Dissecting the role of plant immunity in plant-aphid interactions

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Abstract

Aphids are economically important phloem-feeding insects that cause loss in plant productivity worldwide. This occurs through the removal of photoassimilates and the vectoring of hundreds of plant viruses. Plants possess a complex immune system in order to defend themselves from a range of pathogens including bacteria and fungi. I aimed to discover if this immune system was also involved in defence against aphids.

I found that aphids have proteins that trigger plant immune responses. The aphid *Myzus persicae* contains several protein elicitors with varying molecular weights. These proteins are perceived by the plants *Nicotiana benthamiana* and *Arabidopsis thaliana*. In *A. thaliana* the perception of a 3 to 10 kDa elicitor fraction requires the leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1, as a mutant in this gene was deficient in immune responses activated by this elicitor. Plant recognition of the elicitor is unlikely to depend on a single non-arginine-asparate (non-RD) RLK.

In addition, aphids possess the means to modulate the plant immune response. I helped to identify three aphid effectors that modulate plant processes. I then investigated the role of one of these effectors, a *M. persicae* chemosensory protein (CSP) known as Mp10, in suppressing the immune responses triggered by the aphid elicitors. Mp10 is likely to disrupt the function of plant genes near the top of the immune signalling cascade in *N. benthamiana* in order to suppress elicitor-triggered immunity. Surprisingly, the homologs of this CSP in other aphids also show the same ability to suppress plant immune responses, suggesting an important role for Mp10 in plant-aphid interactions. This is the first report of a role for elicitor recognition by plants in aphid defence, the use of plant cell surface receptors to detect insects, and aphids' attempts to suppress plant immunity.

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Chapter 1- Introduction

1.1 Insects

Insects are a class of animals that are extremely numerous, ubiquitous, and very diverse in lifestyle. It is estimated that nearly 60% of all described eukaryotics species are insects (Stork, 1988). Insects inhabit practically all land surfaces (Daly *et al.*, 1998) including the Arctic and Antarctic (Sugg *et al.*, 1983; Danks, 2004). They also feed in a variety of ways including scavengers that feed on dead plant and animal matter, entomophagous predators and parasitoids that feed on other insects, parasites that feed on the blood or flesh of larger animals, and phytophagous insects that feed on green plants by either chewing tissue or “sucking” sap or cell contents. Insects benefit human life in several ways including through the ecosystem services they provide such as pollinating crops. However, they are also harmful to human well-being through the diseases they vector to humans, animals and plants, and direct damage caused to crops. Nearly one million insect species have so far been described, and nearly half of them feed on plants (Wu and Baldwin, 2010). Within plant feeding insects most species feed on a few related species, while approximately 10% can feed on multiple plant families (Schoonhoven L *et al.*, 2005). The mechanisms that allow some insects to feed on many plant species and others to feed on a few are not well understood.

1.2 Aphids

Aphids are plant-feeding insects within the order Hemiptera, suborder Sternorrhyncha. There are approximately 4500 species of aphids (Sabater-Munoz *et al.*, 2006) and they are predominately found in northern temperate regions (Blackman and Eastop, 2000). Aphids are small in size (1-10 mm) and have distinguishing features of a long pair of antennae, a proboscis that they use to pierce the plant and suck phloem sap through, and siphunculi which are located on the dorsal fifth abdominal segment and consist of a pair of upward-pointing tubes (Brisson and Stern, 2006) (Figure 1.1A). In keeping with other hemimetabolous insects, aphids undergo an incomplete metamorphosis from juvenile to adult stage (The International Aphid Genomics Consortium, 2010). Aphids evolved at least 280 million years ago (Dixon, 1998) and began to diversify into most of the extant species number at about the same time as the angiosperms, approximately 140 million years ago (Von Dohlen and Moran, 2000).

Aphids are major agricultural pests, as over 250 species feed on crop plants and can also vector viruses (Blackman and Eastop, 2000), leading to losses in crop production estimated to be hundreds of millions of dollars each year (Oerke *et al.*, 1994).

In addition to the vectoring of viruses, aphids can also damage crop plants in three other ways. Firstly, aphids remove photoassimilates from the host plant and therefore deprive it of resources that would have been used for growth and reproduction. An extreme example of this is the peach-potato aphid *Myzus persicae,* whose feeding on peach tree flowers can lead to poor fruit development (Dedryver *et al.*, 2010). Secondly, the injection of aphid saliva into the host plant when feeding can be phytotoxic, and lead to leaf decolouration and necrosis amongst other symptoms (Dedryver *et al.*, 2010). Finally, aphids secrete a sticky, sugar-rich fluid called honeydew as a by-product of feeding. The honeydew can provide a food source for soot moulds to grow on the surface on plant leaves, hindering plant photosynthetic activity (Wood *et al.*, 1988).

Aphids have evolved complex life cycles which demonstrate a high degree of phenotypic complexity (Blackman and Eastop, 2000). In many species reproduction is parthenogenic for much of the year, producing live offspring (nymphs) which are genetically identical to the mother, yet individuals with the same genotype can develop into several alternative phenotypes due to differing environmental conditions (polyphenism). The important environmental conditions are the seasons and plant health. The seasons, and particularly the day length, trigger changes from asexual to sexual forms and also the production of male aphids. The plant response to aphid infestation or stress can trigger changes to the dispersive, winged form (alates). Alates passively disperse on the wind to colonise new host plants. Figure 1.1B shows the holocyclic life cycle (sexual and asexual reproduction occur) of the pea aphid *Acyrthosiphon pisum*, as an example of the aphid lifecycle. Aphid colonies can grow very rapidly due to a short generation time. During the asexual phase of the lifecycle females can be carrying both their own daughters and embryos developing within the daughters (granddaughters) (Blackman and Eastop, 2000). The lifecycle and rapid colony growth rate can prove to be advantageous to the aphid, as clones well-adapted to a set of environmental conditions can quickly spread without their genotype being altered through sexual reproduction. Yet, the sexual reproduction at the end of a year provides fresh genetic variation for adaption to the environmental conditions of the following year. Holocyclic aphids have primary hosts on which they lay eggs (oviposit) and secondary hosts on which they feed. This leads to the double-barrelled common names of many aphid species, such as the currant - lettuce aphid (*Nasonovia ribisnigri*). Some aphids are thought to persist without sexual reproduction (anholocycly), due to environmental conditions or lack of primary hosts. Populations thought to be anholocyclic include *M. persicae* in northern England and Scotland (Fenton *et al.*, 1998) and the melon-cotton aphid *Aphis gossypii* in southern France (Dogimont *et al.*, 2010).

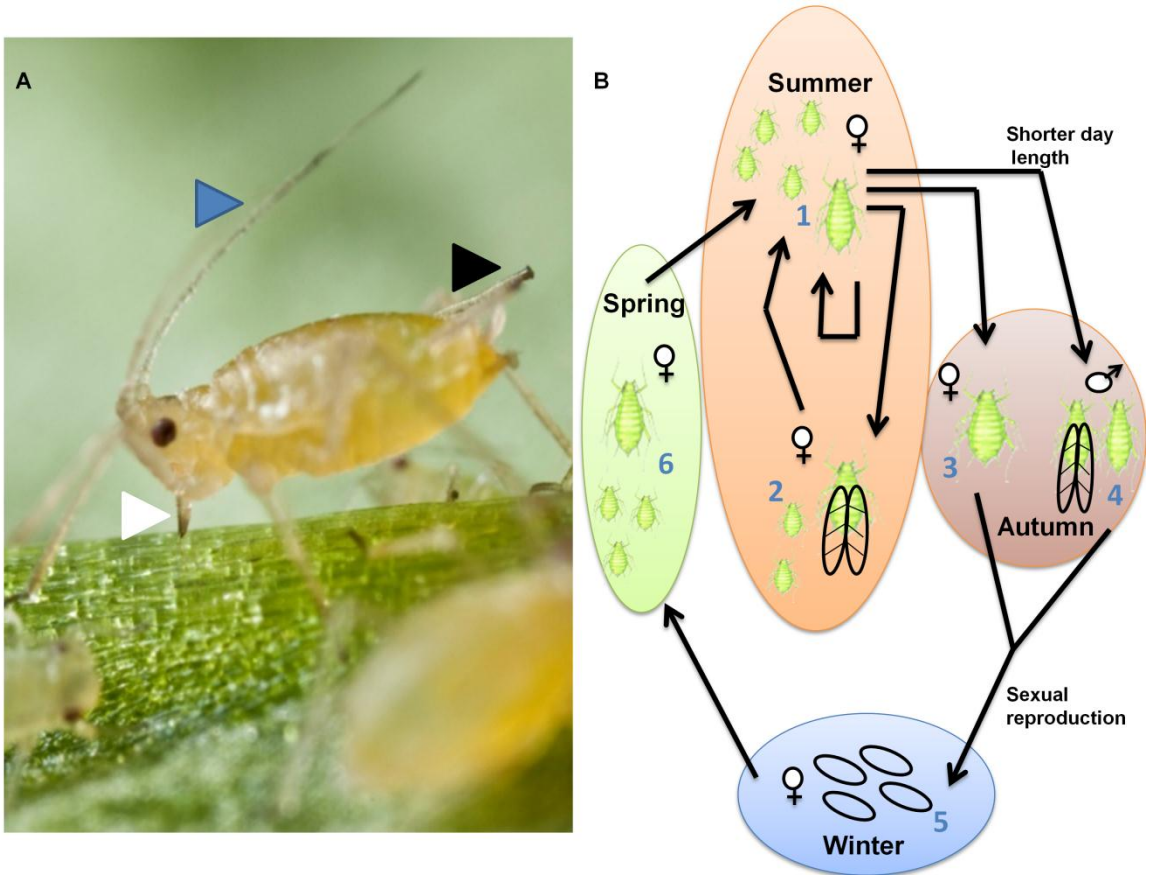

Figure 1.1 – Important aspects of aphid biology.

(A) *M. persicae* inserting its stylet into a plant (white arrow). Antenna (blue arrow) and siphunculi (black arrow) are also visible. Photo by A. Davis, JIC.

(B) An example of a holocyclic life cycle, taken from *A. pisum*. During the spring and summer months, asexual females give birth to live clonal offspring. These offspring undergo four molts during larval development to become (1) unwinged or (2) winged asexually reproducing adults. The production of winged individuals, capable of dispersing to new plants, is induced by crowding or stress during the prenatal stages. After repeated cycles of asexual reproduction, shorter autumn day lengths trigger the production of (3) unwinged sexual females and (4) males, which can be winged or unwinged in *A. pisum*, depending on genotype. After mating, oviparous sexual females deposit (5) overwintering eggs, which hatch in the spring to produce (6) wingless, asexual females and thus complete the cycle. Modified from (The International Aphid Genomics Consortium, 2010), an open access article.

1.3 Systems for studying aphid-plant interactions

Many aphid and host plant species have been studied over the years. More recently, resources and techniques for studying the molecular biology of aphids have been developed for a few species in order to study them in more detail with the hope of gaining knowledge that is applicable to other species. One such species is *A. pisum,* which was the first aphid to have its genome sequenced (The International Aphid Genomics Consortium, 2010), and ribonucleic acid (RNA) interference (RNAi) techniques have been developed in order to better understand the role of certain aphid genes (Mutti *et al.*, 2006; Shakesby *et al.*, 2009). *A. pisum* has been used to study a variety of biological phenomena including insect-bacterial symbiosis and polyphenisms (Brisson and Stern, 2006). One of the legume host plants of *A. pisum*, *Medicago truncatula*, has a published genome (Young *et al.*, 2005) which allows the plant-insect interaction to be studied at the molecular level from both sides. *A. pisum-M. truncatula* interactions have primarily been studied in terms of resistance genes to the aphid (e.g. (Klingler *et al.*, 2009; Stewart *et al.*, 2009)) but the recent release of insertion mutant lines in *M. truncatula* (Cheng *et al.*, 2011b) will allow other aspects of the plant-insect interaction to be better studied.

Another aphid species for which molecular biology resources and techniques are being developed is *M. persicae*. This allows many aspects of *M. persicae* biology to be studied at the molecular level including the plant-aphid interaction. *M. persicae* is an important aphid species because it is a major pest worldwide; it is responsible for transmission of many viruses, and it has a broad host range (Blackman and Eastop, 2000), including *Arabidopsis thaliana* (de Vos *et al.*, 2007) . It is thought to have developed resistance to more insecticides than any other insect (Anstead *et al.*, 2005). The genome sequence for *A. thaliana* is available (The Arabidopsis Information Resource), as are many molecular tools and so the *M. persicae-A. thaliana* system can be used to identify plant genes that control behaviour and fecundity of the aphid (Hunt *et al.*, 2006). The *M. persicae* genome is currently being sequenced by the Hogenhout group in collaboration with The Genome Analysis Centre (TGAC), Denis Tagu, Lin Field, Brian Fenton, Georg Jander and Alex Wilson. There are also many expressed sequence tags (ESTs) available for *M. persicae* in the National Centre for Biotechnology Information (NCBI) database from other sequencing efforts. RNAi techniques for *M. persicae* have been developed (Pitino *et al.*, 2011; Bhatia *et al.*, 2012), and they possess an advantage over the methods used for *A. pisum* because the host plant delivers the double-stranded RNA (dsRNA) or small interfering RNA (siRNA) to the aphid. This allows the plant-insect

interaction to be studied *in vivo* without the need for injection or artificial diets. In addition to *A. thaliana*, *M. persicae* can also feed on *Nicotiana benthamiana. N. benthamiana* has a draft genome available (http://solgenomics.net/) (Bombarely *et al.*, 2012) and is amenable to methods such as *Agrobacterium tumefaciens*-mediated transient gene expression and virus induced gene silencing (VIGS) (e.g. (Mantelin *et al.*, 2011)), and thus allows the rapid study of aphid and plant factors in the plant-insect interaction.

