The battle between pepper and aphid: genetics and mechanism of host plant resistance Mengjing Sun 2019

The battle between pepper and aphid:

genetics and mechanism of host plant resistance

Mengjing Sun

Propositions

1. Generalist aphids need to be adapted to the crop species before a meaningful resistance screening can be carried out.
   (this thesis)

2. Aphids are able to manipulate plant metabolism and thus affect plant resistance.
   (this thesis)

3. Bacteria are essential to ensure the proper evolution of their hosts.

4. Early recognition of human diseases is as important as drug discovery.

5. In order to achieve a sustainable world we have to use synthetic biology.

6. Although baby day-care is not accepted by the general public, it is very much needed in China.

7. Social interactions are at risk if people are unable to drink alcohol.

Propositions belonging to the thesis entitled:

The battle between pepper and aphid: genetics and mechanism of host plant resistance

Mengjing Sun

Wageningen, 19 November 2019

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences (EPS)

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Thesis

submitted in fulfilment of the requirement for the degree of doctor
at Wageningen University & Research
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public on Tuesday 19 November 2019
at 11 a.m. in the Aula.

Mengjing Sun
The battle between pepper and aphid: genetics and mechanism of host plant resistance, 126 pages

PhD thesis, Wageningen University & Research, Wageningen, the Netherlands (2019)
With references, with summary in English.

ISBN: 978-94-6395-078-7
DOI: https://doi.org/10.18174/497898

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CHAPTER 1

General introduction

Aphids, a worldwide problem in crop cultivation

Aphids belong to the order of Hemiptera in the superfamily Aphidoidea (Figure 1). They are the most widely spread pest insects. More than 200 aphid species worldwide have been reported as economically important pests and most crops suffer from one or more aphid species (Blackman & Eastop, 2000). As a major concern of farmers and gardeners, aphids cause serious (economic) damage to crop production every year (Goggin, 2007, Tagu et al., 2008).

Figure 1. Different aphid species on pepper leaves. A. Green peach aphid *Myzus persicae*, **B**. cotton aphid *Aphis gossypii*, **C**. foxglove aphid *Aulacorthum solani*.

The damage caused by aphids includes direct damage such as chlorosis, stunted plant growth and reduction of photosynthesis (Blackman & Eastop, 2000, Van Emden & Harrington, 2017), as well as indirect damage caused by mould growth on exuded honeydew and diseased plants because of the transfer of viruses (Figure 2). Aphids can transmit pathogenic viruses that may lead to plant mortality when the virus disease is serious (Kennedy et al., 1962, Ng & Perry, 2004). Aphids are renowned for their high reproduction rates (Guerrieri & Digilio, 2008), which is the result of an asexual reproduction cycle in which adult females give birth to nymphs that can immediately start feeding (Delmotte, 2001).

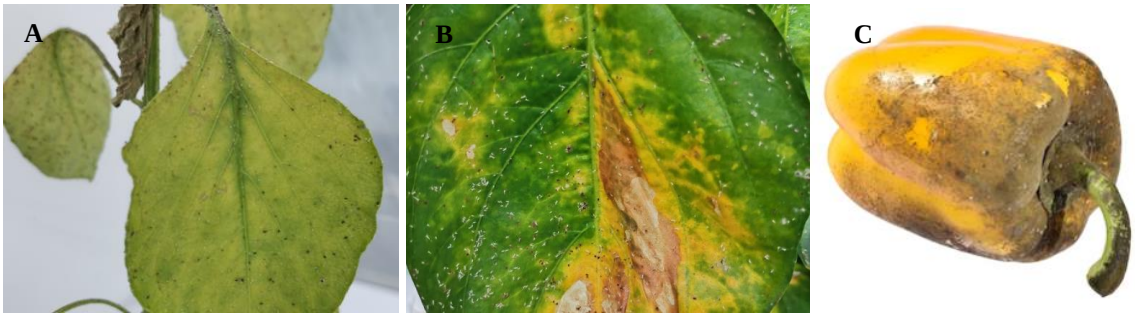

Figure 2. Damages due to aphids infestation. A. leaf chlorosis on pepper, **B**. pepper leaf damaged by aphid-transmitted virus, **C**. damaged pepper fruit because of mould growth (www.shutterstock.com).

Aphids are phloem feeding insects. During feeding, they use their specialized mouthparts, the stylets, to penetrate plant tissue and to take up nutrients from the plant phloem for a prolonged period (Kaloshian & Walling, 2005, Dedryver et al., 2010) (Figure 3). When aphids penetrate a plant, they first secrete a small amount of gelling saliva to form a sheath around their stylet. The secretion of gelling saliva will last until they start feeding from the phloem

(Tjallingii, 2006, Walling, 2008). Before ingesting plant phloem, aphids secrete watery saliva into the phloem sieve element. This watery saliva has been suggested to prevent the triggering of sieve element occlusion induced in the plant, which helps to improve aphid feeding (Furch et al., 2007, Walling, 2008).

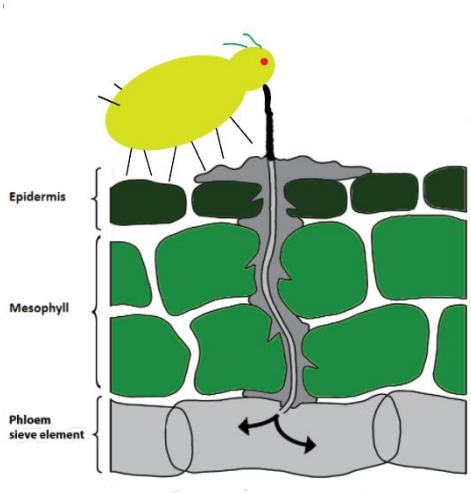

Figure 3. Aphids use their stylets to penetrate plant tissue and feed from the phloem sieve element (adapted from ten Broeke, 2013). During feeding, they secrete gelling saliva (dark grey) to protect the stylet and watery saliva (black arrow) to improve feeding.

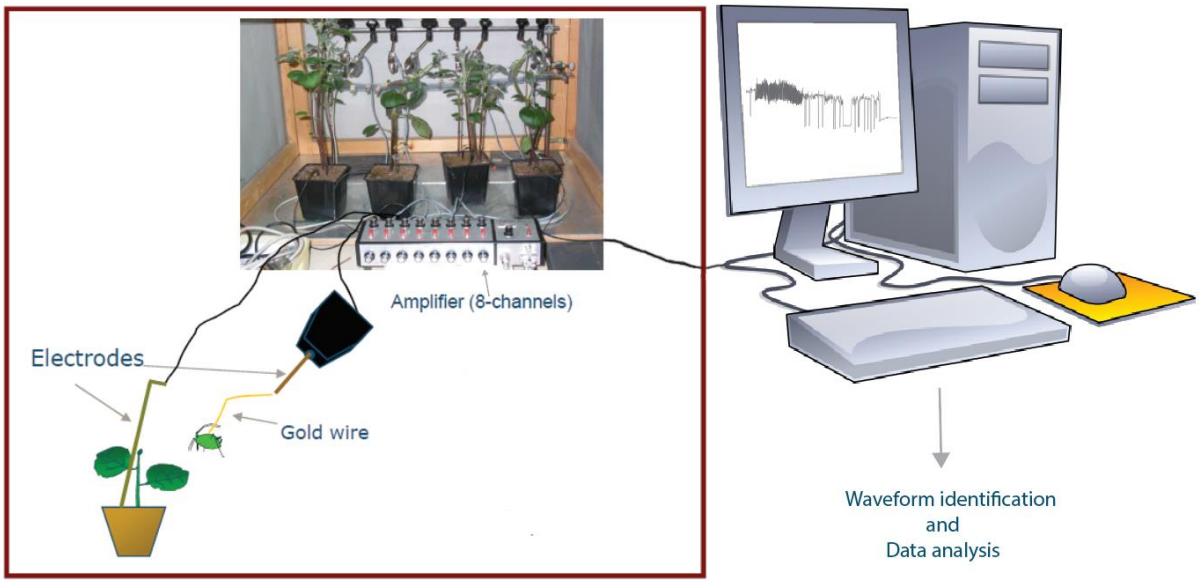

Figure 4. The EPG set-up. One electrode is connected to the back of an aphid, and the other electrode is inserted into the soil of the tested plant. When the aphid penetrates the plant tissue the electrical circuit is closed and the EPG signal is recorded in the computer.

In order to better understand the feeding behaviour of phloem feeding insects including aphids, the electrical penetration graph (EPG) technique was developed (McLean & Kinsey, 1964). In the EPG set-up an aphid is made part of an electrical circuit (Figure 4). Once the aphids stylet penetrates the plant, an electrical circuit is closed and a fluctuating voltage is produced, which is then amplified and recorded (Tjallingii, 1988). The voltage fluctuations depend on aphid activities, which are seen as different waveforms in the EPG recordings. Distinct waveforms are correlated with specific probing and feeding activities of the aphid on the tested plant (Tjallingii & Esch, 1993,

Tjallingii et al., 2010). With the EPG technique, plant tissues containing resistance factors can be identified and differences in aphid behaviour on resistant and susceptible plants can be explored (Walker, 2000, Alvarez et al., 2006, Chandran et al., 2013).

Plant resistance to aphids

To control aphids in crop cultivation, chemical pesticides have been widely used. However, due to the long-time use of insecticides, more and more species (and populations) of aphids have been reported to develop insecticide resistance (Wang et al., 2002, Anstead et al., 2005, Bass et al., 2014). Because of this, and the increasing concern about the negative impact of insecticides on the environment, the use of host plant resistance is commonly seen as a more desirable strategy to control aphid populations (Broekgaarden et al., 2011).

In general, the mechanisms of host plant resistance against insects including aphids can be antixenosis and antibiosis (Painter, 1951, Smith & Boyko, 2007, Züst & Agrawal, 2016). Antixenosis, also known as non-preference, affects aphid settling or feeding through repellence or deterrence (Goggin, 2007). It depends on morphological and chemical plant adaptations that affect host selection by the aphids (Smith et al., 1993). For example, plants of the potato wild relative *Solanum berthaultii* can release the aphid alarm pheromone E-β-farnesene to repel aphids (Gibson & Pickett, 1983). Antibiosis-based resistance is known to interfere with the biology of the insect by impairing insect survival, growth, development and fecundity, again resulting from chemical or morphological adaptations (Smith, 2005). For instance, a tomato wild relative *Solanum habrochaites* can produce 2-tridecanone, which is toxic to the cotton aphid *A. gossypii* (Williams et al., 1980). Similarly the glandular trichomes of potato wild relatives *S. berthaultii* and *S. tarijense* and tomato wild relative *S. galapagense* have been proven to be important for resistance against green peach aphid *M. persicae* (Alvarez et al., 2006, Vosman et al., 2018), respectively.

Host resistance to aphids has been identified in several crops or their wild relatives, including resistance to cotton aphid *A. gossypii* in melon (Pitrat & Lecoq, 1980), resistance to the black currant-lettuce aphid *Nasonovia ribisnigri* in lettuce (Eenink et al., 1982a), resistance to Russian wheat aphid *Diuraphis noxia* in wheat (Marais & Du Torr, 1993), resistance to green peach aphid *M. persicae* in tomato (Leite et al., 1999), and resistance to soybean aphid *Aphis glycines* in soybean (Wu et al., 2004).

Genetic and molecular mechanisms underlying plant resistance to aphids

Quantitative trait loci (QTLs) for plant resistance

A breeding program for resistant varieties can be accelerated with the help of molecular approaches, such as detecting quantitative trait loci (QTLs) (Young, 1996, Moose & Mumm, 2008). Some QTLs controlling plant resistance to aphids have been identified in several crops (Table 1).

Table 1. Host plant resistance QTLs against aphids reported in crop plants.

Plant species	Aphid species	Identified QTLs	References
Alfalfa	*Acyrthosiphon kondoi*	AKR, AIN	(Klingler et al., 2005, Klingler et al., 2009)
Alfalfa	*Acyrthosiphon pisum*	RAP1, AIN	(Stewart et al., 2009, Guo et al., 2012)
Apple	*Dysaphis devecta*	Sd-1, Sd-2 and Sd-3	(Stoeckli et al., 2008)
Apple	*Dysaphis plantaginea*	Sm-h	(Stoeckli et al., 2008)
Barley	*R. padi*	QTLs on chromosome 2H and 5	(Cheung et al., 2010, Moharramipour et al., 1997)
Chrysanthemum	*Macrosiphoniella sanbourni*	NoaE2G1, NoaE2G7, NoaE1H3, NoaE2H7, NoaE2H8	(Wang et al., 2014)
Cucumber	*A. gossypii*	QTL on chromosome 5	(Liang et al., 2016)
Lettuce	*N. ribisnigri*	Nr	(Van Helden et al., 1993)
Maize	*Rhopalosiphum maidis*	aph, aph2 and Bx10c	(So et al., 2010, Meihls et al., 2013)
Melon	*A. gossypii*	Vat	(Pauquet et al., 2004)
Peach	*M. persicae*	Rm1, Rm2 and MP.SD	(Pascal et al., 2002, Lambert & Pascal, 2011, Sauge et al., 2012, Pascal et al., 2017)
Soybean	*A. glycines*	Rag1, rag1b, rag1c, Rag2, Rag3, rag3b, rag3c, rag4, Rag5, Rag6	(Hill et al., 2012, Kim et al., 2014a, Hill et al., 2017, Zhang et al., 2017, Hanson et al., 2018)
Soybean	*Aulacorthum solani*	Raso1, Raso2	(Ohnishi et al., 2012, Lee et al., 2015)
Tomato	*M. euphorbiae*	Mi	(Vos et al., 1998)
Wheat	*D. noxia*	Dn1, Dn2, Dn3, Dn4, Dn5, Dn6, Dn7, Dn8, Dn9, Dny	(Liu et al., 2001, Liu et al., 2002, Peng et al., 2007, Liu et al., 2014)
Wheat	*Rhopalosiphum padi*	QRp.slu.4BL	(Crespo-Herrera et al., 2014)
Wheat	*Schizaphis graminum*	Gb1,Gb2, Gb3, Gb5, Gb6, Gb7, Gbx1, Gba, Gbb, Gbc, Gbd, Gbz,	(Zhu et al., 2005, Lu et al., 2010, Azhaguvel et al., 2012, Liu et al., 2014)

Among all the resistance QTLs identified in crops, only two genes have been cloned. One is the tomato *Mi-1.2* gene, which has been found to confer resistance to certain strains of *M. euphorbiae* (Rossi et al., 1998, Goggin et al., 2001). The other one is the *Vat* gene cloned from melon, conferring resistance to *A. gossypii* (Vos et al., 1998). These two genes, as well as the resistance QTLs *Nr* from lettuce and *Rag1*/*Rag2* from soybean are dominant (Dieleman & Eenink, 1980, Li et al., 2007, Hill et al., 2009). All of these have been applied in commercial breeding programs. However, there is also a possibility that they can be overcome by virulent aphid populations. For example, aphid biotype 1 and 2 have overcome the *Rag1* resistance just a few years after the commercial release of a soybean variety carrying *Rag1* (Kim et al., 2008).

Molecular model describing plant resistance to aphids

The zigzag model, which is based on the gene-for-gene principle (Flor, 1971), is used to describe the molecular interactions between plants and their pathogens, as well as the interaction between plants and insects (Jones & Dangl, 2006, Hogenhout et al., 2009, Douglas, 2018) (Figure 5).

During feeding, aphids secrete conserved molecules present in their saliva which can be recognized by plants. These conserved molecules are known as herbivore-associated molecular patterns (HAMPS). Pattern recognition receptors (PRRs) from plants recognize HAMPs and activate HAMP-triggered immunity (PTI) (Jones & Dangl, 2006, Zipfel, 2008). The PRRs identified in plants are divided into two classes, receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The crucial difference between RLKs and RLPs is that RLKs contain a cytoplasmic kinase domain while RLPs do not have any obvious signalling domain (Couto & Zipfel, 2016).

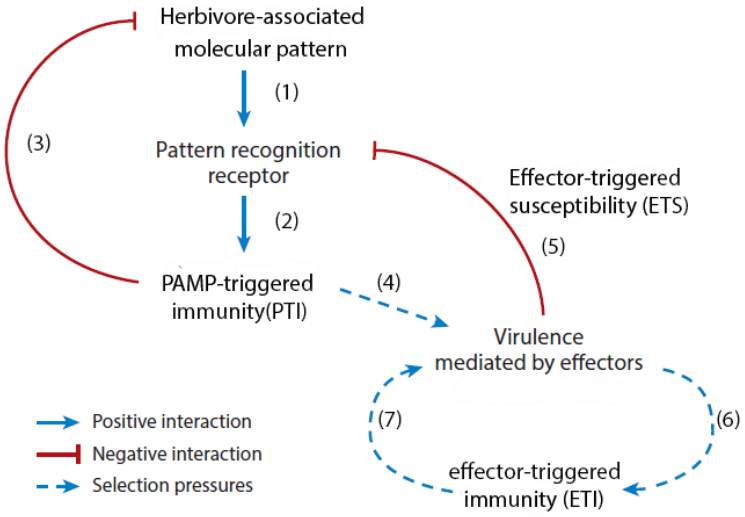

Figure 5. The molecular model describing plant resistance to aphids (adapted from Douglas, 2018). (1) During feeding aphids secrete herbivore-associated molecular patterns (HAMPs) into their host plant, and these HAMPs can be recognized by pattern recognition receptors (PRRs); (2) Once the HAMPs are recognized, PRRs induce HAMP-triggered immunity (PTI); (3) Host plants impair the performance of the aphids in various ways; (4) However, in order to circumvent/suppress defence responses in plants, aphids may secrete effector proteins; (5) These effectors may induce effector-triggered susceptibility by blocking PTI; (6) Some host plant may respond by producing resistance proteins (R proteins) that can recognize these effectors and then induce effector-triggered immunity (ETI); (7) This process may be repeated during evolution.

In order to counteract PTI induction in plants, aphids may secrete effector proteins, which leads to effector-triggered susceptibility (ETS) (Rodriguez & Bos, 2013, Elzinga et al., 2014). In their turn, some plants may react to these effector proteins with the production of resistance proteins (R proteins) that can recognize them and then induce effector-triggered immunity (ETI) (Hogenhout & Bos, 2011, Jaouannet et al., 2014). Most R proteins inducing ETI are of the NBS-LRRs type, containing a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain (Cook et al., 2015, Białas et al., 2017). The NBS domain is conserved and has been shown to be responsible for binding and hydrolysing ATP (Tameling et al., 2002). The LRR domain determines the specificity and mediates the interaction with the effectors (Sarris et al., 2016).

Defense signalling causing plant resistance to aphids

When plants are able to induce defense responses against insects, the signalling pathways involved in PTI and ETI usually overlap (Tsuda & Katagiri, 2010, Peng et al., 2018). During the defense responses in plants, multiple signalling pathways may be elicited, including phytohormone induced pathways (Louis & Shah, 2013, Yates & Michel, 2018).

Plant hormones play an important role in defense responses against aphids. The induction of phytohormones may stimulate downstream defense processes including the production of plant secondary metabolites (Voelckel et al., 2004, Boyko et al., 2006), expression of pathogenesis-related (PR) proteins (Moran & Thompson, 2001, Mewis et al., 2006) and strengthening of cell walls (Park et al., 2006). Aphid feeding often induces the salicylic acid (SA) pathway, which is associated with plant defense responses to microbial pathogens (Walling, 2000). It has been shown that SA signalling is required for *Mi-1.2* mediated resistance against *M. euphorbiae* in tomato (Li et al., 2006). In addition, expression of the Enhanced Disease Susceptibility 5 gene (*EDS5*), which is required for SA synthesis, is induced in *Arabidopsis* infested by *M. persicae* (Pegadaraju, 2005). The release of Methyl salicylate (MeSA), a volatile compound derived from SA, can induce plant defenses and impair aphid behaviour such as of *R. padi* on barley (Glinwood et al., 2007) and *M. euphorbiae* on tomato (Digilio et al., 2012). However, the application of functional analogues of SA, like benzothiadiazole, does not prevent or impair the colonization of *Arabidopsis* by *M. persicae* (Moran & Thompson, 2001). Similarly, the induction of the SA pathway by a pathogen on tobacco does not influence subsequent feeding by *Myzus nicotianae* (Ajlan & Potter, 1992). Taken together, these results suggest that SA-mediated plant defenses do not have consistent effects on aphid performance and the function of SA signalling needs to be studied in each specific plant-aphid interaction.

The jasmonic acid (JA) signalling pathway is known to play an important role in plant defense responses against aphids as well (Züst & Agrawal, 2016). For instance, exogenous application of JA impaired aphid development on tomato (Agrawal, 1998, Cooper et al., 2004), and *M. persicae* performed much worse on an *Arabidopsis* mutant overexpressing JA than on wild type (Ellis et al., 2002). As SA and JA are natural antagonists (Mur et al., 2006), aphids may be able to use this hormonal 'crosstalk' to suppress a potentially more detrimental JA response by conversely inducing SA pathway in host plants (Mewis et al., 2005, de Vos et al., 2007, Erb et al., 2012). However, defense signalling is more complex than this and does not solely rely on the production or inhibition of these two hormones (Vos et al., 2005).

Although less is known about the ethylene (ET) signalling pathway, it has been shown that ET is important in plant defense responses to aphid infestation as well. The role of ET signalling is specific to different plant-aphid interactions. For example, up-regulation of ET production could make celery and *Arabidopsis* susceptible to *M. persicae* (Moran et al., 2002, Divol et al., 2005), while it could increase wheat resistance against *D. noxia* (Boyko et al., 2006) and melon resistance against *A. gossypii* (Anstead et al., 2010).

The involvement of other hormones, such as abscisic acid (ABA) and gibberellic acid (GA), in plant defense responses to aphid infestation is even less documented (Morkunas et al., 2011). However, that does not mean that these hormones are not related to plant defenses. There are several studies focusing on the function of ABA and GA in defense signalling. For instance, several highly upregulated genes under ABA and GA control are found in

aphid-resistant sorghum and wheat (Boyko et al., 2006, Park et al., 2006). Exogenous application of ABA can enhance *M. persicae* resistance in *Nicotiana tabacum* (Zhao et al., 2016).

In summary, it seems that the type of phytohormone that is induced during aphid–host interactions is host-species-specific. The effect of specific phytohormones needs to be individually studied.

Mechanism of plant resistance to aphids

As aphids are phloem-feeding insects, it is reasonable to assume that some mechanisms of resistance are located in the phloem. Triggered by an influx of calcium, phloem protein (P protein) plugging and callose deposition may be induced by aphids. Phloem protein plugging is a fast process that occurs within a few minutes in response to aphid feeding. It has been best studied in legumes, and forisomes, which are phloem based proteins, were shown to play a crucial role. Forisomes are presumed to control phloem transport by forming reversible sieve tube plugs after aphid infestation (Peters et al., 2006), and their function is demonstrated by a stop of mass flow observed in artificial sieve tubes (Knoblauch et al., 2012). Compared with P protein plugging, callose deposition is a slower process in reaction to aphid infestation (Van der Westhuizen et al., 2002, Garzo et al., 2018). Callose, a ß-1,3-glucan, is an important component in plant defense to mechanical wounding and pathogen infection (Donofrio & Delaney, 2001, Luna et al., 2011). Recently, several studies showed its relevance in plant resistance against aphids. For example, stronger callose deposition is found in barley resistant to *D. noxia* (Saheed et al., 2009), in *Arabidopsis* resistant to *M. persicae* (Shoala et al., 2018), as well as in maize and wheat resistant to *Sitobion avenae* (Li et al., 2018). Callose is produced by callose synthases (CalS), which are encoded by a family of callose synthase genes (Richmond & Somerville, 2000, Verma & Hong, 2001). Among these callose synthase genes, the *CalS1* and *CalS12* genes have been implicated in the plant defense response against aphids (Kempema et al., 2007, Kuśnierczyk et al., 2008, Shoala et al., 2018).

The involvement of reactive oxygen species (ROS) in pathogen and insect resistance is also well documented (Moloi & van der Westhuizen, 2006, Kerchev et al., 2012). ROS may have direct adverse effects on pests (Liu et al., 2010), but may also act as a signalling component to activate downstream defense metabolites or enzymes (Divol et al., 2005, Boyko et al., 2006). It has been found that accumulation of ROS in plants can improve aphid resistance (Moloi & van der Westhuizen, 2006) while inhibition of ROS production reduced aphid resistance (Lei et al., 2014). As ROS are involved in a large network associated with plant defense responses, it is conceivable that the dynamic change of ROS might be controlled by multiple enzymes in the host plant. ROS can be produced by various enzymes, such as NADPH oxidases (Torres et al., 2002), peroxidases (Bindschedler et al., 2006) and oxalate oxidase (Hu et al., 2003), and can be removed by ROS-scavenging enzymes or metabolites like catalase (Mhamdi et al., 2010) and superoxide dismutase (Mittler et al., 2004). The mechanisms regulating ROS metabolism might be distinct in different plant-aphid interactions.

Occurrence of virulent biotypes of aphids

Although the use of host plant resistance is a promising method to manage aphid populations, the rapid evolution of aphids is a big challenge. Aphids may overcome resistance and a new population that has overcome host resistance is called a virulent population (Power & Irwin, 1992). A virulent aphid population may even be found in a cultivation area where resistant cultivars are not or rarely grown (Goggin et al., 2001). Virulent populations

have been often reported among specialist aphids such as *D. noxia* (Haley et al., 2004), *A. glycines* (Kim et al., 2008) and *A. pisum* (Kanvil et al., 2014). For generalist aphids, this is not so common and only a few examples are known, virulent populations of *M. euphorbiae* on *Mi*-mediated resistant tomato (Goggin et al., 2001, Pallipparambil et al., 2010), virulent populations of *A. gossypii* on melon (Lombaert et al., 2009) and virulent populations of *M. persicae* on peach (Cabrera-Brandt et al., 2015) have been found to overcome or partially overcome crop resistance. Regardless of the virulent aphid population of specialists or generalists, preventing their emergence requires a better understanding of the interaction between plant and herbivore, resulting in effective plans for sustained use of host plant resistance (O'Neal et al., 2018).

Pepper (*Capsicum* spp.) a worldwide horticulture crop

Pepper (*Capsicum* spp.) originates from South and Central America, where cultivation started probably around 5000 B.C. (Eshbaugh, 1993). After Columbus discovered the Americas at the end of the 15th century, he introduced *Capsicum* species into Europe where they were quickly accepted and from there they spread to almost all the tropical, subtropical, and temperate zones in the world, including Africa, India, Indonesia and China (Lembeck, 1987, Palevitch & Craker, 1996, Bosland et al., 2012). Twenty-five distinct *Capsicum* species have been identified so far (Baral & Bosland, 2002), five of which are domesticated: *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* (Pickersgill, 1991).

Since cultivation started, pepper has been used in many ways as vegetable, spice, condiment, ornamental, and medicinal plant (Palevitch & Craker, 1996). Pepper is one of the most popular snack and cooked vegetables in the human diet (Wahyuni et al., 2011, Wahyuni et al., 2013). Among the cultivated pepper species, *C. annuum* is the dominant species used as vegetable (Govindarajan & Salzer, 1985, Kraft et al., 2014). Because of the diverse size, shape and colour of fruits, pepper is more and more widely used as ornamental plant (Stommel & Bosland, 2007, Nascimento et al., 2012, Stommel et al., 2018). Pepper is used as spice and condiment because of the pungent component, capsaicin (Sherman & Billing, 1999). Capsaicin has also been used to treat human diseases such as arthritis and postherpetic neuralgia (Deal et al., 1991, Watson et al., 1993), making pepper an important medicinal plant as well (Bernstein, 1987, Palevitch & Craker, 1996).

Because of its economic value and wide cultivation, pepper ranks as one of the top cultivated vegetables in the world and pepper production keeps expanding year by year (Maharijaya, 2013). The annual global production area and fresh yield of pepper in 2016 were about 3.8 million hectares and 60 million tons respectively (FAOSTAT, 2016). With such a wide cultivation area, it is not surprising that pepper production is constrained by various abiotic and biotic factors worldwide. Abiotic stresses such as drought, salinity, flooding and extreme temperatures may impair the growth and development of pepper (Boyer, 1982). Compared with abiotic stress, the negative effects of biotic stresses in pepper production are even more severe (Maharijaya, 2013). Insects, fungi, bacteria, nematodes and viruses are important pathogens that lead to yield loss in pepper. Improving pepper resistance to biotic and abiotic stress is crucial for keeping pepper production at a stable and high level.

The problem of *M. persicae* in pepper cultivation

The generalist *M. persicae*, or green peach aphid, is one of the most threatening pests in pepper cultivation. Besides causing direct damages it is also a major virus vector. Many pepper viruses are transmitted by *M. persicae*,

including Pepper mottle virus, Pepper severe mosaic virus and Pepper yellow mosaic virus (Black et al., 1991, Kenyon et al., 2014). In spite of the common agreement about the usage of host plant resistance in integrated pest management (Broekgaarden et al., 2011), only two studies on the identification of pepper accessions resistant to *M. persicae* have been published (Bosland & Ellington, 1996, Frantz et al., 2004). Both studies did not identify useful plant material that can be directly used in breeding programs. Bosland & Ellington (1996) found one *C. pubescens* plant that showed antixenosis resistance to *M. persicae*, but detailed information on this accession was not provided and moreover no hybridization between *C. pubescens* and *C. annuum* has been reported yet. Although Frantz et al detected significant differences among 50 pepper accessions in a choice test to *M. persicae* (Frantz et al., 2004), no strong resistance was found. Therefore, there is still an urgent need for the identification of pepper accessions resistant to *M. persicae*.

Objectives and outline of this thesis

Given the fact that *M. persicae* causes serious problems in pepper and that there is no good resistance source for pepper breeding, several questions are addressed in this PhD thesis: (1) Can we find a good source of *M. persicae* resistance in pepper accessions that are crossable to *C. annuum*? (2) If we find such accessions, what then is the resistance mechanism? (3) If aphid resistance is a complex trait, how many QTLs are involved in the resistance? What is their individual contribution, and which genes are underlying the resistance QTL? (4) Is the resistance effective to all *M. persicae* populations? And (5) if there are *M. persicae* populations that are able to overcome the resistance, how do they do it?

In **Chapter 2** the level of resistance to *M. persicae* in 74 pepper accessions from different geographical regions was evaluated. After four rounds of evaluation, a number of pepper accessions were identified as highly resistant to *M. persicae*. The resistance mechanism in one of them was studied.

In **Chapter 3**, QTLs conferring resistance to *M. persicae* in pepper were identified. We confirmed the effects of the major resistance QTL and narrowed it down to a genomic region predicted to encode four analogues of the LRR-RLK subfamily of resistance genes.

In **Chapter 4** we identified a *M. persicae* population virulent on the pepper accession that we previously identified as resistant, and described in detail different aspects of the interaction between the two aphid populations (avirulent and virulent) and two different pepper accessions, including the biochemical process by which the resistance might work.

In **Chapter 5**, RNA-seq was carried out to explore in detail how the pepper resistance is induced by the avirulent *M. persicae* population and how this is suppressed by the virulent population. A ROS accumulation assay and pre-infestation of conspecific populations strongly suggest that the virulent *M. persicae* population overcomes pepper resistance by manipulating plant defense responses, especially ROS metabolism.

Finally, in **Chapter 6** I provide a general discussion about the most important findings described in this thesis, including plans for further research.

CHAPTER 2

Reduced phloem uptake of *Myzus persicae* on an aphid resistant pepper accession

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This chapter has been published as:

Sun M., Voorrips R., Steenhuis-Broers G., Van 'T Westende W. and Vosman B. (2018) Reduced phloem uptake of Myzus persicae on an aphid resistant pepper accession. BMC plant biology, 18, 138.

Abstract

The green peach aphid (GPA), *Myzus persicae*, is economically one of the most threatening pests in pepper cultivation, which not only causes direct damage but also transmits many viruses. Breeding aphid resistant pepper varieties is a promising and environmentally friendly method to control aphid populations in the field and in the greenhouse. Until now, no strong sources of resistance against the GPA have been identified. Therefore the main aims of this study were to identify pepper materials with a good level of resistance to GPA and to elucidate possible resistance mechanisms. We screened 74 pepper accessions from different geographical areas for resistance to *M. persicae*. After four rounds of evaluation we identified one *Capsicum baccatum* accession (PB2013071) as highly resistant to *M. persicae*, while the accessions PB2013062 and PB2012022 showed intermediate resistance. The resistance of PB2013071 resulted in a severely reduced uptake of phloem compared to the susceptible accession, as determined by Electrical Penetration Graph (EPG) studies. Feeding of *M. persicae* induced the expression of callose synthase genes and resulted in callose deposition in the sieve elements in resistant, but not in susceptible plants. Three aphid resistant pepper accessions were identified, which will be important for breeding aphid resistant pepper varieties in the future. The most resistant accession PB2013071 showed phloem-based resistance against aphid infestation.

Introduction

Pepper (*Capsicum* spp.) belongs to the *Solanaceae* family and is one of the economically most important and widely cultivated vegetable crops. The annual global production area and yield of pepper are 3.7 million hectares and 37 million tons, respectively (FAOSTAT, 2015). The genus *Capsicum* originates from Central and South America and 25 distinct species have been reported (Baral & Bosland, 2002), among which five are domesticated: *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* (Pickersgill, 1991).

Aphids (*Aphididae*) are the most wide-spread pest insects. More than 100 aphid species are reported as economically important pests and most crops suffer from one or more species (Blackman & Eastop, 2008). The green peach aphid (GPA), *Myzus persicae*, is one of the most threatening pests in pepper and many other crops. It is a generalist that causes many types of damages in pepper, including chlorosis, necrosis, wilting, defoliation and flower and fruit abortion. It produces honeydew when feeding on plants, which may affect fruit quality and reduce photosynthetic capacity by stimulating mold development. However the most serious damage is done indirectly by the viruses that GPA may vector, including Potato virus Y, Pepper mottle virus, Pepper severe mosaic virus, Pepper yellow mosaic virus, Peru tomato mosaic virus (Kenyon et al., 2014).

As phloem-feeding insects, aphids use their specialized mouthparts, the stylets, to penetrate plant tissue and to take up nutrients without inflicting serious damage (Tjallingii, 2006, Dedryver et al., 2010). To study aphid probing and feeding behaviour, the electrical penetration graph (EPG) technique can be used (Tjallingii, 1988). In the EPG technique an aphid and a plant are wired into an electrical circuit, and aphid activity on the plant is recorded as waveforms that are specific for different probing and feeding activities (Tjallingii, 1985, Tjallingii et al., 2010). The EPG technique can be applied to explore the nature of the differences in aphid behaviour on resistant and susceptible plants, for instance to determine where in the leaf an aphid encounters a specific plant resistance factor (Alvarez et al., 2006, Tjallingii, 2006, Chandran et al., 2013, Khan et al., 2015).

In several cases it has been observed that aphids show a significantly shorter period of phloem feeding on resistant than on susceptible plants (Chandran et al., 2013, Khan et al., 2015). One possible explanation is occlusion of the phloem vessels in response to feeding (Will et al., 2007, Gaupels et al., 2008), which may be caused by callose deposition (Will et al., 2007, Hao et al., 2008). Callose, a ß-1,3-glucan, is an important component in the defense response to mechanical wounding, pathogen infection and insect infestation (Donofrio & Delaney, 2001, Saheed et al., 2009, Luna et al., 2011). In *Arabidopsis thaliana* callose deposition was induced and the expression of related synthase genes was enhanced in response to whitefly infestation (Kempema et al., 2007). In rice, callose deposition was suggested as an important resistance factor against the brown plant hopper (Hao et al., 2008).

Callose is produced by callose synthases (CalS), which are encoded by a family of callose synthase genes. Twelve, ten, six, nine and eight synthase genes were identified and characterized in *A. thaliana* (Richmond & Somerville, 2000, Verma & Hong, 2001), rice (Hazen et al., 2002), barley (Schober et al., 2009), wheat (Fu et al., 2014) and grapevine (Yu et al., 2016b), respectively. These genes were studied in detail in *A. thaliana*. The *CalS7* gene was reported to be expressed specifically in the phloem vessels and was responsible for callose deposition induced by mechanical wounding (Xie et al., 2011). The *CalS12* was mainly shown to be required for wound and papillary callose formation in response to pathogen attack (Jacobs et al., 2003, Nakashima et al., 2003) and to aphid feeding (Lü et al., 2011). The expression of *CalS1* was found to be up-regulated after infestation with aphids and whiteflies (Kempema et al., 2007, Kuśnierczyk et al., 2008). Besides the role of callose formation and deposition in plant resistance, the breakdown of callose might be another factor. Callose degradation, which is governed by some ß-1,3-glucanases, was shown to cause susceptibility in the interaction between the brown plant hopper and rice (Hao et al., 2008) as well as in the interaction between bird cherry-oat aphid and barley (Mehrabi et al., 2016).

