolaat (Hoofdstuk 3). Na besmetting met het agressieve isolaat werd een versterking van de symptomen en een toename van de schade waargenomen in planten die eerst geïnoculeerd waren met een mild isolaat dat tot een ander genotype (EU, LP) behoorde dan het agressieve ‘challenge’ isolaat (CH2). Een kwantitatieve genotypespecifieke TaqMan toets onthulde dat de accumulatie van het ‘challenge’ isolaat in deze planten slechts tijdelijk werd afgeremd. Echter, wanneer er een mild isolaat van het CH2 genotype werd gebruikt was de bescherming zeer efficiënt en kon het agressieve isolaat niet of amper worden gedetecteerd in het gewas. Deze resultaten tonen aan dat de interactie tussen PepMV

isolaten grotendeels afhangt van de homologie in RNA sequentie en dat 'post-transcriptionele gene silencing' (PTGS) een belangrijke rol speelt in crossprotectie.

Vermits plantvirussen obligate intracellulaire parasieten zijn die cellulaire functies, bouwstenen en voedingsbronnen van hun gastheer wegkapen voor hun eigen replicatie en transport, induceren ze doorgaans een brede waaier van veranderingen in genexpressie en celfysiologie in hun gastheer. In **Hoofdstuk 6** gebruikten we een 'custom-designed Affymetrix tomato GeneChip array' met detector sets voor meer dan 22,000 tomatengenen om de transcriptionele veranderingen in tomatenplanten te bestuderen in respons op inoculatie met de twee sterk gelijkende (99.4% identiteit in nucleotidensequentie) CH2 isolaten (mild en agressief) die gekarakteriseerd werden in Hoofdstuk 3. Onze resultaten tonen aan dat de twee isolaten verschillende transcriptoom responsen induceren in de tomatengastheer, ondanks het feit dat ze tot gelijkaardige virustiters accumuleren. PepMV inoculatie resulteerde in een brede maar transiënte repressie van gastheergenen, waarbij onder meer het primair metabolisme sterk aangetast werd. De intensiteit van de verdedigingsrespons was sterker bij inoculatie met het agressieve isolaat en de verdediging bleek via de salicylzuur signaaltransductie te verlopen, eerder dan via jasmonaat. Verder tonen onze resultaten aan dat PepMV de PTGS pathway differentieel reguleert, wat suggereert dat PepMV-gecodeerde 'silencing suppressors' een rol spelen in het infectieproces, alsook de 'ubiquitination pathway'. Daarnaast werd een verstoring van de pigmenthuishouding door differentiële regulatie van de pathways voor biosynthese van flavonoïden, anthocyanen en lycopeen vastgesteld, wat mogelijk gecorreleerd is met de typische PepMV-geïnduceerde marmering van tomatenvruchten.

Hoofdstuk 7 tenslotte, de algemene discussie, is een PepMV pathogeen profiel, waarin de resultaten die in dit werk bekomen werden besproken worden en geïntegreerd in een overzicht van de huidige kennis over deze succesvolle pathogeen van tomaat.

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Inge

06/02/2010

Curriculum vitae

Inge M. Hanssen was born on June 19th 1977 in Hasselt, Belgium. After graduating from the O.L.Vrouw-Lyceum in Genk in 1995, she studied Bio-Engineering at the Catholic University of Leuven (KULeuven), where she graduated with honor as master in Applied Biological and Agricultural Sciences (bio-ir) in 2001. She specialized in plant protection and tropical agriculture and performed her undergraduate thesis research in Can Tho University, Can Tho, Vietnam, on integrated pest management in citrus orchards in the Mekong Delta, under the supervision of Prof. J. Coosemans, Laboratory of Phytopathology, KULeuven. In 2001 she started working as a consulting plant pathologist at the research institute Scientia Terrae v.z.w. in Sint-Katelijne-Waver, Belgium, where she collaborated with Fons Vanachter on diagnosis of plant diseases and advising horticultural growers on plant disease management. In addition, from 2003 until 2005 she coordinated a research project on microbial food safety of fresh cut vegetables, in collaboration with the Flemish vegetable auction organization LAVA cvba and the food processing company UNIVEG. From 2006 onwards she coordinated several research projects on Pepino mosaic virus (PepMV) in the Flemish tomato industry, conducted by the research consortium Scientia Terrae, Research Centre Hoogstraten and Research Station for Vegetable Production, Sint-Katelijne-Waver. In the framework of these activities, she started her PhD research in 2007 under supervision of Dr. Ir. Bart P.H.J. Thomma at the Laboratory of Phytopathology, Wageningen University, headed by Prof. Dr. Ir. P.J.G.M. de Wit. Currently she is responsible for the research program 'Phytopathology and Biorational Control' within the Scientia Terrae Research Institute, where she coordinates both governmentally financed and contract research projects on (mainly viral) plant pathogens.

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Front cover:
Freshly washed tomato fruits infected with *Pepino mosaic virus* displaying the typical fruit marbling symptoms (Picture: Christophe Heusdens).

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