1.4 Aphid molecular biology

Certain aspects of the molecular biology of aphids have been well studied. The control of aphid species as agricultural pests is predominantly by chemical insecticides. Over time some aphid biotypes have developed resistance to insecticides, in a situation analogous to bacteria developing resistance to antibiotics. The molecular bases of these insecticide resistances have been well studied in *M. persicae*, which has developed resistance to many insecticides including organophosphates through overproduction of detoxifying carboxylesterases, insensitivity to dimethyl carbamates through mutation of the acetylcholinesterase protein, and resistance to pyrethroids through mutation of the voltage-gated sodium channel (Devonshire *et al.*, 1998). These resistance mechanisms do not affect the insecticide activity of another class of chemicals, neonicotinoids, which target nicotinic acetylcholine receptors. For this reason, neonicotinoids have become the main class of insecticide used to control *M. persicae* (Puinean *et al.*, 2010). However, reports of neonicotinoid resistant *M. persicae* clones have emerged on several continents (Nauen and Denholm, 2005; Foster *et al.*, 2008; Puinean *et al.*, 2010; Bass *et al.*, 2011). These resistances are associated with overproduction of detoxification enzymes, in part due to gene amplification, and mutation of the target site (Puinean *et al.*, 2010; Bass *et al.*, 2011). The long term effectiveness of insecticides to control aphids in agriculture is unclear, and so other methods of aphid control, informed by chemical and molecular biology, are being pursued.

One such approach makes use of knowledge of aphid chemical ecology. Pheromones are chemical signals directed at members of the same species. Aphids produce an alarm pheromone upon attack by predators, the main component of which is the sesquiterpene (*E*)-β-farnesene (EBF) (Bowers *et al.*, 1972). There are various responses by aphids to receiving the pheromone, but typical responses include stopping feeding, moving away from the signal and dropping from the host plant (Pickett *et al.*, 1992). Aphid predators have also learned or evolved to use alarm pheromone as a foraging cue (Vet and Dicke, 1992). Transgenic *A. thaliana* plants have been made which

express high levels of pure EBF and show an affect on the behaviour of *M. persicae* and its parasitoid *Diaeretiella rapae* (Beale *et al.*, 2006). Another study found habituation in the response to EBF of aphids exposed to the EBF transgenic *A. thaliana* for many generations, which led to higher levels of predation by ladybird the *Hippodamia convergens* (de Vos *et al.*, 2010). This suggests that the value of constitutive EBF emission from plants may be in increased predation as opposed to repellence. The results of current field trials of transgenic spring wheat producing EBF (http://www.rothamsted.ac.uk/Content.php?Section=AphidWheat) will shed more light on how effective this aphid control strategy is in practice.

The sequencing of the *A. pisum* genome has helped improve our understanding of many areas of aphid molecular biology. For example, it allowed the identification of the odorant-binding protein (OBP) and chemosensory protein (CSP) complement of *A. pisum* (Zhou *et al.*, 2010). OBPs are involved in chemical perception in insects and OBP3 and 7 been shown to bind EBF (Qiao *et al.*, 2009; Sun *et al.*, 2012). The role of CSPs are less well understood and are likely to be different from OBPs, for example Olfactory-specific D2 (OS-D2) from the vetch aphid *Megoura viciae* is unlikely to bind twenty-eight compounds known to elicit an electrophysiological response in electroantennograms or in single olfactory neurone preparations (Jacobs *et al.*, 2005). The genome also allowed the identification and characterisation of a wide range of aspects of aphid biology such as ion channels (Dale *et al.*, 2010), detoxification enzymes (Ramsey *et al.*, 2010), and circadian clock genes (Cortés *et al.*, 2010).

1.5 Aphid feeding behaviour

The range of host plants for an aphid species is variable. *A. pisum* has a narrow host range, because it feeds on leguminous plants such as pea (*Pisum sativum*) (Blackman and Eastop, 2000). In contrast *M. persicae* is considered to have the broadest host range of an aphid, since it is able to colonize over 40 different plant families (Blackman and Eastop, 2000). Aphids feed on the phloem sap in the sieve elements of a host plant using their stylets. This feeding style is distinct from that of other insect herbivores that either consume leaf tissue (e.g. lepidopteran larvae) or feed on epidermal cells (e.g. thrips). The phloem is the plants long-distance transport system that moves photoassimilates by mass flow, using a turgor difference between the sources (tissues that make carbohydrates) and sinks (tissues that use carbohydrates usually for growth) (Münch, 1930). The contents of phloem sieve tube sap include carbohydrates, amino acids, lipids and

minerals, which aphids use as their diet with the assistance of symbiotic micro-organisms (Douglas, 2006).

The aphid stylets are formed by two mandibular and two maxillary parts. The mandibular stylets enclose a larger channel for food (0.7 µm) and a smaller channel for saliva (0.3 µm) (Ponsen, 1987). The maxillary stylets each contain a neuronal canal. Recently, an anatomical feature named the acrostyle has been described at the distal end of the maxillary stylets, and it may be involved in virus transmission, protein binding, controlling fluid dynamics, or mediate mechanics of the stylet (Uzest *et al.*, 2010).

The aphid stylets need to negotiate their way from the plant surface to the sieve elements of the phloem, and do so using an intercellular pathway (Tjallingii, 2006). During this process the stylets briefly puncture most of the cells along the pathway and then seals them afterwards (Tjallingii and Esch, 1993) until they eventually reach the sieve elements. It has been proposed that aphids use sucrose concentration and pH to find the sieve elements within the plant tissue (Hewer *et al.*, 2010; Hewer *et al.*, 2011).

The insertion of the stylets into the plant is coupled with secretion of gelling saliva, which is one of the two types of saliva aphids secrete. A reaction with oxygen in the air or surrounding plant tissue causes the saliva to rapidly gel. A small amount of gelling saliva is secreted onto the plant surface before the stylet insertion, and this forms the salivary flange. Gelling saliva is also secreted along the stylet pathway to form a sheath (Tjallingii, 2006). There are a few possible roles of the sheath in aphid feeding, including stabilization of the stylets (Pollard, 1973), protecting stylets from plant defence compounds (Hogenhout and Bos, 2011) and the minimization of calcium ion (Ca^{2+}) leakage into the sieve element when it is punctured, thereby stopping the sieve elements from being plugged (Tjallingii, 2006).

Watery saliva, the second type of aphid saliva, is injected into punctured cells along the stylets pathway (Martin *et al.*, 1997) and is mostly secreted once a sieve element has been reached (Tjallingii, 1994). When an aphid reaches a sieve element a period of watery salivation will follow that may last up to a few minutes (Prado and Tjallingii, 1994). One of the roles of this saliva has been shown to be the suppression of sieve element occlusion (Will *et al.*, 2007). After this, the aphid will start to ingest phloem sap mixed with watery saliva. The role of the saliva is probably to keep the food channel in the stylets open by interacting with sieve element proteins in the sap (Tjallingii and Cherqui, 1999). Aphids can feed continuously from a single sieve element for many hours and in some cases even days (Tjallingii, 1995).

1.6 Morphology of aphid salivary glands

Aphid salivary glands are paired, and each pair has a large principal gland that is bilobed and a smaller accessory gland that is spherical in shape. Cells in both glands have canaliculi, which are channels that cross the cuticular lining of the salivary duct and transport secretory granules released by the cells (Ponsen, 1972). Ducts from both glands on one side unite to form a principal duct, with both principal ducts uniting to form a common salivary duct that discharges into the salivary canal (Ponsen, 1972). The roles of the principal and accessory salivary gland in producing watery and sheath saliva are unclear. The principal gland is innervated and it has been suggested that it may play a role in the production of the sheath saliva (Tjallingii, 2006), however proteins thought to be in the watery saliva have also been found to be produced there (Mutti *et al.*, 2008). The innervations of the principal salivary gland does suggest that the aphid has an element of control over what is secreted from it, but there is currently no experimental evidence to back up this hypothesis (Tjallingii, 2006). The accessory gland has no apparent signs of neural input (Tjallingii, 2006) and has been shown to be involved in the transfer of (luteo)viruses to plants (Gray and Gildow, 2003), and from this it has been inferred that the accessory glands are responsible for watery saliva. Watery saliva has been shown to be responsible for the inoculation of viruses (Prado and Tjallingii, 1994).

Whilst the roles of the two salivary gland types are currently unclear, there is more evidence for roles in the cells within the principal salivary gland. These cells have morphological differences. Weidemann (Weidemann, 1968) identified 9 different cell types in the principal salivary gland of *M. persicae.* These 9 cell types consisted of seven types of main cell (*Hauptzellen*) and two types of cover cell (*Deckzellen*). The morphological difference in cell types within the principal salivary gland might suggest that the cells have differentiated to serve different functions. The recent use of immunolocalisation techniques on aphid salivary glands has added more weight to this idea. The sheath salivary proteins S66, S69 and S154 (Baumann and Baumann, 1995) have been localised to the posterior cells of the principal salivary gland only (Cherqui and Tjallingii, 2000) in the spring grain aphid *Schizaphis graminum*. In *A. pisum*, the secreted salivary gland protein C002 and the enzyme laccase, which is also thought to be secreted in aphid saliva, have been localised to different subsets of the secretory cells (Mutti *et al.*, 2008). These pieces of evidence raise the possibility of specialisation within the secretory cells of the principal salivary gland to producing specific proteins.

1.7 Content of aphid saliva

Aphid saliva is composed of enzymes and other proteins, along with a mix of ions, amino acids and hemolymph (Miles, 1999). Sheath saliva is mainly composed of protein, phospholipids and conjugated carbohydrates, whereas watery saliva has a more complex composition including a wide range of enzymes (Miles, 1999). The composition of aphid saliva seems to vary within and between species, and so the published literature has lots of contradictions (Tjallingii, 2006). The most common technique of collecting aphid saliva is from Parafilm membrane feeding systems (e.g. (Harmel *et al.*, 2008)). However, this method has limitations as the content of the saliva changes depending on the artificial diet used (Cherqui and Tjallingii, 2000; Cooper *et al.*, 2010), and it is possible that the saliva collected from artificial diets is different to that secreted into plants due to differences in sensory aspects of aphids feeding on plants versus diets (Tjallingii, 2006).

Structural proteins are found in the sheath saliva (Cherqui and Tjallingii, 2000) and include the proteins identified by Baumann and Baumann (Baumann and Baumann, 1995) with estimated molecular masses of 154 and 66/69 kDa (Cherqui and Tjallingii, 2000). Initial efforts to characterise aphid watery saliva centred on identifying enzymatic activities. The enzymes identified in aphid saliva fall into the two broad classes of hydrolases and oxidoreductases (Miles, 1999). Hydrolases include pectinases, cellulases and glucosidases and are thought to have varied roles such as aiding aphid feeding by breaking down pectin (Ma *et al.*, 1990) or hydrolysing glucose in digestion (Harmel *et al.*, 2008). Oxidoreductases include phenol oxidases and peroxidases and are thought to be involved in the detoxification of phytochemicals produced by the plant (Miles and Oertli, 1993). Whilst some functions for these enzymes in plant-aphid interactions have been hypothesised, their individual roles are not currently well understood.

More recently, comparisons of watery saliva composition of different aphid species using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) have been conducted, although results are not consistent. Whilst Cooper and colleagues (Cooper *et al.*, 2011) found similarities in gel bands between aphid species causing similar symptoms on the same monocotyledonous plant hosts, Will and colleagues (Will *et al.*, 2009) found large variation in gel bands between aphid species on the same dicotyledonous plant host, and even variation between two differently coloured morphs of *A. pisum*. These differences may have resulted from the process of aphid adaption to monocotyledonous and dicotyledonous plant hosts. Alternatively they may be due to differences in methodology such as the different diet compositions used to collect the saliva, or the limitations of comparing bands on one-dimensional gels.

The *A. pisum* genome sequence and sequence data from other aphids have facilitated the use of proteomic studies to more accurately identify the proteinaceous components of aphid saliva. Proteomic studies into secreted saliva of three aphid species have been published; two aphids (*M. persicae* and *A. pisum*) that feed on dicotyledonous plants and one aphid (the Russian wheat aphid, *Diuraphis noxia*) that feeds on monocotyledonous plants. These studies provide identities based on amino sequence for some of the secreted proteins in aphid saliva and many sequences for uncharacterised proteins. A few proteins were identified in the saliva of *M. persicae* feeding on an artificial diet (Harmel *et al.*, 2008). The identified proteins were glucose oxidase (GOX), glucose dehydrogenase (GLD), nicotinamide adenine dinucleotide (NAD)H dehydrogenase, alpha-glucosidase and alpha-amylase. Proteins with no homology to previous characterised sequences were also identified. Carolan and colleagues (Carolan *et al.*, 2009) identified nine proteins from *A. pisum* saliva. These were two metalloproteases, a glucose-methanol-choline (GMC)-oxidoreductase, a homolog to regucalcin and five proteins without homology to previously described sequences. Amongst the five non-homologous proteins was a large, abundant protein similar to a protein identified in (Harmel *et al.*, 2008) and hypothesised to play a role as a sheath protein. This work was followed up by transcript and proteomic analysis of *A. pisum* salivary glands (Carolan *et al.*, 2011). Nearly 1000 proteins were identified in the salivary glands, including eight out of the nine previously identified in the saliva. The absence of a saliva protein from the salivary gland proteomics may be due to technical reasons such as relative protein levels, or may suggest that proteins other than salivary gland proteins are able to enter the saliva.