Due to the severe negative effects of aphids on crop yield and quality, chemical pesticides have been widely used to control aphids. However, with more and more reports on aphids developing resistance to pesticides (Silver et al., 1995, Wang et al., 2002) and growing concern about the environmental impact of insecticides, breeding aphid resistant pepper varieties is a desirable alternative which will become an indispensable part of integrated pest management. Plant resistance mechanisms against insects, including aphids, are classified as antixenosis, antibiosis and tolerance (Painter, 1951, Smith & Boyko, 2007, Van Emden & Harrington, 2007, Züst & Agrawal, 2016). Antixenosis, or non-preference, affects insect settling or feeding through repellence or deterrence (Goggin, 2007). Antibiosis-based resistance impairs insect survival, growth, development and fecundity, caused by chemical or morphological adaptations of the plant (Williams et al., 1980, Smith, 2005, Van Emden & Harrington, 2007). Tolerance reduces damage to the plant after insect feeding, in spite of the presence of insect population densities similar to those on susceptible plants (Painter, 1951, Smith, 2005). A number of genes conferring resistance to aphids have been identified in crops, including among others in wheat (Boyko et al., 2004), soybean (Kim et al., 2010b), lettuce (Eenink et al., 1982a) and cowpea (Githiri et al., 1996). However, only two genes have been cloned, the tomato *Mi-1.2* gene which confers resistance to the potato aphid *Macrosiphum euphorbiae*, to the whitefly *Bemisia tabaci* and to three species of root-knot nematodes (Vos et al., 1998, Rossi et al., 1998, Nombela et al., 2003), and the melon *Vat* gene, which confers resistance to the cotton aphid *Aphis gossypii* (Pauquet et al., 2004), as well as to non-persistent viruses when vectored by *A. gossypii* (Pauquet et al., 2004). Both genes are of the NBS-LRR type (Vos et al., 1998, Pauquet et al., 2004) and work according to the gene-for-gene principle which means that the *R* gene in the plant recognizes an effector secreted by the aphid, and activates an aphid-specific

defense response (Smith & Boyko, 2007). Until now only a few studies to identify donors of resistance genes that may be used in pepper breeding have been published (Bosland & Ellington, 1996, Frantz et al., 2004). One *C. pubescens* plant showed antixenosis rather than antibiosis resistance to the GPA (Bosland & Ellington, 1996), but detailed information on this accession was not provided, and no hybridization between *C. pubescens* and *C. annuum* has been reported yet. Franz *et al.* detected significant differences among 50 pepper accessions in choice tests with GPA, however no strong resistance was found (Frantz et al., 2004). De Costa *et al*. identified a pepper cultivar which was resistant against the *A. gossypii*, but it is unknown if it is also resistant to GPA (Da Costa et al., 2011). Therefore, there is still an urgent need for pepper accessions resistant to GPA.

This research was carried out to identify accessions with a good level of resistance to GPA and to shed light on the possible resistance mechanism. We evaluated a collection of *C. annuum*, *C. chinense*, *C. frutescens* and *C. baccatum* accessions for GPA resistance and identified resistant accessions in *C. baccatum.* The resistance, mainly affecting aphid reproduction, is most likely phloem based and accompanied by callose deposition.

Material and methods

Plant materials and growing condition

The plant materials used consisted of accessions of *C. annuum*, *C. chinense*, *C. frutescens* and *C. baccatum* that were obtained from the Centre for Genetic Resources, the Netherlands (CGN) and from the collection of Wageningen University and Research. Based on the results of an initial evaluation of about 50 accessions, additional material from *C. baccatum* were screened. The accession codes, names, species and geographical origin of all materials used can be found in the Tables S1 and S2.

Two weeks after sowing, plants were transplanted into 14 cm pots with potting compost and grown in a standard greenhouse at 19-21 °C, 60–70% relative humidity and a 16–8 h light–dark photoperiod at Wageningen University & Research, Wageningen, NL. Plants were watered every other day and no aphid control was applied during growth and testing.

Aphid population

The GPA (*M. persicae*) population used originated from the population used by (Chen et al., 2012). Initially it was reared on Chinese cabbage (*B. rapa*) cv. Granaat; later the rearing was transferred to *C. annuum* accession CGN19226 and subsequently to *C. baccatum* accession PB2013046. The aphid rearing was maintained in a standard greenhouse under the same conditions as the pepper plants.

Evaluation of *Capsicum* accessions for GPA resistance in a clip cage test

All evaluations were carried out in the greenhouses of Wageningen University & Research, Wageningen, NL and were performed in four experiments during summer and autumn. The first experiment, including 50 accessions (Table S1), was done when plants were eight weeks old. Plants were tested in a complete block design with four blocks in the same glasshouse compartment, with one plant of each accession per block and two clip cages

containing per cage 10 1-day-old GPA nymphs that were obtained from a rearing on Chinese cabbage. The clip cages were placed on the abaxial side of the top two fully expanded leaves of the plants. After seven days the numbers of surviving and dead aphids as well as new nymphs produced in each clip cage were counted. The second experiment was conducted similarly to the first with the following changes. Ten accessions were selected from the 50 tested in the first experiment (Table S1). They were re-tested in a complete block design with 10 blocks when they were seven weeks old, one plant per accession in each block, again per plant with two clip-on cages with 10 1-day-old nymphs, originating from a rearing on *C. annuum* accession CGN19226.

In the third experiment only *C. baccatum* accessions were evaluated, together with *C. annuum* CGN19226 as susceptible control (Table S2) in a complete block design with four blocks under conditions similar to the first two experiments. They were evaluated with two clip cages per plant, containing 5 1-day-old GPA nymphs per cage obtained from a rearing on CGN19226, when plants were seven weeks old. During the fourth experiment, eight selected accessions from the third experiment (including the susceptible *C. annuum* CGN19226) were re-tested in a complete block design with five blocks (Table S2). Similar to the third experiment plants were evaluated with two clip cages containing 5 1-day-old GPA nymphs originated from a rearing on the susceptible *C. baccatum* accession PB2013046, when the plants were seven weeks old. After eight days all clip cages were observed.

For statistical analysis, the observations from two clip cages per plant were combined. Survival was determined by dividing the number of living aphids by the total number of aphids (dead and alive) in the clip cage. The number of new nymphs was divided by the average number of living aphids present, calculated as (2*living aphids + dead aphids)/2. Additionally, data used for ANOVA analysis were transformed to obtain a more or less constant residual variance: survival as arcsin(sqrt(x)) and nymphs as sqrt(x). Significance of differences in the means was evaluated using the LSD test ($P<0.05$) on the transformed data.

Population development

A population development experiment was used to further confirm resistance/susceptibility of the accessions. Ten plants of each selected accession were randomized in one greenhouse compartment. Approx. 40 days after sowing each plant was infested with 5 wingless GPA adults and 10 nymphs and enclosed in an aphid-proof sleeve. After 19 days, the number of adult aphids was counted and the number of nymphs was estimated according to a visual scale (0=none, 1=few (<50), 2=many nymphs (>50)). For ANOVA analysis, the number of adults per plant was transformed to log(x). Significance of differences of means was tested by LSD test ($P<0.05$).

Electrical Penetration Graph

The Electrical Penetration Graph (EPG) technique was used to monitor GPA probing and feeding behaviour on the most resistant (PB2013071) and a susceptible (PB2013046) *C. baccatum* accession. For each accession, 10 seven-week-old plants were each probed with two adult aphids placed on the abaxial side of the top two fully expanded leaves. Experimental setup was as described by (Alvarez et al., 2013). Recording lasted for six hours at 20 ± 2°C under constant light. The EPG patterns were transformed into waveforms using the Stylet+a software version 1.20 (http://www.epgsystems.eu/). Extraction of resistance parameters from the waveforms was carried out using EPG-Calc 6.1.3 (Giordanengo, 2014). T-tests were used to determine the significance of the differences

between the accessions for various EPG parameters. The Fisher exact test was used to determine the significance of the difference in percentage of aphids that reached E2 during six hours' recording.

Callose deposition

Histological analysis of *in situ* callose deposition was performed essentially as described by (Kissoudis et al., 2016) on the resistant (PB2013071) and susceptible (PB2013046) *C. baccatum* accession. The second fully expanded leaf with petiole was cut with scissors from each plant and immediately put into a 6 cm-diameter petri dish with 1.5% water-agar medium. Twenty randomly selected wingless aphids were put gently into the petri dish, which was sealed by Parafilm M (Bemis NA, USA). Four plants/replicates were used for each treatment or control. After 24 hours, three to four leaf disks (1.3 cm in diameter) containing highest number of aphids were sampled from the detached leaf and directly placed in 96% ethanol with their abaxial side up to remove chlorophyll. After washing in 0.07 M K_2HPO_4 (pH=9), leaf disks were stained for 2 h in 0.1% (w/v) aniline blue in 0.07 M K_2HPO_4 (pH=9) at room temperature. Samples were subsequently mounted on glass slides with 70% glycerol. Callose fluorescence was observed qualitatively under UV light, and photos were taken using the Zeiss Axiophoto digital imaging microscope (Carl Zeiss AG, Germany). Control leaf samples without aphids were treated in the same way; leaf disks were taken from areas comparable to the areas taken from the infested leaves. In total 12 leaf disks were observed for accession PB2013071 and 14 for accession PB2013046 after 24h GPA treatment; and 12 leaf disks were observed for both accessions as control.

Gene expression analysis

The expression level of callose related genes was analyzed by quantitative real-time PCR. Seven-week-old plants received three clip cages containing 15 randomly selected wingless aphids per cage. Leaf disks were collected from the clip cage areas 1.5, 6 and 24 hours after the start of aphid infestation. After gently brushing aphids away, disks were flash-frozen in liquid nitrogen and stored at -80 ºC until use. Leaf disks under an empty clip cage were also collected after 1.5, 6 and 24 hours and used as reference. Additionally, leaf disks without clip cage and aphid infestation were collected just before the infestation stated (time point 0h). Four biological replicates were used per treatment with aphid infestation and three per treatment with empty clip cages. For the reference without clip cages (time point 0h) also three biological replicates were used. In all cases, two plants were pooled together as one biological replicate.

The sequences of *CalS* family genes were obtained from the Pepper Genome Platform (http://peppergenome.snu.ac.kr/) (Kim et al., 2014b) and the Pepper Genome Database (http://peppersequence.genomics.cn/page/species/index.jsp) (Qin et al., 2014) through BlastP queries (McGinnis & Madden, 2004) referring to the sequences from *Arabidopsis* (https://www.arabidopsis.org/index.jsp). Genes were identified and named according to phylogenetic tree of *CalS* family genes among *Arabidopsis*, grapevine and pepper which was constructed by MEGA5 (Tamura et al., 2011). Besides the *CalS* family genes, the *basic ß-1,3-glucanase* gene (CA03g30020, *BGLU*) was obtained from the Pepper Genome Platform (http://peppergenome.snu.ac.kr/). The pepper *actin* gene (CA12g08730) was used as an internal reference for normalization of gene expression (Bin et al., 2012). Gene specific primers were designed using Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are listed in TableS4.

Total RNA was isolated with the RNeasy plant mini kit (Qiagen, USA) according to the suppliers' recommendations. After treatment with DNase I (Invitrogen, USA), 1 µg RNA template was reversely transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Quantitative real-time PCR was conducted using the iQ™ SYBR Green Supermix (Bio-Rad, USA) and the CFX96 Touch™ Real-Time system (Bio-Rad, USA).

The PCR mix contained 5 µl 2x iQ™ SYBR GREEN Supermix, 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM) and 2 µl cDNA template with 10-time dilution, into a final volume of 10 µl. Quantitative RT-PCR was performed in duplicate using the following program: 95 ºC for 3 min followed by 40 cycles of 95 ºC for 15 sec, and 60 ºC for 1 min. As the primers were designed on the gene sequences from *C. annuum*, the QPCR products were sequenced to validate the region of amplification in *C. baccatum*. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Independent-samples t-tests on log2-transformed data were used to determine the significance of the differences between certain time points after GPA infestation and no GPA infestation ($P< 0.05$).

Results

Selection of pepper accessions resistant to GPA

Evaluation of 50 accessions, representing 4 *Capsicum* species, for GPA resistance showed large and highly significant differences (Table S1) for the two resistance parameters used: survival of the original nymphs and the number of next generation nymphs produced. Survival rate ranged from 6% to 97%, while the average number of new nymphs produced by each living adult during infestation varied from 0 to 0.8.

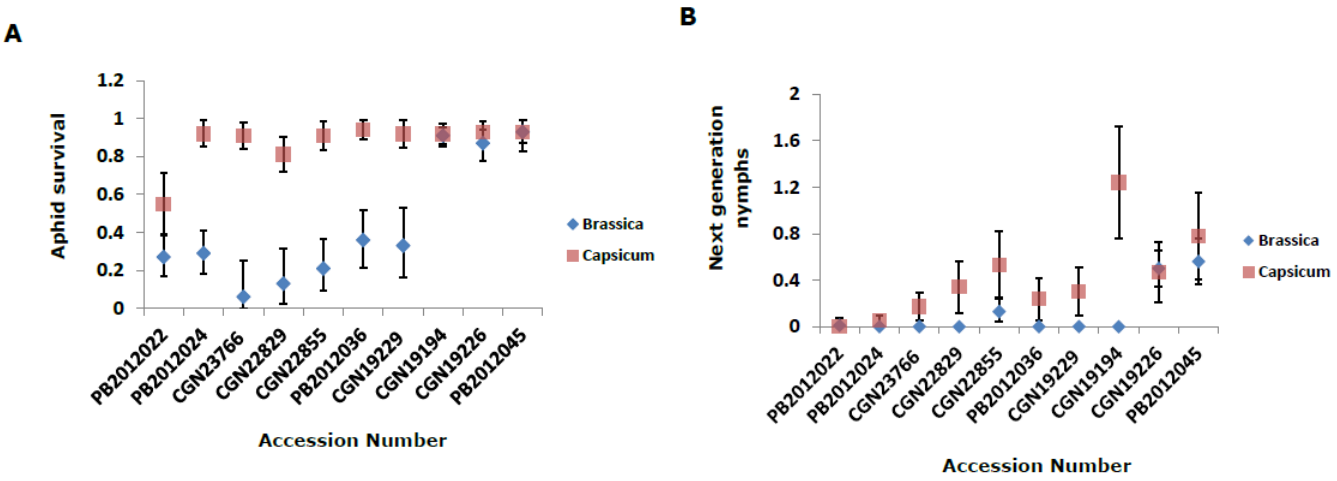

Figure 1. Performance of *M. persicae* after adaptation to different host plants. Ten selected accessions were infested with aphids reared on Chinese cabbage (*Brassica rapa* cv. Granaat; blue icon) or pepper (*C. annuum* CGN19226; red icon). Performance parameters used: survival of the original nymphs (A) and the number of next generation nymphs produced (B). Survival was determined by dividing the number of living aphids by the total number of aphids (dead and alive) in the clip cage. The number of next generation nymphs was divided by the average number of living aphids present, calculated as (2*living aphids + dead aphids)/2. Each bar represents the mean values ± SD. More details on the statistics can be found in Table S1.

After transferring the GPA rearing from Chinese cabbage to *C. annuum* accession CGN19226, ten selected accessions (Table S1 and Figure 1) were re-tested with the GPA colony that had been adapted to pepper. These included seven accessions showing a low aphid survival and also a low production of second generation nymphs in the first experiment. The accessions *C. annuum* CGN19226 and *C. frutescens* PB2012045 were chosen as susceptible standards as they are from different species and origins. Accession *C. annuum* CGN19194 was selected as no second generation nymphs were produced on it, while the number of surviving adults was high, suggesting that this accession may possess a resistance mechanism affecting reproduction only. In this second experiment the two susceptible standards were again completely susceptible. Accession CGN19194 was also highly susceptible; the reduced reproduction observed in the first test was not confirmed in the second one using the aphids adapted to pepper. Among the seven accessions selected as resistant in the first experiment, the five *C. chinense* accessions respectively showed varying levels of resistance based on the two resistance parameters between the two experiments (T-test, $P<0.01$). However, the two *C. baccatum* accessions (PB2012022 and PB2012024) continued to show an impaired reproduction in the second experiment, which was the same as that in the first experiment.

Based on the results of the initial screening, we decided to focus further efforts on the screening of *C. baccatum* accessions (Table S2). In the third experiment accession *C. annuum* CGN19226 was used as susceptible standard. Evaluation of 38 accessions showed significant variation for aphid survival and aphid fecundity: survival of original nymphs varied from 0.49 to 0.98 and the number of new nymphs produced per aphid ranged from 0 to 0.89. The accessions PB2013071, PB2013062 and CGN23260 were among the most resistant although they were not significantly different from a number of others, based on aphid survival and next generation nymphs produced. Accession PB2012022 showed a slightly higher nymph survival, but no next generation nymphs, confirming previous results. The accession *C. baccatum* PB2013046 was as susceptible as the susceptible standard *C. annuum* accession CGN19226. For this reason we transferred the GPA rearing to PB2013046 and re-tested eight accessions for resistance using GPA reared on this susceptible *C. baccatum* accession (Table S2 and Figure 2). In this fourth experiment, we classified PB2013071, PB2013062, CGN23260 (no reproduction, relatively low survival: <0.7) together with PB2012022 and CGN22834 (also no reproduction, somewhat higher survival: >0.7) as resistant, CGN22858 (some reproduction, low survival) as an intermediate resistant, and PB2013046 together with CGN19226 (high reproduction, high survival) as susceptible accessions. In this experiment, the accession *C. baccatum* PB2013071 was again the most resistant, as it continued showing the lowest survival and no reproduction. The accession *C. baccatum* PB2013046 was again as susceptible as *C. annuum* accession CGN19226. The correlation coefficient between the number of new nymphs produced by *C. annuum* and *C. baccatum* adapted aphids (third and fourth experiment) was 0.83, which was calculated on the basis of the eight accessions tested with both populations.

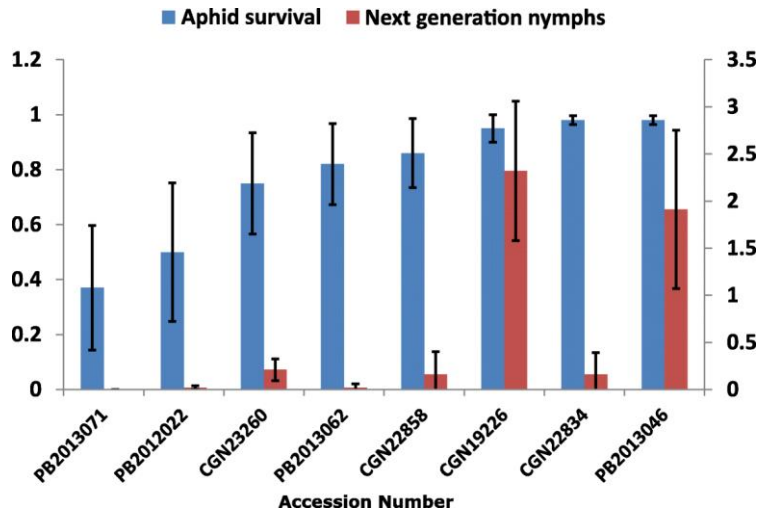

Figure 2. Performance of *M. persicae* on eight accessions after adaptation on *C. baccatum*. Aphids were reared on accession PB2013046. Performance parameters used: survival of the original nymphs (blue column) and the number of next generation nymphs produced (red column). Survival was determined by dividing the number of living aphids by the total number of aphids (dead and alive) in the clip cage. The number of next generation nymphs was divided by the average number of living aphids present, calculated as (2*living aphids + dead aphids)/2. Each bar represents the mean values ± SD. More details on the statistics can be found in Table S2.

GPA population development on selected accessions

The three selected resistant *C. baccatum* accessions (PB2013071, PB2013062 and PB2012022), the susceptible *C. baccatum* accession (PB2013046) and the susceptible *C. annuum* accession (CGN19226) were used for further confirmation of resistance and susceptibility using a population development experiment. Results are shown in Table 1. PB2013046 is confirmed as a susceptible accession on which aphids show a high survival rate and strong fecundity, which was even higher than the *C. annuum* susceptible standard (CGN19226). Accession PB2013071 showed the highest level of resistance, while the accessions PB2013062 and PB2012022 were intermediate.

Table 1. Population development of the aphid *M. persicae* on five *Capsicum* accessions

Accession Number	Adults		Nymphs[1]
PB2013071	33	a	0.7
PB2012022	158	b	1.2
PB2013062	337	c	1.6
PB2013046	2655	d	2.0
CGN19226	1633	d	2.0

[1] Average number of nymphs according to visual scale: 0=none, 1=few (<50), 2=many (>50)
Mean values of adult count followed by the same letter are not significantly different (LSD- test on log-transformed scale at P<0.05)

EPG analysis on accessions PB2013071 and PB2013046

Results for the parameters extracted from the EPG recordings are presented in Table 2. No significant difference was found between the resistant accession PB2013071 and the susceptible accession PB2013046 for parameters

related with non-probing, pathway phase, derailed stylet mechanics and xylem phase. However, significant differences were seen during the phloem phase E1 (salivation into the phloem) and E2 (phloem sap ingestion) (T-test, $P<0.05$). The total duration of E1 on PB2013071 was more than two times as long as on PB2013046, while the total duration of E2 on PB2013071 was only about one-eighth of that on PB2013046. However, there was no significant difference in the number of aphids that successfully reached phloem ingestion E2: 75% on PB2013046 and 47% on PB2013071 (Fisher exact test, $P=0.101$). The total number of individual cell punctures (potential drops) and average number of potential drops per minute of pathway phase were both more on PB2013071 than PB2013046 (T-test, $P<0.01$).

Table 2. *M. persicae* EPG parameters measured on a susceptible (PB2013046) and a resistant (PB2013071) *C. baccatum* accession. Data are based on 20 and 17 aphids tested on PB2013046 and PB2013071, respectively. Mean values are shown.

Class	Trait Definition [a]	PB2013046	PB2013071	P-value
Non-probing (NP)	Number of NP	15.7	17.3	0.5645
	Total duration of NP (min)	21.3	21.3	0.9879
Probes	Number of Probes	14.8	16.3	0.5870
	Total duration of Probes (min)	338.6	338.6	0.9874
Pathway phase (C)	Number of C (pathway periods)	24.4	27.8	0.3528
	Total duration of pathway period (min)	125.0	155.1	0.1206
Derailed stylet (F)	Number of periods with F form	3.3	1.5	0.0650
	Total duration of F period (min)	72.9	59.8	0.6230
Xylem phase (G)	Number of periods with G form	2.1	1.7	0.5651
	Total duration of G period (min)	32.9	33.4	0.9686
	Time to first G phase (min)	162.8	153.2	0.8326
Phloem phase (E)	Number of salivation periods (E1)	6.1	9.4	**0.0265**
	Time to first E1 (min)	105.1	82.2	0.4575
	Total duration of E1 (min)	28.6	80.0	**0.0001**
	Total duration of phloem uptake (E2, min)	78.9	10.5	**0.0032**
	Time to first E2 (min)	258.5	324.0	**0.0414**
	Number of E1 followed by E2	1.2	0.3	**0.0049**
	Total duration of E1E2 (min)	86.6	5.5	**0.0008**
	Time to first E1E2 (min)	225.4	324.4	**0.0037**
Potential drops (Pd)	Number of potential drops	83.7	156.4	**0.0000**
	Number of Pd per min of Pathway C	0.7	1.0	**0.0025**
Aphids reaching E2	Percentage of aphids reaching E2	75 %	47 %	0.1010

[a] Trait definition according to Giordanengo and Philippe (Giordanengo, 2014).

Callose deposition

Callose deposition is considered important for plant resistance against pathogens and insects (Ton & Mauch-Mani, 2004, Hao et al., 2008). We studied the accumulation of callose in resistant and susceptible plants after GPA feeding. Detached leaves were infested with GPA for 24 h, after which three or four leaf disks were prepared for the callose deposition study. Representative results are shown in Figure 3 and additional images can be found in

Figure S1. Callose signals were detected in the vascular tissue of all sampled leaf disks from accession PB2013071, but not in accession PB2013046 treated by GPA or in leaf disks of both accessions without aphids infestation.

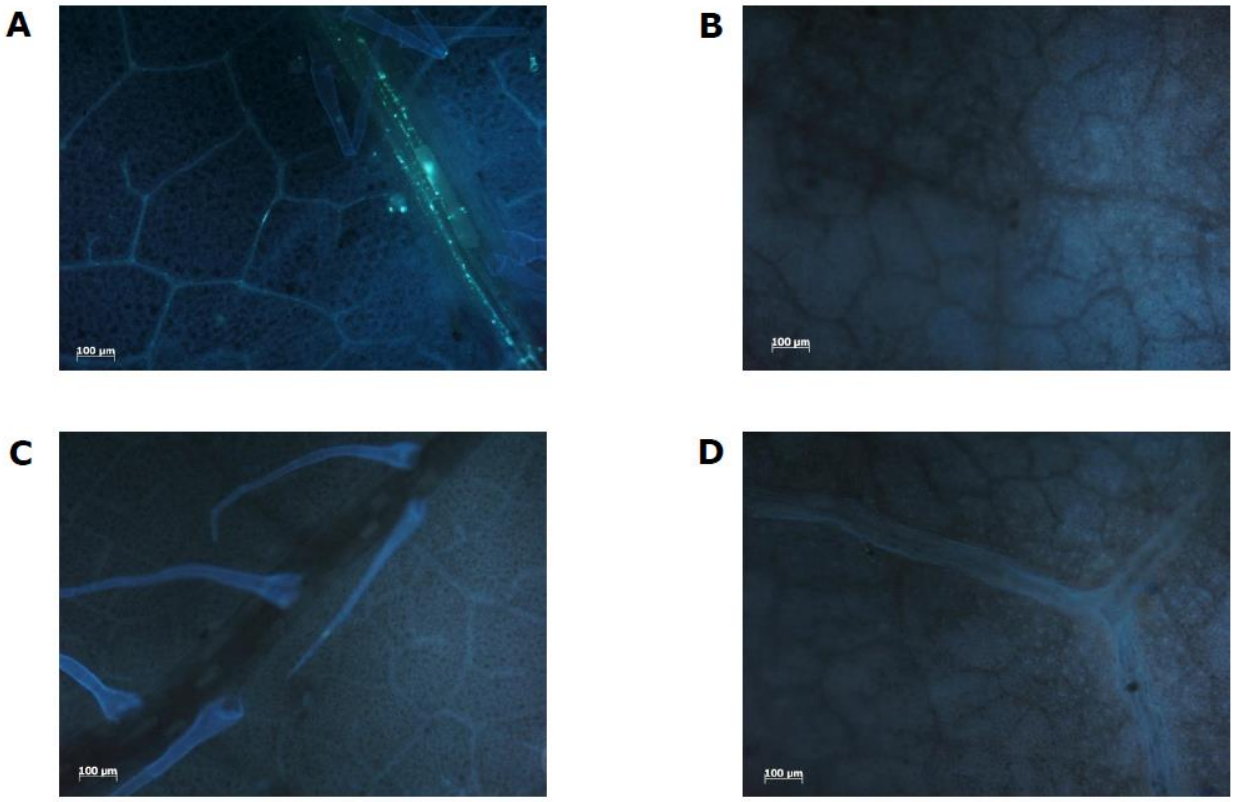

Figure 3. Histochemical staining of callose in the GPA-infested leaves (A, B) and GPA-free leaves (C, D). Resistant accession PB2013071 (A, C); susceptible accession PB2013046 (B, D). Staining was carried out 24 hours after the start of the infestation.

Identification and expression of callose related genes

Nine putative *Callose Synthase* (*CalS)* genes were identified in the *C. annuum* sequences and named with reference to the most homologous gene in *Arabidopsis*, *CaCalS1*, *CaCalS3*, *CaCalS5*, *CaCalS7*, *CaCalS8*, *CaCalS9*, *CaCalS10*, *CaCalS11* and *CaCalS12*. The length of open reading frames (ORFs) and gene IDs in both pepper genome sequences are listed in Table S3. A neighbour-joining tree of CalS proteins among pepper, *Arabidopsis* and grapevine is shown in Figure S2.

To shed light on the regulation of the callose deposition we compared the expression of *callose synthase* genes (*CalS* family genes) and the *basic ß-1,3-glucanase* gene (*BGLU*) in GPA-infested leaves with those of non-infested leaves. Nine putative *CalS* family genes were analyzed by real-time PCR. Among these nine genes, only *CalS1* (Figure 4A) and *CalS7* (Figure 4B) showed a clear change in transcript accumulation upon aphid infestation. In the leaves of PB2013071 infested with GPA, no difference in expression was detected for both genes after 1.5h, but expression was significantly up-regulated at 6 h and 24 h after the start of the infestation compared to empty cages (T-test, $P<0.05$). The expression level of *CalS1* increased 5.6-fold (T-test, $P=0.0004$) and that of *CalS7* increased 3.9-fold (T-test, $P=0.0088$) 24 h post-infestation compared to empty cages. In the leaves of PB2013046

infested with GPA, expression of the *CalS1* and *CalS7* genes remained stable during 24 hours, except that *CalS7* after 1.5 h showed significantly lower expression level in GPA infested leaves compared to GPA free leaves (T-test, P=0.0017). The expression of the *CalS1* and *CalS7* gene in leaves of both accessions after 1.5h, 6h, 24h with empty clip cages remained constant (ANOVA, P>0.05).

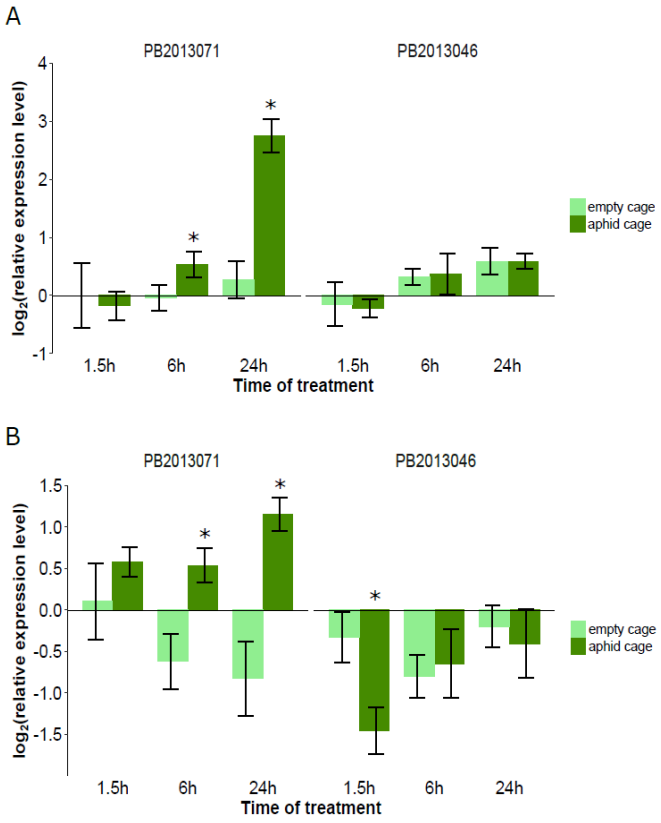

Figure 4. Expression analysis of *Callose synthase* genes *CalS1* (A) and *CalS7* (B) after aphid infestation. Gene expression was quantified relative to the value obtained from leaf samples without clip cage or aphid infestation (time point 0h). Data was $\log_2$-transformed. Each bar represents the mean values of three or four biological replicates, each with two technical replicates. The *actin* gene was used as the reference gene. * indicates a significant difference in level of gene expression between the GPA treated sample and the GPA-free (empty clip cage) sample at that time points (T-test, P<0.05). Each bar represents the mean values ± SD.

The *BGLU* gene was up-regulated in PB2013071 at all three time-points during the 24 hours of aphid infestation compared to empty cages (Figure 5) (T-test, P<0.05). The ratio of transcripts with and without aphid infestation increased to 2.3 at 1.5 h (T-test, P=0.0070), to 3.9 at 6 h (T-test, P=0.0062) and to 6.4 at 24 h (T-test, P=0.0141) after the start of the infestation. In contrast, there was no significant difference in expression of the *BGLU* gene in PB2013046 between plants with GPA treatment for 1.5 h, 6 h and 24 h and plants with empty cages at the same time points. In leaves that received empty clip cages, the expression of the *BGLU* gene increased after 1.5h, 6h, 24h, in both accessions (ANOVA, P<0.05).

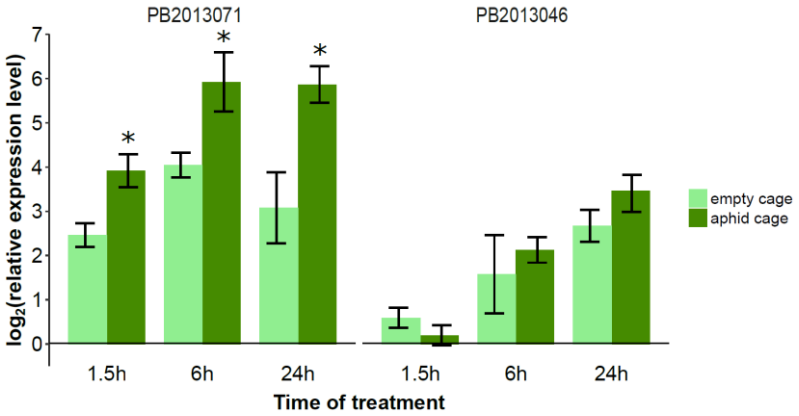

Figure 5. Expression analysis of the *BGLU* gene after aphid infestation. Gene expression was quantified relative to the value obtained from leaf samples without clip cage or aphid infestation (time point 0h). Date was log2-transformed. Each bar represents the mean values of three or four biological replicates, each with two technical replicates. The *actin* gene was used as the reference gene. * indicates a significant difference in level of gene expression between the GPA treated sample and the GPA-free (empty clip cage) sample at that time points (T-test, P<0.05). Each bar represents the mean values ± SD.

Discussion

Importance of rearing history during evaluation of GPA performance

When the initial evaluations were performed using GPA reared on cabbage or pepper, large differences were seen in aphid survival: GPA survival was relatively low when cabbage reared GPA were used and high when pepper reared GPA were used. The effect of GPA rearing history varied among *Capsicum* accessions. There was hardly any effect on *C. annuum* accessions, whereas on *C. chinense* accessions the effect of the rearing was pronounced. It has been reported before that the host plant on which an aphid colony is reared can affect the performance of aphids. For example, the grain aphid *Sitobion avenae* reared on wheat performed less well on the cocksfoot than on wheat (De Barro et al., 1995), and *A. gossypii* that adapted to cotton or cucumber could not survive and reproduce after reciprocal host transfer (Liu et al., 2008).

About the background of host adaptation in our test system we can only speculate. (1) As there are differences in metabolite content between cabbage and pepper, aphids may have to develop/adjust their detoxification system to adapt to the host plant, which may take several generations. For instance, the enzymatic detoxification system, a family of glutathione S-transferases, was reported to be involved in adaptation of GPA to different species containing different glucosinolates (Francis et al., 2005). (2) Another hypothesis involves a change in endosymbiont composition after transferring from one plant species to the other. Mutualistic symbionts play an instrumental role in plant-insect interactions (Sugio et al., 2014). Host plant specialization of pea aphid *Acyrthosiphon pisum* was reported to be influenced by the facultative pea aphid U-type symbiont (PAUS) (Tsuchida et al., 2004). Also, the abundance of *Buchnera aphidicola*, the primary endosymbiotic bacterium of GPA, was found to affect GPA host acceptance and stylet penetration on host plants (Machado-Assefh et al., 2015). In our case, the rearing on *C. annuum* may have changed the aphid metabolism, introduced a new endosymbiont species or increased the abundance of an already present symbiont species, improving their performance on *C.*

chinense. Based on the observations made, it is highly recommended that evaluations of germplasm are carried out using insect populations that are adapted to the species, or that re-testing is conducted with adapted aphids to confirm results of resistance screenings especially when aphids are reared on evolutionary distant plant materials, as on Chinese cabbage in our case.

A wide diversity in GPA resistance among *Capsicum* accessions

The high multiplication rate of aphids makes them a pest in many crops (Leather & Dixon, 1984). Even in the presence of natural enemies (predators and parasitoids) it is often difficult to control the growth of aphid populations. Varieties that are highly or even partly resistant to aphids can make a big difference by reducing the multiplication rate of the aphids and thus give natural enemies more chance to control them (Thomas & Waage, 1996). To develop such varieties, resistance sources need to be identified in crossable species and in this paper we describe the identification of such sources. Accessions from four inter-crossable *Capsicum* species were evaluated for resistance against the GPA and considerable variation was observed. After four rounds of evaluation, we identified a number of *C. baccatum* accessions with a relatively high and stable level of aphid resistance. A GPA population development experiment among five selected accessions confirmed their resistance. Resistance primarily seems to affect the production of next generation nymphs and to a lesser extent the survival of the aphid itself. Accession *C. baccatum* PB2013046 showed susceptibility with the highest GPA survival rate and fecundity while *C. baccatum* PB2013071 showed the strongest resistance, with a significantly lower GPA survival than on the susceptible accession and a severely impaired population development. Accessions *C. baccatum* PB2013062 and *C. baccatum* PB2012022 showed intermediate levels of resistance. These three accessions are the first *C. baccatum* accessions in which resistance to GPA is demonstrated and may be used for breeding resistant varieties in the other *Capsicum* species as well. The species *C. baccatum* has been used for pepper breeding as donor of anthracnose (Yoon et al., 2004, Park et al., 2009) and powdery mildew resistance (De Souza & Café-Filho, 2003). With respect to insect resistance, two *C. baccatum* accessions were reported as a good source for thrips (*Thrips parvispinus* and *Frankliniella occidentalis*) resistance (Maharijaya et al., 2011) and three *C. baccatum* accessions were identified as tolerant but not resistant to cotton aphid (*A. gossypii*) (Frantz et al., 2004). To our knowledge, this is the first report of a strong antibiosis type of resistance to GPA in *Capsicum*.

Impaired phloem uptake on a resistant accession

The Electrical Penetration Graph (EPG) technique allows an in-depth study of the feeding behaviour of piercing-sucking insects (Tjallingii, 1988) and is able to reveal possible constraint encountered by such insects when trying to feed on plants (Tjallingii, 2006, Tjallingii et al., 2010). The EPG analysis revealed significant differences in parameters related with the phloem phase of GPA feeding on the resistant versus susceptible pepper accession. In comparison to the susceptible accession PB2013046, on the resistant PB2013071 the phloem salivation periods were longer and more frequent, and the phloem uptake periods were much shorter, suggesting that the resistance is most likely located in the phloem. In other words, aphids feeding on resistant accession PB2013071 have difficulties to initiate and sustain phloem sap ingestion. Aphids feeding on accessions containing a phloem based resistance are likely to grow more slowly, have lower fecundity and are more likely to die early due to the problems they experience with taking up sufficient nutrition. This is in line with our observations. Besides the possibility to

control aphid population, phloem based resistance may reduce the transmission of persistent viruses because generally aphids cannot acquire persistent viruses during short-time feeding (Gray & Banerjee, 1999). It is likely that the percentage of plants infested with persistent viruses will also decrease when the number of aphids carrying virus is low (Radcliffe & Ragsdale, 2002).

No significant differences were observed in the pre-phloem phase, with the exception of the number of potential drops. Potential drops indicate that the aphid's stylets puncture cells along the pathway to the phloem (Tjallingii & Esch, 1993). The number of potential drops was much higher on the resistant accession PB2013071 than on the susceptible accession PB2013046. One biotype of soybean aphid (*Glycine max*) was also shown to have a higher number of potential drops when feeding on resistant genotypes than on susceptible genotypes (Chandran et al., 2013). It has been reported that potential drops are related with aphid transmission of non-persistently transmitted viruses (Mart et al., 1997, Symmes et al., 2008). However, it is unknown if they are indicative for a specific plant resistance component. In spite of the difference in number of potential drops, the total duration of the pathway phase was not different between the two accessions. We examined the number of cell layers between the epidermis and the phloem in the two accessions, which might have a relation with the number of cells punctured while passing to the phloem; however we did not observe a difference between the two accessions in this respect (results not shown). Therefore, it remains unclear if the higher number of potential drops on the resistant plant is important for resistance.

Induced callose deposition in the resistant accession

One possible mechanism of phloem-based resistance might be occlusion of the phloem vessels in response to aphid feeding, which may result from callose deposition. Callose induction and formation is a defense response to phloem-sucking pests that plugs the sieve element to obstruct feeding (Botha et al., 2004, Saheed et al., 2007, Hao et al., 2008, Kuśnierczyk et al., 2008, Mondal et al., 2017). Our data clearly show callose deposition 24 h after the start of the aphid infestation on detached leaves from the resistant accession PB2013071, but not on the susceptible accession PB2013046 and also not on non-infested leaves of either accession. This suggests that callose deposition may be one of the mechanisms behind the phloem-based resistance. The fact that callose deposition was studied on detached leaves and not on intact plants may have resulted in a weaker callose response. We did not assess the resistance on detached leaves, but studies on lettuce with the aphid *Nasonovia ribisnigri* (Broeke et al., 2016) suggest that the expression of resistance may be partially reduced in detached leaves compared to intact plants. It is also reported that callose deposition is observed in epidermal and mesophyll cell walls in the interaction of *A. gossypii* with melon plants carrying resistance gene *Vat* (Villada et al., 2009).

As a strong callose signal was found in leaf veins of resistant pepper plants after GPA feeding and not in susceptible plants, it was hypothesized that one or several *CalS* family genes or *ß-1,3-glucanase* gene(s) might be involved in this difference between resistant and susceptible plants after GPA infestation. We carried out quantitative real-time PCR to examine whether callose deposition could be due to increased *CalS* gene expression upon aphid attack. Among the nine putative *CalS* family genes, the *CalS1* gene was found to be significantly up-regulated at 6h and 24h post-infestation of GPA feeding in the leaves of PB2013071, while the level of gene transcripts remained constant in the leaves of PB2013046 during the initial 24 hours of aphid infestation. The *CalS1* gene has been

reported in *Arabidopsis* to accumulate after whitefly and aphid infestation (Kempema et al., 2007, Kuśnierczyk et al., 2008). Besides the *CalS1* gene, we detected that transcripts of *CalS7* in the infested leaves of resistant accession PB2013071 also significantly increased after 6h and 24h compared to non-infested leaves, but less than the transcripts of *CalS1*. The *CalS7* gene is the only phloem-specific *callose synthase* gene and it is responsible for callose biosynthesis in developing sieve elements as well as for callose deposition after mechanical wounding in mature phloem (Xie et al., 2011). Here we report for the first time an induction of *CalS7* transcription upon infestation with a phloem-feeding insect. The expression of the two *CalS* genes increased after aphid attack in leaves of the resistant accession but not in leaves of the susceptible accession. We speculate that the *CalS1* and/or *CalS7* genes are responsible for callose deposition in leaves of the resistant accession PB2013071 after GPA feeding. As in *A. thaliana CalS1* also can be induced by other phloem-feeding insects like the whitefly *B. tabaci* (Kempema et al., 2007) and cabbage aphid *Brevicoryne brassicae* (Kuśnierczyk et al., 2008), the *CalS1* gene might have a common role in callose deposition induced by phloem-feeders. As the *CalS7* gene is expressed specifically in phloem vessels (Xie et al., 2010), the sampling of entire leaf disks rather than just leaf veins for real-time PCR may lead to an underestimation of the level of induction in phloem punctured by the insect. The role of these two *CalS* genes in callose deposition needs to be further studied. As transformation of pepper is difficult (Kothari et al., 2010), it may not be so easy to do this by silencing the two *CalS* genes. It may be more effective to carry out a genetic (fine) mapping study to identify genes involved in the resistance.

The BGLU protein, also known as pathogenesis-related (PR) protein 2, is responsible for hydrolyzing callose (ß-1,3-glucan) in order to destabilize the cell wall of pathogens as well as to activate some immunity elicitors which can stimulate defense responses against pathogen attack (Linthorst & Van Loon, 1991). In pepper plants BGLU has been reported to play an important role during defense against pathogens (Jung & Hwang, 2000, Zheng et al., 2004, Wang et al., 2013).The BGLU protein or *BGLU* gene transcript has also been found to accumulate in leaves of wheat (Van der Westhuizen et al., 1998) and *Arabidopsis* (De Vos et al., 2005) after aphid infestation. The *BGLU* gene is considered as a marker of the salicylic acid (SA)-dependent defense response in plants (Uknes et al., 1992, Moran & Thompson, 2001). Also, some ß-1,3-glucanases of the same family as BGLU were proposed as susceptibility factors in the interaction between brown plant hopper and rice (Hao et al., 2008) as well as between bird cherry-oat aphid and barley (Mehrabi et al., 2016). It is thought that the feeding barrier for insects caused by callose deposition can be weakened in susceptible plants due to accumulation of *ß-1,3-glucanase*, while callose deposition can be maintained in resistant plants when the expression of *ß-1,3-glucanase* gene is low. However, in contrast to this hypothesis we found that expression of the *BGLU* gene increased during the 24 hours of GPA feeding in the leaves of resistant accession PB2013071, but not in leaves of susceptible accession PB2013046. There may be a delicate balance between the expression level of the callose synthesis and callose degrading genes. The *BGLU* accumulation might be caused by the plant's need to degrade callose in the phloem, as callose deposition may affect the transport of assimilates. The fact that also under the empty clip cages the expression of the *BGLU* gene increased may be related to the involvement of the *BGLU* gene in the general defense response (Sardesai et al., 2005, Xue et al., 2010, De Zutter et al., 2017). Putting a clip cage on a leaf may inflict such a response.

The accumulation of the *CalS1* and *CalS7* gene transcripts seems not to coincide with impaired phloem uptake as recorded by EPG. The gene expression increased after 6 hours infestation whereas aphids already show difficulty

in phloem feeding before that time. One possible explanation is that callose deposition is regulated at the protein level in the early stage of the defense response. In bean, callose can be induced within 5-10 min after injury through the activation of proteases (Nakashima et al., 2003). We found that aphids tried to start phloem probing after about 1.5 h on resistant as well as susceptible plants (EPG parameter: time to first E1). However, no callose deposition was detected 1.5 h after the start of the aphid infestation (results not shown), which suggests that callose deposition is not involved in the early response of PB2013071 to aphid feeding. Another possible mechanism of phloem vessel occlusion is plugging by phloem proteins (P-proteins), which can block sieve tubes of higher-level plants rapidly (Cronshaw & Sabnis, 1990, Kehr, 2006, Furch et al., 2007, Zhang et al., 2011, Ernst et al., 2012). P-proteins based occlusion is thought to be a faster and earlier response than callose deposition (Furch et al., 2007). It may be speculated that specific P-proteins are involved in the early response to aphids on the resistant accessions, while callose deposition is induced later to prevent aphid feeding in a more stable and long-lasting way.

Conclusion

In conclusion, we identified three *C. baccatum* accessions that are resistant to the green peach aphid and one *C. baccatum* accession that is susceptible. Accession PB2013071 shows the highest aphid resistance, which seems to be phloem based according to the EPG recordings. The resistance is accompanied by callose deposition in the sieve elements, which may be at least partially causal. The up-regulated expression of the *CalS1* and *CalS7* genes in the resistant accession is in line with this observation.

Supplementary Materials

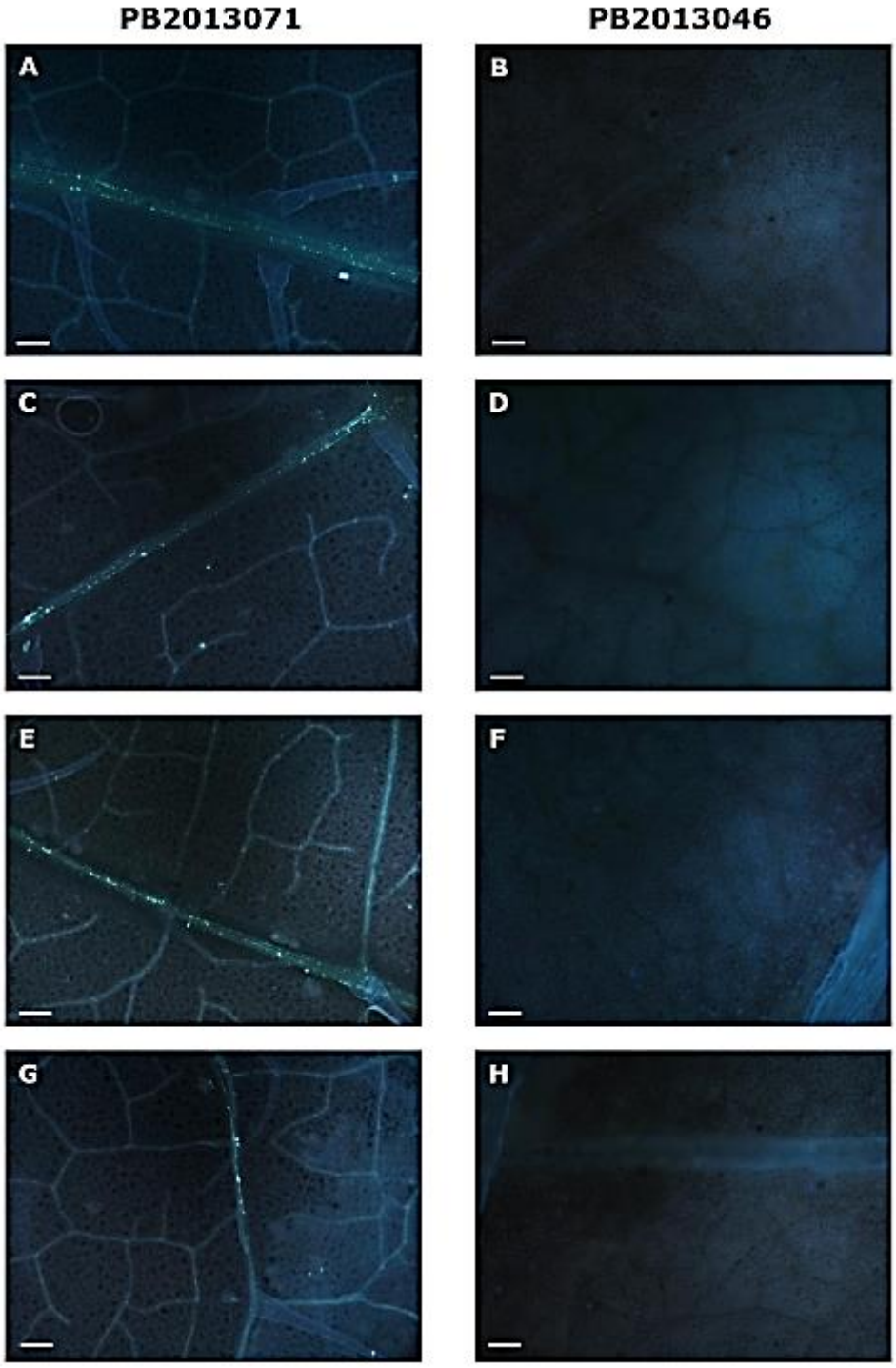

Figure S1. Histochemical staining of callose in 24h GPA-infested leaves. Resistant accession PB2013071 (A, C, E, G); susceptible accession PB2013046 (B, D, F, H). Bars=100µm.

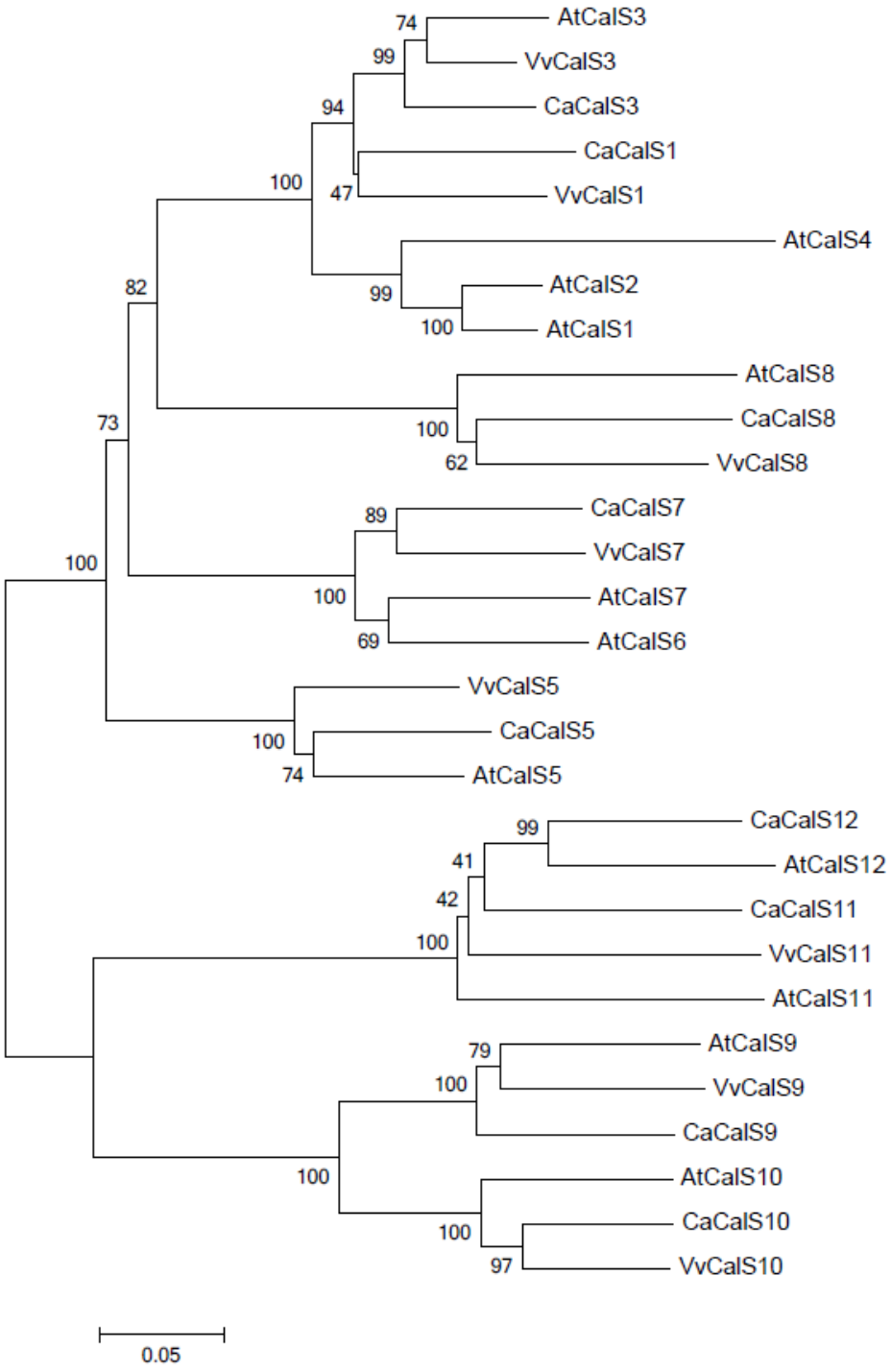

Figure S2. Phylogenetic analysis of pepper (Ca), *Arabidopsis* (At) and grapevine (Vv) CalS proteins, using the MEGA (Tamura et al., 2011) neighbour-joining algorithm.

Table S1. Evaluation of *Capsicum* accessions for resistance against the aphid *Myzus persicae*.

Accession Nr.	Name	Species	Experiment 1[3]		Experiment 2[4]	
			Survival[1]	Nymphs[2]	Survival	Nymphs
CGN23766	Small Bell Chilly	*C. chinense*	0.06 a	0.00 a	0.91 bc	0.17 bc
CGN22829	Miscucho colorado; PI 152225; 1 SCA no.6	*C. chinense*	0.13 ab	0.00 a	0.81 b	0.34 cd
2012022		*C. baccatum*	0.27 abcd	0.01 ab	0.55 a	0.00 a
CGN22855	PI 281428 Selection	*C. chinense*	0.21 abc	0.13 abcde	0.91 bc	0.53 de
2012024		*C. baccatum*	0.29 abcd	0.00 a	0.92 bc	0.05 ab
2012036		*C. chinense*	0.36 bcdef	0.00 a	0.94 c	0.24 cd
CGN19229	No. 1882 selection; PI 281417 selection	*C. chinense*	0.33 bcde	0.00 a	0.92 bc	0.30 cd
CGN22786	SA 344; PI 260434	*C. baccatum*	0.39 bcdefg	0.00 a		
CGN17023	Aceituno; PI 281443; No.1852	*C. chinense*	0.36 bcdef	0.01 ab		
CGN23763	RU 72-77	*C. baccatum*	0.51 cdefgh	0.01 ab		
CGN16972	1 GAA; PI 263258	*C. baccatum*	0.52 cdefghi	0.00 a		
CGN22775	7104	*C. frutescens*	0.54 defghij	0.00 a		
CGN22096	I 5429	*C. baccatum*	0.51 cdefgh	0.00 a		
CGN23278	PI 337524	*C. baccatum*	0.63 efghijk	0.00 a		
CGN17042	No. 1553; PI 238061	*C. baccatum*	0.67 efghijkl	0.01 ab		
CGN22831	Pimento	*C. annuum*	0.64 efghijk	0.01 ab		
CGN16975	AC 1979	*C. annuum*	0.69 fghijkl	0.00 a		
CGN17221	Morron Selection; PI 257284 Selection	*C. chinense*	0.71 ghijkl	0.01 ab		
CGN22185	No.4692; PI 159249; 1SCA	*C. baccatum*	0.72 ghijkl	0.02 ab		
CGN23206	RU 72-51	*C. baccatum*	0.76 hijklm	0.00 a		
CGN22181	Yellow Bouquet	*C. baccatum*	0.73 hijkl	0.01 ab		
CGN17219	No.4661 Selection; PI 159236 Selection	*C. chinense*	0.73 ghijkl	0.01 ab		
CGN23092	Local; Krasnyi; VIR 1829	*C. chinense*	0.76 hijklm	0.04 ab		
CGN22830	Chili Serrano; PI 281367; No. 999	*C. annuum*	0.81 hijklm	0.00 a		
CGN22817	Lombok	*C. frutescens*	0.79 hijklm	0.00 ab		
CGN19199	AC 1448	*C. frutescens*	0.72 ghijkl	0.08 abcd		
2012025		*C. baccatum*	0.81 hijklm	0.03 ab		
CGN19224	MI 1/81; Pili Pili	*C. chinense*	0.78 hijklm	0.05 abc		
2012018		*C. baccatum*	0.81 hijklm	0.00 a		

Table S1 (continued)

Accession Nr.	Name	Species	Experiment 1		Experiment 2	
			Survival	Nymphs	Survival	Nymphs
CGN17009	Hungarian Wax 4202-3	*C. annuum*	0.77 hijklm	0.03 ab		
CGN19194	Cayenne Long Red Narrow	*C. annuum*	0.91 klm	0.00 a	0.92 bc	1.24 f
CGN22131	Local	*C. chinense*	0.82 hijklm	0.13 abcde		
CGN19188	Calcom	*C. annuum*	0.81 hijklm	0.11 abcd		
CGN22790	AC 1249	*C. frutescens*	0.86 klm	0.07 abcd		
CGN23211	SA 218; PI 260459, Malagueta	*C. frutescens*	0.77 hijklm	0.15 bcde		
CGN20503	Bisbas	*C. annuum*	0.89 klm	0.10 abcd		
CGN16994	RU 72-194	*C. chinense*	0.85 jklm	0.18 bcdef		
CGN22862	No.1720; PI 281426; 1GAA	*C. chinense*	0.81 hijklm	0.18 bcdef		
CGN19189	California Wonder 300	*C. annuum*	0.97 m	0.07 abcd		
CGN22173	Sweet Banana	*C. annuum*	0.93 lm	0.10 abcd		
CGN23210	SA 137; PI 257155	*C. frutescens*	0.74 hijkl	0.35 defg		
CGN24363	Vindunger Mutande	*C. frutescens*	0.70 fghijkl	0.36 defg		
CGN22168	RU 72-357	*C. frutescens*	0.83 ijklm	0.34 cdefg		
CGN21554	Ndungu; Pili Pili	*C. frutescens*	0.93 lm	0.11 abcd		
CGN16995	RU 72-241	*C. chinense*	0.90 klm	0.34 defg		
CGN17020	No.965; PI 281353	*C. frutescens*	0.85 jklm	0.57 fg		
CGN22792	SA 252; PI 260478, Aji Chuncho	*C. frutescens*	0.77 hijklm	0.69 g		
CGN22779	C 307	*C. frutescens*	0.88 klm	0.80 g		
CGN19226	Bruinsma Wonder	*C. annuum*	0.87 klm	0.50 efg	0.93 c	0.47 de
2012045		*C. frutescens*	0.93 lm	0.56 fg	0.93 c	0.78 ef

[1] Survival refers to fraction of the aphids that survived on an accession after 7 days.

[2] Nymphs means average number of new nymphs reproduced by every estimated living adult.

[3] Aphids used in Experiment 1 were reared on Chinese cabbage (*B. rapa*) cv. Granaat.

[4] Aphids used in Experiment 2 were reared on *C. annuum* accession CGN19226.

Means followed by the same letter within the same column are not significantly different (LSD- test on transformed scales at $P<0.05$).

Table S2. Evaluation of *C. baccatum* accessions for resistance against the aphid *M. persicae*.

Accession Number	Name	Experiment 3[3]		Experiment 4[4]	
		Survival[1]	Nymphs[2]	Survival	Nymphs
PB2013071		0.51 ab	0.000 a	0.37 a	0.00 a
PB2013062		0.57 abc	0.000 a	0.82 cd	0.02 ab
CGN23260	PI 260567	0.63 abcde	0.000 a	0.75 bc	0.21 c
CGN22834	RU 72-48	0.73 abcdefg	0.000 a	0.98 e	0.16 bc
PB2013074		0.76 abcdefgh	0.000 a		
CGN16972	1 GAA; PI 263258	0.77 abcdefgh	0.000 a		
PB2012022		0.82 bcdefghij	0.000 a	0.50 ab	0.02 ab
PB2012024		0.83 bcdefghij	0.000 a		
CGN22096	I 5429	0.85 cdefghij	0.000 a		
CGN21513	PI 260580	0.89 efghij	0.000 a		
CGN23763	RU 72-77	0.94 ghij	0.000 a		
PB2012018		0.92 fghij	0.006 ab		
CGN22858	RU 72-93	0.59 abcd	0.018 abc	0.86 cde	0.16 bc
CGN21514		0.85 cdefghij	0.018 abc		
CGN17025	No. 1553; PI 281306	0.94 ghij	0.019 abc		
PB2013061		0.52 ab	0.020 abc		
CGN22786	SA 344; PI 260434	0.66 abcdef	0.025 abc		
CGN21582	AC 2129	0.85 cdefghij	0.029 abc		
CGN17042	No. 1553; PI 238061	0.97 hij	0.029 abc		
CGN22872	PM 593	0.90 efghij	0.030 abc		
CGN17174	Aji; VIR 252	0.94 ghij	0.051 abcd		
CGN23566	AC 2060	0.64 abcdef	0.058 abcd		
CGN17241	AC 2200	0.98 ij	0.090 abcd		
CGN21512	PI 260561	0.93 ghij	0.092 abcd		
CGN23278	PI 337524	0.77 abcdefgh	0.098 abcd		
CGN21479	AC 1986	0.88 defghij	0.099 abcd		
CGN22871	PM 325	0.93 ghij	0.101 abcd		
CGN22185	No.4692; PI 159249; 1SCA	0.98 j	0.112 abcd		
CGN23206	RU 72-51	0.89 efghij	0.128 abcd		
CGN22181	Yellow Bouquet	0.97 hij	0.128 abcd		
PB2012025		0.90 efghij	0.179 bcd		
CGN21515		0.49 a	0.229 cd		
CGN19233	Pen 3.4	0.89 efghij	0.265 cd		
CGN19202	Cluster Rod	0.93 ghij	0.269 cd		
CGN16905		0.96 ghij	0.324 de		
CGN19226	Bruinsma Wonder (*C. annuum*)	0.98 hij	0.342 de	0.95 de	2.32 d
CGN16973	SA 361	0.79 abcdefghi	0.370 de		
PB2013046		0.94 ghij	0.890 e	0.98 e	1.91 d

[1] Survival refers to fraction of the aphids that survived on an accession after 7 days in Experiment 3 and after 8 days in Experiment 4.

[2] Nymphs means average number of new nymphs reproduced by every estimated living adult.

[3] Aphids used in Experiment 3 were reared on *C. annuum* accession CGN19226.

[4] Aphids used in Experiment 4 were reared on *C. baccatum* accession PB2013046..

Means followed by the same letter within the same column are not significantly different (LSD- test on transformed scales at $P<0.05$).

Table S3. Callose synthase (*CalS*) genes in *C. annuum*.

Gene[a]	ORF(bp)	Gene ID in CM334[b]	Gene ID in Zunla1[c]
CaCalS1	5856	CA01g26370	Capana01g003121
CaCalS3	5847	CA01g11310	Capana01g001537-1540
CaCalS5	5460	CA12g07860-07870	Capana12g002111
CaCalS7	5730	CA07g15510	Capana07g001971
CaCalS8	5865	CA07g16450	Capana07g002034
CaCalS9	6393	CA01g11290-11300	Capana01g001540
CaCalS10	5265	CA03g25210	Capana03g001312
CaCalS11	5316	CA02g16100	Capana02g001499
CaCalS12	5307	CA07g13560	Capana07g001693

[a] The name of each *C. annuum CalS* (*CaCalS*) gene was assigned on the basis of homology with the *A. thaliana CalS* (*AtCalS*) and *V. vinifera CalS* (*VvCalS*) genes.

[b] Pepper Genome Platform (http://peppergenome.snu.ac.kr/)

[c] Pepper Genome Database (http://peppersequence.genomics.cn/page/species/index.jsp)

Table S4. Primer sequences used in real-time PCR.

Gene Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
CalS1	GCAACCCAAGGGTAGCTTATC	AACTCTGCATTTCACGAGCA
CalS3	CGTGTCGCGTATCTATGTCG	CGTGTCGCGTATCTATGTCG
CalS5	ACGTTTAGAGCGTGACAATG	TTCAAAAAGCACTCCAGCAG
CalS7	ATCCTGCTGCTGGTGAAGAT	ATGTCTTGAATTGACGAACGCC
CalS8	TCGAGTGGCTTATCTTTGTCG	CAGTCCTTTCCTTCCTTTTCC
CalS9	CGAGGACCCTAATGTCTCCA	TCTTGGCTTCTATCAATCGTC
CalS10	TTACGGAGGAGTTGAGAAGGA	GGGTAGCTGAGGGAACTGCT
CalS11	TGGCTTGGACTCTTCTTTGG	AGCGAACTTGCGACTTCTTC
CalS12	CGGTAGATGAAGAACCATACAACA	GCCAGTCAAGCAGGTCATAAT
BGLU	CATTGATATAGCAGGGGGTCA	CAATGTTGGAGCCTCTTAAAGC
Actin	TGAGCAGGAGCTTGAAACTG	CTTGTCCATCAGGCAATTCA

CHAPTER 3

Aphid resistance in *Capsicum* maps to a locus containing LRR-RLK gene analogues

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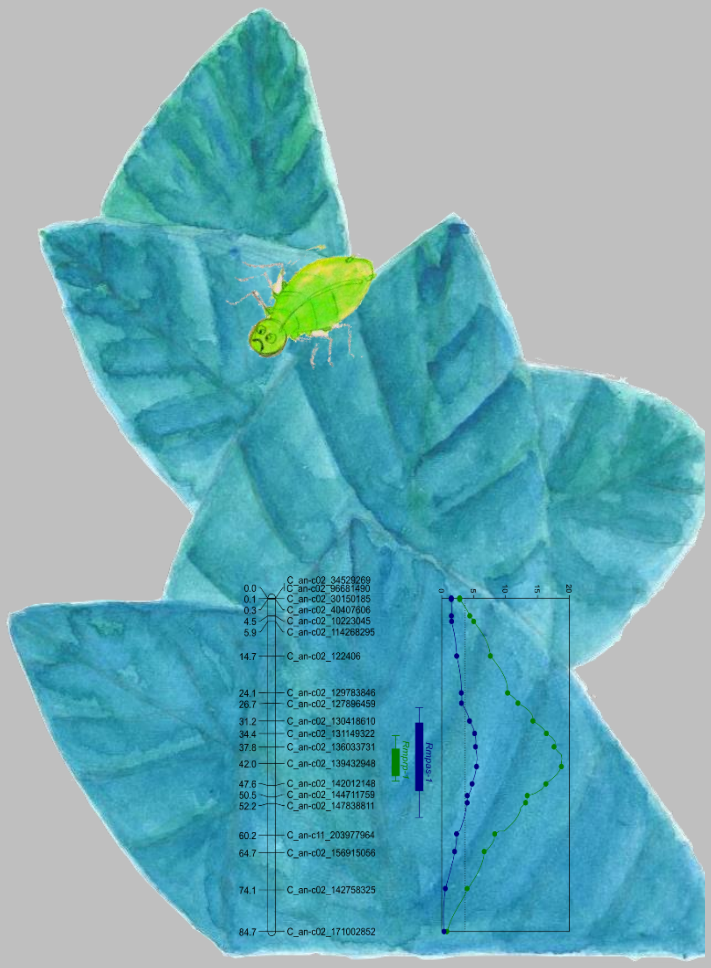


This chapter has been accepted by the journal of Theoretical and Applied Genetics.

Abstract

Myzus persicae is one of the most threatening insect pests that adversely affects pepper (*Capsicum*) cultivation. Resistance to aphids was previously identified in *Capsicum baccatum*. This study aimed at elucidating the genetics of the identified aphid resistance in *C. baccatum*. A QTL analysis was carried out for *M. persicae* resistance in an F_2 population derived from an intraspecific cross between a highly resistant and a susceptible plant. Survival and reproduction were used as resistance parameters. Interval mapping detected two QTLs affecting aphid survival (*Rmpas-1*) and reproduction (*Rmprp-1*) respectively, both localized in the same area and sharing the same top marker on chromosome 2. Use of this marker as co-factor in multiple-QTL mapping analysis revealed a second, minor QTL (*Rmprp-2*) only affecting aphid reproduction on chromosome 4. Further fine mapping confirmed the effects of *Rmpas-1* and *Rmprp-1* and narrowed the major QTL *Rmprp-1* down to a genomic region predicted to encode four analogues of the LRR-RLK subfamily of resistance genes. This work provides not only initial information for breeding aphid resistant pepper varieties, but also forms the basis for future molecular analysis of gene(s) involved in aphid resistance.

Introduction

Pepper (*Capsicum* spp.) is one of the economically most important and widely cultivated vegetable crops. However, its cultivation is constrained by aphids (Frantz et al., 2004, Herman et al., 2008). Aphids can damage pepper plants in many ways, leading to chlorosis, defoliation, wilting, and flower abortion. Aphids also can cause a reduction in the photosynthetic capacity and fruit quality through the stimulation of moulds that grow on the honeydew. Aphids damage pepper plants most seriously in an indirect way by vectoring a large number of viruses (Black et al., 1991, Kenyon et al., 2014, Kennedy et al., 1962).

Among the different aphid species feeding on pepper, the green peach aphid (GPA), *Myzus persicae*, is one of the most threatening (Fereres et al., 1993). The control of GPA population development is currently mainly carried out using insecticides. However, given the fact that more and more aphid species have developed resistance against the pesticides (Devonshire et al., 1998, Foster et al., 2000, Bass et al., 2014) and that insecticides negatively affect the environment, varieties resistant to the GPA may be a more promising alternative. Resistance to aphids not only reduces the size of the aphid population on pepper (Frantz et al., 2004, Sun et al., 2018), but may also decrease the percentage of plants infected with viruses (Radcliffe & Ragsdale, 2002). In several plant species quantitative trait loci (QTLs) for resistance against aphids have been detected. Aphids for which resistance QTLs have been found include the soybean aphid *Aphis glycines* (Kim et al., 2010b), the pea aphid *Acyrthosiphon pisum* (Stewart et al., 2009), the cotton aphid *Aphis gossypii* (Boissot et al., 2010, Liang et al., 2016), the potato aphid *Macrosiphum euphorbiae* (Rossi et al., 1998) and the Russian wheat aphid *Diuraphis noxia* (Aykut Tonk et al., 2016). However, only a few QTLs for resistance to GPA were found, most of which were in peach (Sauge et al., 2012, Sauge et al., 2004, Lambert & Pascal, 2011, Pascal et al., 2017) and *Arabidopsis* (Pfalz et al., 2009, Thoen et al., 2017, Kloth et al., 2017). No aphid resistance QTL has been described in pepper so far.

Until now two genes affecting aphid performance have been cloned based on identified QTLs (Pauquet et al., 2004, Vos et al., 1998). Both of them, the tomato *Mi-1.2* gene and the melon *Vat* gene, conferring resistance to *M. euphorbiae* and *A. gossypii* respectively, are of the NBS-LRR type, sharing motifs with many disease resistance genes (Belkhadir et al., 2004, Broekgaarden et al., 2011). This type of genes code for proteins that can recognize an effector from an external pathogen or pest and activate a specific immune response in the host plant according to the gene-for-gene principle, which is called effector-triggered immunity (ETI) (Van Der Biezen & Jones, 1998, Jones & Dangl, 2006). Next to ETI, the innate immune system of plants consists of another layer, the microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) (Silva Couto & Zipfel, 2016, Jones & Dangl, 2006) or the herbivore-associated molecular pattern (HAMP)-triggered immunity in case of herbivores (Bonaventure et al., 2011). The MAMPs and HAMPs can generally be recognized by pattern recognition receptors (PRRs) in plants, which often belong to the family of receptor-like kinases (RLKs) (Jones & Dangl, 2006, Zipfel, 2014). Several resistance genes from the RLK family have been identified, which provide protection against pathogens (Gómez-Gómez & Boller, 2000, Krol et al., 2010, Fradin et al., 2009), nematodes (Mendy et al., 2017) and insects (Liu et al., 2015). However, no PRR gene has been found that is involved in plant resistance against aphids. Only one RLK gene was reported to act as a co-receptor of other unknown PRRs and play a role in aphid resistance in *Arabidopsis* (Prince et al., 2014). Fine mapping resistance QTLs may help to identify more genes involved in aphid resistance and provide clues to whether they are NBS-LRRs or RLKs type of genes.

In a previous paper we have described the screening and characterisation of several sources of GPA resistance in *Capsicum* species (Sun et al., 2018). Three accessions of *Capsicum baccatum* were shown to be resistant or intermediately resistant to GPA by negatively affecting aphid survival and reproduction. Among them, accession PB2013071 showed the highest level of resistance, which severely impaired phloem uptake by the aphid and induced callose deposition in the sieve elements during aphid feeding. Our current study aimed at elucidating the genetics of GPA resistance in accession PB2013071 through a QTL mapping approach followed by fine mapping. This work will enable us to discover the gene underlying GPA resistance, which is useful information for breeding aphid resistant pepper varieties.