The largest set of proteomic work on aphid saliva was carried out on *D. noxia*. After an initial study to optimise saliva collection identified an alkaline phosphatise, a zinc-binding dehydrogenase, a RNA helicase and several unidentified proteins (Cooper *et al.*, 2010), a much larger study compared the saliva of several biotypes of *D. noxia* with differing virulence (Nicholson *et al.*, 2012). This study identified a total of 34 proteins in the saliva, including many found in previous studies of *A. pisum* and *M. persicae*. The presence of the same proteins in aphids feeding on such different hosts suggests that they may be essential to aphid feeding on plants in general. *D. noxia* is different to the other two aphids whose saliva has been studied because it feeds on monocotyledons and is phytotoxic. Many differences in *D. noxia* saliva were found when compared to *M. persicae* and *A. pisum*, although more data is needed to determine whether this is due to type of plant host, phytotoxic ability, or differences in experimental method. A difference in saliva composition was also observed between the different *D. noxia* biotypes used, providing evidence that aphid virulence may depend on salivary proteins.

1.8 Factors affecting plant-aphid interactions

The majority of aphid species are specialists and only feed on a small number of plant species. Biotypes occur within some species which are adapted to a subset of plants; for example *A. pisum* generally only feeds on legume plants, but individual biotypes only feed on one or two species within the legumes (Via, 1991). In contrast, some aphid species, such as *M. persicae,* can feed on a large number of plant species in different families (Blackman and Eastop, 2000). Whilst aphid selection of host plants is thought to involve discrimination based on visual (David and Hardie, 1988; Gish and Inbar, 2006) and olfactory cues (reviewed in (Pickett *et al.*, 1992)), little is known as to the nature of barriers to aphid colonization imposed by non-host plants (Goggin, 2007). A number of theories have been presented, although none seem to account for all circumstances. Explanations include the presence of aphid symbionts, the nutritional content of the plant, the ability of aphids to detoxify plant metabolites, and the ability to manipulate plant defences.

Aphids have established a symbiosis with an obligate symbiont, *Buchnera aphidicola*, which is likely to aid its host in the synthesis of certain essential amino acids missing in the diet (Wilson *et al.*, 2010). Aphids can also harbour one or more facultative symbiont. Studies on the affect of aphid facultative symbionts in *A. pisum* have shown that they can protect against entomopathogenic fungi and parasitoid wasps, lessen the detrimental effects of heat, and can have an affect on host plant suitability (Oliver *et al.*, 2010). The studies on host-plant use have centred on different biotypes of *A. pisum*, and have given contradictory results. One study found that removing the facultative symbiont *Regiella insecticola* decreased aphid performance on white clover (*Trifolium repens*) but not on vetch (*Vicia sativa*) (Tsuchida *et al.*, 2004). However another, similar study found no effect of *R. insecticola* on *A. pisum* performance on white clover (Leonardo, 2004). A third study using five *A. pisum* clones found that *R. insecticola* increased the performance on red clover (*Trifolium pratense*) of one out of five clones, demonstrating a genetic basis in the aphid for the effect of symbiont presence (Ferrari *et al.*, 2007). A study using a different facultative symbiont, *Hamiltonella defensa*, found no evidence that it played a direct role in host plant utilization of the *A. pisum* (McLean *et al.*, 2011). The black bean aphid *Aphis fabae* shows lower fitness on the purple deadnettle (*Lamium purpureum*) compared to broadbean *Vicia faba*, and infection with *R. insecticola* or *H. defensa* further decreases fitness (Chandler *et al.*, 2008). There remains no clear evidence that symbionts play a major role in host-plant colonization (McLean *et al.*, 2011) and no examples where symbionts allow the colonization of a host plant outside of the aphid

species host range. The cause of the interaction between aphid genotype and symbiont presence is also unclear.

An aphid's diet of phloem sap is low in amino acids and therefore aphid growth and reproduction may be limited by amino acid content (Dixon, 1998). However, it is unclear as to whether this plays a role in determining colonization of a plant by an aphid. The amino acid composition of the phloem has been related with aphid host plant resistance (Chiozza *et al.*, 2010), although examples exist of the amino acid composition in the phloem not playing a role in aphid host range or performance (Wilkinson and Douglas, 2003; Hunt *et al.*, 2006; Hunt *et al.*, 2010). One study concluded that secondary plant compounds were more important that amino acids in determining aphid fitness (Tosh *et al.*, 2003). In order to reject the plant on the basis of its phloem content the aphid must first reach the phloem, and recent research suggests that plant preference is decided before the phloem is reached (Powell *et al.*, 2006).

Plants defend themselves from insects using a variety of methods, including producing toxic metabolites. It might therefore follow that an increased capacity to detoxify these metabolites may lead to an increased number of available hosts. Ramsey and colleagues (Ramsey *et al.*, 2010) compared the number of detoxifying enzymes in a specialist and generalist aphid by using the *A. pisum* genome and *M. persicae* EST data respectively. Their finding that *M. persicae* has around 40% more genes encoding cytochrome P450s, the largest and most diverse class of detoxification enzymes, may provide some support for the theory but this single correlative example is not conclusive proof.

Toxic metabolites are only one part of plant defences against aphids and therefore it is more likely that manipulating the defences as a whole is more important than detoxifying one part of them. Aphid saliva has been hypothesised as an important part of the colonization of plants for a long time (e.g. (Miles, 1999)). Recently, several studies have provided evidence that the watery saliva of an aphid may be an important factor in colonizing a plant (e.g. (Carolan *et al.*, 2009; Will *et al.*, 2009; Nicholson *et al.*, 2012)). Aphid feeding behaviour elicits different responses compared to chewing herbivores (De Vos *et al.*, 2005) or even when compared to silverleaf whiteflies (*Bemisia tabaci*) that feed from the phloem in a similar way to aphids (Kempema *et al.*, 2007). The feeding behaviour that aphids use to colonize plants shares similarities with the intimate association between plants and biotrophic plant pathogens. The secretion of saliva into plants by aphids has been likened to pathogens secreting effectors into plants (Walling, 2008), and the feeding of aphids on plants activates some of the same defence pathways as pathogen attack (Walling, 2000). It is probable that proteins in aphid saliva play similar roles to pathogen effectors in enhancing the ability of an aphid to colonize a plant

(Hogenhout *et al.*, 2009), and the existence of classical nucleotide-binding site leucine-rich repeat (NBS-LRR) plant defence genes that confer resistance to aphids gives them more in common with pathogens than with chewing herbivorous insects (Tagu *et al.*, 2008). Therefore it is likely that plant immunity responds to aphids in a similar way as to biotrophic plant pathogens. This hypothesis will be investigated in this thesis. The following sections summarise current knowledge about plant immunity against pathogens and highlights parallel concepts in the plant-insect interaction field.

1.9 Terminology

A debated term in the field of plant-pathogen interactions is “effector”. A recent, broadly inclusive definition of effector is “a molecule secreted by plant-associated organisms that alters host-cell structure and function” (e.g. (Hogenhout *et al.*, 2009)). Under this definition effectors include molecules that aid pathogen colonization of the plant (traditionally refered to as virulence factors and toxins). However, such a broad definition includes molecules that have the opposite effect on the plant-pathogen interaction. These molecules include those that trigger plant immune responses dependent on cell surface receptors, variously described in the literature as pathogen-associated molecular patterns (PAMPs), microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and herbivory-associated molecular patterns (HAMPs). It also includes molecules that trigger immune responses dependent on intracellular receptors (also termed avirulence (Avr) proteins or recognised effectors). Therefore, in the context of this thesis, effector provides a useful initial term for aphid molecules that may have a role in the plant-aphid interaction. These molecules can then be given more specific terms once more information about their function is available, and the particular distinction I will make in this thesis is for molecules that trigger plant defence responses, which I will term elicitors.

My definition of an elicitor is a molecule produced directly or indirectly by an organism that triggers plant immune or defence responses. Therefore molecules referred to in the literature as PAMPs, MAMPs, DAMPs, HAMPs and Avrs act as elicitors. Elicitors was originally a term used to describe a molecule capable of inducing phytoalexin production in plants (e.g (Keen *et al.*, 1983)) but later has been used for molecules stimulating any type of plant defence (e.g. (Nürnberger, 1999)). I think that it is a helpful term for three reasons. Firstly, the term makes no comment on where the molecule that triggers plant defence originates from. This is advantageous when referring to aphids that may produce molecules otherwise known as MAMPs from their bacterial symbionts,

HAMPs in their saliva, and DAMPs from the action of their saliva on the plant. Therefore a more general term makes the nomenclature simipler. Secondly, the term makes no comment on where in the plant cell the molecule interacts with a plant gene to trigger resistance. Thirdly, it is a term commonly used in both the plant-pathogen field (e.g. (Bethke *et al.*, 2011; Galletti *et al.*, 2011)) and plant-insect interactions field (e.g. (De Vos and Jander, 2009; Gilardoni *et al.*, 2011)) and therefore avoids confusion when bringing the work of these two fields together. If I identify the origin of an elicitor and whether it is recognised at the plant cell surface membrane or intracellulary then the elicitor will be given a name consistent with PAMP, MAMP, DAMPs, HAMP and Avr. When referring to the literature I shall keep the designation of PAMP, DAMP and HAMP for established elicitors who are usually referred to as belonging to one of these classes of molecules and whose origin is known (e.g. chitin remains a PAMP). Avr proteins or recognised effectors may trigger and suppress defence responses depending on the presence of a plant resistance (*R)* gene. In this instance I shall use the term effector for these aphid molecules, and reserve elicitor for molecules that do not show defence suppression.

An additional term that I wish to define is induced resistance. Induced resistance is separate to resistance. Plant resistance can be defined as the outcome of genetically inherited qualities that result in a plant being less damaged by a pathogen/pest than a susceptible plant lacking these qualities (Smith and Clement, 2012). Induced resistance is a broad term used in the literature to describe a variety of plant defence responses triggered by biotic and abiotic agents that lead to reduced plant susceptibility and hence reduced amounts of plant disease, but not complete loss of the ability of the pathogen to colonize the plant (Walters *et al.*, 2013). Induced resistance can be effective against both pathogens and herbivores (Balmer *et al.*, 2012). The plant responses include PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (described in more detail below), but also other responses such as systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is the activation of defence in distal tissues upon infection of a pathogen (Fu and Dong, 2013), whereas ISR occurs following root colonization by non-pathogenic soil microbes (Shoresh *et al.*, 2010). Induced resistance is a helpful term as it is general and therefore does not state what changes in the plant lead to the triggered reduction in susceptibility to the pathogen or herbivore. The term is also used in both the plant-pathogen and plant-insect interaction fields (e.g. (Zipfel *et al.*, 2004; Halitschke *et al.*, 2011; Boyd *et al.*, 2013)).

1.10 The plant immune response

Plants are sessile and therefore cannot evade attackers. However, they do possess a complex immune system, which is thought to be composed of multiple levels. The first active level of the immune system is a mechanism to recognize non-self or modified-self through the interaction of molecules with plant cell surface receptors. The recognition of PAMPs by transmembrane pattern recognition receptors (PRRs) at the plant cell membrane allows the plant to recognize the presence of a non-self organism, and the recognition of DAMPs by PRRs allows the plant to determine that it is being damaged. PAMPs are conserved molecules present across broad classes of microbe, are essential for the pathogen's lifecycle, and induce a plant immune response upon detection. They can be proteins, such as bacterial flagellin (Felix *et al.*, 1999) or carbohydrate based, such as bacterial peptidoglycans (PGNs) (Gust *et al.*, 2007). Single mutants in PRRs show enhanced susceptibility to virulent, weakly virulent and non-adapted bacteria (Nicaise *et al.*, 2009).

The perception of PAMPs and DAMPs by the plant is mediated by receptor-like kinases (RLKs) or receptor-like proteins (RLPs). There are over 600 predicted RLKs in the *A. thaliana* genome and over 1100 in the rice (*Oryza sativa)* genome (Shiu *et al.*, 2004), although only a small number of these have been functionally characterised. Included in these numbers are receptor-like cytoplasmic kinases (RLCKs) that lack an extracellular domain, some of which can be located at the plasma membrane through their interaction with other proteins (e.g. BOTRYTIS-INDUCED KINASE1 (BIK1) (Laluk *et al.*, 2011). RLKs consist of a ligand-binding extracellular domain, a transmembrane segment and a cytoplasmic domain. The extracellular domain amongst plant RLKs is very divergent, and has been used to classify them into subfamilies (Shiu and Bleecker, 2001). The cytoplasmic domain possesses a conserved serine/threonine kinase domain.

RLKs are able to mediate signal transduction of an extracellular signal, from the changing environment the plant cell is situated in, into the cytoplasm of a cell to allow an appropriate response to be generated. In addition to PAMP perception, RLKs have been found to play a role in a wide range of plant processes including development (De Smet *et al.*, 2009) and responses to abiotic stress (Ouyang *et al.*, 2010). There are more than 10 subfamilies of RLKs (Gou *et al.*, 2010). The largest subfamily of RLKs is the LRR family, which itself is further divided into more than 13 subfamilies (Shiu and Bleecker, 2001). Only 30 out of more than 200 of these genes have defined biological functions (Gou *et al.*, 2010). The LRR-RLK subfamily includes the PAMP PRRs FLAGELLIN-SENSITIVE2 (FLS2), ELONGATION FACTOR-TU RECEPTOR (EFR), and

(XANTHOMONAS RESISTANCE 21) XA21 (Segonzac and Zipfel, 2011), and the DAMP PRRs PEP1 RECEPTOR1 and 2 (PEPR1 and PEPR2) (Yamaguchi *et al.*, 2010). PRRs involved in PAMP and DAMP recognition are not exclusively found in the LRR-RLKs. The lysine motif (Lys-M) RLK CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) can also be considered a PRR (Segonzac and Zipfel, 2011), and the wall-associated kinase (WAK) RLK CELL WALL-ASSOCIATED KINASE1 (WAK1) acts a DAMP receptor by perceiving oligogalacturonides (OGs) (Brutus *et al.*, 2010).