Materials and methods

Plant materials, growing conditions and aphid population

The plant materials were obtained from the collection of Plant Breeding at Wageningen University & Research, Wageningen, NL. Aphid resistant accession PB2013071 and susceptible accession PB2013046 of *C. baccatum* were described previously (Sun et al., 2018). An F_2 mapping population of 192 plants was obtained after selfing a single F_1 plant obtained from the cross between PB2013046 as female parent and PB2013071 as male parent. First-generation inbred lines of the resistant (PB2014009) and susceptible (PB2014005) parent were obtained by self-pollination. Eight F_3 lines, derived from four F_2 plants that were homozygous for the resistance allele and four F_2 plants that were homozygous for the susceptibility allele in the 2-LOD confidence interval of the major QTL were obtained by selfing and used for QTL validation. For fine mapping of the resistance gene we used F_3 line PB2016027 which was obtained after selfing an F_2 plant that was heterozygous for the 2-LOD interval of the major QTL. In the F_3 line we selected 230 recombinants for fine mapping. Five plants from F_3 line PB2016027 that were

homozygous for the resistance allele and 5 plants that were homozygous for the susceptibility allele in the 2-LOD interval of the major QTL were also kept and used for evaluation and validation of the QTL.

For all experiments, seedlings were transplanted into 17 cm pots with potting compost two weeks after sowing and grown in a standard greenhouse at 19-21 °C, 60–70% RH and an L16:D8 photoperiod at Unifarm, Wageningen University & Research, Wageningen, NL. During growth and testing, plants were watered every other day without any pest control and fertilized with 2.5 mg l-1 Kristalon Blauw (pH=5.5, N-P-K, 4-1-7; Hydro Agri, Rotterdam, Netherlands) every two weeks.

The GPA (*M. persicae*) population used was the same as the one we used previously (Sun et al., 2018). It was reared on *C. baccatum* accession PB2013046 and was maintained under the same conditions as the pepper plants.

Resistance evaluation by clip cage tests

Resistance evaluations were carried out when the plants were seven weeks old in the greenhouse of Unifarm, Wageningen University & Research, Wageningen, NL. Every plant under evaluation received three clip cages containing five 1-day-old nymphs. After twelve days the living and dead aphids as well as the new nymphs produced in each clip cage were counted.

For the phenotyping of the F_2 population, in October 2015 192 F_2 plants were randomized and equally divided over two greenhouse compartments next to each other with the same climate conditions. For the major QTL validation, five plants of eight F_3 lines either homozygous for the resistance or susceptibility allele over the 2-LOD QTL interval were randomized in one compartment in August 2016. For fine mapping of the major resistance gene, in July 2017 230 recombinants from F_3 line PB2016027 together with ten homozygous plants (five with the resistance allele and five with the susceptibility allele over the 2-LOD QTL interval) from the same line were randomized and equally divided over the two compartments with the same climate conditions. Five plants of the two first-generation parental inbred lines, PB2014009 and PB2014005, were included as reference in every evaluation and randomized together with the other materials.

For statistical analysis, the observations from the three clip cages on a plant were always combined. Clip cages with less than four aphids (dead or alive) were discarded, as were data on plants with less than two remaining clip cages. Two resistance parameters, survival of the original nymphs and the number of next generation nymphs produced per aphid, were analysed. Survival data was obtained by dividing the number of living aphids by the total number of aphids. The number of next generation nymphs was divided by the average number of living aphids present, which was calculated as (2*living aphids + dead aphids)/2. Data was transformed as follows: survival as arcsin(sqrt(x)) and next generation nymphs as sqrt(x). Transformed data were used in statistical analysis and QTL mapping. In the two experiments where the plants were divided over two compartments no significant compartment effect was detected ($P > 0.05$ in both cases) and consequently the compartment effect was ignored in further statistical analyses and QTL mapping. Data from the QTL validation experiment with either the resistance or susceptibility allele were analysed using ANOVA combined with a LSD test ($P<0.05$). For comparing the parental inbred lines in the QTL mapping and fine mapping experiments a T-test was used. The Pearson correlation was used to evaluate the correlation between aphid survival and reproduction.

DNA extraction, molecular markers (SNPs) and genetic linkage map construction

Samples of newly expanded leaves of plants were collected and stored at -80 ºC. until DNA extraction. Collected samples were ground using a TissueLyser II (Qiagen, USA). After being ground, genomic DNA was extracted using the CTAB method (Fulton et al., 1995). The DNA quantity and quality were determined by NanoDrop 1000 V.3.7 (Thermo, USA).

DNA from the F_1 plant used for making the F_2 population was sequenced by one lane Illumina HiSeq2500 (60 Gb, 2 x 125 nt Paired End). Reads were mapped to the reference genome *C. annuum* 'CM334' v.1.55 (Kim et al., 2014b) (http://peppergenome.snu.ac.kr/) using BWAmem (Li, 2013). Single nucleotide polymorphisms (SNPs) were identified using Freebayes (Garrison & Marth, 2012). A total of 167 evenly spaced SNP markers (Table S1) were selected and named based on their physical position on the *C. annuum* genome sequence 'CM334' v.1.55 (Kim et al., 2014b).

DNA solutions of F_2 plants were prepared for genotyping through the KASP™ technology (KBioscience, UK), which was carried out by the dr. van Haeringen laboratorium B.V., Wageningen, NL. A linkage map was constructed using the JoinMap 4.1 software (Van Ooijen, 2006). Map distances were calculated using the Kosambi mapping function.

QTL mapping

Potential resistance QTLs associated with aphid survival and reproduction were identified using the MapQTL 6.0 software (Van Ooijen, 2011). Interval mapping analysis was first performed to look for regions with potential QTL effects. Multiple-QTL mapping (MQM) was applied to find additional QTLs using the top marker in the major QTL region as co-factor. A permutation test was used to determine the LOD threshold for aphid survival and reproduction corresponding to a genome-wide confidence level of 0.05. The QTL graphs were drawn using MapChart 2.3, including a 2-LOD confidence interval (Voorrips, 2002).

Fine mapping

Leaf samples of 1118 plants from one F_3 line (2016027) were genotyped using the KASP™ technology (KBioscience, UK) with 21 SNP markers in the 2-LOD confidence interval of the major QTL. Seven additional SNP markers were designed and used for further genotyping through the LightScanner System (Idaho Technology, USA) (Wittwer et al., 2003). Primers were designed using primer3 (Untergasser et al., 2007). The PCR products that were used for the LightScanner were amplified according to the protocol of the manufacturer. All used markers are listed in Table S1.

Results

Aphid performance on the F_2 population and parents

We monitored GPA performance on the F_2 population and the first-generation parental inbred lines (resistant line PB2014009 and susceptible line PB2014005) . The two inbred lines showed significant differences in aphid performance for the two parameters used: survival of the original nymphs (42 ± 13% vs 97 ± 4%; T-test, $P<0.001$),

and number of next generation nymphs produced by per aphid (2.2 ± 0.82 vs 13.8 ± 1.68; T-test, P<0.001), on resistant and susceptible line, respectively. The F_2 population showed a large variation for GPA performance based on the two parameter used: 20%-100% survival of the aphids placed on the plant and 0.2-13.3 new nymphs produced per aphid (Figure 1). The correlation between the two used parameters was R=0.72 (Pearson correlation, P<0.001).The average aphid survival (84%) and the average number of new nymphs per aphid (5.70) in the F_2 population were somewhat skewed toward the susceptible and resistant inbred line, respectively.

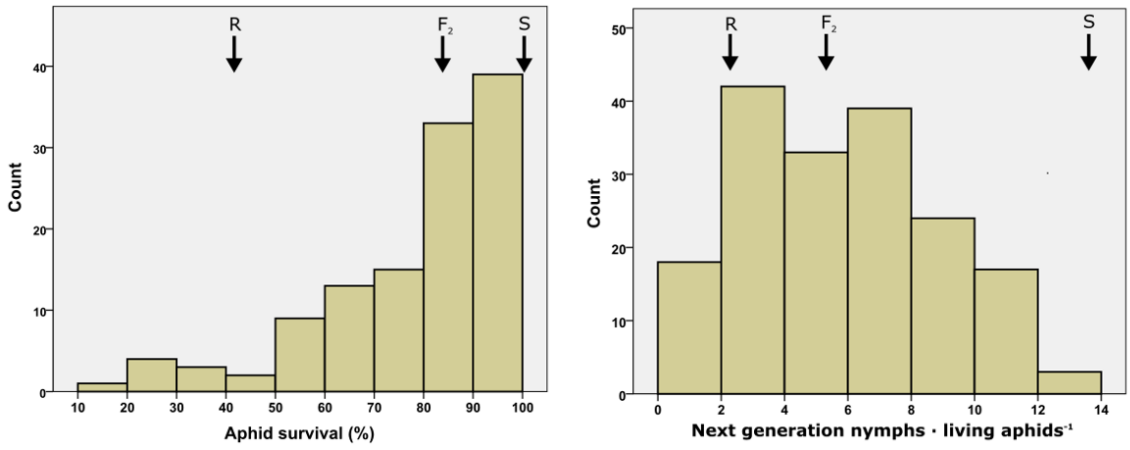


Figure 1. Aphid resistance in the F_2 population. Frequency distribution of survival of original aphids (left) and the aphid reproduction (right) in the 176 F_2 plants for which genotyping and phenotyping data were obtained. The population was derived from a cross between an aphid resistant and susceptible *C. baccatum* plant. Black arrows indicate the approximate means of the resistant (R) and susceptible (S) parental inbred line and the F_2 population.

Linkage map

A genetic linkage map was constructed with 176 F_2 individuals (16 out of 192 plants were discarded because of incomplete data) and 167 SNP markers (Figure S1, Table S2). The constructed 12 linkage groups, which correspond to the number of pepper chromosomes, varied in length from 85 cM (LG2) to 139 cM (LG11), with a total length of 1319 cM and an average distance of 8 cM between markers. The largest gap between two markers was 33 cM and located on LG8. The assignment of linkage groups was according to the chromosomal location of the SNP markers that were most frequently found in a group, and also according to the BLAST result of SNP markers to the recently released genome of *C. baccatum* 'PBC81' (Kim et al., 2017). Linkage group 3 of *C. baccatum* contained segments of chromosome 3 and 9 of *C. annuum*, LG5 of chromosomes 3 and 5, and LG9 contained segments of chromosome 3, 5, and 9 of *C. annuum*. The other linkage groups seemed to be homologous to the chromosomes of *C. annuum* (Figure S1).

QTL mapping

Interval mapping of aphid survival and reproduction resulted in the identification of QTLs for both of them, which we designated *Rmpas-1* and *Rmprp-1*, respectively. Both QTLs were located on chromosome 2 (LG2, Figure 2), with marker C_an-c02_139432948 as top marker. The LOD scores at this marker were 5.5 and 18.8, with an explained phenotypic variance of 14.7% and 41.9% for survival and reproduction, respectively (Table 1). The 2-

LOD intervals span 25.5 cM (between markers C_an-c02_127896459 and C_an-c02_147838811) for survival and 13.2 cM (between markers C_an-c02_131149322 and C_an-c02_142012148) for reproduction. One additional, minor QTL for reproduction was detected on chromosome 4 (LG4, Figure S2) using Multiple QTL Model (MQM) mapping with marker C_an-c02_139432948 as co-factor. This minor QTL (*Rmprp-2*) explained 6.4% of phenotypic variation (Table 1). No epistasis was found between the major QTL *Rmprp-1* and the minor QTL *Rmprp-2*.

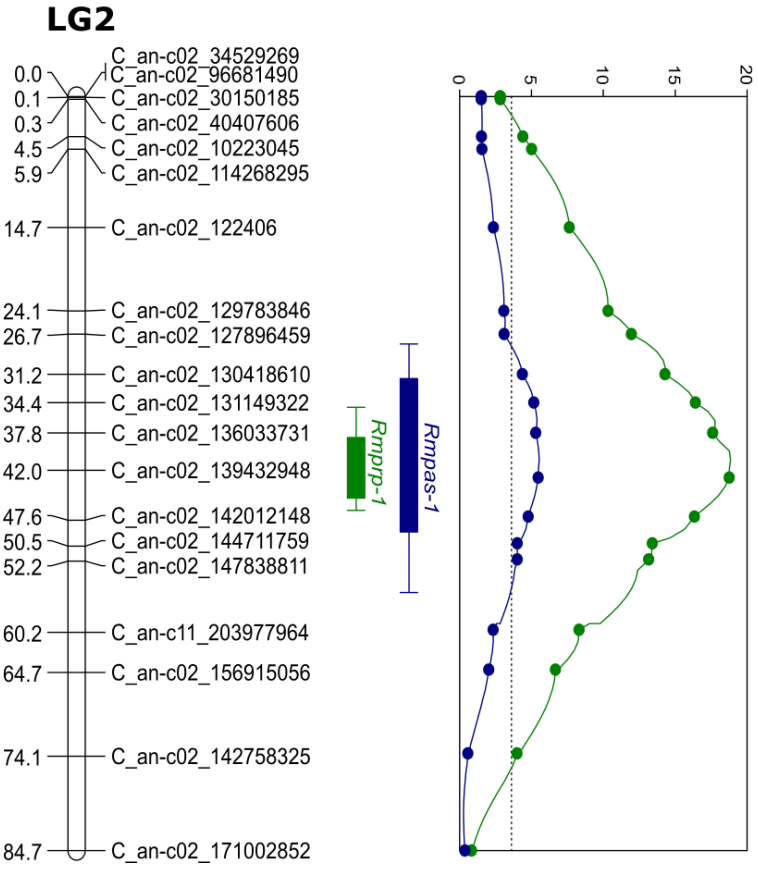


Figure 2. Linkage map, LOD profiles and 1-LOD and 2-LOD support intervals for aphid resistance QTLs on linkage group 2. Blue and green lines represent the profiles for survival of original aphids and aphid reproduction, respectively. The dotted line at LOD 3.5 represents the LOD threshold. LG2, linkage group 2, is assigned to chromosome 2 of the pepper genome. *Rmpas-1*and *Rmprp-1* represent major resistance QTL for aphid survival and reproduction, respectively. The y-axis of the LOD profile shows the LOD score. The number at the end of the marker name indicates its physical positions on the CM334 v.1.55 genome (Kim et al. 2014).

Table 1: QTL effect of resistance-related traits after the infestation with *M.* persicae determined in the F2 population.

Traits	QTL name	Marker at QTL peak	Chromosome	Position (cM)[a]	LOD	Additive effect [b]	Dominance effect[c]	% Explained variance
Aphid survival [d]	*Rmpas-1*	C_an-c02_139432948	2	42.0	5.47	0.16	0.32	14.7
Aphid reproduction^{-1} [e]	*Rmprp-1*	C_an-c02_139432948	2	42.0	18.76	0.66	-0.09	41.9
	Rmprp-2	C_an-04_30341348	4	46.3	4.06	0.26	0.25	6.4

[a] Genetic position of the QTL in the linkage group.

[b] Positive values indicate that alleles from susceptible accession result in higher phenotypic values than those from resistant accession

[c] positive values indicate that the heterozygote condition results in higher phenotypic values than the midparent value

[d] Based on arcsin(sqrt(x)) transformed data

[e] Based on sqrt(x) transformed data

Confirmation of the resistance QTLs on chromosome 2 in F_3 lines

The effect of the major QTL on both aphid survival and reproduction was validated in a set of eight F_3 lines, originating from four F_2 plants homozygous for the resistance allele (lines 2016037, 2016060, 2016120 and 2016119) and four plants homozygous for the susceptibility allele (lines 2016023, 2016029, 2016124 and 2016178) in the 2-LOD interval around the top marker on chromosome 2 (Figure 2). Aphids feeding on all lines with the resistance allele in the QTL region produced significantly fewer new nymphs than aphids feeding on lines without the resistance allele on chromosome 2 (Figure 3). For aphid survival, the difference between lines with the resistance allele and lines with the susceptibility allele was not as clear as the difference in aphid reproduction. There was no significant difference in aphid survival on line PB2016199 which has the resistance allele in the QTL region and the lines PB2016023 and PB2016029 which have the susceptibility allele.

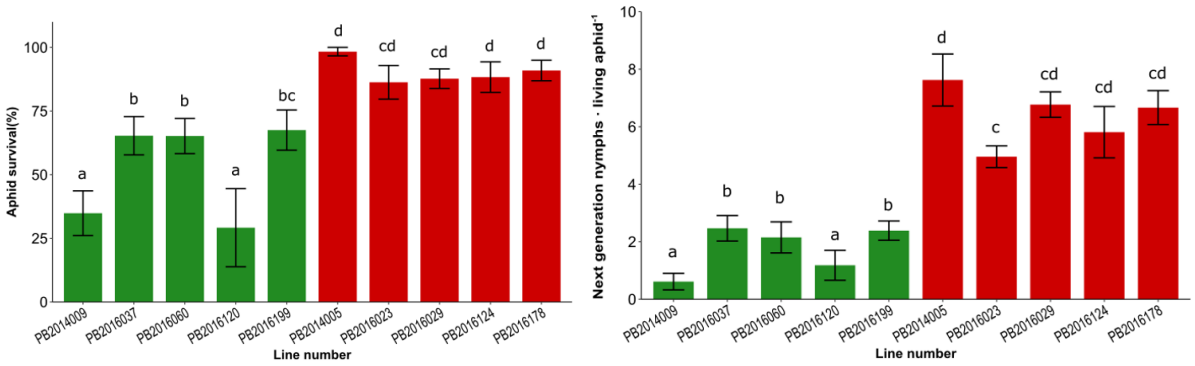


Figure 3. Performance of *M. persicae* on F3 lines and two parental inbreds. Four F3 lines (PB2016037, PB2016060, PB2016120 and PB2016199) originating from four F2 plants homozygous for the resistance allele and four F3 lines (PB2016023, PB2016029, PB2016124 and PB2016178) homozygous for the susceptibility allele in the 2-LOD interval of the major QTL *Rmpas-1* and *Rmprp-1* on chromosome 2. The inbred lines PB2014009 and PB2014005 were obtained by self-pollination of the resistant and susceptible parent of the F2 population, respectively. Each bar represents the mean values ± standard error. Green and red bars represent plants with genotypic background homozygous for resistance and susceptibility allele, respectively. Same letters indicate that values are not significantly different (LSD- test on transformed scales at P<0.05).

Fine mapping of QTL *Rmprp-1*

To fine map the major QTL *Rmprp-1* affecting aphid reproduction, we genotyped 1118 plants from F_3 line PB2016027 with marker C_an-c02_131149322 and marker C_an-c02_142012148 flanking the 2-LOD interval, together with 19 extra markers (Table S1) to identify recombinants. Five plants that were homozygous for the resistance or susceptibility allele of *Rmprp-1* and five plants of both parental inbred lines were phenotyped together with the 230 recombinants. The two sets of homozygous F_3 plants showed a significantly different reproduction: 3.2 ± 0.9 vs 7.8 ± 0.6 new nymphs per aphid (T-test, P<0.001). There was no significant difference in reproduction between homozygous susceptible plants from F_3 line PB2016027 and plants from inbred line PB2014005 (7.8 ± 0.6 vs 8.6 ± 1.4 (T-test, P=0.254)). Plants from inbred line PB2014009 were more resistant than homozygous resistant plants from F_3 line PB2016027 (0.9 ± 0.9 vs 3.2 ± 0.9 (T-test, P=0.003)).

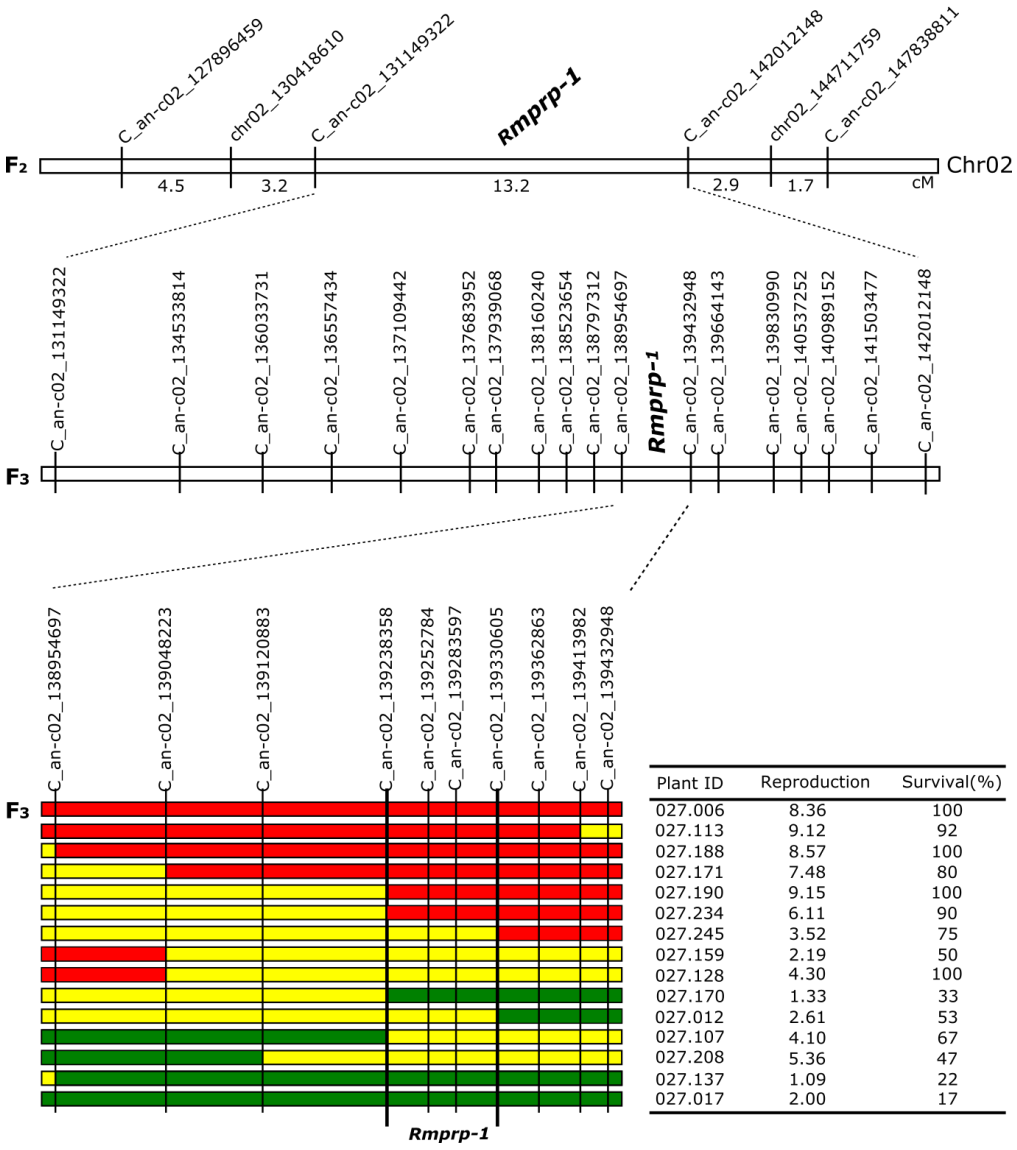


Figure 4. Fine mapping of the major resistance QTL *Rmprp-1*. The genotype in the area between marker 138954697 and marker Chr02_139432948 of F_3 plants is shown in colour codes (red = homozygous susceptible allele, yellow = heterozygous, and green = homozygous resistant allele). The number at the end of the marker name indicates its physical positions on the CM334 v.1.55 genome (Kim et al. 2014). The three columns behind the coloured bars indicate ID number of the F_3 plant, average number of next generation nymphs produced per aphid and survival of the original aphids (%).

Among 1118 plants from F_3 line PB2016027, 230 plants had a recombination between the 2-LOD flanking markers. The estimated distance between C_an-c02_131149322 and C_an-c02_142012148 was 11.9 cM (in the F_2 population: 13.2 cM) and the relative order of the markers and genetic distances between them was consistent with their position on the physical map of chromosome 2 of *C. annuum* 'CM334', and also with their position on chromosome 2 of the recently released *C. baccatum* 'PBC81' genome sequence (Kim et al., 2017) based on BLAST results. After phenotyping all the 230 recombinants, aphid resistance QTL *Rmprp-1* was mapped to an area between marker C_an-c02_138954697 and marker C_an-c02_139432948 (Figure 4). As there were still 15 recombinants in this area, seven additional SNPs markers were developed to genotype these 15 recombinants. This resulted in a further fine mapping of *Rmprp-1* to a 92.3 kb area between marker C_an-c02_139238358 and marker C_an-c02_139330605 (Figure 4). The physical distance between these two markers is 92.3 kb in the genome of *C.*

annuum (Kim et al., 2014b) and 96.2 kb in the genome of *C. baccatum* 'PBC81'. In the genome of 'PBC81' (www.ncbi.nlm.nih.gov/nuccore/CM008444.1?report=graph), six genes are identified in the target region, and four of them are annotated as 'probable LRR receptor-like serine/threonine-protein kinase' (Table 2).

Table 2. Annotated genes in the area of major QTL *Rmprp-1* in pepper genome 'PBC81'.

Gene ID	Location (bp) [a]	CDS length (bp) [b]	Functional annotation
CQW23_04318	129922179-129941715	3036	LRR receptor-like serine/threonine-protein kinase
CQW23_04319	129951251-129954463	810	unknown
CQW23_04320	129971136-129975767	552	LRR receptor-like serine/threonine-protein kinase
CQW23_04321	129981447-129990787	2076	LRR receptor-like serine/threonine-protein kinase
CQW23_04322	129991551-130002579	3003	LRR receptor-like serine/threonine-protein kinase
CQW23_04323	130011293-130014738	663	unknown

[a] Physical position of the gene on chromosome 2 based on the assembled genome from NCBI.
[b] CDS indicates coding sequence.

Discussion

Aphid resistance QTLs

A SNP-based linkage map of *C. baccatum* was constructed for QTL mapping of aphid resistance. The map contains 12 linkage groups, which is identical to the number of pepper chromosomes, and covers a total length of 1319 cM, which is similar to some other published maps of either *C. annuum* or *C. baccatum* (Eun et al., 2016, Lee et al., 2016, Mahasuk et al., 2016). Comparing our linkage map to the reference physical genome of *C. annuum* 'CM334' (Kim et al., 2014b) pointed at translocations between chromosomes 3 and 5, and chromosomes 3 and 9, which is consistent with previous observations (Lee et al., 2016, Mahasuk et al., 2016). However, we did not find the translocation between chromosome 1 and 8, which was previously detected (Lee et al., 2016). The reason for this might be the small number of markers on these two chromosomes in our study. Although the markers used were evenly distributed based on their physical position on the reference genome, there were still a number of gaps in the genetic map, some of which were around 30 cM. It is possible that minor QTLs located in these gaps have not been detected.

This is the first time that an aphid resistance QTL has been mapped in pepper. Two parameters were used to quantify resistance: survival of original aphids put on the plant and number of new nymphs produced by each of these aphids. The two parameters were significantly correlated (Pearson correlation, $R=0.72$, $P<0.001$). Therefore, it is not surprising that the major QTLs, *Rmpas-1* and *Rmprp-1*, share the same region on chromosome 2.

The major QTL *Rmprp-1* explained about 42% of the variance for the production of next generation nymphs. In addition, a minor QTL *Rmprp-2* was detected on chromosome 4, which explains approx. 6% of the variance. The QTL *Rmprp-1* has been validated using F 3 lines as reported here, and also using F_4 and F_5 lines (data not shown). However, the minor QTL *Rmprp-2* still needs validation. As. As the dominance effect of the major QTL *Rmprp-*

1 is negative (-0.09; Table 1) and much smaller than its additive effect (0.66), the QTL for aphid reproduction can be regarded as partially dominant for resistance. The QTL for aphid survival (*Rmpas-1*) was also located on chromosome 2 and explains about 15% of the variance in the F_2; it was also validated using F_3 lines. No other QTLs for aphid survival were detected. The unexplained F_2 variance might be due to environmental variation and/or to several undetected small effects QTLs segregating in this F_2 population. Segregation of undetected small effects QTLs may cause some F_3 plants to deviate in their phenotype from the expected values based on the major QTL. This is for instance supported by the observation that a similar GPA survival was found with one F_3 line that was homozygous resistant (line PB2016199) and two F_3 lines which were homozygous susceptible (line PB2016023 and PB2016029) for the QTL on chromosome 2. Although the major QTL explained only 15% of the F_2 variance for aphid survival, in the QTL validation experiment the difference between the F_3 lines with the resistance and the susceptibility alleles was about 50% of the difference between the parental inbred lines. This suggests that the low percentage of explained F_2 variance for aphid survival may be due to environmental variation. Environmental variation may affect aphid survival more than reproduction. One reason could be that adaptation of the nymphs to their new environment (clip cage, new genotype) may affect survival, while reproduction usually takes place about 8 days after start of the clip cage test, when aphids are already adapted to their new 'home'. A second reason could be that handling of the small nymphs may cause varying amounts of damage/stress leading to increased aphid mortality. As the reproduction is calculated based on the average number of living aphids the effects of handling on mortality are partially compensated leading to a lower environmental variation and a higher fraction of explained variance. This QTL, with a large effect on reproduction and a smaller effect on adult survival is the first QTL described in pepper which is related with antibiosis resistance. Antibiosis based resistance is helpful in reducing the build-up of an aphid population (Züst and Agrawal 2016). Preventing aphid population build-up may also interfere with the spread of some viruses (Radcliffe and Ragsdale 2002).

As the detected QTLs are located on chromosomes 2 and 4, where no translocations have been found between *C. baccatum* and *C. annuum*, it looks feasible to transfer the resistance QTLs from *C. baccatum* to commercial *C. annuum* cultivars by hybridization. However, because of the post-fertilization barriers between *C. baccatum* and *C. annuum* (Eshbaugh, 1970), the necessary interspecific crosses are likely to need some effort (Yoon et al., 2005).

Resistance QTL *Rmprp-1* is mapped to a cluster of receptor-like kinase genes

The major resistance QTL *Rmprp-1* was fine mapped to a 96 kb region based on the recently released *C. baccatum* "PBC81" genome located between markers C_an-c02_139238358 and C_an-c02_139330605 on chromosome 2. During fine mapping of QTL *Rmprp-1*, the 230 plants from F_3 line 2016027 were not only phenotyped for reproduction but also for survival. The two traits showed a similar correlation as in the F_2 ($R=0.73$, Pearson correlation $P<0.001$). It seems likely that QTL *Rmpas-1* and *Rmprp-1* are based on the same causal gene, although it cannot be excluded that they are due to two different but very closely linked genes. The current annotation of the 96 kb region predicts the presence of six putative genes. Two genes are annotated as "unknown", the other four as receptor-like kinase genes with a leucine-rich repeat domain (LRR-RLKs), suggesting that the gene underlying the resistance QTL for aphid reproduction (and probably aphid survival) may belong to the LRR-RLK family.

The LRR-RLK family is the major group of plant receptor like kinases (Shiu & Bleecker, 2001). This type of genes encodes a protein that contains an receptor domain for signal perception and a single-pass transmembrane

domain for protein anchoring, together with a cytoplasmic serine/threonine protein kinase domain for signal transduction (Shiu & Bleecker, 2001). One of the most important functions of identified plant LRR-RLKs is responding to environmental stress and subsequent induction of plant defences (Sakamoto et al., 2012, Shiu & Bleecker, 2001). These LRR-RLKs can recognize microbe-associated molecular patterns (MAMPs) and are required for MAMP-triggered immunity (Silva Couto & Zipfel, 2016). Among the LRR-RLKs in the Solanaceous crops, the LRR-RLK FLS2 is found to perceive MAMP flg22 from bacteria and activate an immunity response in tomato (Robatzek et al., 2007). Two paralogs SlSERK3A and SlSERK3B in tomato have distinct but also overlapping functions in bacterial and nematode innate immunity (Peng & Kaloshian, 2014). To our best knowledge, no solid evidence is available at present that any LRR-RLK gene recognizes herbivore-associated molecular patterns (HAMPs) from aphids and contributes to aphid resistance, although a cucumber LRR-RLK was found to be the most likely candidate gene in the defence response to *Aphis gossypii* (Liang et al., 2016).

As four annotated LRR-RLK genes are located in the 96 kb area on chromosome 2 and sequence similarity among the genes is high, we can consider them as a gene cluster (Graham, 1995). The LRR-RLK genes often occur in clusters consisting of several homologous genes (Shiu & Bleecker, 2001, Wei et al., 2015, Zhou et al., 2016). Clustering of LRR-RLK genes, which is probably caused by gene duplications, was suggested to be a consequence of adaptation to fast-evolving biotic stresses (Lehti-Shiu et al., 2009). Multiple genes in a cluster may allow fast selection for the detection of diverse biotic attackers. However, the high similarity among these genes and their close proximity make it difficult to identify the one that is conferring the resistance to GPA. Further efforts are needed to identify and validate the gene conferring aphid resistance and to elucidate the resistance mechanism.

Conclusion

In this study we have mapped for the first time QTLs conferring resistance to GPA in pepper. The QTL region was narrowed down to a gene cluster with four analogues of the LRR-RLK subfamily. This work will significantly speed up the breeding of aphid resistant pepper varieties.

Supplementary Materials

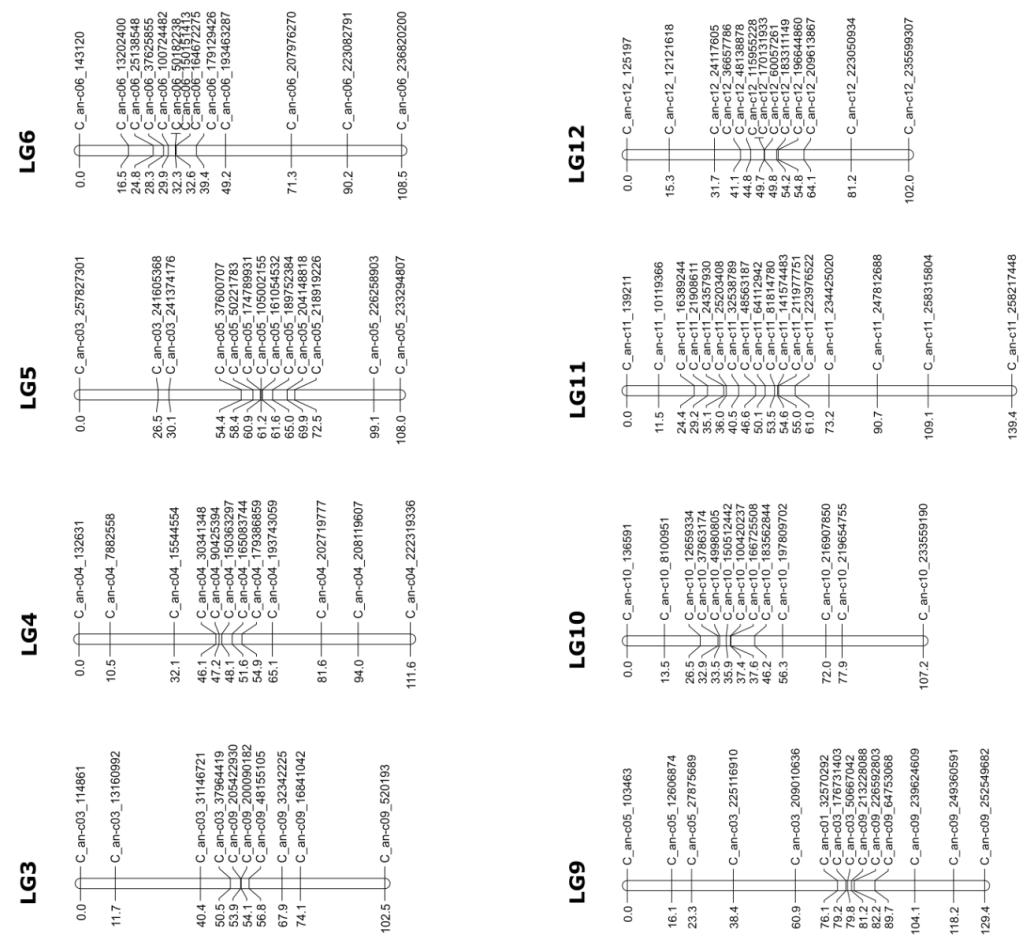


Figure S1. Genetic linkage map of *Capsicum baccatum*. The map is based on 167 SNP markers segregating in an F_2 population of 192 plants, which was derived from a cross between an aphid resistant and susceptible *C. baccatum* plant. The 12 linkage groups LG1 – LG12 correspond to chromosomes 1 – 12 of *C. baccatum* (Kim et al, 2017).

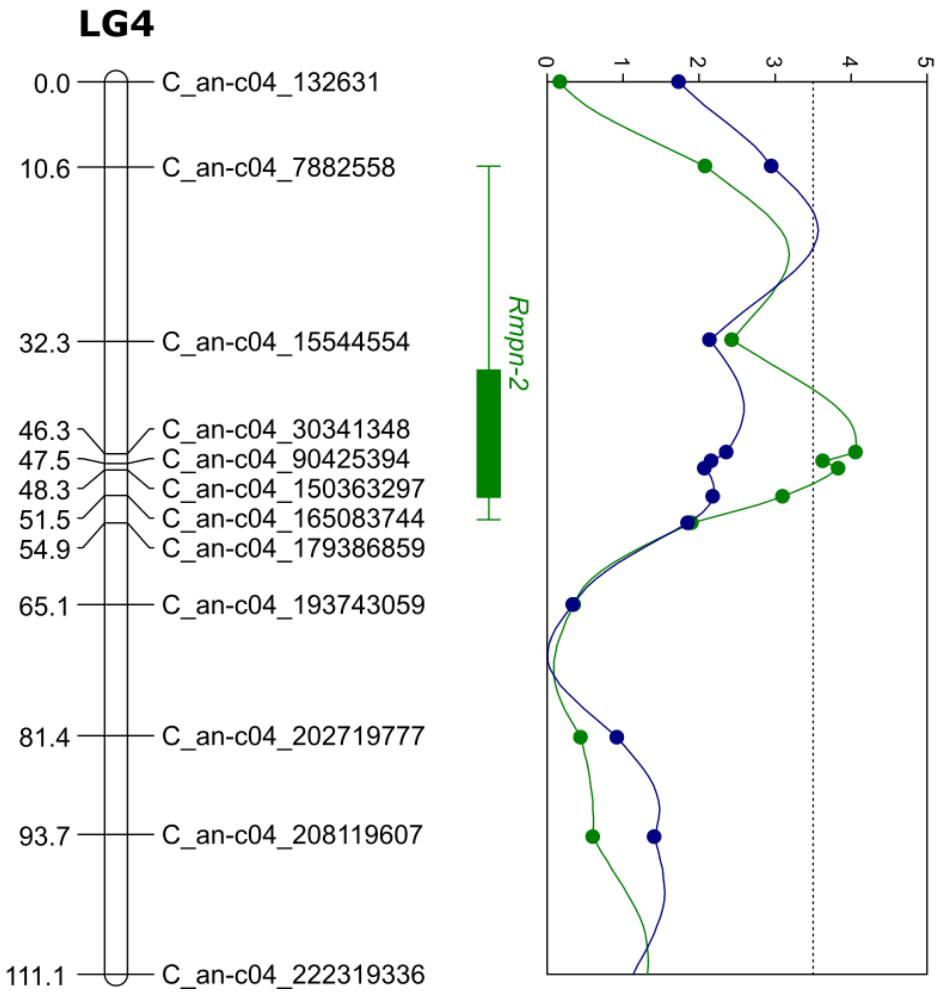
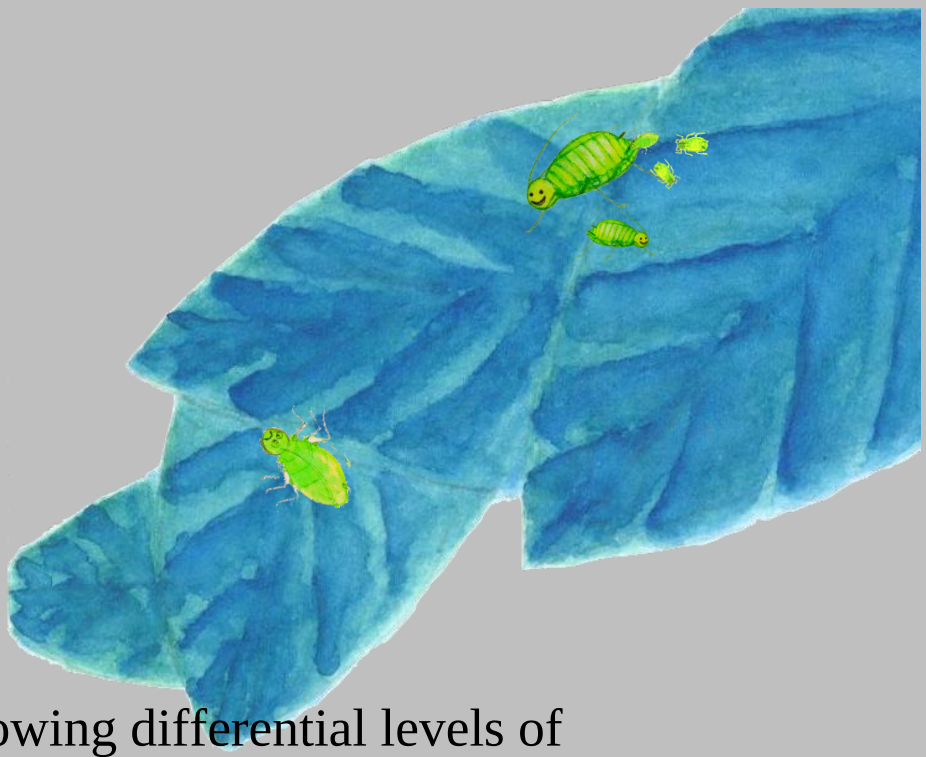


Figure S2. Linkage map, LOD profiles and 1-LOD and 2-LOD support intervals for the minor aphid resistance QTL on linkage group 4. Blue and green lines represent the profiles for survival of the aphids that were placed on the plant, and number of new nymphs produced per aphid, respectively. The number at the end of the marker name indicates its physical positions on the CM334 v.1.55 genome (Kim et al. 2014). The dotted line at LOD 3.5 represents the LOD threshold. LG4, linkage group 4, is assigned to chromosome 4 of the pepper genome. *Rmprp-2* represents a minor resistance QTL for aphid reproduction. The y-axis of the LOD profile shows the LOD score.



CHAPTER 4

Aphid populations showing differential levels of virulence on *Capsicum* accessions

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This chapter has been published as:

Sun M., Voorrips R. & Vosman B. (2019) Aphid populations showing differential levels of virulence on *Capsicum* accessions. *Insect Science*, DOI: https://doi.org/10.1111/1744-7917.12648.

Abstract

The green peach aphid, *Myzus persicae*, is one of the most threatening pests in pepper cultivation and growers would benefit from resistant varieties. Previously, we identified two *Capsicum* accessions as susceptible and three as resistant to *M. persicae* using an aphid population originating from the Netherlands (NL). Later on we identified an aphid population originating from a different geographical region (Switserland, SW) that was virulent on all tested *Capsicum* accessions. The objective of the current work is to describe in detail different aspects of the interaction between two aphid populations and two selected *Capsicum* accessions (one that was susceptible (PB2013046) and one that was resistant (PB2013071) to population NL), including biochemical processes involved. Electrical penetration graph (EPG) recordings showed similar feeding activities for both aphid populations on PB2013046. On accession PB2013071 the aphid population SW was able to devote significantly more time to phloem ingestion than population NL. We also studied plant defense response and found that plants of accession PB2013046 could not induce an accumulation of reactive oxygen species and callose formation after infestation with either aphid population. However, plants of PB2013071 induced a stronger defense response after infestation by population NL than after infestation by population SW. Based on these results, population SW of *M. persicae* seems to have overcome the resistance of PB2013071 that prevented feeding of aphids from NL population. The potential mechanism by which SW population overcomes the resistance is discussed.

Keywords: plant-aphid interaction, EPG, ROS accumulation, callose deposition, virulence, plant immunity

Introduction

Aphids are among the most important plant pests worldwide, damaging crops directly by feeding from the phloem and indirectly by transmitting many harmful viruses (Dixon, 1977, Powell et al., 2006). The generalist green peach aphid, *Myzus persicae*, is one of the most important pest insects in pepper crops (*Capsicum* spp.), causing chlorosis, leaf defoliation, flower and fruit abortion (Blackman & Eastop, 2000). Many pepper viruses are mainly vectored by *M. persicae*, including Pepper mottle virus, Pepper severe mosaic virus and Pepper yellow mosaic virus (Black et al., 1991, Kenyon et al., 2014). Chemical pesticides have been widely used to control aphids. However, due to the long-time use of these chemicals, more and more species (and populations) of aphids are reported to be developing resistance to pesticides (Wang et al., 2002, Cheng et al., 2004, Bass et al., 2014). With increasing concern about the negative environmental impact of insecticides, host plant resistance is commonly seen as a desirable goal in plant breeding and is projected to play an indispensable role in integrated pest management (Broekgaarden et al., 2011). In many cases, resistance factors like Quantitative Trait Loci (QTLs) or genes controlling plant resistance have been successfully used in breeding programs, such as the resistance in lettuce to the black currant-lettuce aphid *Nasonovia ribisnigri* (Eenink et al., 1982b), the resistance in wheat to the Russian wheat aphid *Diuraphis noxia* (Cleveland et al., 2003), the resistance in soybean to soybean aphid *Aphis glycines* (Wu et al., 2004) and the resistance in melon to cotton aphid *Aphis gossypii* (Pitrat & Lecoq, 1980). One type of plant resistance mechanism was hypothesized to work according to the gene-for-gene principle, which means that a resistance gene (*R* gene) in the resistant plant recognizes an effector secreted by the aphid and then activates defense responses against the attacking aphid (Stotz et al., 1999, Kessler & Baldwin, 2002). Later on the more

comprehensive zigzag model was developed (Jones & Dangl, 2006, Smith & Boyko, 2007, Yates & Michel, 2018). During aphid infestation, plants can recognize conserved molecules (known as pathogen or herbivore-associated molecular patterns or PAMPs/HAMPS) by pattern recognition receptors (PRR) and activate PAMP-triggered immunity (PTI) (Jones & Dangl, 2006, Smith & Boyko, 2007). In order to colonize plants, aphids may secrete effectors to prevent the plant defense response, which is known as effector-triggered susceptibility (ETS) (Rodriguez & Bos, 2013, Elzinga et al., 2014). At their turn plants may respond with the production of R proteins that are able to recognize effectors, leading to effector-triggered immunity (ETI) (Hogenhout & Bos, 2011, Jaouannet et al., 2014). Both PTI and ETI result in an incompatible plant-aphid interaction (Tsuda & Katagiri, 2010). The incompatible interaction between host and insect may be observed as a microscopic hypersensitive response in the host plant after insect infestation, involving phloem protein plugging (Tjallingii, 2006, Medina-Ortega & Walker, 2015), callose deposition (Villada et al., 2009, Luna et al., 2011) and/or accumulation of reactive oxygen species (ROS) (Moloi & van der Westhuizen, 2006, Villada et al., 2009, Lei et al., 2014). Phloem protein plugging is a fast process which has been best studied in legumes, involving forisomes (Peters et al., 2006). So far there is only limited information on protein plugging of sieve elements in other species (Knoblauch et al., 2014, Garzo et al., 2018). The deposition of callose, a ß-1,3-glucan, has been reported as an important and long-lasting reaction to wounding, pathogen infection and insect infestation (Stone & Clarke, 1992, Donofrio & Delaney, 2001, Hao et al., 2008, Van der Westhuizen et al., 2002). Phloem protein plugging and callose deposition induced by phloem-feeding insects are triggered by an influx of calcium. They prevent the uptake of sieve-tube sap by the insect and is suggested to be a resistance factor against several insects (Van der Westhuizen et al., 1998, Liu et al., 2017, Sun et al., 2018). The accumulation of ROS is an earlier and faster reaction than callose deposition after pathogen or insect attack (Piedras et al., 1998, Miller et al., 2009). ROS accumulation is believed to play an important role in plant resistance to invading aphids (Moloi & van der Westhuizen, 2006, Kerchev et al., 2012, Shoala et al., 2018). Not only does it protect plants directly (Liu et al., 2010), it also acts as signal to activate downstream defense enzymes (Moloi & van der Westhuizen, 2006, Kuśnierczyk et al., 2008). The incompatible host-aphid interaction also can be detected by monitoring aphid probing and feeding behaviour using the electrical penetration graph (EPG) technique (Alvarez et al., 2006, Chandran et al., 2013). The EPG technique provides information about the aphid's activity on the plant through different waveforms (Tjallingii, 1988, Tjallingii et al., 2010) and these waveforms have been used to deduce the physical location of resistance factors encountered by aphids (Alvarez et al., 2006).

Although breeding resistant varieties is a promising method to manage aphid populations, one challenge is to prevent the evolution of new aphid populations which can overcome the resistance (Haley et al., 2004, Hill et al., 2010, ten Broeke et al., 2013a). An aphid population that can overcome host resistance is called a virulent population. Virulent populations are often found with specialist aphids such as *Diuraphis noxia* (Haley et al., 2004), *A. glycines* (Kim et al., 2008) and *A. pisum* (Kanvil et al., 2014). For generalist aphids, there are only a few reports showing that certain populations of *Macrosiphum euphorbiae* (Hebert et al., 2007, Pallipparambil et al., 2010), *A. gossypii* (Lombaert et al., 2009) and *M. persicae* (Cabrera-Brandt et al., 2015) can overcome or partially overcome crop resistance. To prevent the emergence of virulent or semi-virulent aphid populations it is important to understand how they overcome the resistance. Previous studies which revealed the existence of virulent aphid populations mostly payed attention to the variation in aphid behaviour on resistant plants. A more detailed study

on the interaction, which involves not only aphid behaviour but also constitutive and induced plant resistance mechanisms, may help to understand the mechanism by which a virulent aphid population overcomes resistance.

Recently, we identified *Capsicum* accessions susceptible and resistant to a *M. persicae* population from the Netherlands (Sun et al., 2018). These accessions were also challenged with a *M. persicae* population originating from a different geographical region (Switzerland). Aphid feeding activity and plant defense responses were studied in the various aphid-plant combinations in order to elucidate in detail different aspects of the interaction between the pepper accessions and the two aphid populations.

Materials and Methods

Plant materials

The plant materials used are *C. baccatum* accessions (PB2013046, PB2012022, PB2013062 and PB2013071, obtained from the collection of Wageningen University & Research, NL) and a *C. annuum* accession (CGN19226, obtained from the Centre for Genetic Resources, NL). About two weeks after sowing, plants were transplanted into 14 cm pots with potting compost and grown in a standard greenhouse at 19-21 °C, 60–70% relative humidity and a 16–8 h light–dark photoperiod at Wageningen University & Research, NL. Plants were watered every other day. No insect control was applied during growth and testing of the plants.

Aphid populations

Two populations of *M. persicae* were used in this study. One population was collected in the Netherlands in the 1980s and reared for many years on Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* cv. Granaat) at Wageningen University & Research, NL. The other population originates from Switzerland where it was collected in 1982. It was reared on peas until 2013, when it was transferred to *C. annuum*. The populations are referred to as NL and SW, respectively. We refer to them as populations, as it is unclear if they were started from one single aphid. They may in fact be two different clones. For the experiments discussed here, both populations were reared since 2015 on *C. baccatum* accession PB2013046 under the same conditions as used for growing of the pepper plants.

Evaluation of aphids performance by a clip cage test

The evaluations were performed in 2016 in a greenhouse of Wageningen University & Research, NL, when the plants were seven weeks old and still in the vegetative stage. Five plants of each accession were used per aphid population. All plants were randomized in one greenhouse compartment. Each plant received three clip cages (2.5 cm diameter), containing five one-day-old nymphs from either the NL or SW population. The one-day old nymphs were produced by putting adult aphids on a clean leaf for 24 hours and collecting all nymphs produced during that period, which were then used for infestation. After twelve days the number of surviving and dead aphids as well as new nymphs produced in each clip cage were counted. Statistical analysis was carried out as described previously (Sun et al., 2018). The observations from the three clip cages per plant were combined. Aphid survival was determined by dividing the number of living aphids by the total number of original aphids (dead and alive) that were found back in the clip cage. The number of next generation nymphs per original aphid was calculated by

dividing the number of next generation aphids by the average number of living aphids present in the clip-cage, which was calculated as (2*living aphids + dead aphids)/2. In this formula we assume that dead original aphids contributed to the offspring during half of their life. Given that some aphids were able to escape from the clip cages because of the uneven leaf surface, data from clip cages with less than four aphids (dead and alive) were not included in the analysis. For statistical analysis data were transformed to stabilize the residual variance: survival as arcsin(sqrt(x)) and nymphs produced per average living adult as sqrt(x). Significance of accession effects (five tested accessions) was evaluated using ANOVA and the LSD test ($P<0.05$) was used to assess pairwise differences between accessions, and between the two aphid populations using the T-test ($P<0.05$).

Monitoring of aphid probing and feeding behaviour

The Electrical Penetration Graph (EPG) technique was used to monitor probing and feeding behaviour of the two aphid populations on *C. baccatum* accessions PB2013071 and PB2013046, which were resistant and susceptible to the aphids of the NL population, respectively. Seven-week-old plants were probed with one adult aphid per plant placed on the abaxial side of the second fully-expanded leaf from the top. For each recording a new aphid and plant were used. The EPG setup was as described by (Alvarez et al., 2013). EPG recordings lasted for six hours and were carried out under constant light and at a temperature of 20 ± 2°C. We made 14 recordings (one per aphid) with each population on accession PB2013071, and 13 recordings with each population on accession PB2013046, after removing incomplete recordings because of aphid escape, respectively. The Stylet+ analysis software version 1.20 (http://www.epgsystems.eu/) was used to convert EPG recordings into different waveforms. EPG parameters were calculated online using EPG-Calc 6.1.3 (Giordanengo, 2014). When a waveform was not produced, its duration was set to 0 (zero). T-tests were used to determine the significance of both the differences between the accessions treated by the same aphid population and the differences between two aphid populations feeding on the same accession. Parameters that represent a fraction (such as parameter "% of E1 to E") were transformed as arcsin(sqrt(x)) to stabilize variances. Other parameters were transformed to Ln(x+1) if needed. All the T-tests were done in R v3.4.1 (https://www.R-project.org/) with default packages.

DAB staining for ROS accumulation

DAB (3,3'-Diaminobenzidine) staining was performed according to the protocol of (Daudi & O'Brien, 2012) on plants of the accessions PB2013071 and PB2013046 after infestation with the two aphid populations. Seven-week-old plants received three clip cages containing 15 randomly selected wingless adult aphids per cage or three empty clip cages. Per accession we used four biological replicates (four plants) per aphid population. Leaf disks were collected from the clip cage areas after 6 hours of aphid infestation, and disks under empty clip cages were collected at the same time for reference. Feeding aphids were removed from the leaves with a brush, and disks were then placed in 1 mg／mL 3,3'-diaminobenzidine (DAB)-HCl (Sigma-Aldrich, USA) followed by vacuum infiltration for 20 min. After that, the disks were gently shaken and incubated overnight at room temperature in the dark. The next day they were cleaned with 96% ethanol in a 65℃ water bath for 3 h or in boiling water for 30-40 min. Ethanol was replaced when needed. After chlorophyll was removed, samples were washed in 30% ethanol and then mounted on glass slides with 30% glycerol. The presence of ROS was manifested by brown polymerized deposits. Photos were taken using a Zeiss Axiophoto digital imaging microscope (Carl Zeiss AG, Germany).

Callose deposition

Histological analysis of *in situ* callose deposition was carried out on accessions PB2013071 and PB2013046, infested with two aphid populations when plants were seven weeks old. Plants received either an empty clip cage or a cage with aphids. Three leaves each with one clip cage from three independent biological replicates per treatment were collected 24 hours after the start of aphid infestation. Fifteen randomly selected wingless adult aphids were used in one clip cage. Leaf disks under an empty clip cage were collected after 24 hours and used as reference. Aphids on disks were gently brushed away, and then leaf disks were washed and stained according to (Kissoudis et al., 2016, Sun et al., 2018). Samples were subsequently mounted on glass slides with 50% glycerol. Callose fluorescence was observed under UV light and photos were taken using the Zeiss Axiophoto digital imaging microscope (Carl Zeiss AG, Germany). The number of fluorescent callose spots in each disk was counted. For statistical analysis, the significance of differences in the average number of callose spots from three treatments (NL population, SW population and uninfested reference) was evaluated using ANOVA with the LSD test ($P<0.05$).

Results

Aphid performance

The aphid populations NL and SW, which were collected in the Netherlands and Switzerland respectively, can survive and reproduce well on accessions PB2013046 and CGN19226 (Figure 1, Table S1). More than 90% of the one-day-old nymphs of each population survived and developed into adults, and on average each aphid produced more than 10 offspring after turning into adults. However, reproduction on accession PB2013046 was significantly higher than on accession CGN19226 for both aphid populations (Figure 1A, LSD, $P<0.05$, Table S1).

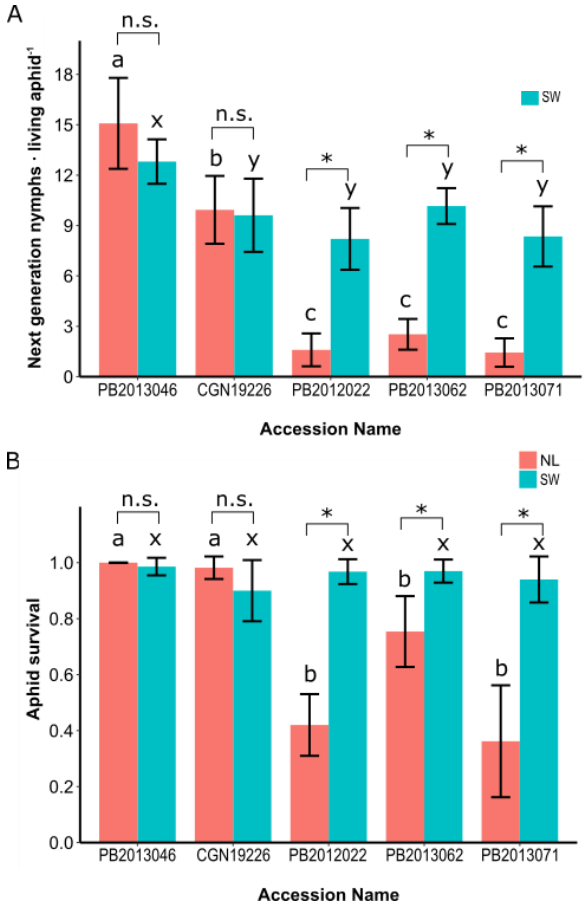

Figure 1. Performance of *Myzus persicae* populations NL and SW on five pepper accessions. (A) Average number of next generation nymphs produced per living adult after 12 days. **(B)** The fraction of aphids initially put on a plant that survived 12 days. Each bar represents the mean values ± SD of five plants per accession. Within each panel, pink bars labelled with the same letter (a, b or c) are not significantly different from each other and *m.m.* for the blue bars (x and y), (LSD test, P=0.05). Within each set of two bars a significant difference is indicated by * and a non-significant one by n.s. (T-test, P=0.05).

On the other three accessions (PB2012022, PB2013062 and PB2013071) the population of NL aphids produced fewer next-generation nymphs than on the accessions PB2013046 and CGN19226, whereas SW aphids produced significantly fewer nymphs than on PB2013046, but not compared with CGN19226 (Figure 1A, LSD, P<0.05, Table S1). Moreover, NL aphids on these three accessions produced significantly fewer next generation nymphs than SW aphids (Figure 1A, T-test, P<0.05, Table S1). Aphids of the NL population showed a significantly lower survival on these three accessions than on the other two accessions (PB2013046 and CGN19226) (Figure 1B, LSD, P<0.05, Table S1), while aphids of SW population showed a similar survival level on all accessions.

EPG analysis

The EPG technique was used to study aphid feeding behaviour on the pepper accessions PB2013071 and PB2013046 (resistant and susceptible to the NL population, respectively) using both aphid populations. Table 1 and Table S2 show the results for some EPG parameters.

Table 1. EPG parameters (mean value) measured for *M. persicae* populations NL and SW on accessions PB2013071 and PB2013046. The following codes are used: no-penetration period (NP), pathway phase (C), derailed stylet (F), xylem ingestion (G), phloem phase (E), phloem salivation (E1) and phloem ingestion (E2). Time values are in minutes.

	Parameter value				P value from T-test			
	Population NL		Population SW		Population NL	Population SW	PB2013071	PB2013046
	PB2013071 (n=14)	PB2013046 (n=13)	PB2013071 (n=14)	PB2013046 (n=13)	PB2013071 VS PB2013046	PB2013071 VS PB2013046	Population NL VS Population SW	Population NL VS Population SW
Number of probes	13.8±2.4	11.2±2.5	10.4±2.7	12.0±2.8	0.2687	0.5956	0.1948	0.7673
Total duration of probes	342.9±3.2	348.2±2.5	333.7±6.1	341.7±4.5	0.1129	0.5038	0.3994	0.2880
Number of NP	12.9±2.4	10.2±2.5	9.4±2.7	11.1±2.8	0.2542	0.5785	0.1830	0.7470
Total duration of NP	16.9±3.2	11.4±2.6	16.5±3.8	17.6±4.6	0.1125	0.8793	0.9362	0.3222
Number of C	26.3±2.5	16.7±2.9	15.8±3.1	14.5±2.8	**0.0025**	0.7041	**0.0030**	0.4814
Total duration of C	162.1±6.3	122.5±7.5	127.7±8.5	105.7±7.6	**0.0482**	0.4003	0.1458	0.4588
Number of F	3.2±1.8	2.9±1.5	0.5±0.8	1.2±1.2	0.7945	0.1162	**0.0090**	**0.0422**
Total duration of F	64.3±8.6	111.9±7.8	48.1±8.8	77.8±9.3	0.0823	0.3674	0.5846	0.2625
Total duration of G	45.3±5.7	19.0±4.6	9.4±4.2	12.4±5.1	**0.0199**	0.7306	**0.0018**	0.4841
Time to first G	176.0±10.2	264.8±10.2	334.7±8.1	325.3±8.6	**0.0357**	0.7341	**0.0000**	0.1022
Total duration of E	71.2±6.6	94.8±9.2	156.7±10.4	145.8±10.3	0.3830	0.7995	**0.0178**	0.1913
Time to first E	63.9±6.8	154.6±9.4	48.0±6.3	121.1±10.3	**0.0042**	**0.0329**	0.3481	0.3893
Total duration of E1	71.2±6.6	19.8±4.7	75.1±8.1	20.2±3.3	**0.0007**	**0.0111**	0.8566	0.9541
Number of single E1	9.1±1.9	2.5±1.8	4.6±1.9	1.2±1.0	**0.0000**	**0.0045**	**0.0026**	0.0817
Total duration of single E1	69.9±6.6	13.5±4.0	42.6±6.7	5.5±2.6	**0.0003**	**0.0117**	0.1236	0.1084
Number of E1 followed by E2	0.9±1.6	0.7±0.8	2.0±1.4	1.8±1.0	0.8265	0.3123	0.2346	**0.0062**
Total duration of E1 followed	1.3±2.0	6.3±3.2	32.5±7.5	14.7±3.3	**0.0151**	0.2896	0.0717	0.0518
% of E1 to E	99.9±0.6	50.4±6.7	67.7±6.4	30.1±5.7	**0.0020**	**0.0160**	**0.0149**	0.2034
Total duration of E2	0.1±0.4	74.9±9.5	81.6±10.7	125.6±10.4	**0.0107**	**0.0500**	**0.0257**	0.2038
Time to first E2	340.4±7.8	284.8±9.5	231.9±11.0	187.7±10.4	**0.0108**	0.3352	**0.0092**	**0.0207**
Total duration of sE2[a]	0.0±0.0	74.8±9.5	76.3±10.4	124.5±10.4	**0.0000**	**0.0298**	**0.0263**	0.2153
Time to first sE2[a]	360.0±0.0	284.9±9.5	266.0±11.0	195.3±10.7	**0.0044**	0.1377	**0.0152**	**0.0371**
Number of potential drops	152.9±6.7	69.9±6.2	106.9±7.5	65.4±6.1	**0.0000**	**0.0390**	**0.0296**	0.7674

[a] sE2 represents sustained E2, phloem ingestion lasting for longer than 10 min.

Comparison between pepper accessions

For both aphid populations many differences were observed between the two accessions during the phloem feeding phase. More time was spent on phloem salivation and much less time on phloem ingestion by aphids on accession PB2013071 than on accession PB2013046. The time until the first phloem event was shorter for aphids on accession PB2013071 than for those on accession PB2013046. In addition, the total number of potential drops (individual cell punctures) for both aphid populations was higher on accession PB2013071 than on accession PB2013046.

For some parameters, the aphids of the NL population showed clear and significant differences in performance on the two accessions while the aphids of the SW population did not show a significant difference. These included the total time spent in the intercellular apoplastic pathway phase, the number of this pathway phases and the total time spent on xylem sap ingestion (all larger on accession PB2013071 than on accession PB2013046) and the time until first xylem probing (shorter on accession PB2013071).

Comparison between aphid populations

Aphids of the SW population were more successful than the aphids of the NL population when feeding on accession PB2013071, which is also more resistant to the NL population in terms of survival and reproduction. Although no significant differences between both aphid populations were detected in the overall duration of phloem salivation and the time until first phloem event, aphids of SW population spent much more time on phloem ingestion and needed less time until the first phloem ingestion compared to aphids of the NL population. The SW population also had a smaller number of intercellular apoplastic pathway phases, derailed stylets, xylem ingestion and potential drops compared with the NL population.

While on accession PB2013046 only minor differences between the two aphid populations were observed: aphids of the NL population had more penetration difficulties (higher number of F) and needed a longer time until the first phloem ingestion compared with aphids of the SW population.

ROS accumulation

To investigate possible differences in the accumulation of reactive oxygen species (ROS) in plants when aphids of the NL or SW population were present, leaf disks (under the clip cages) where aphids had been feeding for six hours were collected and stained for ROS accumulation. No ROS accumulation was seen in leaf disks from the accession PB2013046 with either aphid population or without aphids (Figure S1). On accession PB2013071 (resistant to the NL population), dark staining was observed on leaf disks infested with aphids from the NL population and stained spots were mostly distributed along leaf veins. Conversely, only a very weak staining signal was seen on leaves of this accession infested with SW aphids (Figure 2) and no staining was observed at all on leaf disks under empty clip cages.

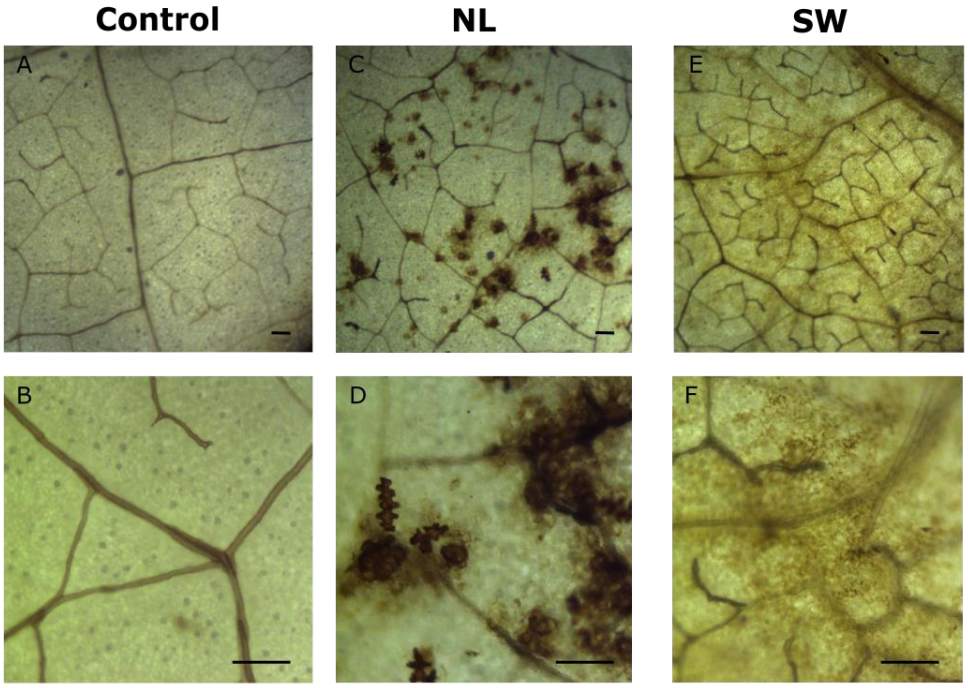

Figure 2. ROS accumulation in leaves of pepper accession PB2013071 in response to *M. persicae* populations NL and SW. DAB staining was used to show ROS accumulation after 6 hrs in leaves under empty clip cages **(A,B)** and under clip cages after a 6h infestation with aphids of the NL **(C,D)** or SW **(E,F)** population. Bars=200 µm. Photos B, D, F were taken with higher magnification on the same leaf disk than photos A, C, E, respectively.

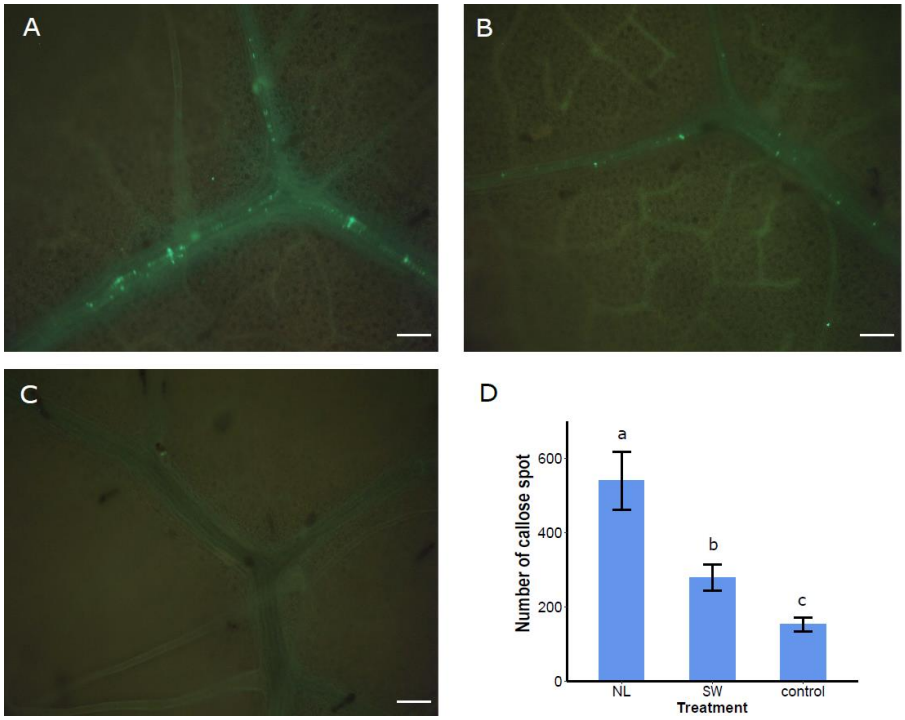

Figure 3. Callose deposition induced by *M. persicae* populations NL and SW on accession PB2013071. (A-C) Callose depositions in pepper leaves under clip cages after a 24h infestation with aphids of the NL **(A)** or SW **(B)** population and under an empty cage **(C)**. **(D)** Shows the number of callose spots counted per leaf disk under the clip cage area. Bars represent means ± SD. Different letters indicate statistically significant differences between treatments (LSD-test at $P<0.05$).

Callose deposition

The formation of callose was examined to explore differences in defense after infestation with aphids of the NL or SW population on accessions PB2013071 and PB2013046 (resistant and susceptible to the NL population, respectively). No callose signal was detected in plants of the accession PB2013046 with either aphid population or in leaf disks without aphids infestation (results not shown). A clear callose signal was found in the vascular system of plants of the accession PB2013071 after 24h of infestation with either aphid population (Figure 3A and 3B). More fluorescent signal was detected in leaf disks infested with aphids of the NL population compared to the SW population (Figure 3D, LSD, $P<0.05$).

Discussion

Resistance in accession PB2013071 seems to be overcome by aphids of the SW population

The five accessions can be classified into resistant or susceptible based on differences in the performance of the aphid population from the Netherlands (NL) for both parameters used: survival of the original nymphs and the number of next generation nymphs produced. When using the SW population on plants of the three accessions resistant to the NL population (PB2012022, PB2013062 and PB2013071), we found that aphids of the SW population always had a higher survival and produced more offspring than those of the NL population. This difference between the two aphid populations was not seen on plants of accessions PB2013046 and CGN19226, on which both aphid populations behaved the same. Similar results were obtained in other studies involving other aphids and host plants; different populations of an aphid species performed differently on resistant, but not on susceptible plants (Pallipparambil et al., 2010, ten Broeke et al., 2013a, ten Broeke et al., 2013b). During EPG recordings, many differences were observed in the feeding of aphids from the two populations on accession PB2013071 that is resistant to the NL population, and these differences were apparent in all phases except the non-probing phase, although not for all parameters. The most important difference between the two populations was seen during the phloem feeding phase. Both aphid populations were able to start phloem ingestion, but only aphids of the SW population were able to continue feeding for a prolonged time, resulting in a large difference in the length of the E2 phase. Probably because of successful phloem feeding, aphids of the SW population were able to propagate on accession PB2013071, as was shown by the performance experiment. In contrast, for aphids of the NL population it was almost impossible to take up phloem sap. These aphids often switched to xylem ingestion, perhaps to prevent starvation (Helden & Tjallingii, 1993, Crompton & Ode, 2010). Compared to aphids of the SW population, an attack by aphids of the NL population induced a stronger defense response in accession PB2013071. This induction was accompanied by a clearer ROS accumulation and more callose deposition. As one of the functions proposed for ROS is that it acts as a local toxin and discourages the attacker (Chen & Schopfer, 1999, Liu et al., 2010), it might be expected that strong ROS accumulation in resistant pepper leaves is induced directly in the phloem vessels, and this is indeed suggested by the distribution of stained spots along leaf veins in our case. Also in the leaves of accession PB2013071 more callose deposits were found upon infestation with NL aphids than with SW aphids. More callose deposition may lead to more serious occlusion of the phloem vessels and cause more difficulties to aphids during prolonged feeding (Hao et al., 2008, Sun et al., 2018). However, the fast plant

reaction that prevents NL aphids from feeding might be caused by phloem proteins (Tjallingii, 2006, Furch et al., 2009). Coagulation of phloem proteins may cause the occlusion (plugging) of sieve elements and the aphid food canal (Garzo et al., 2018, Peng & Walker, 2018). Further experiment are needed to elucidate what is going on during this fast response in pepper-aphid interaction. Based on all these data presented in our study, the resistance mechanism in accession PB2013071 seems to be much less effective against the SW population than against the NL population. Compared to aphids of the NL population, those of the SW population were able to initiate sustained phloem ingestion and only induced a mild defense response, suggesting that the aphids of the SW population are (semi)-virulent on PB2013071 and have for a large part overcome the resistance. Such differences in virulence between populations were also reported for other aphid species and on other host plants (Tolmay et al., 2007, Lombaert et al., 2009, ten Broeke et al., 2013b). However, in our case population SW can only be termed semi-virulent because accession PB2013071 still shows some residual resistance to the SW aphids.