Several PRRs and their respective ligands have been identified (Monaghan and Zipfel, 2012). The best studied PAMP/PRR system is flg22/FLS2 in *A. thaliana*, and whilst there are still many apects of the system that need to be further elucidated it serves as a model of how PAMP recognition and signalling in plants is currently thought to occur. Flg22 is 22 amino acids in length and is the minimum active epitope of bacterial flagellin (Felix *et al.*, 1999). In the absence of flg22 FLS2 interacts with other proteins including the RLCKs BIK1 and AVRPPHB SUSCEPTIBLE1 (PBS1)-like (PBLs) (Zhang *et al.*, 2010). In the presence of flg22, the binding of flg22 to FLS2 is thought to lead to a complex forming between FLS2, the LRR-RLK BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE (BAK1) and BIK1, although the interaction between BAK1 and BIK1 is still a matter of debate and may occur via FLS2 rather than directly (Lu *et al.*, 2010, Zhang, 2010 #431). BAK1 is a member of the somatic embryogenesis receptor-like kinase (SERK) family and is also known as SERK3. Other members of the SERK family, such as BAK1-LIKE (BKK1) (SERK4), may also be part of the receptor complex (Roux *et al.*, 2011). Phosphorylation events of FLS2, BAK1 and BIK1 occur very rapidly after flg22 binding to FLS2, and BIK1 partially dissociates from the complex upon phosphorylation (Lu *et al.*, 2010, Zhang, 2010 #431). At least two mitogen-activated protein kinase (MAPK) cascades are then activated (Nicaise *et al.*, 2009). A Ca^{2+} burst is also triggered (Jeworutzki *et al.*, 2010) and this potentially activates Ca^{2+}-dependent protein kinases (CDPKs) (Boudsocq *et al.*, 2010). The CDPKs act both with the MAPKs and independently of them to induce defence gene expression (Boudsocq *et al.*, 2010). The Ca^{2+} burst is capable of activating the nicotinamide adenine dinucleotide phosphate (NADP)H-oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) (Ogasawara *et al.*, 2008), which is required for the PTI reactive oxygen species (ROS) burst (Nühse *et al.*, 2007; Zhang *et al.*, 2007), although the mechanism of activation of RBOHD in PTI is not fully resolved. These initial signalling events lead to later responses such as callose deposition and seedling growth inhibition (Gómez-Gómez *et al.*, 1999).

BAK1 is not only present in the recognition of flg22, but also in the recognition of the bacterial PAMPs elongation factor Tu (EF-Tu), cold shock protein, PGN, lipopolysaccharides (LPS), and the *Phytophthora infestans* elicitin INF1 (Heese *et al.*,

2007; Shan *et al.*, 2008), as well as the DAMP AtPep1 (Krol *et al.*, 2010). Besides its role in PTI signalling, BAK1 is also involved in regulating brassinosteriod (BR) responses (Li *et al.*, 2002; Nam and Li, 2002), light signalling (Whippo and Hangarter, 2005) and cell death (Kemmerling *et al.*, 2007). The key role for BAK1 in regulating PTI responses makes it a target for pathogens trying to disrupt plant immunity, and plant pathogen effectors that target BAK1 have been identified (e.g. AvrPtoB (Cheng *et al.*, 2011a)). However, targeting BAK1 may have disadvantages for some pathogens, for example biotrophic pathogens would not benefit from the misregulated cell death seen in the absence of BAK1 (Kemmerling *et al.*, 2007).

Whilst BAK1 is a key component of mostly protein PAMP recognition, data are beginning to emerge that suggest the Lys-M-RLK CERK1 may act as a regulator of carbohydrate PAMP recognition responses, although the picture is still unclear. CERK1 is necessary for PGN perception in *A. thaliana*, along with Lys-M proteins LYSM DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN1 (LYM1) and LYM3 (Willmann *et al.*, 2011). A previous study by another group found that BAK1 is involved in *A. thaliana* PGN perception (Shan *et al.*, 2008). This is intriguing as CERK1 chitin responses in *A. thaliana* are BAK1 independent (Gimenez-Ibanez *et al.*, 2009). In *O. sativa*, OsCERK1 and the Lys-M RLP CHITIN ELICITOR BINDING PROTEIN (OsCEBiP) form a complex that mediate chitin perception and immunity to fungal infection (Shimizu *et al.*, 2010), further suggesting an analogous role for CERK1 to that of BAK1. Yet, in *A. thaliana* CERK1 has been shown to bind to chitin both *in vitro* and *in vivo* (Iizasa *et al.*, 2010; Petutschnig *et al.*, 2010) and can therefore be viewed as a PRR in its own right (Segonzac and Zipfel, 2011). A recent study has found that an additional gene, Lys-M RLK4 (LYK4), may be involved in chitin signal transduction in *A. thaliana* (Wan *et al.*, 2012). Whether CERK1 is a PRR or a facilitator of PAMP recognition through Lys-M RLPs, or both, its contribution to PTI is shown by the fact that it is targeted by the bacterial effector AvrPtoB (Gimenez-Ibanez *et al.*, 2009).

The importance of PTI as a defence response to pathogens means that diverse plant higher plant families such as Brassicaceae, Solanaceae and Poaceae can respond to PAMPs. Boller and Felix claim that all major groups of higher plants can respond to flg22 (Boller and Felix, 2009), although no data to support this has yet been published. Downstream PTI signalling components have not been characterised well outside of *A. thaliana*, although a recent study showed that in *N. benthamiana* orthologous genes to those identified to play a role in *A. thaliana* PTI are also involved and the pattern of signalling events is broadly the same (Ca^{2+} burst, followed by ROS burst and MAPK activation, and then defence gene expression) (Segonzac *et al.*, 2011). This suggests a degree of conservation of the PTI pathway amongst higher plants. Consistent with the

idea of a co-evolutionary arms race between plant defences and pathogens, some plants have been found to have family specific PRRs, such as EFR in the Brassicaceae and XA21 in *O. sativa*. Unique PRRs also open up the prospect of producing more resistant crop plants, as the resistance to bacteria pathogens gained by expressing EFR in two solanaceous plant species shows (Lacombe *et al.*, 2010).

The next active level of the immune system after PAMP/DAMP recognition is the interaction between effectors produced by the invading pathogen and *R* genes. *R* genes produce proteins that confer resistance to a plant by mediating direct or indirect recognition of a pathogen effector (van der Hoorn and Kamoun, 2008), and often contain NBS and LRR domains (Jones and Dangl, 2006). The perception of the recognized pathogen effector (traditionally referred at as an Avr protein because its presence meant a pathogen could not successfully colonize a host) by a plant's resistance gene is known as the gene-for-gene hypothesis (Flor, 1971) and many examples of such interactions have been characterized (e.g. RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) and AvrRps4 (Hinsch and Staskawicz, 1996; Gassmann *et al.*, 1999)). In contrast with PRR recognition of PAMPs at the plant cell membrane the interaction between R proteins and recognized effectors occurs largely intracellularly.

The method of perception of the Avr protein by the plant is an area of debate. Whilst some R proteins are thought to bind directly with the Avr protein (e.g. (Dodds *et al.*, 2006)) other R proteins are thought to act indirectly through either the guard model (van der Biezen and Jones, 1998) or the decoy model (van der Hoorn and Kamoun, 2008). The guard model proposes that R proteins monitor the pathogen target (the "guardee") and react to changes in it caused by the binding of the Avr protein. Alternatively the decoy model proposes that the pathogen host has proteins that mimic the target of the effector and are specialised in its recognition. The R protein responds to binding of these "decoy" proteins but the pathogen does not gain any advantage, as it is implied it would from being bound to its intended target.

The role of many effectors secreted by the pathogens is to manipulate host cell function and they can do this in a number of ways including suppressing immunity through targeting protein turnover and the phosphorylation pathway (Block *et al.*, 2008), suppressing hypersensitive cell death (Bos *et al.*, 2006), and mimicking or modulating plant hormones (Hogenhout and Loria, 2008; Sugio *et al.*, 2011).

The plant immune signalling events described above can be combined to build a scheme of the evolutionary relationship between plants and organisms that wish to feed from it, such as pathogenic microbes. Initially in a pathogen's attempt to colonize a plant PAMPs are recognized by PRRs and this leads to an immune response which can halt further colonization (PTI). However, the immune response to PAMPs may be interfered

with by effectors released by the pathogen, and this can lead to the plant becoming susceptible again (effector-triggered susceptibility (ETS)). Effectors may subsequently be recognized by a NBS-LRR protein, triggering a much larger immune response that may lead to a hypersensitive cell death response (HR) at the site of infection (ETI). At this point natural selection in the pathogen-plant arms race drives pathogens to lose or change the effector gene that was recognised, or acquire extra effectors that suppress the immune response of the initial effector. Likewise, natural selection drives the plant to develop new *R* genes so that the effectors can be recognized again. The different levels of the plant immune system come together to form the 'zigzag' model (Jones and Dangl, 2006) that summarizes the above interactions between pathogen and plant. The model takes its name from the increase and decrease in the amplitude of the plant defence responses during successive phases of a pathogen attack.

1.11 Animal immunity

Animal immune systems have been the subject of much study over recent years, and comparisons with plant immune systems have highlighted some interesting similarities and differences. Vertebrates have two kinds of immune response: innate immune responses that are triggered at the beginning of an infection and do not depend on prior exposure to the pathogen, and adaptive immune responses that are highly specific to the pathogen that induce them and involve immunoglobulin genes and T cell receptor genes. Non-vertebrates and plants only possess innate immune responses (Alberts *et al.*, 2008).

Animals and plants show similarities in their innate immune response. For example they can both perceive MAMPs and DAMPs through PRRs at the plasma membrane, and they also respond to some of the same microbial molecules such as PGN and LPS (Dow *et al.*, 2000; Raetz and Whitfield, 2002; Akira *et al.*, 2006; Gust *et al.*, 2007). An example of a molecule where the PRRs in both plants and animals have been identified is flagellin. Flagellin is perceived by the LRR domains of both FLS2 in plants (Gómez-Gómez and Boller, 2000) and TOLL-LIKE RECEPTOR5 (TLR5) in animals (Hayashi *et al.*, 2001). Whilst both receptors recognize flagellin, the conserved regions of the molecule that are recognized are different in the two systems (Smith *et al.*, 2003). The similarities in MAMP receptors in plants and animals have been proposed to be due to convergent evolution (Ausubel, 2005), although more examples of how both sets of organisms perceive the same MAMPs are needed before clear conclusions can be made.

Whilst ETI in plants has been the subject of much study, recent data suggest that animals are also likely to have ETI (Stuart *et al.*, 2013). The clearest current example of

animal ETI is the *Escherichia coli* effector CNF1 (cytotoxic necrotizing factor 1), which triggers an effective immune response in *Drosophila melanogaster* that is independent of PRRs (Boyer *et al.*, 2011). A similar response is seen in mammalian systems (Boyer *et al.*, 2011). CNF1 recognition does not occur directly, but is due to modification of Rac2 (Ras-related C3 botulinum toxin substrate 2) by the effector. Rac2 then interacts with the proximal immune adaptor protein IMD (immune deficiency) to activate IMD innate immune signalling (Boyer *et al.*, 2011). As more examples of ETI in animal immunity emerge it will be possible to make more detailed comparisons of how they compare to plant ETI.

An interesting difference between animal and plant innate immunity may exist in intracellular sensing of MAMPs. Mammals have been reported to mount an innate immune response to MAMPs in the cytosol. Flagellin and rod protein from bacteria activate the nucleotide-binding LRR (NLR) receptor NLR family CARD domain-containing 4 (NLRC4) (Miao *et al.*, 2010). This response leads to inflammatory cell death known as pyroptosis. A recent investigation found no evidence for a similar cytosolic immune response to MAMPs in *N. benthamiana* (Wei *et al.*, 2012).

1.12 Insect interactions with plant immunity

Aphid interactions with plants have been proposed to follow a similar pattern to the ‘zigzag’ model of plant-pathogen interactions (Hogenhout and Bos, 2011) (Figure 1.2), with PAMPs being replaced by HAMPs. The following sections of this introduction give a summary of what we know about plant-insect, and particularly plant-aphid, interactions at each step in the model.