Pepper accession PB2013071 shows residual resistance to the SW population

The EPG analysis revealed that aphids of the SW population to some extent experienced difficulties in taking up the phloem sap on the plants of accession PB2013071. The phloem salivation periods were longer and the phloem uptake periods were shorter on accession PB2013071than on accession PB2013046. Differences were also detected in the level of ROS accumulation and callose deposition between both accessions after the infestation with the SW aphid population. No ROS accumulation and no callose deposits were found in the leaves of accession PB2013046 after infestation, whereas weak signals were clearly present in the resistant accession. These observations suggest that there still are resistance components in PB2013071 showing some residual effectivity against the SW population. Similar studies by others show that virulent aphids or pathogens are sometimes not able to overcome resistance completely and show a reduced virulence, therefore they are called as semi-virulent (Stewart et al., 2003, Tan et al., 2008, Humphries et al., 2013, Humphries et al., 2016).

The interaction between two aphid populations and pepper accession PB2013071

The interaction between aphids and their host plants is often hypothesized to follow the gene-for-gene principle (Flor, 1971, Stotz et al., 1999, Kessler & Baldwin, 2002), which has been developed into the more comprehensive zigzag model (Jones & Dangl, 2006, Smith & Boyko, 2007, Yates & Michel, 2018). When aphids attack a plant, herbivore-associated molecular patterns (HAMPs) from aphid saliva might be recognized by pattern recognition receptors (PRRs), causing PAMP-triggered immunity (PTI) (Hogenhout & Bos, 2011). Insects may develop effectors that suppress PTI which is called effector-triggered susceptibility (ETS) (Rodriguez & Bos, 2013, Elzinga et al., 2014, Wang et al., 2015). In their turn, plants may develop R proteins that recognize effectors in the saliva of the aphids and thus through effector-triggered immunity (ETI) restore resistance (Bos et al., 2010, Chaudhary et al., 2014).

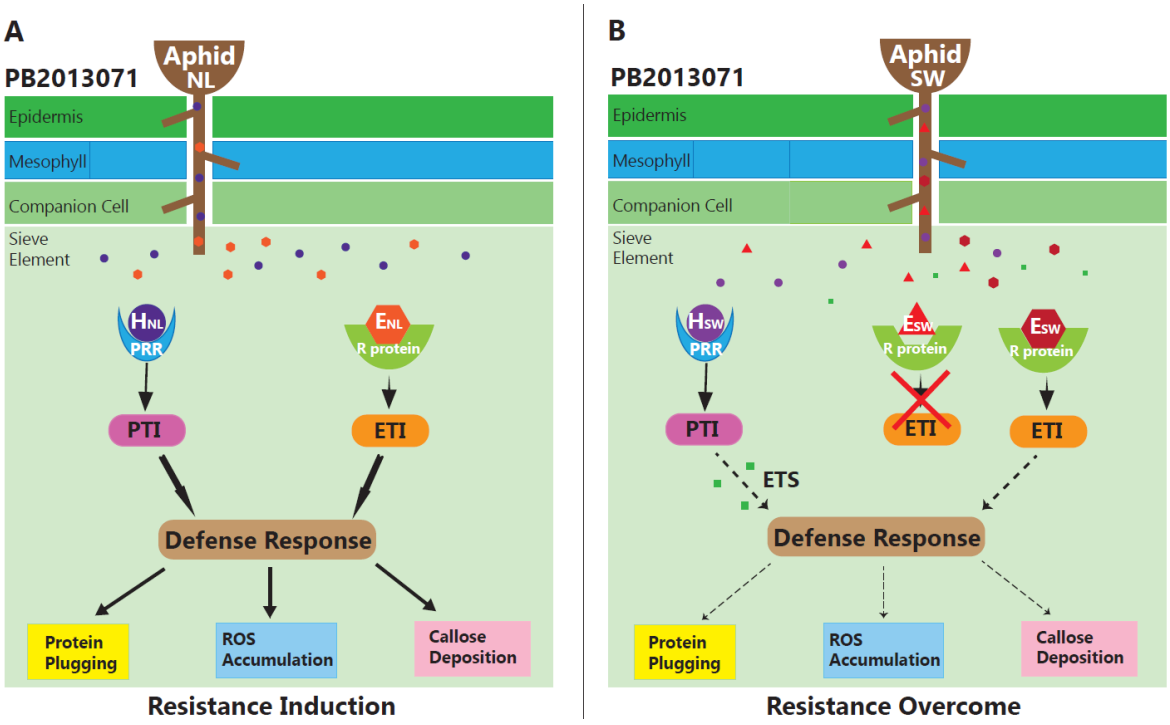

Figure 4. Model explaining different interactions with pepper accession PB2013071 induced by two different *M. persicae* populations. Aphids of the NL and SW populations use their stylets to ingest phloem sap of accession PB2013071. Saliva is secreted during probing and feeding. The herbivore-associated molecular patterns (HAMPs, such as H_{NL} and H_{SW}) from the saliva of both aphid populations might be recognized by pattern recognition receptors (PRRs) from accession PB2013071, and induce PAMP-triggered immunity (PTI). The PTI involves a defense response, which may include plugging of the phloem by proteins, ROS accumulation and callose deposition. To circumvent/suppress plant defences, aphids may produce specific effectors resulting in effector-triggered susceptibility (ETS). In turn, the plant may respond by producing specific resistance (R) proteins that recognize the effector (such as E_{NL} and E_{SW}) of the aphid, resulting in effector-triggered immunity (ETI). The defense responses involving in ETI normally overlap with those in PTI. **(A)** Resistance of accession PB2013071 to NL aphids might be caused by induction of PTI, due to recognition of H_{NL}, or by induction of ETI, due to recognition of E_{NL}. **(B)** Accession PB2013071 is only partially resistant to SW aphids because both PTI and ETI are (at least partially) suppressed, perhaps due to ETS triggered by some SW effectors, or failure of R proteins to recognize SW effectors, or suppression of ETI. In Figure 4A and 4B, H and circles indicate HAMPs; E and polygons (triangles, squares and hexagons) indicate effectors. Black arrows and dashed arrows mean induced and (partially) suppressed responses of PB2013071 respectively.

If we apply this model to our system we may hypothesize that accession PB2013071 is resistant to the NL aphid population through PTI or ETI (Figure 4A) while it is partially susceptible to the SW aphids because PTI is suppressed by effectors from SW aphids (Pitino & Hogenhout, 2013, Rodriguez et al., 2017), ETI is not activated because of lack of effectors that can be recognized by R protein (Drurey et al., 2017), and/or the resistance response is suppressed at a later stage (Postma et al., 2012, Białas et al., 2017, Zhuo et al., 2017) (Figure 4B). Further and more detailed studies are necessary to elucidate the mechanism behind the differential interaction between the two *M. persicae* populations and accession PB2013071.

Conclusion

Two populations of *M. persicae* (NL and SW) perform similarly with respect to survival and reproduction on two *Capsicum* accessions susceptible to the NL population, but significantly different on three *Capsicum* accessions resistant to that population. The performance difference between the two aphid populations is accompanied by differences in feeding and probing activity as well as in levels of defense response (ROS accumulation, callose deposition), strongly suggesting that the SW population has (partially) overcome the resistance that is effective against the NL population.

Supplementary Materials

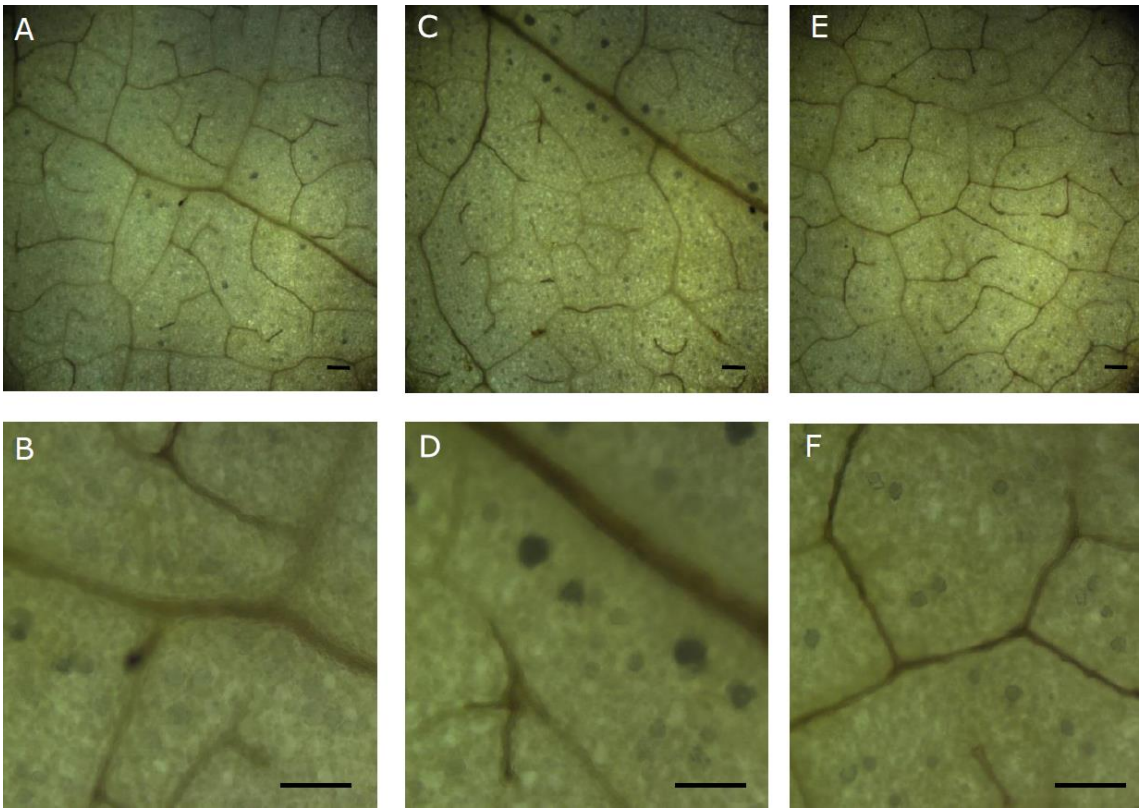

Figure S1. ROS accumulation in leaves of pepper accession PB2013046 after infestation by *M. persicae* populations NL and SW. DAB staining was used to show ROS accumulation in leaves of accession PB2013046 after 6 hrs under empty clip cages **(A,B)** and after 6 hrs infestation with aphids of the NL **(C,D)** or SW **(E,F)** population. Bars=200 µm. Photos B, D, F were taken with higher magnification on the same leaf disk than photos A, C, E, respectively.

Table S1. Evaluation (mean value ± standard deviation) of *Capsicum* accessions for resistance against two *M. persicae* populations NL and SW.

accession	Population NL		Population SW	
	Nymphs [1]	Survival [2]	Nymphs	Survival
PB2013046	15.08±1.08	1.00±0.00	12.81±0.53	0.99±0.01
CGN19226	9.94±0.81	0.98±0.02	9.61±0.87	0.90±0.04
PB2012022	1.59±0.39	0.42±0.04	8.20±0.73	0.97±0.02
PB2013062	2.53±0.37	0.75±0.05	10.16±0.43	0.97±0.02
PB2013071	1.44±0.34	0.36±0.08	8.35±0.72	0.94±0.03

[1] Survival refers to fraction of the aphids that survived on an accession after 12 days.
[2] Nymphs means average number of next generation nymphs produced per aphid on an accession after 12 days.

Table S2. Proportion of individuals that produced the waveform type (PPW) in EPG recording. Two *M. persicae* populations NL and SW were used for EPG on two pepper accessions PB2013071 and PB2013046.

Waveform type	Population NL		Population SW	
	PB2013071 (n=14)	PB2013046 (n=13)	PB2013071 (n=14)	PB2013046 (n=13)
Probe	14/14	13/13	14/14	13/13
No-penetration period (NP)	14/14	13/13	14/14	13/13
Intercellular apoplastic stylet pathway (C)	14/14	13/13	14/14	13/13
Derailed stylet mechanics (F)	12/14	12/13	5/14	7/13
Xylem ingestion (G)	12/14	7/14	3/14	3/13
Phloem phase (E)	14/14	13/13	14/14	13/13
Phloem salivation (E1)	14/14	13/13	14/14	13/13
Passive phloem ingestion (E2)	2/14	8/13	9/14	12/13
Sustained E2 (sE2)	0/14	8/13	7/14	11/13

CHAPTER 5

The ability to manipulate ROS metabolism affects aphid virulence on pepper

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This chapter has been submitted for publication.

Abstract

Myzus persicae has severe economic impact on pepper (*Capsicum*) cultivation. Previously, we identified two populations of *M. persicae*, NL and SW, that were avirulent and virulent, respectively on *C. baccatum* accession PB2013071. The transcriptomics approach used in the current study, which is the first study to explore the pepper-aphid interaction at the whole genome gene expression level, revealed genes whose expression are regulated in pepper accession PB2013071 upon infestation with these two *M. persicae* populations. The NL population induced ROS production genes, while the SW population induced ROS scavenging genes and repressed ROS production genes. We also found that the SW population can induce the removal of ROS which accumulated in response to pre-infestion with the NL population, and that pre-infestation with the SW population significantly improved the performance of the NL population. This paper supports the hypothesis that *M. persicae* can overcome the resistance in accesion PB2013071 probably because of its ability to manipulate plant defense response especially the ROS metabolism and such ability may benefit avirulent conspesific aphids.

Introduction

Aphids, which belong to the order of Hemiptera, are one of the most destructive plant pests worldwide. Among the more than 4,000 described aphid species, over 100 species are reported as economically important pests and are able to damage plant health (Blackman & Eastop, 2008). Aphid infestation can result in direct damage such as chlorosis, necrosis, wilting, defoliation and, more importantly, indirect damage resulting from aphid transmitted viruses (Eastop, 1977). Most aphid species reproduce asexually under suitable conditions, which leads to rapid population expansion and therefore to difficulties in population control (Blackman & Eastop, 2008).

Given the fact that aphids have severe negative effects on crop cultivation, the frequent use of chemical pesticides is the major management strategy (Perring et al., 1999). However, with growing concern about the negative impact of pesticides on the environment, integrated pest management such as promoting aphid resistant varieties is more and more encouraged (Flint & Van den Bosch, 2012). Several resistant crop varieties have been applied to alleviate aphid problems, such as melon varieties resistant to cotton aphid *Aphis gossypii* (Pitrat & Lecoq, 1980), lettuce varieties resistant to the black current-lettuce aphid *Nasonovia ribisnigri* (Eenink et al., 1982b) and soybean varieties resistant to the soybean aphid *Aphis glycines* (Wu et al., 2004). Although using resistant varieties is a beneficial strategy to control aphids, the durability of crop resistance is threatened by the evolution of new aphid biotypes which have overcome the resistance (ten Broeke et al., 2013a, Hill et al., 2010). Understanding the interaction between aphids and their host plants, including how the resistance response is induced in resistant plants and how aphids adapt to host plant resistance, may help to keep crop resistance more durable during agricultural application.

Plants may induce defense responses against aphid feeding. The defense responses induced in resistant plants include calcium influxes (Vincent et al., 2017), accumulation of reactive oxygen species (ROS)(De Ilarduya et al., 2003, Sun et al., 2019), phloem occlusion by specific proteins(Kehr, 2006, Peng & Walker, 2018, Garzo et al., 2018) and callose deposition (Kempema et al., 2007, Sun et al., 2018). ROS have been suggested to play an important role in plant defense responses against biotic stresses. They not only may have a direct toxic effect on aphids (Liu et al., 2010), but have also been suggested to mediate defense gene activation and interact with other

signalling components (Waszczak et al., 2018, Apel & Hirt, 2004). Accumulation of ROS in plants could enhance aphid resistance (Moloi & van der Westhuizen, 2006, Shoala et al., 2018) while impairment of ROS production reduces aphid resistance, e.g. makes the plant more susceptible (Lin et al., 2016). As ROS are involved in a large network associated with plant defense responses, it is conceivable that their levels may be affected/regulated by multiple enzymes. It has been proposed that ROS can be produced by various enzymes, such as NADPH oxidases (Torres et al., 2002), peroxidases (Bindschedler et al., 2006) and oxalate oxidase (Hu et al., 2003), and may be removed by ROS-scavenging enzymes like catalase (Mhamdi et al., 2010) and superoxide dismutase (Mittler et al., 2004), or by antioxidants like glutathione (Noctor & Foyer, 1998).

Pepper (*Capsicum* spp.) is one of the most important and widely cultivated horticultural crops. However, pepper cultivation is hampered by aphids and the viruses they transmit (Pernezny et al., 2003, Kenyon et al., 2014). One accession of *Capsicum baccatum* (PB2013071) has been recently identified as a good resistance source to a population of *Myzus persicae* (the NL population (Sun et al., 2018)). The resistance of this accession was later found to be (partly) overcome by another population of *M. persicae* (the SW population (Sun et al., 2019)). That resistance was overcome by the SW population was reflected in longer phloem feeding and a much weaker induction of the defense response in plants of accession PB2013071. To understand the plant-aphid interaction better and to explore why the resistance of PB2013071 is overcome, we (1) analysed gene expression in the compatible and incompatible interaction to identify differentially expressed genes that specifically responded to the virulent and avirulent aphid population respectively, with an emphasis on genes involved in ROS production and scavenging; (2) studied ROS accumulation to investigate whether the SW population of *M. persicae* is able to promote plant susceptibility by suppressing ROS accumulation; and (3) studied the ability of both *M. persicae* populations to induce plant susceptibility for conspecific aphids.

Materials and Methods

Plant material and aphid populations

C. baccatum accessions PB2013071 and PB2013046 were obtained from the collection of Plant Breeding, Wageningen University & Research, NL and are described in (Sun et al., 2018).

Two weeks after sowing, seedlings were transplanted into 14 cm pots with potting compost and grown in a standard greenhouse at 19-21 °C, 60–70% relative humidity with an L16:D8 photoperiod at Unifarm, Wageningen University & Research, NL. Plants were watered every other day. Seven-week-old plants of PB2013071, which were still in vegetative stage, were used in all the experiments.

The two *M. persicae* populations (NL and SW) used were described previously (Sun et al., 2019). The NL population is avirulent on pepper accession PB2013071, while the SW population is virulent. Both populations were reared on susceptible *C. baccatum* accession PB2013046 in cages in different greenhouse compartments under the same conditions as used for growing the plants.

RNA-seq experiment

Plants of accession PB2013071 were infested for 6h with aphids of the NL (NL-infested) or SW population (SW-infested), or received an empty clip cage (control), after which RNA was extracted (Figure S1). Each treatment included three biological replicates and three plants were pooled for each replicate. All the used plants were grown in the same greenhouse compartment at the same time. The first three fully expanded leaves from the top of every plant each received one clip cage either with 10 randomly selected apterous adults of the NL or SW population, or one empty clip cage. After 6h treatment, aphids were gently brushed away from the leaves, and leaf disks under clip cages from one biological replicate (three plants) were quickly sampled and pooled. Leaf disks were flash-frozen in liquid nitrogen and stored at -80 ºC until use. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, the Netherlands) according to the suppliers' recommendations. RNA quality and quantity were evaluated by NanoDrop 1000 V.3.7 (Thermo, USA), Qubit fluorometric quantitation (Thermo, USA) and agarose gel electrophoresis before sending for RNA-seq analysis.

Library construction and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd (Beijing, China). After cDNA library construction, sequencing was performed on an Illumina HiSeq 2500 system (Illumina, USA) and 2*150 bp pair-ended reads were generated. In total at least 6GB data were generated per replicate.

Bioinformatic analysis of RNA-seq data

After the quality of raw data was evaluated by FASTQC (Andrews, 2010), sequence reads of each biological replicate were mapped to the PBC81 *C. baccatum* reference genome (http://peppergenome.snu.ac.kr/) (Kim et al., 2017) using STAR (Dobin et al., 2013). The number of reads per gene was counted with Salmon (https://combine-lab.github.io/salmon/) (Patro et al., 2017) and transcript abundance was calculated using the FPKM (Fragments Per Kilobase of transcript per Million fragments) method (Trapnell et al., 2010). The SARTools pipeline (Varet et al., 2016), which is based on DESeq2 package in R (Love et al., 2014), was employed to detect differentially expressed genes (DEGs) between the control treatment and the NL-infested treatment or SW-infested treatment, as well as for the direct comparison between NL and SW-infested plants. In this pipeline, a false discovery rate (FDR) analysis (Benjamini & Hochberg, 1995) was implemented to correct the p-values of the multiple t-tests in these comparisons. Genes with a FDR ≤ 0.01 and $|\log_2(\text{FoldChange})| \geq 1$ were classified as differentially expressed. Blast2GO v5 Basic (https://www.blast2go.com/) (Conesa et al., 2005) was used to carry out gene ontology (GO) analysis to predict the function of DEGs.

Gene expression validation

Six genes with differential expression levels based on the RNA-seq data were selected for validation with quantitative real-time PCR. The pepper *UBI3* gene (ubiquitin-conjugating protein) was used as an internal reference for normalization of gene expression (Wan et al., 2011). Gene specific primers were designed using Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are listed in Table S1. Each first-strand cDNA was synthesized using the iScriptTM cDNA Synthesis Kit (Bio-Rad, USA) with 1 µg RNA. Quantitative RT-PCR was performed in duplicate as described previously (Sun et al., 2018). QPCR products were sequenced to verify that the correct fragment was amplified in PB2013071. The relative transcription level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Reactive Oxygen Species (ROS) accumulation experiment

In order to explore whether the SW population is able to suppress ROS accumulation induced in PB2013071, five different treatments were designed. Three leaves each with one clip cage from five independent biological replicates (plants) were used for each treatment. Four of the five treatments consisted of a pre-inoculation with either the NL population (15 randomly selected apterous adults in a clip cage) or an empty cage for three days. In both cases the clip cage, and if present the aphids, were then removed and replaced at the same place with either an empty clip cage or a clip cage containing 15 apterous adults of the SW population. After this second infestation, lasting again 3 days, the leaves were harvested for observation. In this way four combination treatments were applied (pre-infestation – infestation):, NL – empty, NL – SW, empty – SW and empty – empty. The fifth treatment consisted of only the pre-infestation with NL after which the leaves were harvested directly.

After the treatment leaf disk areas under the clip cage were collected and aphids were gently brushed away if needed. DAB (3,3'-Diaminobenzidine) staining of leaf disks was performed as described (Sun et al., 2019). The photos of mounted glass slides with leaf disks were taken with a Canon EOS 100D camera (Canon Inc., Japan). The percentage of the area of brown polymerized deposits, which reflect ROS accumulation on each leaf disk was quantified using ImageJ(Collins, 2007). The average percentage from three clip cages which were collected from the same plant was used as the data for one biological replicate. Data were transformed as arcsin(sqrt(x)) to stabilize the residual variance. The significance of the difference in level of ROS accumulation between the five treatments was evaluated using ANOVA with the LSD test ($P<0.05$).

Effect of pre-infestation on subsequent aphid performance

A no-choice assay with clip cages was carried out to study whether pre-infestation by the NL population had an effect on the performance of SW population on pepper plants of accession PB2013071 and vice versa.

Seven plants of accession PB2013071 were pre-infested with three clip cages containing 15 randomly selected apterous adults of the NL population. Another seven plants were similarly pre-infested with the SW population. Two control groups of seven plants received three empty clip cages. The cages were placed on first three fully expanded leaves from the top of every plant. Cages were kept on the plants for three days, after which the original adult aphids together with offspring were gently removed with a soft brush. Every pre-infested plant then received three clip cages containing five 1-day-old nymphs of the aphid population that was different from the population used for pre-infestation: NL pre-infested plants received SW aphids and vice versa. Plants of two control groups were infested with aphids of either the NL or SW population. The clip cages were put on the same spots on the leaves where the removed clip cages had been. After twelve days the living and dead aphids as well as the next generation nymphs produced in each clip cage were counted. The observations from three clip cages per plant were combined. Aphid survival and reproduction were determined as described by (Sun et al., 2019). For statistical analysis, aphid survival and reproduction were transformed as arcsin(sqrt(x)) and sqrt(x), respectively, to stabilize the residual variance. A t-test was used to compare the difference in aphid survival and reproduction on pre-infested and control plants ($P<0.05$).

Results

Transcriptome profiling

Pepper accession PB2013071 shows resistance to aphids of the *M. persicae* NL population, but is susceptible to aphids of the *M. persicae* SW population. On this accession aphids of the NL population show a reduced survival and a poor reproduction. Aphids of the SW population encounter much fewer problems in survival and reproduction (Sun et al., 2019).

RNA isolated from plants of accession PB2013071 treated with aphids of NL population (NL-infested), SW population (SW-infested) or empty clip cages (control) for 6h was subjected to RNA-seq analysis. On average 7.7 GB clean data (6.7-9.2 GB) per sample were generated (detailed information in Table S2).

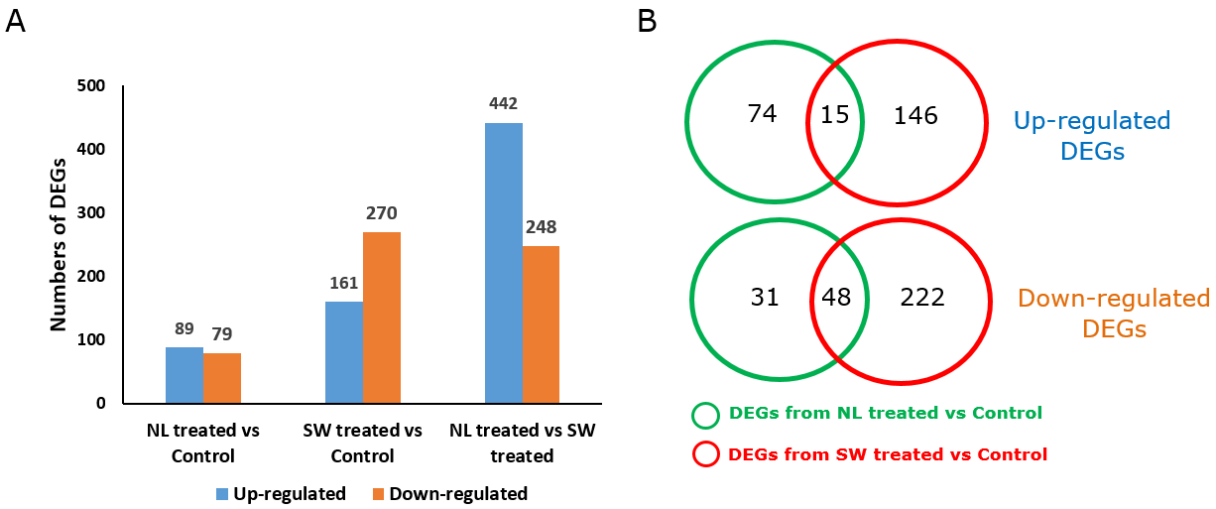


Figure 1. Differentially expressed genes (DEGs) identified in response to two *M. persicae* populations. Plants received clip cage with aphids of the NL (NL treated) or SW(SW treated) population, control plants received an empty clip cage. After a 6 hrs incubation leaf disks were harvested and analysed using RNAseq. The criteria used for assigning significance were: FDR≤0.01 and |log2FoldChange|≥1. **(A)** number of up-regulated or down-regulated DEGs; **(B)** number of DEGs specifically or co-expressed in comparison between control and NL-treated plant as well as in comparison between control and SW-treated plant.

Using a False Discovery Rate (FDR) of 0.01 and a $|\log_2(\text{FoldChange})| \geq 1$, there were 168, 431 and 690 differentially expressed genes (DEGs) detected between NL-infested and control plants, between SW-infested and control plants and between NL-infested and SW-infested plants, respectively (Figure 1A, Table S3).

To validate the results obtained from RNA-seq, the expression level of six genes was measured by QPCR in all nine samples. For each gene, the Fragments Per Kilobase of transcript per Million fragments (FPKM) values of transcriptome data exhibited similar expression patterns for all the three treatments compared with the QPCR results (Figure S2). The correlation coefficient between RNA-seq and QPCR ranged from 0.86 to 1, indicating that the RNA-seq expression data is reliable.

Genes differentially expressed in response to feeding by both aphid populations

Among all the DEGs identified from the comparison between NL-infested and control plants and between SW-infested and control plants, only 15 genes were up and 48 genes were down-regulated in both comparisons (Figure 1B, Table S4). GO enrichment analyses of the 15 common up-regulated genes responding to both aphid populations showed that five and six genes were predicted to be involved in cellular components (GO:0005575) and biosynthetic processes (GO:0009058), respectively. Among the 48 down-regulated genes, genes involved in photosynthesis (GO:0009522, GO:00095223, GO:0009535, GO:0016168) were over-represented (26 genes out of 48), and genes encoding chlorophyll a-b binding proteins were the main group (21 genes).

Differentially expressed genes specific for feeding of the NL or the SW population

Of all genes that were differentially regulated in response to feeding of the two aphid populations, most genes are regulated in a population specific way, showing that transcriptional responses are largely aphid population specific (Figure 1B).

When compared with control plants, 105 genes were only significantly up- or down-regulated in the plants that were treated with aphids of the NL population, and 368 genes only after the feeding by the SW population (Figure 1B, Table S5). In a direct comparison between plants that were treated with the NL and SW population 690 genes were differentially expressed. This list was used to narrow down the number of genes specifically involved in response to different aphid populations. There were 63 genes responding specifically to feeding by the NL population and 203 specifically responding to feeding by the SW population (Figure 2, Table S6). GO enrichment analyses of the 63 genes responding to the NL population indicated that they were mainly involved in oxidoreductase activity (GO:0016491, 19 genes), ion binding (GO:0043167, 19 genes), biosynthetic process (GO:0009058, 11 genes) and response to stress (GO:0006950, 10 genes). The 203 genes specifically responding to SW population were predicted to be mainly involved in different activities such as working in integral component of membrane (GO:0016021, 36 genes), oxidation-reduction processes (GO:0055114, 24 genes), ATP binding (GO:0005524, 19 genes), protein kinase activity (GO:0004672, 18 genes) and phosphorylation (GO:0006468, 18 genes).

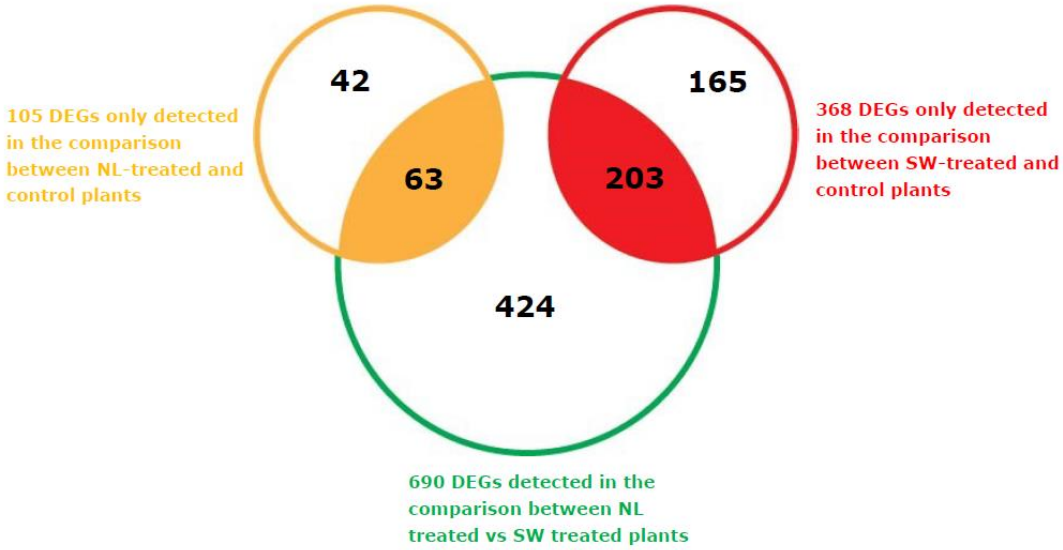


Figure 2. Venn diagram of DEGs that specifically respond to the feeding by the NL or the SW population. DEGs were first compared between the NL-treated vs control plants and SW-treated vs control plants. One-hundred and five genes were differently regulated in the comparison between NL-treated and control plants(yellow circle) and 368 genes were differently regulated in the comparison between SW-treated and control plants (red circle). The lists of 105 and 368 genes were then compared with the list of 690 DEGs which were detected in the direct comparison between NL-treated and SW-treated plants. The 63 and 203 genes that appeared in both comparisons were considered here to specifically respond to the feeding by the NL and the SW populations respectively.

Differentially expressed genes involved in oxidoreductase activity

We previously observed that aphids of the NL population induce a strong accumulation of reactive oxygen species (ROS), while the SW population induces only a very weak ROS response in plants of PB2013071 (Sun et al., 2019). As the ROS staining assay in the previous study was performed after six hour aphid incubation, and also the transcriptome analysis of the current study was carried out after six hours of infestation, we looked for differences in plant DEGs involved in ROS metabolism, especially in DEGs that specifically responded either to the NL or to SW population (Table S6). Given that ROS production and scavenging is a dynamic oxidation-reduction process, we looked for the DEGs which are assigned with predicted functions related with oxidation-reduction process, which is reflected in the GO annotations GO:0016491, GO:0016709, GO:0016717, GO:0055114 and GO:0003824.

Among the 63 DEGs that specifically respond to infestation by the NL population, there were 18 up-regulated and 4 down-regulated genes with oxidoreductase activity (Table 1). Similarly, among the 203 DEGs responding only to the SW population, 14 up- and 16 down-regulated were annotated as having oxidoreductase activity (Table 2). Among all DEGs involved in oxidoreductase activity (Table 1 and 2), only one gene was present in both lists: Peroxidase 12 was up-regulated in NL-infested plants while down-regulated in SW-infested plants.

Table 1. Differentially expressed genes (DEGs) involved in oxidation-reduction process in resistant *C. baccatum* PB2013071 specifically responding to the NL aphid population.

Gene ID	control-average FPKM	NL treated-average FPKM	log2Fold Change	Up-Down-Regulation	FDR	Gene annotation
rna5965	113	475	2.07	Up	1.88E-26	Peroxidase N1
rna10318	9013	23158	1.36	Up	1.86E-08	Peroxidase 12
rna10320	1003	2596	1.37	Up	7.59E-07	partial Peroxidase 12
rna8690	1	25	4.07	Up	0.0048	Peroxidase 5
rna15242	1570	4540	1.53	Up	0.00034	linoleate 9S-lipoxygenase 5
rna19402	99	328	1.73	Up	0.00036	carotenoid 9,10(9',10')-cleavage dioxygenase 1
rna29042	66	219	1.74	Up	0.0062	DMR6-Like oxygenase
rna34704	78	279	1.84	Up	1.30E-08	Omega-6 fatty acid desaturase
rna29538	136	465	1.78	Up	0.00074	Omega-6 fatty acid desaturase
rna21402	52	164	1.66	Up	0.0014	delta(12)-fatty-acid desaturase
rna28453	64	180	1.49	Up	0.0018	L-ascorbate oxidase
rna24520	1176	2963	1.33	Up	0.00084	Cytochrome P450 76A1
rna10853	667	1733	1.38	Up	0.0033	Cytochrome P450 82A3
rna28819	604	1588	1.39	Up	0.0014	3-oxoacyl-[acyl-carrier-protein] reductase
rna19820	157	463	1.56	Up	0.0059	NAD(P)H: quinone oxidoreductase
rna29543	1241	3277	1.4	Up	0.0025	7-alpha-hydroxysteroid dehydrogenase
rna21511	69	215	1.64	Up	0.0061	Arogenate dehydrogenase
rna25969	68	155	1.19	Up	0.0069	Aldehyde dehydrogenase
rna11857	3276	794	-2.04	Down	2.03E-13	Geranylgeranyl diphosphate reductase
rna16367	810	1121	-1.22	Down	1.54E-06	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase
rna22917	68	18	-1.90	Down	0.000033	Cytochrome P450 83B1
rna14689	279	97	-1.52	Down	0.00042	Chlorophyllide a oxygenase

The criteria used for assigning significance were: FDR≤0.01 and $|\log_2 FoldChange|>1$.

Table 2. Differentially expressed genes (DEGs) involved in oxidation-reduction process in in resistant *C. baccatum* PB2013071 specifically responding to the SW aphid population.