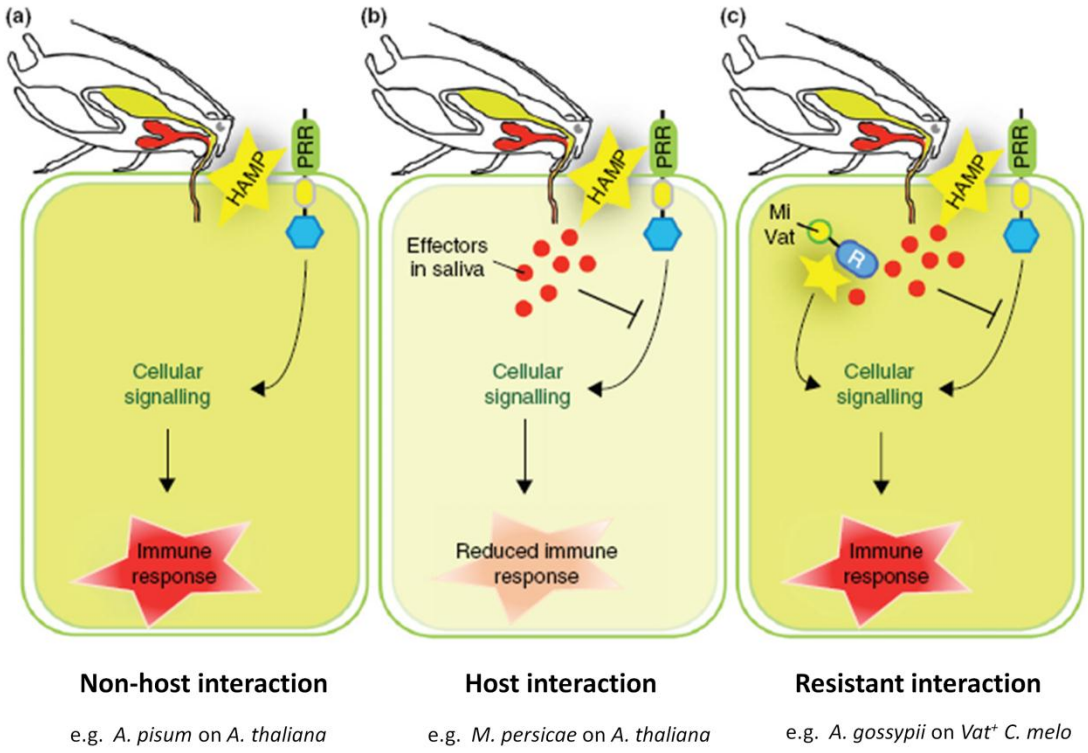

Figure 1.2 – Model of aphid interactions with the plant immune system.
(A) Aphids produce elicitors (here represented as HAMPs) which are perceived by plant cells using PRRs. The subsequent signalling leads to effective plant resistance against the aphid (HAMP-triggered immunity (HTI)). This occurs when aphids are on non-host plants.
(B) Effectors present in aphid saliva can suppress HTI, allowing successful colonization of the plant by the aphid. This occurs on susceptible host plants.
(C) Plant *R* genes recognise effectors used to suppress HTI, leading to a strong immune response and enhanced levels of resistance (ETI). This occurs on resistant host plants. *Mi-1* and *Vat* have been identified as aphid resistance genes. Image adapted from (Hogenhout and Bos, 2011) and used with the permission of the publisher (Elsevier).

1.13 Insect elicitors of plant defence

There are many examples of molecules from chewing insects that act as elicitors of plant defences. These include fatty acid-amino acid conjugates (FACs) (Alborn *et al.*, 1997), GOX (Musser *et al.*, 2005; Diezel *et al.*, 2009), β-glucosidase (Mattiacci *et al.*, 1995) and inceptins (Schmelz *et al.*, 2006) from lepidopterans, and caeliferins (Alborn *et al.*, 2007) and lipases (Schafer *et al.*, 2011) from grasshoppers. These molecules are present in the insect regurgitant or saliva and therefore are perceived by plants when the insects feed.

Some of them, such as inceptins, are produced from damage to the plant by the insect, and can be considered DAMPs. Lipids in the eggs of the cabbage butterfly *Pieris brassicae* trigger defence responses in *A. thaliana* similar to PTI responses (Little *et al.*, 2007; Bruessow *et al.*, 2010; Gouhier-Darimont *et al.*, 2013).

In contrast, aphid elicitors of plant defence are not very well characterised. GOX, identified as a lepidopteran elicitor of plant defence (Musser *et al.*, 2005), is found in aphid saliva (Harmel *et al.*, 2008) and therefore may act as an elicitor. Will and van Bel (Will and van Bel, 2008) postulated that enzymes in the aphid saliva sheath would digest plant cell walls to produce OGs, which trigger plant defence. OGs are a DAMP that trigger responses similar to the PAMP flg22 (Denoux *et al.*, 2008). No direct evidence has currently been found for this, but aphid salivary enzymes have been shown to trigger plant defences. Polyphenol oxidases in the saliva of the grain aphid *Sitobion avenae* and *S. graminum* elicit jasmonic acid (JA) and terpene signalling pathways in wheat (Ma *et al.*, 2010). Indirect defences to aphids can also be triggered by enzymes in saliva. When pectinases in the saliva of *S. avenae* are applied to wheat they trigger the release of volatiles that attract the parasitoid wasp *Aphidius avenae* (Liu *et al.*, 2009).

The best example to date of an aphid salivary elicitor of plant defence is from *M. persicae*. Applying the saliva of *M. persicae* to *A. thaliana* plants leads to an induction of defence against the aphid, shown by a reduction in aphid performance (De Vos and Jander, 2009). The plant defence response did not depend on PHYTOALEXIN DEFICIENT4 (PAD4), a gene involved in *A. thaliana* defence to *M. persicae*, or the known defence signalling pathways involving JA, salicylic acid (SA) or ethylene (ET). The saliva component was not identified but was found to be a heat sensitive peptide between 3 and 10 kDa.

1.14 Plant signalling in response to insects

Whilst much research has been conducted on plant defence compounds against insect attack, relatively little is known about how plants perceive signals derived from the insect (Wu and Baldwin, 2010). However, when the early plant signalling components and responses upon elicitor perception from herbivorous chewing insects are compared to those triggered by plant pathogens elicitors, some similarilities are beginning to emerge. There is an example of an insect elicitor inducing a Ca^{2+} burst (Schafer *et al.*, 2011) in addition to examples of insect feeding or elicitors inducing ROS bursts (e.g. (Maffei *et al.*, 2006)), as well as MAPK activation (Wu *et al.*, 2007; Schafer *et al.*, 2011). However, there are still several parts of the signalling pathway that are unaccounted for (for example no

receptors for elicitors have been identified (Wu and Baldwin, 2010)), and those parts whose involvement have been identified are not well understood. Whilst several elicitor receptors, co-receptors and regulators in the plant membrane have been identified in the plant-pathogen field, no genes involved in the perception of the elicitor at the plant membrane have been confirmed for insects. BAK1 is a likely candidate to be involved in insect elicitor perception, as it is involved in the perception of many proteinaceous elicitors. *Bak1* was silenced in the wild tobacco species *Nicotiana attenuata*, but this did not appear to affect perception of caterpillar elicitors (Yang *et al.*, 2011). LECTIN RECEPTOR KINASE 1 (LecRK1) is a recently identified RLK in *N. attenuata* and is involved in plant defence against the tobacco hornworm *Manduca sexta* (Gilardoni *et al.*, 2011), although it is unclear if this gene is acting as a receptor to elicitors from the insect. The authors currently hypothesise that LecRK1 acts downstream of the signalling cascade upon perception of the insect elicitor (Gilardoni *et al.*, 2011). Another lectin receptor kinase, LecRK-I.8 has recently been shown to play a role in plant responses to insect egg elicitors (Gouhier-Darimont *et al.*, 2013). Clear evidence for LecRK-I.8 being an egg elicitor receptor has not been presented, and the partial responsiveness to the elicitor that remains in the *lecrk-I.8* mutant shows that if LecRK-I.8 is an elicitor receptor then it is not the sole receptor.

Knowledge of elicitor-triggered signalling in aphid-plant interactions is less well developed than that of chewing herbivore elicitors, partly due to the better characterisation of chewing herbivore elicitors. However, similar plant signalling reponses to those described above for chewing herbivores have been documented for aphid feeding. Aphid feeding induces a ROS burst in several plant species (Moloi and van der Westhuizen, 2006; Kusnierczyk *et al.*, 2008; Kerchev *et al.*, 2012), as well as a Ca^{2+} burst in *A. thaliana* (Kusnierczyk *et al.*, 2008). *A. thaliana* plants mutant in RBOHD, required for PTI ROS generation, are more susceptible to *M. persicae* (Miller *et al.*, 2009), suggesting it could potentially play a role in signalling responses to aphid elicitors. Two different aphid species have been shown to induce the PTI reporter gene *PHYTOALEXIN DEFECIENT3* (*PAD3)* (Kusnierczyk *et al.*, 2008; De Vos and Jander, 2009) in *A. thaliana*. Taken together, these data point to a common perception of aphids by plants that shares similarilities with plant perception of microbial pathogens.

1.15 Plant defence responses to insect attack

Plants display a vast array of defences against insects and pathogens, ranging from physical to chemical defences, constitutive to induced defences, and direct to indirect

defences. Defence responses to aphids also bear similarities to those used against pathogens (Walling, 2000). For example, *M. persicae* infestation of potato plant, *Solanum tuberosum* L. Cv. Désirée, results in the synthesis of products from the 9-lipoxygenase pathway which is also induced by pathogens (Gosset *et al.*, 2009). *M. persicae* also increases gene expression of pathogenesis-related proteins when feeding on *A. thaliana* (Moran and Thompson, 2001) and these proteins are usually elicited by biotrophic pathogen attack but not by other herbivorous insects (de Vos *et al.*, 2007). Secondary metabolites, such as glucosinolates in *A. thaliana*, have also been shown to play a role in defence against aphids (de Vos *et al.*, 2007) and pathogens (Clay *et al.*, 2009). *M. persicae* feeding causes *A. thaliana* to alter its glucosinolate profile to gain a greater defensive benefit (Kim *et al.*, 2008), and the plant pathogen PAMP flg22 causes callose deposition that requires glucosinolates (Clay *et al.*, 2009).

It is probable that all plants possess toxic secondary metabolites, either induced or constitutive, as part of their defence response against herbivores (Howe and Jander, 2008). Secondary metabolites is a term used for the many chemical compounds in plants that are not involved in the biochemical processes of plant growth and reproduction (Hartmann, 2007). However, they do enhance the fitness of the plant through defence against pathogens and pests. Different plant species produce their own suite of secondary metabolites, with some metabolites being restricted to certain phylogenetic group. For example, the glucosinolates mentioned above are found in the Brassicaceae (Fahey *et al.*, 2001). Many classes of secondary metabolites besides glucosinolates may play a role in plant defence against aphids including terpenes (Aharoni *et al.*, 2003), alkaloids (Cai *et al.*, 2004), and nonprotein amino acids (Adio *et al.*, 2011). In *A. thaliana* the secondary metabolite camalexin, produced from a metabolic pathway that branches from the indole glucosinolates (Glawischnig, 2007), has been found to play a role in plant-aphid interactions (Kusnierczyk *et al.*, 2008). Camalexin has also been shown to be involved in some plant-pathogen interactions, such as those involving necrotrophic fungi (Thomma *et al.*, 1999).

A. thaliana has been used to identify genes involved in plant interactions with aphids. Much work has centred on the interaction of certain clones of *M. persicae* with only a few accessions of *A. thaliana*, and therefore whether the results of these studies can be generalised to all *M. persicae-A. thaliana* interactions is still to be shown. Plant defences to aphids can be anti-xenotic, a deterrent against settling and feeding on the plant, or antibiotic, limiting the insects reproduction, survival or growth (Goggin, 2007). The best studied gene in the *A. thaliana-M. persicae* interaction is *PAD4*. *PAD4* encodes a protein with similarity to lipases (Jirage *et al.*, 1999) that is involved in a phloem-mediate form of defence (Pegadaraju *et al.*, 2007) to a clone of *M. persicae*, which

involves both antibiotic and anti-xenotic effects. Antibiosis was shown through increased aphid reproduction on *pad4* mutants and decreased reproduction on *35S:PAD4* transgenic lines when compared to the wildtype in assays where the aphids were given no choice on host plant (Pegadaraju *et al.*, 2007). In addition, *M. persicae* population sizes were larger when fed on artificial diets supplemented with petiole extract from plants mutant in *PAD4* (Louis *et al.*, 2010a). Anti-xenosis was shown using choice assays on the same plants lines, results in the preference of mutants over wildtype and wildtype over *PAD4* expressing transgenic lines (Pegadaraju *et al.*, 2007). Interestingly, PAD4 is also involved in defence against biotrophic pathogens and increasing the accumulation of SA and camalexin (Zhou *et al.*, 1998; Jirage *et al.*, 1999; Feys *et al.*, 2001) as well as playing a role in ETI to pathogens (Louis *et al.*, 2012). PAD4 seem to play a different role in aphid defence compared to plant-pathogen defence (de Vos *et al.*, 2007), as mutants in SA, camalexin and ETI were investigated and found to be unchanged in *M. persicae* defence (Pegadaraju *et al.*, 2005; Louis *et al.*, 2012). *PAD4* expression in *A. thaliana* is influenced by the sugar trehalose (Singh *et al.*, 2011). Trehalose also plays a role in *M. persicae* defences in *A. thaliana* that is independent of PAD4, as mutants in the trehalose-synthesizing enzyme TREHALOSE PHOSPHATE SYNTHASE11 (TPS11) have improved *M. persicae* performance (Singh *et al.*, 2011). PAD4 may regulate the activity or stability of another potential lipase involved in *A. thaliana* defence against *M. persicae*, MYZUS PERSICAE-INDUCED LIPASE1 (MPL1), although it seems more likely that the two possible lipases participate in antibiosis through parallel pathways rather than the same one (Louis *et al.*, 2010b). Given the role of PAD4 in *M. persicae-A. thaliana* interactions, it is surprising that it seems to have no influence on the nymph development of a closely related insect, *B. tabaci* biotype B (Walling, 2008). It is possible that *B. tabaci* either do not trigger PAD4-mediated defence, or are able to counteract it in some way.

1.16 Aphid responses to plant defences

The presence of plant defences provides the selection pressure for aphids to evolve methods of avoiding them. The production of secondary metabolites is an example. The secondary metabolite gramine is produced by plants in the Gramineae in order to deter and/or kill a number of herbivorous insects including some aphid species (Cai *et al.*, 2009). *S. avenae* has been shown to increase activity of peroxidases and polyphenolic oxidases in response to gramine in order to degrade it (Cai *et al.*, 2009). Peroxidases and polyphenolic oxidases are present in aphid saliva, however it unclear whether it is salivary enzymes that respond in this instance.

Aphids may also overcome plants defences by manipulating the plant phytohormone-mediated signalling pathways. The main three pathways that are associated with defence against pest and pathogens are the JA pathway, the SA (derived from the shikimate-phenylpropanoid pathway) pathway, and the ET pathway (Walling, 2008). There is cross talk between this pathways that can act antagonistically (in the case of SA and JA) or synergistically (in the case of JA and ET) (Koornneef and Pieterse, 2008). The current 'decoy' hypothesis is that aphids are manipulating the antagonistic cross talk between SA and JA by inducing SA signalling in order to repress the potentially more damaging effects of the JA induced responses (Thompson and Goggin, 2006). Although experimental evidence supporting this hypothesis is not available for aphids (de Vos *et al.*, 2007), SA has been shown to be involved in plant defence against aphids (Li *et al.*, 2006), and supporting evidence for the hypothesis has been shown in whiteflies, which feed in a similar way to aphids (Kempema *et al.*, 2007). There is support for a similar manipulation of plant defences by microbial pathogens (Zhao *et al.*, 2003; Brooks *et al.*, 2005).

1.17 Aphid candidate effectors

The responses of plants at a molecular level to aphid feeding suggest that aphid saliva contains effectors to modulate host plant processes to the benefit of aphid colonization. Most work on aphid effectors has not passed the hypothesis stage. Ramsey and colleagues (Ramsey *et al.*, 2007) examined *M*. *persicae* ESTs from salivary glands and identified 30 potential secreted proteins that were not predicted to remain in the cell membrane. They suggested that these proteins may be required for phloem feeding and plant defence suppression. Carolan and colleagues (Carolan *et al.*, 2011) identified over 300 putative effectors in *A. pisum* based on protein presence in the salivary glands and predicted secretion signal. These genes covered a wide range of functions and some shared homology with previously identified genes in other phytopathogenic organisms such as nematodes and fungi. To date, none of the hypotheses put forward by this work have been tested. Similarly, Nicholson and colleagues (Nicholson *et al.*, 2012) identified 34 proteins in the saliva of *D. noxia*, some of which had the potential to be effectors but none of which have any functional data. For example, it was postulated that the trehalase found in the saliva might be used to degrade trehalose, a molecule with a role in *A. thaliana*-*M. persicae* interactions (Singh *et al.*, 2011). However, the role of trehalose on aphid interactions outside of *A. thaliana* and tomato (*Solanum lycopersicum*) (Singh and Shah, 2012) has not been investigated.

The strongest candidate effectors from these approaches are the GMC oxidoreductases GOX and GLD. GOX has been identified in proteomic analysis of *M. persicae* saliva, and its activity was also found in the saliva (Harmel *et al.*, 2008). GLD was identified in *M. persicae* saliva (Harmel *et al.*, 2008) and several GLD paralogs were found in *D. noxia* saliva (Nicholson *et al.*, 2012) as well as *A. pisum* salivary glands (Carolan *et al.*, 2011), suggesting that it may be a common saliva constituent of aphids in general. GOX is a common part of caterpillar saliva (Eichenseer *et al.*, 2010) and has been shown to suppress plant defences against the corn earworm *Helicoverpa zea* in *Nicotiana tabacum* (Musser *et al.*, 2002). As mentioned previously, GOX also induces plant defences (Musser *et al.*, 2005). GLD is proposed to function in a similar way to GOX (Nicholson *et al.*, 2012).

However, aphid saliva has been shown to have a potential effector function. Will and colleagues (Will *et al.*, 2007) found a function for watery saliva proteins in suppressing host plant defences in the interaction between *M. viciae* and *V. faba*. This effector function was due to the saliva proteins' ability to bind Ca^{2+}. They showed that aphids changed their salivation behaviour when the sieve tubes of the plant were experimentally occluded, that the aphids' saliva could contract the forisomes, whose dispersion caused sieve tube occlusion, in the same way as ethylenediaminetetraacetic acid (EDTA), and that aphid saliva proteins were capable of binding Ca^{2+}. The proteins that are acting as effectors have not been identified. Will and colleagues went on to propose that watery saliva may be universally used by aphids to counteract sieve-tube occlusion mechanisms, with the binding of Ca^{2+} as the mostly likely way of doing this (Will *et al.*, 2009). The data provided does not currently support such a generalised theory, as some aphid species were unable to reverse the occlusion of the sieve-tube they were feeding from once it had been occluded (Will *et al.*, 2009).

Proteomic studies of saliva have identified potential Ca^{2+} binding proteins in *A. pisum* and *D. noxia* (Carolan *et al.*, 2009; Nicholson *et al.*, 2012). A Ca^{2+} binding protein (NcSP84) was also identified as a major component of the saliva in another hemipteran insect, the green rice leafhopper *Nephotettix cincticeps*, and was shown to be secreted into the plant (Hattori *et al.*, 2012), suggesting Ca^{2+} binding may be a common strategy amongst phloem-feeding insects. The Ca^{2+} binding protein calreticulin was found in both *D. noxia* saliva and *A. pisum* salivary glands. Jaubert-Possamai and colleagues (Jaubert-Possamai *et al.*, 2007) used RNAi to silence calreticulin in *A. pisum*, achieving a maximum knock-down on day 5 post injection to 60% of control expression levels. They observed no change in aphid growth, survival or reproduction. This may be due to insufficient knock-down in expression to affect aphid feeding, or several Ca^{2+} binding proteins may be used in the aphid saliva, leading to redundancy. Alternatively calreticulin

may play no direct role in the plant-insect interaction but is present in the saliva for other reasons, such as acting as a chaperone.

The first aphid salivary gene identified as a potential effector was *C002*. *C002* was originally identified in *A. pisum* and had no sequence similarity to sequences in public depositories (Mutti *et al.*, 2008). Homologs of the gene have recently been identified in several aphid species (Ollivier *et al.*, 2010; Cui *et al.*, 2012; Liu *et al.*, 2012b). The gene is predominately expressed in the salivary glands of *M. persicae* (Pitino *et al.*, 2011); whilst salivary gland and gut expression have been reported in *A. pisum* (Mutti *et al.*, 2008). This may be a by-product of contamination whilst dissect aphids for RNA extraction, or may reflect a physiological requirement for the aphid to have COO2 present in the gut as well as saliva. *A. pisum* introduces C002 into *V. faba* during feeding (Mutti *et al.*, 2008) and peptides corresponding to this protein have also been detected in mass spectrometry analysis of *M. persicae* saliva (Harmel *et al.*, 2008). *C002* is an important gene in plant-aphid interactions as silencing it through injection of siRNA into the hemolymph leads to death in *A. pisum* on plants but not an artificial diet of sucrose and amino acids (Mutti, 2006). Silencing *C002* in *M. persicae* through feeding from plants producing dsRNA leads to reduced fecundity (Pitino *et al.*, 2011). *A. pisum* with knocked down gene expression for *C002* have altered feeding behaviour including problems finding and feeding from the phloem (Mutti *et al.*, 2008). Sequences of *C002* from four aphids representing the two aphid tribes, Aphidini (*A. pisum* and *M. persicae*) and Macrosiphini (the brown citrus aphid *Toxoptera citricida* and *A. gossypii*), were subjected to phylogenetic analysis and showed that the gene under positive selection; the ratio of non-synonymous to synonymous substitutions in the nucleotide sequence (dN/dS ratio) of *C002* was 0.57 between *A. pisum* and *M. persicae* sequences, compared to a median value of 0.238 (Ollivier *et al.*, 2010). However, no specific sites of positive selection were found for *C002* (Ollivier *et al.*, 2010). These results were confirmed by another recent study (Pitino and Hogenhout, 2013) that provided evidence that sites of positive selection may exist in alignment gaps between *C002* from different species. Analysis of *C002* transcripts in two biotypes of *D. noxia* showed a total of nine different variants, only one of which was shared between the two biotypes (Cui *et al.*, 2012). Given the important role C002 in aphid feeding, the large amount of diversity in sequence within and between aphid species, and the high rate of evolution, C002 may be an adaptive response to different plant genotypes and species as part of a co-evolutionary arms race. In this sense C002 is similar to eukaryotic pathogen effectors whose evolution is driven by positive selection, with multiple alleles maintained in the population (Ma and Guttman, 2008).

1.18 Plant recognition of insect effectors

Due to the importance of insects as agricultural pests much research has been carried out on plant *R* genes to insects. *R* genes to insects have been found in a wide range of plant species and have been reported for the insect orders Hemiptera, Coleoptera, Thysanoptera, Lepidoptera, and Diptera (Smith and Clement, 2012). Only three *R* genes to insects have been cloned; *Mi-1.2, Virus aphid transmission* (*Vat*) and *Brown planthopper14* (*Bph14)* (Hogenhout and Bos, 2011). These genes all share the fact that they provide resistance to a biotype of hemipteran insect and they encode coiled-coil (CC) NBS-LRR, similar to those that recognise plant-pathogen effectors. No insect effectors recognized by these *R* genes have currently been identified.

Mi-1.2 was isolated from *S. lycopersicum* and confers resistance to three species of hemipteran insects (some clones of the potato aphid *Macrosiphum euphorbiae*, two biotypes of whitefly, and a psyllid) as well as three species of nematode (Milligan *et al.*, 1998; Rossi *et al.*, 1998; Nombela *et al.*, 2003; Casteel *et al.*, 2006). The resistance involves SA and involves one or more MAPKs (Li *et al.*, 2006), and has been proposed to act in a gene-for-gene manor with an aphid protein (Kaloshian, 2004). *Vat* was isolated from melon (*Cucumis melo*) and confers resistance to *A. gossypii* (Dogimont *et al.*, 2010), as well as several potyviruses transmitted by the aphid (Sattar *et al.*, 2012). Recognition of the aphid by the plant may lead to a localised HR (Villada *et al.*, 2009) and is regulated by microRNAs (Sattar *et al.*, 2012). One apparent mechanism for the resistance to aphids in *C. melo* is by enhancing sieve element wound healing and thus blocking an insect's food canal (Walling, 2008). Callose deposition has also been associated with *Vat* resistance (Villada *et al.*, 2009). *Bph14* was isolated from *O. sativa* and confers resistance to the brown planthopper (*Nilaparvata lugens*). *Bph14* shares similar resistance mechanisms to the aphid resistance genes. In a similar way to *Mi-1.2,* the resistance conferred by *Bph14* seems to involve the SA pathway (Du *et al.*, 2009). *Bph14* and *Vat* share an association between callose deposition and resistance.

Some aphid resistance genes exist as quantitative trait loci (QTLs) but have not been identified as individual genes, such as the resistance genes in barley (*Hordeum vulgare*), rye (*Secale cereale*) and wheat to *D. noxia* (Smith and Boyko, 2007). It is likely that several other uncloned aphid resistance genes also encode NBS-LRR proteins. For example, two candidate Toll/Interleukin-1 Receptor (TIR) NBS-LRR were identified in a 115 kilobase region associated with soybean (*Glycine max*) resistance to *Aphis glycines* (Kim *et al.*, 2010). Also, *ACYRTHOSIPHON KONDOI RESISTANCE (AKR)*, a resistance gene to the bluegreen aphid (*Acyrthosiphon kondoi*) in *M. truncatula*, maps to a region

flanked by predicted CC-NBS-LRRs (Klingler *et al.*, 2005). Further work on these genes and others will provide information on how widely aphid resistance is controlled by NBS-LRR proteins.

The co-evolutionary arms race between plant and pathogen described in the 'zig-zag' model predicts that pathogens lose or change the effectors recognized by resistance genes in order to break the host resistance. Whilst this can happen rapidly for some plant pathogen species, the reduced amount of sexual reproduction in their lifecycle means that resistance against aphids can be more durable in some instances. Sexual reproduction in pathogen species significantly increases the risk of resistance-breaking biotypes or races (McDonald and Linde, 2002). An example of the durability of aphid resistance is the *Ag1* gene in raspberry (*Rubus* spp.) that confers resistance to the large raspberry aphid *Amphorophora agathonica*. This gene was extensively used for around 50 years before an adapted biotype appeared (Daubeny and Anderson, 1993). Similarly, *Vat* resistance against *A. gossypii* in southern France has been deployed for 20 years without breaking down (Dogimont *et al.*, 2010). However, such long term resistance does not always occur. *S. graminum* biotypes able to break resistance are normally found within a few years of the release of a new *S. graminum*-resistant sorghum (*Sorghum bicolour*) cultivar (Zhu-Salzman *et al.*, 2003).

1.19 Unique plant defence responses to aphids

Possessing similarities in defences to different types of invader is beneficial to a plant and so similarities between pathogen and herbivore defences might be expected. However, each category of invader of a plant carries its own individual challenges and therefore it is to be expected that plants have more aphid specific defences too. Phloem-located mechanisms of resistance to aphids have been identified in some crop cultivars, although the mechanisms are not clear and the responses are mostly aphid species specific (Tjallingii, 2006). Volatile emission is an indirect defence response that can be aphid specific. Gosset and colleagues (Gosset *et al.*, 2009) found that plants released the alarm pheromone EBF in response to insect infestation, and that aphid infested plants produced a higher proportion of EBF than infestation by the chewing herbivore the Colorado potato beetle (*Leptinotarsa decemlineata*). EBF is not only an aphid alarm pheromone but it also attracts aphid predators (two-spot ladybird beetle (*Adalia bipunctata*) – (Francis *et al.*, 2004)) and parasitoids (marmalade hoverfly (*Episyrphus balteatus*) – (Francis *et al.*, 2005)). Aphid control strategies using EBF have been discussed previously in section 1.4. Aphid salivary enzymes may also play a role in inducing volatile responses, as

pectinases from the saliva of the *S. avenae*, induced parasitoid attracting volatiles when they were exogenously applied to wheat (Liu *et al.*, 2009).