Gene ID	control-average FPKM	SW treated-average FPKM	log2Fold Change	Up-Down-Regulation	FDR	Gene annotation
rna4866	1154	5384	2.22	Up	1.14E-44	Catalase
rna26330	2357	6131	1.38	Up	6.61E-30	alanine aminotransferase
rna8250	161	538	1.74	Up	4.10E-22	Cytochrome P450 71A6
rna12135	37	141	1.91	Up	2.31E-09	cytochrome P450 CYP82D47
rna19682	37	173	2.24	Up	5.39E-09	Ferric reduction oxidase 5
rna5789	106	342	1.68	Up	1.23E-08	cinnamoyl-CoA reductase
rna7913	286	969	1.76	Up	1.24E-07	4-coumarate-CoA ligase 2
rna20326	165	506	1.62	Up	3.00E-07	ferric reduction oxidase 6
rna2114	101	250	1.31	Up	3.21E-07	galactinol-sucrose galactosyltransferase 4
rna21997	23	143	2.63	Up	1.94E-06	Flavonol synthase/flavanone 3-hydroxylase
rna8249	38	124	1.70	Up	0.000046	Cytochrome P450 71A6
rna14917	246	526	1.10	Up	0.00021	quinone-oxidoreductase homolog
rna31209	193	392	1.02	Up	0.0030	Geraniol 8-hydroxylase
rna10118	331	704	1.09	Up	0.0061	cytochrome P450 71A1
rna25419	416	88	-2.23	Down	1.61E-24	cytochrome P450 86A1
rna5501	5302	1710	-1.63	Down	7.44E-23	polyphenol oxidase B
rna17709	897	302	-1.57	Down	2.43E-22	Endoplasmic reticulum oxidoreduction-1
rna27753	224	40	-2.50	Down	2.72E-20	DMR6-Like oxygenase
rna10848	863	235	-1.87	Down	7.46E-19	Allene oxide synthase
rna14282	10875	2580	-2.08	Down	4.37E-18	beta-carotene hydroxylase 2
rna32427	379	126	-1.60	Down	2.07E-17	alkane hydroxylase MAH1
rna35370	1659	797	-1.06	Down	5.16E-16	alcohol-forming fatty acyl-CoA reductase
rna19821	1894	762	-1.32	Down	1.02E-11	NAD(P)H:quinone oxidoreductase
rna23236	10323	1032	-3.32	Down	2.59E-09	protochlorophyllide reductase
rna28288	214	85	-1.35	Down	0.000029	cytochrome P450 CYP736A12
rna5811	59467	28426	-1.07	Down	0.000055	Oxygen-evolving enhancer protein 1
rna23410	304	147	-1.05	Down	0.000056	delta(8)-fatty-acid desaturase
rna20968	355	147	-1.27	Down	0.000057	9-divinyl ether synthase
rna10318	9013	4152	-1.12	Down	0.000112	Peroxidase 12
rna18808	29	4	-2.70	Down	0.000140	cytokinin dehydrogenase 3

The criteria used for assigning significance were: FDR≤0.01 and |log2FoldChange|>1.

Effect of aphid population SW on reactive oxygen species (ROS) metabolism

In order to explore the effect of the SW population on plant ROS metabolism, we analysed ROS in pepper leaves of accession PB2013071 after five different treatments, including pre-infestations with the NL population.

Plants of accession PB2013071 showed a strong ROS accumulation after three days of feeding by aphids of the NL population (Figure 3A), and this accumulation could not be effectively eliminated by the plants themselves after a further three-day period with empty cages (Figure 3B). However, this induced ROS accumulation was mostly eliminated after a subsequent infestation of SW population (Figure 3C). The reduction in ROS accumulation was reflected by a significantly lower percentage of DAB staining area on the leaf (Figure 3F, LSD-test, $P<0.05$).

Although the presence of the SW aphid population induced a weak ROS accumulation in plants of PB2013071 (Figure 3D), there was no significant difference in the percentage of stained area between SW-infested leaf and control leaf that received an empty clip cage (Figure 3D, 3E and 3F, LSD-test, $P>0.05$).

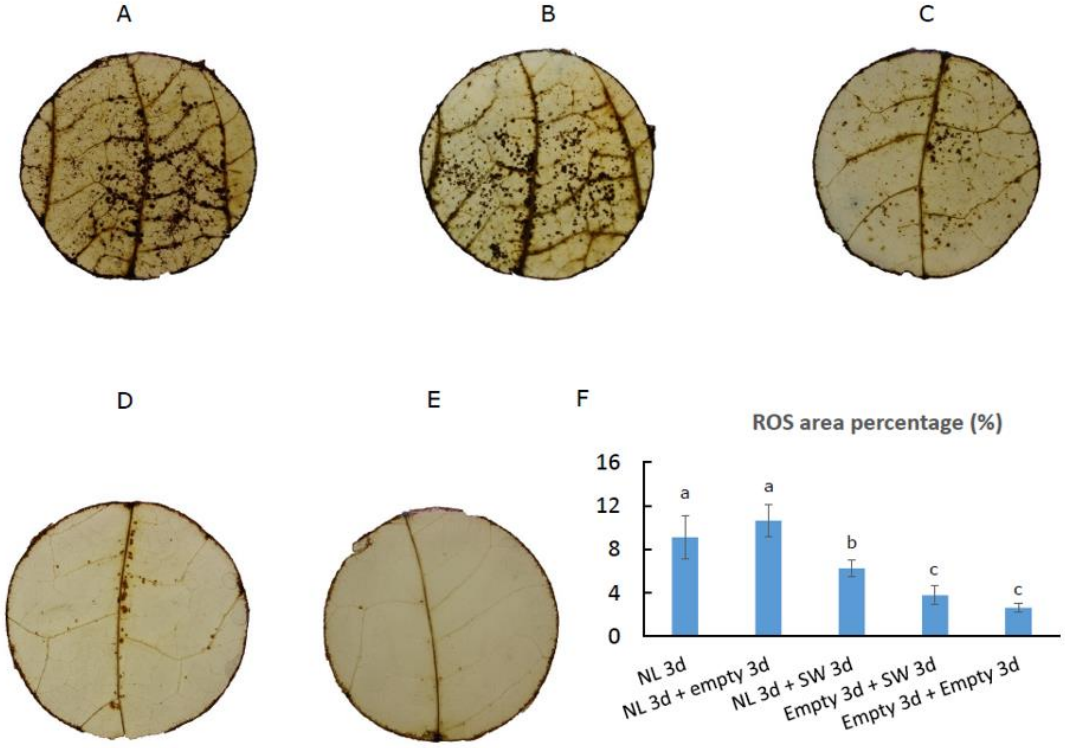


Figure 3. ROS accumulation in leaves of pepper accession PB2013071 in response to *M. persicae* populations NL and SW. DAB staining was used to show ROS accumulation after different treatments. **(A)** Leaf disk after a three-day infestation with the NL aphids population. **(B)** Leaf disk after a three-day infestation with NL aphids followed by 3 days with an empty cage. **(C)** Leaf disk after a three-day infestation with NL aphids followed by 3 days with the SW aphid population. **(D)** Leaf disk after three days with an empty cage followed by 3 days SW population. **(E)** Leaf disk after six days with an empty cage **(F)** shows the percentage of stained DAB area calculated per leaf disk under the clip cage area. Bars represent means ± SD. Different letters indicate statistically significant differences between treatments (LSD-test, $P<0.05$).

Effect of pre-infestation with aphid populations on a subsequent infestation

Besides the RNA-seq analysis and ROS accumulation assays, we also carried out bio-assays to explore whether defense responses induced by the NL population can affect SW performance and whether the manipulation of plant defenses by the SW population could benefit NL population.

Figure 4 shows the effect of pre-infestation with the NL population on the performance of the SW population. There was no significant difference in aphid survival and reproduction between living on NL pre-infested plants and living on control plants (t-test, P>0.05).

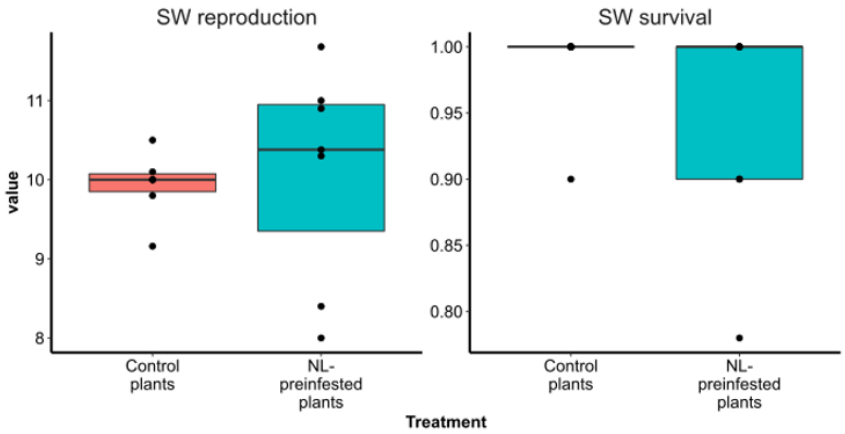


Figure 4. Performance of the SW population on plants of pepper accession PB2013071. Plants were pre-infested with *M. persicae* the NL population (NL-preinfested plants) or received an empty clip cage (control plants) for 3 days, after which the aphids were removed. Next, the plants received clip cages with *M. persicae* of the SW population. Two phenotyping parameters were used: average number of next generation nymphs produced per SW living adult after 12 days (left panel), and the fraction of SW aphids initially put on a plant that survived 12 days (right panel). Seven biological replicates (plants) were used per treatment and are presented as black dots in the box plots. For both phenotyping parameters no significant difference between the treatments was found (t-test, P>0.05).

When the situation was turned around and plants were first infested with aphids of the SW population and the effect on the performance of aphids of the NL population was measured the outcome was different. Aphids of *M. persicae* population NL showed a significantly higher survival and produced significantly more next generation nymphs on SW pre-infested plants than on control plants of PB2013071 (Figure 5, t-test, P<0.01). Pre-infestation with the SW population made it possible for the NL aphids to increase survival from 0.35±0.14 to 0.78±0.17 and to improve reproduction from 2.84±0.87 to 4.92±1.01 nymphs per original aphid.

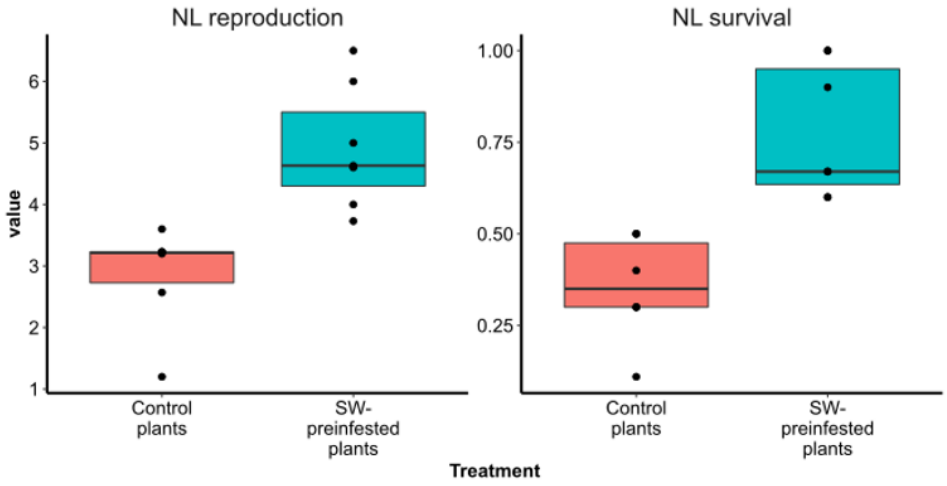


Figure 5. Performance of the NL *M. persicae* population on plants of pepper accession PB2013071. Plants were pre-infested with the SW population (SW-preinfested plants) or received an empty clip cage (control plants) for 3 days, after which the aphids were removed. Next, the plants received clip cages with aphids of the NL population. Two phenotyping parameters were used: average number of next generation nymphs produced per living NL adult after 12 days (left panel), and the fraction of NL aphids initially put on a plant that survived 12 days (right panel). Seven biological replicates were used per treatment and are presented as black dots in box plots. For both phenotyping parameters a significant difference between the treatments was found (t-test, $P<0.05$).

Discussion

Plants show a very different transcriptional response to different conspecific aphid populations

In our previous study we identified two *M. persicae* populations, NL and SW, which are avirulent and virulent on pepper accession PB2013071, respectively. The NL population had difficulties with phloem ingestion and induced a strong defense response, including callose deposition and ROS accumulation in plants of PB2013071, and as a result it was not able to colonize this accession. In contrast, the SW population was able to start sustained phloem intake very easily and only induced a very mild defense response in this accession (Sun et al., 2019). In our transcriptome analysis, plants of accession PB2013071 show a very different response to the two *M. persicae* populations. Of all genes that are differentially regulated in response to feeding of the two aphid populations, 88% are regulated in a population specific way (Figure 1B), and many more genes are regulated only by the SW population than by the NL population (Figure 2; 203 vs 63). This might be simply an effect of the longer sustained feeding of the SW population(Sun et al., 2019), or it could be due to their greater ability to manipulate plant defenses at the gene expression level.

Gene expression among different plant genotypes (resistant/susceptible) in response to one aphid population/clone has been studied frequently (Lee et al., 2017, Studham & MacIntosh, 2013, Smith et al., 2010). Also there are several studies showing the response of one plant genotype to different aphid species (Alvarez et al., 2013, Escudero-Martinez et al., 2017, Jaouannet et al., 2015). However, we found only one report of specific gene expression induced by different populations/biotypes of the same aphid species. That report describes the response of wheat to two different biotypes of the Russian wheat aphid (Botha et al., 2010). It shows that most wheat genes are regulated in a biotype specific way, and that one biotype can regulate many more genes than the other, which

is similar to our transcriptome results. To the best of our knowledge, the current study is the first one to explore the interaction between pepper and conspecific aphid populations at the whole genome gene expression level.

To identify genes regulated differently between infestations with the two aphid populations, we used a combination of two approaches. One was to find a set of DEGs comparing non-infested with NL-population infested plants, and a second set of DEGs comparing non-infested plants with SW-population infested plants, and then filter for genes occurring in only one of these sets (Figure 1B). The other approach was to find DEGs in the direct comparison between plants infested with the NL population and plants infested with the SW population. By combining these two methods we found 63 and 203 genes specifically regulated by infestation with the NL population and SW population, respectively (Figure 2).

Differentially expressed genes are involved in defense signalling pathways in pepper

When plants are able to induce defense responses against aphids, multiple signalling pathways may be elicited, including phytohormone induced pathways(Yates & Michel, 2018). Some genes that are involved in defense signalling pathways are found to specifically respond to feeding by the NL and SW populations respectively (Table S6). For example, linoleate 9S-lipoxygenase (9-LOX, rna15242) is specifically induced by the NL population, and the gene expression level in NL-infested plants is three times higher compared with the one in control plants as well as in SW-infested plants. The 9-LOX gene in pepper can induce responsive genes of salicylic acid (SA) and jasmonic acid pathways (JA), accumulate ROS and therefore enhance resistance to several microbial pathogens (Hwang & Hwang, 2010). Several genes that are involved in abscisic acid (ABA) pathway are only regulated by infestation with the SW population. The ABA-insensitive 5 (rna20904) gene is up-regulated while ABA receptors (rna12809 and rna23680) are down-regulated after SW infestation. It has been reported that over-expression of ABA receptors can promote resistance to bacteria (Lim & Lee, 2015) while a loss of function of ABA-insensitive 5 gene can impair ROS-scavenging activities in *Arabidopsis* (Li et al., 2013). The up-regulation of ABA-insensitive 5 and down-regulation of ABA receptors in pepper after feeding by the SW population may help to promote plant susceptibility. It has been shown that a virulent bacterial effector promotes plant susceptibility in *Arabidopsis* through manipulating the ABA pathway (de Torres-Zabala et al., 2007). As genes involved in the ABA pathway are only regulated by the SW population, it is possible that the SW population promotes the colonization on PB2013071 by targeting ABA pathway-related genes.

Differentially expressed genes are involved in ROS accumulation and scavenging in pepper

As we previously found that NL and SW population could induce a strong and mild ROS accumulation respectively, we speculated that some of the population specific DEGs may be related with ROS induction and scavenging. As ROS induction and scavenging is a dynamic oxidation-reduction process (Bhattacharjee, 2005), we identified DEGs involved in ROS metabolism by looking for genes predicted to be involved in oxidation-reduction processes, which is reflected by the GO annotations: GO:0016491, GO:0016709, GO:0016717, GO:0055114 and GO:0003824 (Tables 1 and 2).

Among the differentially expressed genes that specifically responded to infestation with the NL population are four up-regulated genes encoding peroxidases (Table 1), which may be involved in ROS production (Kawano, 2003). Peroxidase-dependent ROS production has been described in several studies before (Bindschedler et al.,

2006, Daudi et al., 2012). In *C. annuum*, one peroxidase (CaPO2) has been reported to be required for ROS generation and this gene has been found to enhance plant resistance against bacteria (Choi et al., 2007) and fungi (Choi & Hwang, 2012). So far there have been no reports on the involvement of peroxidase in insect resistance in pepper. The peroxidase 5 (rna8690), which is only up-regulated by NL aphids, shares high similarity with CaPO2 in amino acid sequence (Figure S3), suggesting that CaPO2 and rna8690 might be orthologous genes in *C. annuum* and *C. baccatum*. In addition, the activity of peroxidase 12 has been found to be responsible for ROS accumulation in maize and thereby to enhance resistance to the fungus *Ustilago maydis*, and inhibition of peroxidase 12 increased the infection rate of *U. maydis* (Hemetsberger et al., 2012). In our RNA-seq results, peroxidase 12 (rna10318) is the only peroxidase gene that is down-regulated in the plants treated with SW aphids. However, maize and pepper are very distantly related species and peroxidase 12 of maize shares only 50% sequence similarity with that of pepper at the protein level (Figure S4); therefore the function of peroxidase 12 may have changed and further work is needed to establish its exact role in pepper. NADPH oxidases have also been suggested to cause ROS accumulation in plant-biotic interactions(Desikan et al., 1996, Torres et al., 2002), and higher levels of NADPH oxidase activity have been seen in resistant than in susceptible wheat and maize infested with aphids (Moloi & van der Westhuizen, 2006, Sytykiewicz, 2016). A mutation of the NADPH oxidase *RBOHD* gene in *Arabidopsis* results in decreased ROS accumulation and causes increased *M. persicae* susceptibility (Miller et al., 2009). The *RBOHD* gene in PB2013071 is up and down regulated upon infestation by the NL and SW population respectively, but does not pass the criteria of $|\log_2(\text{FoldChange})|$ in our analysis (0.33 and 0.71 respectively), which suggests peroxidase-mediated ROS production may play a more important role in the pepper-aphid interaction than NADPH oxidase-mediated ROS production, at least after six hour of infestation.

Catalase (rna4866) is the most significantly up-regulated gene with oxidation-reduction process in plants of PB2013071 that specifically responded to the infestation with the SW aphid population (Table 2). Catalases are among the fastest enzymes that convert H_2O_2 to oxygen and water as they don't require a reductant(Mhamdi et al., 2010). Suppression of catalase has been found to enhance ROS levels in response to biotic stress in various plant species such as tobacco (Takahashi et al., 1997, Yi et al., 2003) and sorgum(Zhu-Salzman et al., 2004). Conversely, higher levels of catalase activity have been shown to increase susceptibility to pathogens (Valenzuela-Soto et al., 2011, Palanisamy & Mandal, 2014) and also to *M. persicae* (Divol et al., 2005). There are three main isoforms of catalases: class I, II and III (Willekens et al., 1994, Mhamdi et al., 2010). Class I catalases are highly expressed in mature leaves and include *Cat1* of *Nicotiana plumbaginifolia* (Willekens et al., 1994) and *N. tabacum* (Takahashi et al., 1997), which showed about 96% sequence identity to the catalase transcript (rna4866) of PB2013071 (Figure S5). Based on the role of catalase in ROS metabolism and plant defense, the five times higher expression level of catalase (rna4866) in leaves of PB2013071 infested by SW aphids might be one of the most important reasons why the SW population is able to colonize on pepper accession PB2013071. Serine:glyoxylate aminotransferase and alanine aminotransferase are also found to be up-regulated in PB2013071 treated by SW aphid population. They are relevant for glutathione biosynthesis and therefore are involved in ascorbate and glutathione cycle that is the major non-enzymatic ROS scavenging process (Apel & Hirt, 2004, De Gara et al., 2010). The increased activity of serine:glyoxylate aminotransferase has been found to be related with a decrease of ROS accumulation (Yang et al., 2013) and also has been found in the plants that interact with pathogens in a compatible way (Zamany et al., 2012). Additionally, the expression levels of two ferric reduction oxidase genes are also increased. Ferric reduction oxidases participate the process of H_2O_2 production and scavenging (O'Brien et al., 2012), and one ferric reduction

oxidase has been shown to block ROS accumulation in *Arabidopsis* (Einset et al., 2008). Therefore, besides catalase the higher expression of aminotransferase and ferric reduction oxidase genes may also contribute to the suppression of ROS accumulation in PB2013071 after the infestation of SW *M. persicae* population.

In summary, a strong ROS accumulation is induced in PB2013071 after feeding by the NL population, which might be caused by the up-regulation of several genes promoting ROS accumulation, including peroxidases and NADPH oxidases. Several ROS-scavenging genes are up-regulated in PB2013071 after feeding by the SW population, including catalase and aminotransferases, which may explain the mild ROS accumulation in this acccession.

The ability to suppress ROS accumulation may explain why the pepper resistance is overcome by a virulent *M. persicae* population

In previous experiments a strong defense response involving ROS accumulation was induced by aphids of the NL population on plants of PB2013071, but only a very weak response by the SW population (Sun et al., 2019). In the current study we observed in the SW-infested plants of PB2013071 the down-regulation of several genes for enzymes known to have a role in ROS production, and the up-regulation of some genes for enzymes known to have a role in ROS scavenging. The ability of the SW population to manipulate ROS metabolism of pepper plants is reflected not only by the RNA-seq data, but also by the ROS accumulation experiment combined with pre-infestation with the avirulent NL aphid population (Figure 3). The results show that SW aphids can induce removal of most of the ROS accumulated in response to the pre-infestion with NL aphids. The balance between ROS production and scavenging may determine the strength of plant defense response (Scheler et al., 2013, Guan et al., 2017). In several studies ROS accumulation has been observed in plants upon interaction with pathogens or insects, and differences in this ROS accumulation are linked with differences in plant resistance (Moloi & van der Westhuizen, 2006, Lamb & Dixon, 1997, Liu et al., 2010). Additionally, ROS accumulation in host plant is in several cases linked with the virulence or avirulence of pathogens, such as fungi (Molina & Kahmann, 2007), bacteria (Gimenez-Ibanez et al., 2009) and nematodes (Guan et al., 2017). Only very few studies have been published linking differences in ROS accumulation to differences in virulence among pest insects (Elzinga et al., 2014, Ye et al., 2017). Our results on ROS accumulation involving pre-infestation with an avirulent aphid population clearly show the ability of virulent aphids to suppress ROS formation and break down existing ROS.

The ability of virulent aphids to manipulate plant defenses may benefit avirulent aphids

Pre-infestation with the NL population does not significantly change the plant response to the subsequent infestation with the SW population (Figure 4). Based on the fact that plants of PB2013071 induce a strong defense response after infestation with the NL population (Figure 3; Sun et al., 2018), the non-effect of NL pre-infestation to the performance of SW population suggests that the SW population can cope with the plant defense responses induced by other conspecific aphid.

The pre-infestation with the SW population resulted in a significantly better performance of the NL population (Figure 5). The better performance of NL population is reflected in a higher survival of original aphids and higher number of next generation nymphs produced. The phenomenon that feeding by a virulent aphid population induces susceptibility to a conspecific avirulent population has been also observed in the interaction between soybean and

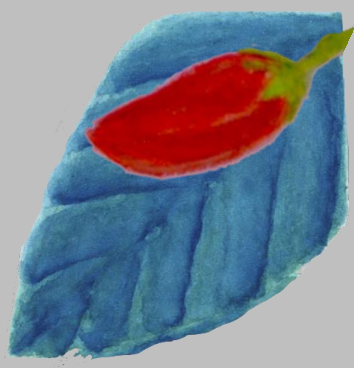
soybean aphid *Aphis. Glycines* (Varenhorst et al., 2015) as well as in the interaction between lettuce and the black current-lettuce aphid *Nasonovia ribisnigri*, although the mechanism of this induced susceptibility remained unclear (ten Broeke et al., 2017). Based on our study we can hypothesize that the benefit caused by virulent aphid populations to the conspecific avirulent aphid population might be due to manipulation of plant defense responses, especially the ROS metabolism. The expression level of pepper genes such as catalase that are induced by virulent aphids may remain high after the pre-infestation, which might help the following avirulent aphids to start phloem feeding successfully. As we removed the virulent aphids, probabaly when they were still feeding, it is possible that secreted virulent effectors continued to induce specific pepper genes.

Conclusion

Two populations of *M. persicae* (NL and SW) were previously shown to be avirulent and virulent on *C. baccatum* accession PB2013071. Our transcriptomics approach revealed genes whose expression was modified upon infestation with these aphid populations, and showed that the SW population regulated genes involved in plant defense responses, especially repressing ROS producing genes and inducing ROS scavenging genes. This paper supports the hypothesis that *M. persicae* can overcome the resistance present in accession PB2013071 probably because of its ability to manipulate plant defense response, especially the ROS metabolism.

Supplementary material

Please find the supplementary materials at the following link:
https://drive.google.com/open?id=13M4LBbCUL6GsKfqK2kXvuxDmrBhC6ADO



CHAPTER 6

General discussion

Direct and indirect damages caused by aphids significantly impair crop production in field and greenhouse cultivation of pepper. Insecticides are widely used to deal with the aphid problem. However, with growing concern about the environmental impact of insecticides and more and more reports on aphid populations developing resistance to insecticides (Silver et al., 1995, Edwards et al., 2008), some insecticides have been banned or are expected to be banned soon. Examples of insecticides that have been banned are ethyl parathion and methamidophos (Stark et al., 1995, Ren et al., 2007). Neonicotinoids, which are very effective against aphids, have been widely used during the last decades (Jeschke & Nauen, 2008), but are now also being banned because of their side effects on honeybees (Hauer et al., 2017, Odemer, 2018). Therefore alternatives are urgently needed, and host plant resistance is certainly one of them. Host plant resistance is an effective, economical, and eco-friendly approach to control aphids. There are two basic mechanisms of host plant resistance, antixenosis and antibiosis. Antixenosis, also known as non-preference, affects aphid settling or feeding through repellence or deterrence (Goggin, 2007). Antibiosis affects life history parameters and may affect aphid survival, growth, development and fecundity (Smith, 2005). In this thesis, I focus on antibiosis-based resistance because in cultivation of a crop a single variety is often grown, thus aphids have no choice but to colonize that variety or to die.

The role of wild relatives in aphid resistance breeding

Wild relatives of a crop species provide breeders with a broad gene pool of potentially useful sources of resistance, which are often not present in the cultivated materials (Hajjar & Hodgkin, 2007). For example, the glandular trichomes, which are important for resistance against *Myzus persicae* in potato and tomato, only appear in their wild relatives (Alvarez et al., 2006, Vosman et al., 2018). So far almost all the disease resistance genes in commercial tomato cultivars have been introgressed from wild genetic resources (Hajjar & Hodgkin, 2007). In potato, up to twelve resistance traits have been introgressed from related wild species (Hirsch et al., 2013). Wild relatives are also very important in pepper breeding. Resistance to anthracnose, thrips and virus has been mostly found in wild pepper accessions (Fery & Schalk, 1991, Kim et al., 2010a, Maharijaya et al., 2011, Retes-Manjarrez et al., 2017).

Germplasm screening among pepper species, including wild relatives, is a good approach to uncover sources of resistance to aphids. In my thesis I evaluated the level of resistance to *M. persicae* in 74 pepper accessions of four *Capsicum* species. Accessions that are resistant to *M. persicae* were found in the species *C. baccatum* (Chapter 2, Sun et al., 2018). Although *C. baccatum* itself is also cultivated (Pickersgill, 1991), it is a taxon separate from the *C. annuum* complex and rarely grown outside South America (Bosland et al., 2012).

Compared with tomato and potato, transferring the resistance from wild pepper relatives to commercial species by introgression breeding may pose a bigger challenge. The hybridization between *C. baccatum* and *C. annuum* is very difficult because of post-fertilization barriers (Eshbaugh, 1970). These barriers are probably due to two major reciprocal translocations between chromosomes 3 and 5 and between chromosomes 3 and 9 (Lee et al., 2016)(Chapter 3). However, hybrids between *C. baccatum* and *C. annuum* can be obtained using embryo rescue (Yoon et al., 2005). Also it is possible to use *C. chinense* as a bridge species between *C. baccatum* and *C. annuum* (Manzur et al., 2015). Both methods can be used to introgress Quantitative Trait Loci (QTLs) for aphid resistance from *C. baccatum* into *C. annuum*.

Genes involved in aphid resistance

QTLs for aphid resistance have been detected in several crops. For instance, resistance to *Aphis glycines* in soybean was mapped to a 115 kb region on the soybean genome and predicted to be controlled by one of the nucleotide binding leucine-rich repeat (NBS-LRR) genes in that region (Kim et al., 2010b). Similarly, a resistance QTL in cucumber against *A. gossypii* was genetically mapped and predicted to be controlled by a leucine-rich repeat receptor-like protein kinase (LRR-RLK) gene (Liang et al., 2016). NBS-LRR and LRR-RLK genes are two important groups of resistance genes against biotic stresses in plants. Compared with NBS-LRR genes (Klingler et al., 2005, Klingler et al., 2009, Ohnishi et al., 2012), LRR-RLK genes conferring aphid resistance have been identified less frequently. However, they have been frequently found to control host resistance against pathogens (Gómez-Gómez & Boller, 2000, Fradin et al., 2009, Krol et al., 2010), nematodes (Mendy et al., 2017) and planthoppers (Liu et al., 2015). In Chapter 3 of this thesis, a major resistance QTL was fine mapped to a genetic region predicted to encode members of the LRR-RLK subfamily according to the recently released *C. baccatum* "PBC81" genome (Kim et al., 2017), which adds evidence that LRR-RLK genes can be involved in host plant resistance against aphids.

Besides plant resistance genes, plant susceptibility genes (*S* genes) can also be used to increase aphid resistance. Insects turn to pests on plants because they have the ability to suppress defense responses of plants and by doing so they enable colonization. Some of these pests can activate certain plant genes, the *S* genes, to avoid or impair plant defense responses, resulting in the susceptibility of the host plant (Giordanengo et al., 2010, Louis & Shah, 2013). Loss of function of such *S* genes can lead to plant resistance to insect pests, and this kind of resistance is suggested to be durable (Fukuoka et al., 2009, Lapin & Van den Ackerveken, 2013, van Schie & Takken, 2014). For example, the resistance based on the loss of function of the *Mlo* gene in barley, which shows resistance to powdery mildew, is already holding for more than 70 years (Freisleben & Lein, 1942, Lyngkjær et al., 2000). Although research on *S* genes started with susceptibility to pathogens, many reports show that plant *S* genes are also important in resistance against insects (Broekgaarden et al., 2015). Examples are: The loss of function of the *fatty acid desaturase 7* gene enhances tomato resistance to *M. persicae* (Avila et al., 2012). Silencing of *Mayetiola destructor susceptibility 1* confers immunity to all Hessian fly biotypes in normally susceptible wheat genotypes (Liu et al., 2013). Rice mutants deficient in brassinosteroids show higher resistance against brown planthopper than the wild-type rice (Pan et al., 2018). In my thesis, the RNA-seq study on the compatible interaction between pepper accession PB2013071 and virulent aphid population SW revealed a list of putative susceptibility genes in pepper to *M. persicae* (Chapter 5, Table S6). *Catalase* is one of them. The expression level of catalase in aphid-infested plants was 4 times higher than in control plants. The *fatty acid desaturase 4* gene was 9 times up-regulated after aphid infection, which also suggests that it might be a pepper susceptibility gene (Chapter 5, Table S6). *Abscisic acid-insensitive 5* might be another one. It was up-regulated only after the infection with the virulent aphid population, and it has been shown that *Abi5* mutant plants have lower ROS-scavenging activities (Li et al., 2013). All of these putative genes need to be functionally analysed before they are confirmed as *S* genes. For instance, candidate *S* genes have been (partially) silenced using RNA interference, which has resulted in plants becoming resistant (Yu et al., 2016a). Recently, a new efficient approach for obtaining mutations in target genes, CRISPR-Cas9, was developed. This allows the accurate knock-out of target *S* genes (Peng et al., 2017, Lu et al., 2018, Zaidi et al., 2018). However, not every potential *S* gene can be silenced to enhance plant resistance, because

some genes are essential for plant growth or loss of function may come with a fitness cost. *Catalase* is one of those genes that cannot be silenced to increase aphid resistance. Without catalase, plants would suffer severe cellular damage and cannot develop (Dat et al., 2001). In order to apply the loss of *S* gene function in practical resistance breeding, the effect knocking out the *S* gene needs to be thoroughly studied.

Phloem-based resistance against aphids

As aphids obtain nutrition only from the phloem, phloem-based resistance could lead to aphid starvation or poisoning and directly affect aphid performance (Stewart et al., 2009). Phloem-based resistance has been demonstrated in many crops such potato (Alvarez et al., 2006), soybean (Chandran et al., 2013), lettuce (ten Broeke et al., 2013b) and wheat (Khan et al., 2015). Successful colonisation of plants by virulent aphids may depend on the ability to overcome phloem-based resistance. Chapter 4 showed that for the avirulent *M. persicae* population it was almost impossible to take up phloem sap while the virulent *M. persicae* population was able to continue phloem feeding for a prolonged time. Similar observations were made in other plant-aphid interactions (Pallipparambil et al., 2010, ten Broeke et al., 2013b).

One possible mechanism of phloem-based resistance is occlusion of the phloem vessels in response to aphid feeding, which may result from callose deposition (Mondal et al., 2017, Varsani et al., 2019). Callose deposition was observed in the phloem of *C. baccatum* accession PB2013071 infested by *M. persicae* populations (Chapters 2 and 4, Sun et al., 2018). Callose deposition was found to be weaker in plants infested with a virulent population compared to plants infested with an avirulent population (Chapter 4, Sun et al., 2019), which also suggests that the ability of aphids to prevent callose deposition may determine the virulence level of aphids on pepper. However, expression of the callose synthase genes only started to increase 6-hour after the start of the infestation (Chapter 2, Sun et al., 2018), which makes it unlikely that callose deposition is important in the early response towards insects.

There may be other mechanisms of phloem-based resistance that respond faster than callose deposition and occlude phloem vessels in response to aphid feeding. In Chapter 4 I showed that the difference in phloem feeding between avirulent and virulent *M. persicae* aphids already appeared during the first 4 hours after the infestation started (Sun et al., 2019). Coagulation of phloem proteins is one of fast reacting resistance mechanisms (Tjallingii, 2006), which can take place in plant phloem vessel only one minute after being damaged (Furch et al., 2007). It has been best studied in legumes, involving forisome proteins (Peters et al., 2006). So far there is only limited information on phloem proteins in other species (Knoblauch et al., 2014, Garzo et al., 2018). Whether phloem protein plugging also contributes to the phloem-based resistance in the pepper PB2013071 against aphids needs further study. Additionally, plants can also induce phloem-based resistance to aphids without occluding phloem vessels. A heat-shock gene *SLI1* in *Arabidopsis* is able to restrict aphid feeding by increasing the firmness and thickness of the sieve tube margin, which functions before phloem occlusion (Kloth et al., 2017). Although *SLI1* is only induced under heat stress, it may be interesting to look for other genes with similar aphid resistance mechanisms but functioning under normal temperatures.

The accumulation of reactive oxygen species (ROS) may also contribute to phloem-based resistance. ROS accumulation was mainly observed along the leaf veins of *C. baccatum* accession PB2013071 infested by *M.*

persicae and confined to small spots, suggesting a very local reaction, which would support the hypothesis that ROS may be acting as a local toxin in phloem vessels (Chen & Schopfer, 1999, Liu et al., 2010). However, it cannot be excluded that it (also) may act as a signal component to activate downstream defense enzymes, similar to what was found in other plant-aphid interactions (Moloi & van der Westhuizen, 2006, Kuśnierczyk et al., 2008). In Chapter 5 I showed that the virulent *M. persicae* population was able to colonize plants of PB2013071 through inducing the expression of ROS scavenging genes while inhibiting the expression of ROS production genes in the host. The manipulation of plant gene expression was supported by the ROS accumulation assay (Chapter 5). How these ROS related genes are induced is unclear yet, but deserves further attention.

Aphid effectors

Aphid effectors, proteins that can modulate plant defense responses, are important components in plant-aphid interactions (Wu & Baldwin, 2010). Aphid effectors have been reported to modulate plant processes beneficial for aphid colonisation, e.g. suppress plant defence, but also elicit effector-triggered immunity when a resistant host plant recognizes the effector (Hogenhout & Bos, 2011, Elzinga & Jander, 2013).

Compared with the mature research on pathogen effector biology in plant-microbe interactions, knowledge on aphid effectors is still in an embryonic stage. In order to understand the detailed role of aphid effectors in plant-aphid interactions, effectors need to be identified. Initially, effectors were isolated and identified from collected aphid saliva, which was obtained through the use of defined artificial diets (Harmel et al., 2008, Carolan et al., 2011). However, later it was found that the composition of aphid saliva injected into artificial diets might not reflect the real composition secreted into plant tissue because aphids are able to adapt salivary secretion in dependence of the stylet milieu (Will et al., 2012). Recently, with the help of transcriptomic approach, the study on aphid effectors has sped up.

Table 1 lists known aphid effectors. Most effectors in the list can enhance aphid fecundity during the colonization. Except C002, all effectors have been only identified in one aphid species but that does not mean that these effectors are species specific. The highest number of effectors were identified in the interaction between *Arabidopsis* and *M. persicae*.

Table 1. Summary of currently identified aphid effectors

Effector	Aphid species	Function	Molecular activity	Reference
C002	*Acyrthosiphon. pisum*; *Myzus. persicae*	Aphid fecundity enhancement	So far unknown	(Mutti et al., 2006, Bos et al., 2010, Pitino et al., 2011)
Mp10	*M. persicae*	Aphid fecundity reduction	Regulation of JA and SA pathway	(Bos et al., 2010, Rodriguez et al., 2014)
Mp42	*M. persicae*	Aphid fecundity reduction	Formation of motile ER aggregation products	(Bos et al., 2010, Rodriguez et al., 2014)
Mp55	*M. persicae*	Plant defense suppression	Suppression of 4-methoxyindol-3-ylmethylglucosinolate, callose and hydrogen peroxide in plant	(Elzinga et al., 2014)
Mp1/PIntO1	*M. persicae*	Aphid fecundity enhancement	Targets trafficking protein VPS52 in plant, and localizes to the sheaths surrounding aphid stylets at feeding sites.	(Pitino & Hogenhout, 2013, Mugford et al., 2016, Rodriguez et al., 2017)
PIntO2	*M. persicae*	Aphid fecundity enhancement	So far unknown	(Pitino & Hogenhout, 2013)
Me10	*Macrosiphum euphorbiae*	Aphid fecundity enhancement	Targets TFT7 in tomato	(Atamian et al., 2013, Chaudhary et al., 2018)
Me23	*M. euphorbiae*	Aphid fecundity enhancement	So far unknown	(Atamian et al., 2013)
Armet	*A. pisum*	Aphid feeding promotion	Induction of anti-pathogen reaction in plant	(Wang et al., 2015)
Me47	*M. euphorbiae*	Aphid fecundity enhancement	Glutathione-S-transferase	(Kettles & Kaloshian, 2016)

Effector Mp55 can suppress a plant defense response by manipulating callose deposition and ROS accumulation, and Mp1 and PIntO2 can improve aphid reproduction on the host plant. Impaired ROS accumulation and callose deposition were also found when virulent *M. persicae* population colonized on accession PB2013071 (Chapter 4, Sun et al., 2019). However, without experimental evidence, it is still unknown whether these identified effectors also affect the virulence of the virulent population on accession PB2013071. Functional analysis of aphid effectors can be achieved through gene silencing in the aphid by RNA interference (Coleman et al., 2014, Tariq et al., 2019), or by transient overexpression of the effector gene in the plant (Bos et al., 2010). Further exploration of the candidate effectors that affect *M. persicae* virulence on accession PB2013071 could be started by comparing transcriptomic data of salivary glands between avirulent and virulent populations. Genes with a higher expression level in the virulent than in the avirulent population would be putative effectors that promote aphid virulence. It is also interesting to know whether effectors from the virulent *M. persicae* population target resistance genes and change the downstream defense pathways, or whether they directly target the genes involving in the downstream defense pathways, and whether pepper resistance genes recognize avirulent effectors to induce a plant defense response.

Aphid symbionts

Aphid endosymbionts play an instrumental role in plant-aphid interaction (Sugio et al., 2014). Symbionts are indispensable for aphids, because they supply essential nutrients which aphids cannot obtain from plant phloem (Douglas, 1998). Besides a basal function in aphid survival support, they are also involved in aphid resistance

against parasitoids and fungi (Schmid et al., 2012, Łukasik et al., 2013), host plant specialisation (Tsuchida et al., 2004) and body colour change (Tsuchida et al., 2010).

It has been hypothesised that aphid symbionts may also play a role in aphid virulence. For instance, the abundance of *Buchnera aphidicola*, the primary symbiont species in *M. persicae*, could improve settlement of *M. persicae* on host plants (Machado-Assefh et al., 2015). Similarly, aphid-associated bacteria from the order *Enterobacteriales* facilitate virulence of *D. noxia* on wheat (Luna et al., 2018). Differences in symbiont gene expression patterns in aphids may also be related with different levels of virulence on host plants (Francis et al., 2010). Given that two *M. persicae* populations used in this thesis were collected from different regions in Europe, it is possible that there are differences in symbiont species, abundance and gene expression between the two populations, and that such differences are related to *M. persicae* virulence on accession PB2013071. Related experiments need to be performed in the future.

Compatible and incompatible plant-aphid interactions

The compatible and incompatible interactions between aphids and their host plants are often hypothesized to follow the zigzag model (Smith & Boyko, 2007, Yates & Michel, 2018), in which the ultimate outcome is plant resistance or susceptibility (Figure 1).

When aphids attack a plant, herbivore-associated molecular patterns (HAMPs) from aphid saliva including aphid symbionts may be recognized by pattern recognition receptors (PRRs), causing pattern-triggered immunity (PTI) (Hogenhout & Bos, 2011, Chaudhary et al., 2014). PRRs often belong to the family of receptor-like kinases (RLKs) (Zipfel, 2014), which are also the candidate genes underlying the resistance QTL for aphid reproduction in pepper (Chapter 3). PRRs can regulate downstream genes and induce defense responses involving phloem protein plugging (Medina-Ortega & Walker, 2015, Garzo et al., 2018), callose deposition and/or ROS accumulation, resulting in an incompatible plant-aphid interaction (Chapter 4)(Shoala et al., 2018, Sun et al., 2019). Callose synthases (CalS) are responsible for callose deposition (Chapter 2)(Kuśnierczyk et al., 2008, Sun et al., 2018). ROS accumulation can be achieved by regulating peroxidases and/or NADPH oxidases (Chapter 5)(Miller et al., 2009, Pandey et al., 2017) (Figure 1A).

In order to colonize plants successfully, aphids may carry effectors that suppress PTI, leading to effector-triggered susceptibility (ETS) (Rodriguez & Bos, 2013, Chaudhary et al., 2018) (Figure 1B). The suppression of PTI could be managed through manipulation of ROS metabolism and impairment of callose deposition, with which aphids are able to sustain phloem ingestion (Chapter 4, Sun et al., 2019). Aphids manipulate ROS metabolism in plants probably by down-regulating ROS producing genes and up-regulating ROS scavenging genes (Chapter 5).

In turn, some plants may produce resistance (R) proteins that recognize aphid effectors and thus restore resistance through effector-triggered immunity (ETI) (Bos et al., 2010, Li et al., 2015). Most R proteins belong to the NBS-LRR family and contains a nucleotide-binding site (NBS) and a leucine-rich repeat domain (LRR) (Białas et al., 2017). Despite the variations in the protein recognition during PTI and ETI, the involved defense responses in plants usually overlap (Peng et al., 2018) (Figure 1A).

However, ETI may not be activated resulting in compatible plant-aphid interaction. The deactivation of ETI may be due to a lack of effectors that are recognized by a R protein (Drurey et al., 2017) or because a R-protein is absent, and/or the resistance response is suppressed at a later stage (Zhuo et al., 2017) (Figure 1B). Which mechanism(s) is(are) involved need to be elucidated through a detailed study of the plant-aphid interaction on a case by case basis.

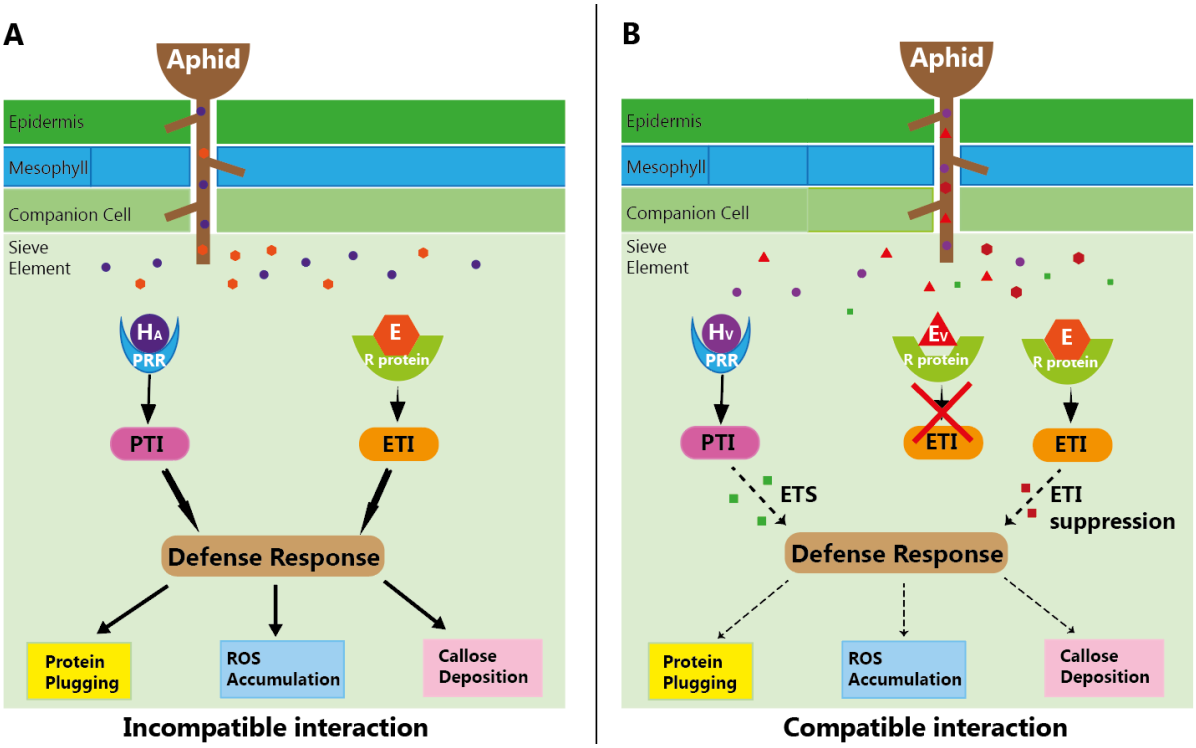


Figure 1. Model explaining incompatible and compatible plant-aphid interactions. Avirulent and virulent aphids use their stylets to ingest plant phloem. Saliva is secreted during probing and feeding. Herbivore-associated molecular patterns (HAMPs, such as H_A and H_V) from the saliva of both kinds of aphids might be recognized by pattern recognition receptors (PRRs), and induce pattern-triggered immunity (PTI). The PTI involves defense responses, which may include plugging of the phloem by proteins, ROS accumulation and callose deposition. To circumvent/suppress plant defences, aphids may carry specific effectors resulting in effector-triggered susceptibility (ETS). In turn, some plants may respond by producing specific resistance (R) proteins that recognize the effectors of the aphid, resulting in effector-triggered immunity (ETI). The defense responses involving in ETI normally overlap with those in PTI. **(A)** Plant resistance to avirulent aphids might be caused by induction of PTI, due to recognition of H_A, or by induction of ETI, due to recognition of effecor (E). **(B)** Plants may be susceptible to virulent aphids because both PTI and ETI are (at least partially) suppressed, perhaps due to ETS triggered by some effectors, or failure of R proteins to recognize effectors, or suppression of ETI.

Management of aphid resistance in crop cultivation

The emergence of virulent aphid populations is a challenge in sustainable crop protection, and this emergence is accelerated under high human-imposed selection pressure in agricultural production (Via, 1990), especially in crop systems where cultivars with only one aphid resistance gene are grown (Karban & Agrawal, 2002). The virulent *M. persicae* population described in this thesis is probably not the result of growing resistant pepper varieties, because it was collected more than 30 years ago and reared on pea plants since then (Chapter 4, Sun et al., 2019). However, it cannot be excluded that the population originates from aphids that have been in contact with pepper

plants prior to their transfer to beans and maintained this virulence ever since. When we evaluated several other *M. persicae* populations collected from cultivated pepper plants in different areas of the Netherlands we found that PB2013071 was resistant to them (data not shown), which suggests that PB2013071 is still good source of aphid resistance that can be very useful in pepper cultivation.

When growing aphid resistant varieties, it is very important to control current virulent populations and avoid the emergence of new virulent populations. Partial resistance has the potential to delay the emergence of virulent populations when implemented as a component of integrated pest management (IPM) (McCreight & Liu, 2012). The combination of multiple resistance genes in one plant variety may also delay the emergence of virulent populations. Soybean plants carrying two resistance genes against *A. glycines* have been shown to control *A. glycines* populations more effectively, compared to varieties with only one of the two resistance genes (Wiarda et al., 2012). Growing multiple varieties differing in aphid resistance mechanisms in one field may also decrease the chance of virulence emergence (Bregitzer et al., 2012). Moreover, if markers for virulence genes in aphid populations can be developed, local aphid populations can be monitored and pepper varieties with the most effective resistance genes can be grown.

Final conclusions

Resistance to aphids can help to reduce the aphid problem in pepper cultivation. In this thesis I show that pepper accessions with a high level of resistance against *M. persicae* can be found. QTL mapping shows that the resistance in the most resistant accession is determined by one major QTL and markers flanking the QTL are identified. These markers allow the introgression of this aphid resistance gene into commercial pepper varieties. The resistance mechanisms in the resistant pepper accession include impairing aphid phloem feeding, accumulation of ROS and formation of callose. Considering that virulent aphid populations are a big challenge in sustainable crop protection, the appearance of a virulent *M. persicae* population needs to be further studied. A new hypothesis advanced in this thesis is that *M. persicae* can overcome resistance in pepper through regulating genes related with ROS metabolism in plant. This hypothesis will have to be verified in the future and if proven can provide a better insight into the virulence mechanism of aphid species on other crops as well.

Summary

Aphids are the one of the most serious pest insects in crops around the world. They penetrate the plant tissue with their stylets and feed from the phloem vessels. Their infestation causes economic losses by negatively affecting crop growth, development and quality of the harvested product, as well as by transmitting viruses. To control aphid population growth in crops, using aphid resistant varieties may be a sustainable and environmentally friendly strategy. The green peach aphid, *Myzus persicae*, is an economically important pest of cultivated pepper plants (*Capsicum*). Unfortunately, there is no pepper variety resistant against *M. persicae* and no sources of aphid resistance have been used in pepper breeding so far. The aim of this PhD-thesis was to obtain more knowledge about aphid resistance in pepper and the pepper-aphid interaction, including identification of new sources of resistance against *M. persicae*, elucidation of resistance mechanisms, identification of Quantitative Trait Loci (QTLs) functioning in aphid resistance and exploration of the virulence mechanism of *M. persicae* on pepper.

In **Chapter 1**, I summarised the relevant research on plant-aphid interactions, especially on the genetic and molecular mechanisms underlying plant resistance to aphids and on the occurrence of virulent aphid populations on plants. Background information about the *M. persicae* problem on pepper is also presented.

In order to find pepper materials showing a good level of resistance to *M. persicae*, 74 pepper accessions from different geographical areas were screened (**Chapter 2**). After four rounds of evaluation, one *Capsicum baccatum* accession (PB2013071) was identified as highly resistant to *M. persicae*, and the accessions PB2013062 and PB2012022 showed intermediate resistance. These three accessions will be important for breeding aphid resistant pepper varieties in the future. A QTL analysis was then carried out for *M. persicae* resistance in an F2 population derived from an intraspecific cross between a susceptible *C. baccatum* accession and the highly resistant accession PB2013071 (**Chapter 3**). Using interval mapping I detected two QTLs affecting aphid survival and reproduction respectively, both localized in the same area and sharing the same top marker on chromosome 2. Further fine mapping confirmed the effects of the two QTLs and narrowed the major QTL affecting aphid reproduction down to a genomic region predicted to encode four members of the subfamily of leucine-rich repeat receptor-like protein kinase (LRR-RLK).

Since the development of virulent aphid populations is a risk when growing resistant crop varieties, as well as a challenge in sustainable crop protection, the potential mechanism by which a virulent *M. persicae* population manipulates a pepper plant was studied. In **Chapter 4**, recordings of electronic penetration graph (EPG) showed that a virulent *M. persicae* population was able to devote significantly more time to phloem ingestion than an avirulent population. I also found that plants induced a stronger defense response after infestation by the avirulent population than after infestation by the virulent population, including stronger accumulation of reactive oxygen species (ROS) and more formation of callose. The transcriptomics approach used in **Chapter 5** revealed genes from which the expression is differentially regulated upon infestation with these two *M. persicae* populations, genes which are probably involved in the compatible and incompatible *M. persicae*-pepper interactions. The avirulent population induced ROS production genes, while the virulent population induced ROS scavenging genes and repressed ROS production genes. I also found that the virulent population was able to induce the removal of ROS which accumulated in response to pre-infestion with the avirulent population, and that pre-infestation with the virulent population significantly improved the performance of the avirulent population. These two chapters

support the hypothesis that a virulent *M. persicae* population can overcome resistance through suppressing plant defense responses, especially by manipulating the ROS metabolism and such ability may benefit avirulent conspesific aphids.

The results presented in Chapters 2 to 5 are discussed in the context of recent literature in **Chapter 6**, with several topics being emphasized: 1) the role of wild relatives in crop breeding programs aimed at aphid resistance and resistance introgression from wild relatives into commercial varieties; 2) genes that can be used in aphid resistance breeding; 3) possible mechanisms of phloem-based resistance in crops that impair aphid feeding; 4) aphid effectors and symbionts that may help aphids to break phloem-based resistance and 5) discussions on how to keep aphid resistance durable in crop cultivation.

Taken together, in this thesis I identify good sources of pepper resistance against *M. persicae*, detect resistance QTLs that can be introgressed into commercial varieties and provide a hypothesis that can help to understand the virulence mechanism of *M. persicae* on pepper.

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Acknowledgements

I am writing the acknowledgements, which means that my PhD is close to the end. Before I started my PhD, I was already considering to finish in time. However I did not set it as my target, because I knew it would be very difficult. I just wanted to learn and to enjoy the whole process. Surprisingly, I do finish my PhD in time and have submitted my thesis in the 46th month. I am excited about this achievement. In the meantime, I clearly know that this achievement could not have been obtained without the help of many people around me.

Thank you, **Ben**! Thanks for giving me the chance to start PhD research in insect resistance in Plant Breeding. I appreciated it very much that you offered me the job despite my limited experience in plant genetics and breeding. I have also appreciated that you patiently guided me to start the project. 'My door is always open for you', you said to me all the time. Because of your patience and kindness, I could come to you anytime I want and I could get help anytime I needed. We made plans for the experiments, discussed the new directions in my project and shared freshly published papers. You have been involved in every part in my PhD research and I would have liked to stay with you longer in the coming years, but at the end I changed my mind for which I am sorry. Your kind support made me feel safe and stable. Like a baby bird needs to fly away when it is strong enough, I think I also need to leave and meet more challenges by myself. Now it is the time. As I am still working in the industry of plant breeding, I am looking forward to working with you again in the future.

Thank you, **Roeland**! Thanks for sending me a lot of suggestions in the set-up of my experiments, data analysis and manuscript writing. I was afraid to talk with you in the beginning of my PhD, because I was not able to understand what you said. But after I got used to your speaking way, I realized you are a very nice person and your feedback is very useful. Thanks a lot for understanding my choice after my PhD. I have appreciated your accompany in the last four years.

Thank you, **Richard**! Thanks for giving me permission to stay in Plant Breeding department as a PhD candidate. Thanks for your time to talk about my project regularly. Thanks for your fast feedback of my manuscripts and thesis. Thanks for your kind words to Monsanto when I put you up as referee. Also thanks for giving me the chance to talk with you personally at the end of my PhD.

Thank you, **Wendy**! Although you are officially not my supervisor, you gave me high-quality supervision in the lab and in the greenhouse at the beginning of my PhD. You guided me to start the first important phenotyping and genotyping experiments. Without your kind support, I cannot carry out my experiments effectively and efficiently. Thanks for helping me to take care of my insects during my holidays. Also thanks for helping me to take the heavy work of 'insect dissecting' during my maternity leave. I have appreciated that you always stand by me when I need help in my experiments. And of course, thanks for being my paranymph!

Thank you, **Pauline**! I cannot imagine what my PhD would have looked like without you. It is my honor to experience the toughest period together with you. I still remember the time when Ben introduced you to me. We met in the thrips climate chamber and you were wearing magnifying glasses. In the last four years, we shared the good and bad news with each other. We talked scientifically and personally. We attended conferences and workshops together. I really enjoyed staying with you. I have appreciated that you gave me a lot of valuable

suggestions on job interviews. Now both of us are working in companies in Wageningen. I hope we can still meet each other and be good friends! Also thank you for being my paranymph!

Thank you, **Arwa**! I feel lucky to stay in the same office with you. When I saw you the first time, I was surprised by your beauty. Later on, I found you are not simply a beautiful woman. You are a professional and skilled researcher, and you work so hard. I think I definitely cannot finish my PhD on time without staying with you. Your spirit of hard work drove me to work hard and take use of time efficiently. I enjoyed sharing with you every small piece of my achievements in my PhD research and in my personal life. Thanks a lot for giving me suggestions when I needed help and supporting me when I was sad. Thanks for the tasty Syrian food. Let's keep in touch in the future! Thank you, **Giorgio**! I am very happy that we three have sit together in the last four years. We talked about politics, shared traveling experiments and made local food. Thanks for giving me travel tips when I planned to travel in Italy. I wish you and Paola can buy a satisfying house in Utrecht and have a happy life in the future!

Thank you, **Sean**! You are so sweet to my pepper plants. They cannot grow well in Unifarm and the insect greenhouse without your care. You gave them water and nutrients, kept track of their growth and paid attention to exterior 'attackers'. Also thank you, André Maassen, Maarten Peters, Teus van den Brink and Bert Essenstam! I have appreciated your kind help in Unifarm during my PhD.

Many thanks to all the colleagues at Plant Breeding, who were always kind to give me a hand in my PhD project. Thank you, **Olga Scholten**! Thanks a lot for your great effort in organizing the research programme 'Groene Veredeling' to fund our research project. You are very nice and sweet colleague! Also thanks to Martijn van Kaauwen, Marian Oortwijn, Doret Wouters, Johan Bucher, Isolde Bertram, Dianka Dees, Fien Meijer-Dekens, Irma Straatman, Linda Kodde, Annelies Loonen and our secretaries. Thank you, **Nicole**! You are like my older sister. I really enjoyed talking with you, and I love your laugh! Thanks to Danielle van der Wee-Uittenbogaard and Letty Dijker for helping me with reimbursement and appointment arrangements. Also thanks to Gerrie Wiegers, Petra van Bekkum and Martin Verbeek for helping me to take care of my insects during my holidays and maternity leave.

Thanks to all my fellow PhDs at Plant Breeding. Thank you, **Charlotte**! I am very glad to stay with you in the last four years, with sharing happy and unhappy things as well as following Dutch courses together. Good luck for your new job in France! Many thanks to Carolina Aguilera Galvez, Miguel Santillan Martinez, Jeroen Berg, Jarst van Belle, Kim Magnee, Sri Sunarti, Katharina Hanika, Jasper Vermeulen, Viviana Jaramillo, Andres Torres, Ashikin Nurashikin Binti Kemat, Atiyeh Kashaninia, Cynara Romero, Anne Giesbers, Shuhang Wang, Lorena Ramirez, Eliana Papoutsoglou. It is my pleasure to work with you and thanks a lot for supporting me during my PhD. Also thanks to Sandeep Sarde and Niccolò Bassetti. I truly enjoyed exchanging research ideas with you in the past several years. I hope you will have a wonderful career in the future!

Many thanks to my Chinese friends and colleagues in Wageningen. Thank you, **Xiao**! You are the first Chinese whom I met at Plant Breeding. Thanks for giving me a lot of suggestions when I had problems. I wish you enjoy your work and have a happy life with Jia in England. Thank you, **Kaile**, **Yiqian** and **Xiaoxue**! I really enjoyed the time that you were around me. I wish everything for you is going well in China. Thank you, **Xuexue** and **Huayi**! It was a pleasure to start my PhD at the same time as you. I wish you find a satisfying job in the future. Thank

you, **Jinbin Wu**! Thanks for guiding me in the lab of Phytopathology. A lot of wishes to you for your research and career. Thank you, **Rufang Wang**! I feel lucky to know you before we came to Wageningen. I wish you finish your experiments with nice scientific papers. Thank you **Ran An**! I have enjoyed living door to door with you for two years. You are a smart and hardworking girl! I wish you achieve your academic targets in the following several years. Thanks to Xinfang Wang, Yanling Liao, Ying Liu, Yi Wu, Li Shi, Xulan Wang, Xing Wang, Peirong Li, Hao Hu, Lina Lou at Plant Breeding. Thanks for accompanying me during lunch.

Special thanks to my friends outside academia. Thank you, **Aojia** and **Guanlin**! Thanks a lot for giving us support in the last several years. We cannot imagine how difficult our life would be without your support. It is our luck that you will still be around us in the future. Thank you, **Yating**! You are our sweet younger sister. It is a pity that we are living far away from each other. We enjoyed travelling with you during our holidays. Let us arrange again, with Indy. Thank you, **Xiaoning (Nina)**! Without you I would definitely not have come to the Netherlands. Thanks for helping me to design my thesis cover. Best wishes to you for your career and for your life with Stefano.

Thank you, my dear **Wei**! Without you I would never ever have started a PhD. You gave me courage to leave China and develop myself in the Netherlands. Thanks for your love and support in our daily life in the past and future. Now we have our sweet daughter Indy. I believe we will have a much better and happier life later in the Netherlands. 爱你哦宝贝!

谢谢我的爸爸妈妈、公公婆婆！感谢你们在背后默默地理解与支持。谢谢妈妈不远万里、不辞辛劳过来帮我照顾宝宝，我和宝宝都非常享受那一段来之不易的时光。真的非常抱歉这么多年一直无法待在你们身旁陪伴你们，接下来可能也还是一样。以后我们一定会尽可能想办法和你们多呆一些时日。照顾宝宝你们不用担心，希望你们平时也可以多有一些自己的空间，多多享受生活，多多和朋友玩耍。身体健康、开心快乐是最重要的!

Many thanks! 非常感谢!

Mengjing Sun (孙梦婧)

September 2019

About the author

Mengjing Sun (孙梦婧), was born on February 26th 1989, in Linyi, China. She started her Bachelor study in Yangzhou University, China in 2006. Since then she has worked with insects for more than 10 years. During her Bachelor study, which lasted for four years, she showed interests in the courses of entomology especially insect ecology and then spent her thesis time in the research on dynamics of insect species on different transgenic cotton plants.

After receiving her Bachelor degree in 2010, she started her Master study in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPP-CAAS). There she changed her research direction from insect ecology to insect molecular biology. She studied the function of several important olfactory genes in the antenna of diamond back moth (*Plutella xylostella*).

In 2013, after finishing her Master study, she started a job as marketing assistant in China representative office of KWS SAAT SE. Working in a plant breeding company inspired her to learn more about plant genetics and breeding.

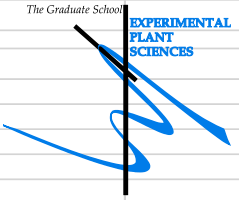
In October 2015 she got the opportunity to start PhD at Plant Breeding in Wageningen Univiersity & Research. In her PhD project, she focused on aphid resistance in *Capsicum baccatum* and pepper-aphid interaction under the supervision of Prof. Richard G.F. Visser, Dr. Ben Vosman and Dr. Roeland Voorrips. The outcome of this PhD project is described in this book. In October 2019 she started a new career as Brassica Breeding Scientist in Bayer (Wageningen).

List of Publications:

1. **Mengjing Sun**, Yang Liu, Guirong Wang. Expression patterns and binding properties of three pheromone binding proteins in the diamondback moth, *Plutella xylostella*. Journal of Insect Physiology, 2013, 59(1): 46-55.
2. **Mengjing Sun**, Yang Liu, William B. Walker, Chengcheng Liu, Kejian Lin, Shaohua Gu, Yongjun Zhang, Jingjiang Zhou, Guirong Wang. Identification and characterization of pheromone receptors and interplay between receptors and pheromone binding proteins in the diamondback moth, *Plutella xylostella*. PloS One, 2013, 8(4): e62098.
3. **Mengjing Sun**, Roeland E. Voorrips, Greet Steenhuis-Broers, Wendy van't Westende, Ben Vosman. Reduced phloem uptake of *Myzus persicae* on an aphid resistant pepper accession. BMC Plant Biology, 2018, 18(1): 138.
4. **Mengjing Sun**, Roeland E. Voorrips, Ben Vosman. Aphid populations showing differential levels of virulence on *Capsicum* accessions. Insect Science, 2019, DOI: https://doi.org/10.1111/1744-7917.12648.
5. **Mengjing Sun**, Roeland E. Voorrips, Wendy van 't Westende, Martijn van Kaauwen, Richard G.F. Visser and Ben Vosman. Aphid resistance in *Capsicum* maps to a locus containing LRR-RLK gene analogues. Theoretical and Applied Genetics, 2019, accepted.
6. **Mengjing Sun**, Roeland E. Voorrips, Martijn van Kaauwen, Richard G.F. Visser and Ben Vosman. The ability to manipulate ROS metabolism in pepper may affect aphid virulence. (under review)

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Mengjing Sun
Date: 19 November 2019
Group: Plant Breeding
University: Wageningen University & Research

1) Start-Up Phase		date	cp
►	**First presentation of your project**		
	Title: Genetics and mechanism of Aphid resistance in Capsicum	11 February 2016	1,5
►	**Writing or rewriting a project proposal**		
	Genetics and mechanism of Aphid resistance in Capsicum	February 2016	2,5
►	**Writing a review or book chapter**		
►	**MSc courses**		
	Subtotal Start-Up Phase		4,0

2) Scientific Exposure		date	cp
►	**EPS PhD student days**		
	Get2Gether 2016	28 & 29 January 2016	0,6
	Get2Gether 2018	15 & 16 February 2018	0,6
►	**EPS theme symposia**		
	EPS theme 4 Symposium 2015	15 December 2015	0,3
	EPS theme 2 Symposium 2016	22 January 2016	0,3
	EPS theme 3 Symposium 2016	23 February 2016	0,3
	EPS theme 4 Symposium 2016	16 December 2016	0,3
	EPS theme 2 Symposium 2017	23 January 2017	0,3
	EPS theme 2 Symposium 2018	24 January 2018	0,3
	EPS theme 4 Symposium 2018	25 September 2018	0,3
►	**Lunteren Days and other national platforms**		
	Consortium meeting 1	31 October 2015	0,1
	Annual meeting 'Experimental Plant Sciences', Lunteren	11 & 12 April 2016	0,6
	Consortium meeting 2	28 June 2016	0,1
	Consortium meeting 3	21 March 2017	0,1
	Annual meeting 'Experimental Plant Sciences', Lunteren	10 & 11 April 2017	0,6
	Consortium meeting 2	28 June 2016	0,1
	Consortium meeting 3	21 March 2017	0,1
	Consortium meeting 4	20 September 2017	0,1
	Consortium meeting 5	2 July 2018	0,1
	Consortium meeting 6	5 November 2018	0,1
	Annual meeting 'Experimental Plant Sciences', Lunteren	8 & 9 April 2019	0,6
	Consortium meeting 7	24 June 2019	0,1
►	**Seminars (series), workshops and symposia**		
	Seminar: Subgenome parallel selection drives diversification and convergent morphotype evolution in Brassica crops	21 October 2015	0,1
	Seminar: Genomics in plant and animal breeding and conservation: strategies for success in plants, birds and livestock	4 November 2015	0,1
	Flying Seminar: Resistance pathway dynamics in plant immunity	21 January 2016	0,1
	Flying Seminar: How do plants read their own shape?	16 March 2016	0,1
	Seminar: Plant meets animal	16 March 2016	0,1
	Workshop: JoinMap course in PBR	18 May 2016	0,3
	Seminar: Plant meets animal	22 June 2016	0,1
	Lecture: rewriting our genes?	30 September 2016	0,2
	Workshop: 'Metabolomics in Chemical Ecology' in NIOO-KNAW	November 2016	0,5
	Seminar: An introduction to sorghum breeding	16 November 2016	0,1
	Seminar: From QTLs to routine DNA-informed breeding	16 November 2016	0,1
	Workshop: 11th Workshop Plant-Insect Interactions	22 November 2016	0,3
	Symposium: 1st WURomics symposium 2016	15 December 2016	0,3
	Seminar: 'The rhizomania complex in sugar beet – virus variation and resistance breaking'	18 April 2017	0,1
	WEES Seminar: Identification of a new sensory neuron membrane gene and why phylogenomics is important	22 June 2017	0,1
	Seminar: Exploring all the options towards engineering an even healthier apple	29 June 2017	0,1
	Guest seminar: Impact of ploidy level and genome evolution on the control of the frequency and distribution of recombination events in Brassicas	4 July 2017	0,1
	Flying Seminar: The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity	11 July 2017	0,1
	Seminar: Public lecture of the candidate for the position of Full Professor of Plant Physiology	15 September 2017	0,1
	WEES Seminar:From sex chromosomes to sex determination in Lepidoptera	25 October 2017	0,1
	Workshop: 12TH Workshop Plant-Insect Interactions	7 November 2017	0,3
	Seminar: Oxford Nanopore Sequencing Seminar	4 December 2017	0,1
	WEES Seminar: Interaction between honeydew-producers for ant protection	30 January 2018	0,1
	Seminar: Pelargonidin in flowers	14 March 2018	0,1

	Seminar: Investigating the virulence mechanism of P. nodorum on wheat	18 April 2018	0,1
	Seminar: A leucine-rich repeat receptor-like protein as PAMP receptor recognising XEG1, a Phytophthora glycoside hydrolase 12	10 September 2018	0,1
	Seminar: Live and Let Die or Live and Let Live - Interactions of Arabidopsis with fungal pathogen	14 September 2018	0,1
	Seminar: Nanopath: Utilising Nanopore sequencing for Septoria surveillance	14 September 2018	0,1
	Seminar: Host adaptation in the fungal cross-kingdom pathogen Fusarium oxysporum	17 October 2018	0,1
	Seminar: Reimagining the future of high-throughput, cell screening using the Beacon platform	25 April 2019	0,1
	Seminar: Phenotypic and molecular characterization of partially mlo-virulent isolates of the barley powdery mildew pathogen	23 May 2019	0,1
	Seminar: Introgression breeding from wild species for crops adaptation to climate change	23 May 2019	0,1
►	**Seminar plus**		
►	**International symposia and congresses**		
	International CRC 973 Symposium (Berlin, Germany)	9-11 April 2018	0,9
	European Plant Science Retreat (Utrecht, the Netherlands)	3-6 July 2018	0,9
	17th EUCARPIA meeting (Avignon, France)	11-13 September 2019	0,9
►	**Presentations**		
	Oral, company consortium meeting	28 June 2016	1,0
	Poster, CEPLAS Summer School	5-9 June 2017	1,0
	Oral, EPS theme 2 Symposium 2018	24 January 2018	1,0
	Poster, Second International CRC 973 Symposium	9-12 April 2018	1,0
	Poster, European Plant Science Retreat	3-6 July 2018	1,0
	Oral, Lunteren meeting 2019	9 April 2019	1,0
	Poster, Lunteren meeting 2019	9 April 2019	1,0
	Oral, company consortium meeting	24 June 2019	1,0
	Oral, 17th EUCARPIA meeting	12 September 2019	1,0
►	**IAB interview**		
►	**Excursions**		
	EPS company visit_tomato world	14 October 2016	0,2
	Rijk Zwaan Inhouse day	2 June 2017	0,3
	Visit Bayer in Mannheim	20 September 2017	0,2
	EPS company visit_Keygene	12 October 2017	0,2
	EPS company visit_Koppert	26 October 2018	0,2
	Subtotal Scientific Exposure		23,3
3) In-Depth Studies		*date*	*cp*
►	**Advanced scientific courses & workshops**		
	Data management plan	15 February 2016	0,4
	Intro to R for statistics	27 & 28 October 2016	0,6
	Data analyses and visualizations in R	11 & 12 May 2017	0,6
	CEPLAS Summer School	5-9 June 2017	1,2
	The Power of RNA-seq	11-13 June 2018	0,9
	SLU-WUR Symposium Plant Breeding and Biotechnology	11-13 June 2019	1,6
►	**Journal club**		
►	**Individual research training**		
	Subtotal In-Depth Studies		5,3
4) Personal Development		*date*	*cp*
►	**General skill training courses**		
	Project and time management	30 March - 11 May 2016	1,5
	PhD Competence Assessment	8 June 2016	0,3
	EPS introduction course	29 September 2016	0,3
	Scientific publishing	13 October 2016	0,3
	Interpersonal communication for PhD students	24 & 25 November 2016	0,6
	Scientific writing	14 March - 16 May 2017	1,8
	Dutch learning	2017	3,0
	Scientific Artwork – Vector graphics and images	2-3 October 2018	0,6
	Start to teach course	27 May - 13 June 2019	1,0
►	**Organisation of meetings, PhD courses or outreach activities**		
►	**Membership of EPS PhD Council**		
	Subtotal Personal Development		9,4
	TOTAL NUMBER OF CREDIT POINTS*		**42,0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was funded by the Ministry of Economic Affairs of the Netherlands (PPS 1409-029) under the Research Programme “Groene Veredeling” (Green Breeding) as well as the breeding companies Syngenta B.V. and BASF Vegetable Seeds. The author is supported by a grant from the China Scholarship Council.

Cover design: Xiaoning(Nina) Zhang and Mengjing Sun

Thesis layout: Mengjing Sun and Wei Du

Printed by: PROEFSCHIFRMAKEN / DIGIFORCE