1.20 Animal immune interactions with insects

A detailed review of all that is known about insect interactions with animal immune systems is beyond the scope of this thesis. However, an overview of the current knowledge in this field is useful in allowing comparisons to be drawn between aphids and insects that feed on animals. Insect-animal interaction research centres on blood-feeding insects of mammals, as these are often vectors of important diseases and viruses such as malaria, leishmaniasis and West Nile virus. The use of a piercing mouthpart in these insects to feed from animal vasculature provides interesting parallels with phloem feeding insects such as aphids. To summarize and compare animal-insect interactions with plant-insect interactions I am going to use the scheme of interactions from the model in figure 1.2.

Blood feeding insects include mosquitos, sand flies, and ticks. All of these three groups of insects are known to trigger immune responses when they feed on mammals (Kamhawi *et al.*, 2000; Schneider *et al.*, 2007; Chmelar *et al.*, 2012), although the presence of adaptive as well an innate immunity in animals makes the picture more complicated than that proposed in the model. Sand flies possess a candidate for an elicitor as defined in this thesis, as all tested species were able to induce immune responses from their saliva proteins in several hosts including mice, dogs and humans (reviewed in (Chmelar *et al.*, 2012)). One protein that caused immune responses was identified in *Phlebotomus papatasi* and called PpSP15 (Valenzuela *et al.*, 2001). However, the mechanism by which sand fly salivary proteins stimulate the immune system remains unknown (Chmelar *et al.*, 2012). The example of sand fly saliva is representative of other blood feeding insects; whilst something is known about the proteins that trigger animal immunity, there is little known about the animal genes involved in perceiving the elicitors and mounting an immune response. Therefore, unlike animal immunity to microbial pathogens, there are no identified animal PRRs to insects elicitors.

Much research has been conducted on insect salivary proteins that act as effectors in animal-insect interactions. Recently, many groups have used transcriptomics to predict the content of blood feeding insect saliva, and a comparison of these efforts in the suborder Nematocera showed that most of the identified protein families had no functional data available (Ribeiro *et al.*, 2010). This draws parallel to the lists of candidate

aphid effectors that have yet to be functionally characterised (Harmel *et al.*, 2008; Carolan *et al.*, 2011; Nicholson *et al.*, 2012). In addition to the transcriptomics studies, functional studies have been conducted on many insect saliva proteins. For example, partial characterisation at the molecular and biochemical level has taken place for more than 50 tick salivary proteins (Chmelar *et al.*, 2012). A lack of knowledge of how insects trigger animal immune responses means there are likely to be many mechanisms of insect effectors that are yet to be discovered.

One host response that blood-feeding insects need to overcome in order to feed is hemostasis, which causes bleeding to stop. Insect effectors target this response in many ways including vasodilators (e,g. sialokinin I from the mosquito *Aedes aegypti* (Beerntsen *et al.*, 1999)), inhibitors of blood coagulation (e.g. Ixolaris from the tick *Ixodes scapularis* (Francischetti *et al.*, 2002)) and platelet aggregation (e.g. Aegyptin from the *Aedes aegypti* (Calvo *et al.*, 2007)). An analogy can be drawn between hemostasis in animals and phloem sieve tube occlusion mechanisms in plants, as both processes are trying to limit the loss of vascular system contents. Therefore it is interesting that phloem feeding insects such as aphids have been proposed to use saliva proteins to reverse sieve tube occlusion (Will *et al.*, 2007), as blood feeding insects use saliva to overcome hemostasis.

Animals are likely to possess ETI. The current ETI examples in animal systems are all from bacterial pathogens (Stuart *et al.*, 2013). As research in this area grows examples of insects triggering ETI may be found.

1.21 Contributions to this thesis

All experiments in this thesis were conducted by me, unless acknowledged in the figure legends (figures 3.6 and 5.1). Individuals who shared knowledge or technical expertise are stated at the start of each results chapter (chapters 3 to 7). Contributions of plasmids and plant seeds are acknowledged in chapter 2.

1.22 Focus and aims of research described in this thesis

Several aspects of aphid biology share parellels with those of biotrophic plant pathogens.These include the long-term and intimate nature of aphids feeding behaviour, the plant responses triggered by aphid feeding, the secretion of proteins into the plant whilst feeding and the identification of classical NBS-LRR plant resistance genes that confer resistance. Whilst interations between biotrophic pathogens and the plant immune

system have been much studied in recent years, the role of the plant immune system in plant-aphid interaction is largely unknown. Given the similarities between aphids and biotrophic plant pathogens, we hypothesised that the plant immune system plays a role in plant-aphid interactions and set out to investigate this at the molecular level. The primary aim of this research was to identify how aphids may trigger and suppress the plant immune system through their saliva.

1.23 Overview of thesis contents

We began our investigation of the interaction between aphids and the plant immune system by screening for aphid salivary effectors (chapter 3). We used *M. persicae* and *N. benthamiana* as a system to test whether aphid salivary gland genes possessed common characteristics of plant pathogen effectors when expressed *in planta*. This screen revealed three genes that possessed one or more of these characteristics. All three of the candidate effector effected aphid fecundity. One of the effectors also caused a chlorosis phenotype and suppressed PTI signalling. These data suggested that aphids possess effectors to manipulate the plant immune system, and that they may contain elicitors of the immune system similar to PAMPs.

To characterise whether aphids contain elicitors of the plant immunity we looked for signalling responses and induction of defences in *N. benthamiana* upon treatment with *M. persicae* extract (chapter 4). Having found these responses, and thus confirmed that *M. persicae* possesses elicitors of plant immunity, we then tested if the LRR-RLK BAK1 was necessary for the signalling, and attempted to purify the elicitors that caused the responses. We identified at least three different aphid elicitor fractions of *N. benthamiana* immunity based on molecular weight, chemical properties and the nature of the plant responses. Two of the elicitor fractions triggered signalling pathways that were BAK1-independent. One of the elicitor fractions was the same molecular weight as a previously described *M. persicae* elicitor triggering defence responses in *A. thaliana*.

A. thaliana is a plant with many research tools available, and its immune system has been well studied. The immune responses of different plant species differ in sensitivity to elicitors. We therefore continued our investigation of *M. persicae* elicitors of plant immunity by looking for signalling responses and induction of defences in *A. thaliana* upon treatment with *M. persicae* extract (chapter 5). *A. thaliana* also responds to *M. persicae* extract with immune signalling responses, but these show differences when compared to PAMPs such as flg22. We identified at least two different aphid elicitor fractions of *A. thaliana* immunity based on molecular weight, chemical properties and the

nature of the plant responses. One of these elicitors had the same properties as the previously reported *M. persicae* elicitor. We were able to show that this elicitor fraction triggered a signalling pathway that required BAK1 but did not require known BAK1-interacting RLKs. We therefore provided further evidence that the saliva of aphids can induce as well as suppress plant immunity.

To discover the RLK that was involved in perceiving the *M. persicae* elicitor we screened a collection of *A. thaliana* mutants in non-RD RLKs for response to the elicitor (chapter 6). However, none of the single non-RD RLK mutants showed a consistent reduction in the response. Therefore, the receptor involved in aphid elicitor perception remains unknown.

Having discovered *M. persicae* elicitors, we then investigated whether one of the effectors, Mp10, was able to suppress the plant immune responses that the elicitors trigger, and if so then how it was doing it (chapter 7). We found that Mp10 was able to suppress aphid elicitor-triggered immune signalling. This seemed to occur at the plasma membrane part of the signalling cascade, before the Ca^{2+} burst. Mp10 homologs in other aphids also suppress immune signalling, suggesting that this is a necessary part of colonizing a plant.

This study is the first to dissect the role of plant immunity in plant-aphid interactions, and provides a framework for further exploration of this topic. We report a role for elicitor recognition by plants in aphid defence and started the characterisation of the aphid elicitors and the role of plant surface receptors in this interaction. We also highlight the differences and similarities in immune response of distantly related plant species to aphids. In addition, we studied aphids' attempts to suppress plant immunity and found it may be common to many aphid species.

Chapter 2 – Materials and methods

2.1 Insect maintenance conditions

***M. persicae* colonies**

An initial stock colony of *M. persicae* (RRes genotype O) (Bos *et al.*, 2010) was continuously reared in 52 cm x 52 cm x 50 cm cages containing up to six Chinese cabbage (*Brassica rapa*, subspecies *chinensis*) plants with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod.

A large number of aphids were subsequently taken from the Chinese cabbage stock cage and placed on *N. tabacum* plants in order to select for individuals able to colonize and reproduce on this plant species. Many of the aphids died but some were able to colonize *N. tabacum* and reproduced. These aphids and their progeny were continuously maintained on *N. tabacum* plants to form a *N. tabacum*-adapted colony. This colony was reared in 52 cm x 52 cm x 50 cm cages containing two to four *N. tabacum* plants under the same conditions as the Chinese cabbage stock cage.

Aphids were also subsequently taken from the Chinese cabbage stock cage and placed on *N. benthamiana* plants in order to select a *N. benthamiana* adapted colony, in the same manner as described above. This colony was reared on at least two *N. benthamiana* plants in pots that were covered by a perforated plastic bag (30 cm by 40 cm, Seal-Packaging Limited, Luton, Bedfordshire, UK) secured by an elastic band. This colony was maintained in a MLR-351H versatile environmental test chamber (Sanyo, Osaka, Japan) with 14 h day (18^{o}C) and 10 h night (15^{o}C) photoperiod.

A. pisum

A stock colony of *A. pisum* was continuously reared in 52 cm x 52 cm x 50 cm cages containing up to four broad bean (*V. faba*) plants with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod.

A. gossypii

A stock colony of *A. gossypii* was continuously reared in 24 cm x 54 cm x 47 cm cages containing a cotton (*Gossypium hirsutum*) plant with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod.

Brevicoryne brassicae

A stock colony of the cabbage aphid *B. brassicae* was continuously reared in 24 cm x 54 cm x 47 cm cages containing a Chinese cabbage plant with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod.

Macrosiphum rosae

The rose aphid *M. rosae* was collected from cultivated rose bushes in my garden in Norwich.

Rhopalosiphum padi

A stock colony of the bird cherry-oat aphid *R. padi* was continuously reared in 24 cm x 54 cm x 47 cm cages containing one pot with several oat (*Avena sativa*) plants with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod.

2.2 Plant growth conditions

A. thaliana

All *A. thaliana* plants used for experiments were germinated and maintained on Scotts Levington F2 compost (Scotts, Ipswich, UK). *A. thaliana* seeds were vernalized for one week at 5-6^{o}C and grown in a controlled environment room (CER) with a 10 h day (90 µmol m^{-2} s^{-1}) and a 14 h night photoperiod and at a constant temperature of 22^{o}C.

***A. thaliana* mutants**

The following *A. thaliana* mutants are all in the Columbia (Col-0) background and were obtained from various laboratories as indicated. The *bak1-5*, *bak1-4*, *bkk1-1*, *efr-1* (*efr*), and *fls2-17* (*fls2)* mutants (Zipfel *et al.*, 2004; Zipfel *et al.*, 2006; He *et al.*, 2007; Schwessinger *et al.*, 2011) were provided by Ben Schwessinger (Dr. Cyril Zipfel's group, The Sainsbury Laboratory (TSL), Norwich, UK). The *pepr1-1, pepr1-2,* and *pepr2-1* (Yamaguchi *et al.*, 2010) were provided by the Nottingham Arabidopsis Stock Centre (NASC) (http://arabidopsis.info/). The *pepr1/pepr2* double mutant (Krol *et al.*, 2010) was obtained from Dr. Dirk Becker (Department of Molecular Plant Physiology and Biophysics, University of Wuerzburg, Wuerzburg, Germany). The collection of transfer deoxyribonucleic acid (T-DNA) insertion mutants in non-RD kinases belonging to the interleukin-1 receptor-associated kinase (IRAK) family (Danna *et al.*, 2011) (Table 2.1) was obtained from Prof. Frederick Ausubel (Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA, USA) and plant lines homozygous for the T-DNA insertion were generated by Dr. Lena Stransfeld (Dr. Cyril Zipfel's group, TSL, Norwich, UK). The double

homozygous T-DNA insertion mutant in At5g56040 and At4g26540 was provided by NASC.

Line number	Mutant name	Gene annotation/ name	Predicted to be cytosolic	T-DNA line	Line number of another allele in the collection
1	*AT4G26540-2*	M3E9.30		SAIL_1220_B03	11
2	*AT1G67000-1*	F1O19.18		SAIL_400_A10	
3	*AT3G47580-1*	F1P2.130		SAIL_252_H12	
4	*AT3G47090-1*	F13I12.140		SALK_101474	13
5	*AT5G38260-2*	MXA21.150		SALK_049448	19
6	*AT1G80870-2*	F23A5.23		SALK_138934	12
8	*AT3G47110-1*	F13I12.160		SALK_101668	
9	*AT5G05160-2*	RUL1		SALK_074276	10
10	*AT5G05160-1*	RUL1		SALK_056624	9
11	*AT4G26540-1*	M3E9.30		SALK_053167	1
12	*AT1G80870-1*	F23A5.23		SALK_049258	6
13	*AT3G47090-2*	F13I12.140		SALK_026298	4
16	*AT5G38250-1*	MXA21.140		SAIL_670_C08	
19	*AT5G38240-1*	MXA21.150		SALK_142662	5
20	*AT1G66920-2*	T4O24.7		SAIL_563_H08	
21	*AT4G18250-1*	T9A21.100		SALK_056431	26
22	*AT5G56040-1*	MDA7.8		SALK_052069	
23	*AT1G66980-1*	SNC4		SALK_122292	28
24	*AT5G39020-2*	MXF12.30		SALK_122162	
26	*AT4G18250-2*	T9A21.100		SALK_036670	21
28	*AT1G66980-2*	SNC4		SALK_139303	23
31	*AT1G66930-1*	T4O24.2		GK-284B09	
32	*AT1G68400-1*	T2E12.5		GK-218D01	
33	*AT3G47570-2*	F1P2.120		GK-415H04	42
38	*AT5G38280-1*	PR5K		GK-254G07	
39	*AT5G39030-1*	MFX12.40		SALK_007613	
40	*AT2G24130-1*	F27D4.4		SALK_025037	44
41	*AT5G51770-1*	MIO24.10	Y	SALK_056450	43
42	*AT3G47570-1*	F1P2.120		SALK_063487	33
43	*AT5G51770-2*	MIO24.10	Y	SALK_075797	41

Table 2.1.

44	*AT2G24130-2*	F27D4.4		SALK_101008	40
46	*AT5G35370-1*	T26D22.12		SALK_123639	50
47	*AT2G19130-1*	T20K24.15		SALK_000051	54
48	*AT5G20050-1*	F28I16.200	Y	SALK_000490	
50	*AT5G35370-2*	T26D22.12		SALK_024084	46
53	*AT4G25390-1*	T30C3.60	Y	SALK_093369	
59	*AT3G26700-1*	MLJ15.17	Y	SALK_142166	
61	*AT2G30940-1*	F7F1.15	Y	SALK_137752	
62	*AT5G18910-1*	F17K4.160	Y	SALK_129579	
63	*AT1G33260-1*	T16O9.6	Y	SALK_049165	
65	*AT3G15890-1*	MVC8.1	Y	SALK_085834	
67	*AT4G10390-1*	F7L13.4	Y	GK-658A06	
68	*AT2G45910-1*	F4I18.11	Y	GK-122F04	
69	*AT1G66910-1*	T4O24.8		WISC_145_096	
70	*AT5G24080-1*	MZF18.3		SAIL_551_D12	71
71	*AT5G24080-2*	MZF18.3		SALK_147104	70
72	*AT2G13800-1*	SERK5		SALK_147275	
73	*AT3G09780-1*	CCR1		SALK_089159	74
74	*AT3G09780-2*	CCR1		SALK_109759	73
75	*AT2G45590-1*	F17K2.12	Y	SALK_087417	
76	*AT5G20480-1*	EFR		SALK_044334	
77	*AT5G46330-1*	FLS2		SAIL_691_C04	

Table 2.1 – The non-RD IRAK T-DNA insertion mutation collection.

N. benthamiana

N. benthamiana plants used for experiments were germinated on Scotts Levington F1 compost (Scotts) and transferred after 12 days to square black plastic pots (base measurement 5 cm x 5 cm, top measurement 7.5 cm x 7.5 cm, height 8 cm) containing Scotts Levington F2 compost (Scotts). All *N. benthamiana* plants were grown in a CER with a 16 h (120 µmol m^{-2} sec^{-1}) day and 8 h night at a constant temperature of 22^{o}C. The *N benthamiana* plant line stably expressing the reporter protein aequorin (SLJR15 – (Segonzac *et al.*, 2011)) was obtained from Dr. Cécile Segonzac (Dr. Cyril Zipfel's group, TSL, Norwich, UK).

V. faba

V. faba plants were grown in Scotts Levington F2 compost (Scotts). Three seeds were planted 1.5 cm from the surface of a square black plastic pot (base measurement 5 cm x 5 cm, top measurement 7.5 cm x 7.5 cm, height 8 cm) filled with compost. The pots were covered in foil and placed in a greenhouse. The foil was removed once shoots appeared. Plants were attached to stakes as they grew.

2.3 RNA methods

RNA extraction

Aphid RNA was extracted by collecting 20 adult aphids from the appropriate stock cage in a 1.5 ml Eppendorf, snap-freezing in liquid nitrogen and storing at -80°C until processing. The aphids were ground in chilled 1.5 ml Eppendorfs using disposable pellet pestles (Sigma-Aldrich, St Louis, USA). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and followed by DNase I treatment (RQ1 DNase set, Promega, Madison, USA). RNA was purified using the RNA cleanup protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany), analyzed for purity on an ethidium bromide (EtBr)-stained 1% agarose gel and Picodrop spectrophotometer (Picodrop Ltd, Saffron Walden, UK) or Nanodrop 2000c spectrophotometer (Nanodrop products, Wilmington, DE, USA). The RNA samples had an A260/A280 ratio of between 1.85 and 2.

cDNA synthesis

Complementary DNA (cDNA) was synthesised from 1 µg RNA using a Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) kit (Invitrogen, Carlsbad, USA) and oligo dT primer following the manufacturer's instructions.

2.4 Cloning

Cloning of candidate effectors

Candidate effector genes were identified by Dr Jorunn I.B. Bos (Department of Disease and Stress Biology (DSB), John Innes Centre (JIC), Norwich, UK) from *M. persicae* salivary gland ESTs (Bos *et al.*, 2010), and primers for these genes were designed to clone the open reading frame (ORF) minus the signal peptide. Primer sequences contained a restriction site and the forward primer contained an ATG at the beginning of the ORF to allow translation. *M. persicae* cDNA was made from adult aphids from the

Chinese cabbage stock cage as described above. The sequences corresponding to the candidate effectors were amplified from the *M. persicae* cDNA using Phusion polymerase (Finnzymes, Vantaa, Finland) and the following program: 30 secs at 98°C, followed by 30 cycles of (10 sec at 98°C, 30 sec at 58°C, 60 sec at 72°C), followed by 10 mins at 72°C. A small amount of the polymerase chain reaction (PCR) product was analyzed for size on an EtBr-stained 1% agarose gel and then the remainder was cleaned up using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Cleaned up PCR products were digested using *Bam*HI, *Spe*I, or *Xba*I (Roche, Burgess Hill, UK). All PCR products were digested with *Bam*HI/*Spe*I, except Mp51 which used *Bam*HI/*Xba*I due to the presence of a *SpeI* site in the sequence. Digested PCR products were ligated into *Bam*HI/*Spe*I or *Bam*HI/*Xba*I digested pCB302-3 vector (Xiang *et al.*, 1999) using T4 DNA ligase (Invitrogen). Ligations were transformed into electrocompetent *E. coli* (DH5α) cells by electroporation, incubated in Super Optimal Broth with Catabolite repression (SOC) media at 37^{o}C, plated on Luria Broth (LB) plates containing 50 µg/ml kanamycin, and grown at 37^{o}C overnight. Colonies were tested for the presence of the insert by amplification using vector specific primers, GoTaq polymerase (Promega) and the following conditions: 3 mins at 98°C, followed by 30 cycles of (1 min at 95°C, 30 sec at 55°C, 90 sec at 72°C), followed by 10 mins at 72°C. A small amount of the PCR product was analyzed for size on an EtBr-stained 1% agarose gel and then the remainder was ethanol precipitated overnight and resuspended in distilled water (dH_2O). The purified DNA (10-20 ng per 100 base pairs (bp) of sequence) was sequenced using BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA, USA) and vector primers, according to the manufacturer's instructions. Ready reactions were submitted to Genome Enterprise Limited (The Genome Analysis Centre, Norwich, UK) for sequencing on Life Technologies 3730XL capillary sequencers. Sequences were analyzed with CodonCode Aligner software (CodonCode Corporation, Dedham, Massachusetts). Good quality sequences were aligned with the corresponding ESTs to look for sequence differences. Clones with the correct sequence were grown overnight at 37^{o}C in LB plus 50 µg/ml kanamycin and plasmid DNA purified using QIAprep Spin Miniprep Kit (Qiagen). Purified plasmid DNA was then transformed into electrocompetent *A. tumefaciens* (GV3101 with pSOUP) by electroporation, incubated in SOC media at 30^{o}C, plated on LB plates containing 50 µg/ml kanamycin and 50 µg/ml rifampicin, and grown at 30^{o}C for 48 hours. Colonies were tested for the presence of the plasmid by amplification using one vector specific primer, one sequence specific primer, and GoTaq polymerase (Promega) as described above. Positive colonies were grown overnight at 30^{o}C in LB plus 50 µg/ml kanamycin and 50 µg/ml rifampicin, and Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) permanent freezer stocks were then made and stored at -80^{o}C.

Candidate effector	Primer name	Representative EST Genbank identifier	Restriction enzyme site	Sequence (5'>3')
Mp3	Mp3-35S-F	DW011743	*Bam*HI	GGAGGATCCATGAAATATG GCTGGTCCACTGTGAAT
	Mp3-35S-R		*Spe*I	GGA ACTAGT CCTTAGAA ATAACAATTTACACG
Mp4	Mp4-35S-F	ES226741	*Bam*HI	GGAGGATCCATGAAAATTTC TCTAATATCTCTTGG
	Mp4-35S-R		*Spe*I	GGAACTAGTGTCACCTTCC ACACCACCAACC
Mp5	Mp5-35S-F	EC389841	*Bam*HI	GGAGGATCCATGCACGAAT CGTTCTTCGGACAAG
	Mp5-35S-R		*Spe*I	GGAACTAGTAATATTGAACA GAATTTTATAGC
Mp6	Mp6-35S-F	EC387921	*Bam*HI	GGAGGATCCATGACACCTG TGCCAGCAGAAGACCAG
	Mp6-35S-R		*Spe*I	GGAACTAGTTAAATATGTGA TGCTTAAATTGGCGC
Mp7	Mp7-35S-F	ES226253	*Bam*HI	GGAGGATCCATGGAAGATA ACCCAGAAGAATGTGAG
	Mp7-35S-R		*Spe*I	GGAACTAGTCGCTACATATA ATATGGATTTTAA
Mp8	Mp8-35S-F	ES226080	*Bam*HI	GGAGGATCCATGGATGTTA TTGTCCGAGCTACCGCG
	Mp8-35S-R		*Spe*I	GGAACTAGTTTACTTGTTGT TGTCAATGCAA
Mp46	Mp46-35S-F	EE264770	*Bam*HI	GGAGGATCCATGCACAAAT TAATAAAAGTCGATC
	Mp46-35S-R		*Spe*I	GGAACTAGTTAATTTTGATT TAATTTTGAGATG
Mp47	Mp47-35S-F	EE264742	*Bam*HI	GGAGGATCCATGGCTCCTG CTGAAACAATAATTGG
	Mp47-35S-R		*Spe*I	GGAACTAGTAATTTCTAAGT ATGACGTAATGC
Mp50	Mp50-35S-F	EE264652	*Bam*HI	GGAGGATCCATGAAGTCTG ACAGTGAAATTGATTTG

Table 2.2.

	Mp50-35S-R		*Spe*I	GGAACTAGTGTGGCTAGTACTAATAATGATG
Mp51	Mp51-35S-F	EE264632	*Bam*HI	GGAGGATCCATGAATGAAATTAACGTCAAACAACTG
	Mp51-35S-R		*Xba*I	GGATCTAGATATAATATTGTTGAGAAATCTCCC
Mp53	Mp53-35S-F	EE264538	*Bam*HI	GGAGGATCCATGGATGTGAGTCAACAACAACAAGGA
	Mp53-35S-R		*Spe*I	GGAACTAGTGAAGTGTGATCGGGGAGATGTCGC
Mp54	Mp54-35S-F	EE261990	*Bam*HI	GGAGGATCCATGGGAAAAGTGCCATCTTCAGATT
	Mp54-35S-R		*Spe*I	GGAACTAGTTAAACTGTGATTCTGCATGGCCA
N/A	pCB302-3-F	N/A	N/A	GAGAACACGGGGGACTCTAGC
	pCB302-3-R		N/A	ACATGCTTAACGTAATTCAACAG

Table 2.2 – Primers for cloning of *M. persicae* candidate effectors.
Above primers designed by Dr Jorunn I.B. Bos (DSB, JIC, Norwich, UK).

Cloning of *Ap10*

Primers were designed to clone the ORF minus the signal peptide of *Ap10*. *A. pisum* RNA was extracted and cDNA synthesised in the way described above. *Ap10* was then amplified from the cDNA using the primers below and subsequently cloned into pCB302-3 as described above.

Primer name	**Representative EST Genbank identifier**	**Restriction enzyme site**	**Sequence (5'>3')**
Ap10-35S-F	NM_001126180	*Bam*HI	GGAGGATCCATGGCGCCGCAAAAAGATGCTG
Ap10-35S-R		*Spe*I	GGAACTAGTGTGCGTATAGGTACTGCATTCTAATG

Table 2.3 – Primers for cloning of *Ap10*.

Gateway cloning of *Mp10* constructs

Primers were designed to clone the ORF minus the signal peptide of *Mp10*. The Mp10-GW-FL primer contained the nucleotide sequence corresponding to the FLAG motif (DYKDDDDK) before the *Mp10* sequence. Primer sequences contained attb1 and attb2 adapters at the ends. *M. persicae* cDNA was made with adult aphids from the Chinese cabbage stock cage as described above. *Mp10* was amplified from the *M. persicae* cDNA using Phusion polymerase (Finnzymes) and the following program: 30 secs at 98°C, followed by 30 cycles of (10 sec at 98°C, 30 sec at 58°C, 60 sec at 72°C), followed by 10 mins at 72°C. A small amount of the PCR product was analyzed for size on an EtBr-stained 1% agarose gel. The *Mp10* fragment was then amplified using attb1 and attb2 adapter primers and a small amount of the PCR product was analyzed for size on an EtBr-stained 1% agarose gel. The *Mp10* fragment was then cloned into pDONR207 (Invitrogen) using BP Clonase II (Invitrogen) following the manufacturer's instructions. Reactions were transformed into electrocompetent *E. coli* (DH5α) cells by electroporation, incubated in SOC media at 37^{o}C, plated on LB plates containing 10 µg/ml gentamicin, and grown at 37^{o}C overnight. Positive clones were identified, using vector primers and GoTaq polymerase (Promega), and sequenced as described above to verify the origin and sequence of the insert. Clones of plasmids with the correct sequence were grown overnight at 37^{o}C in LB plus 10 µg/ml gentamicin and plasmid DNA purified using QIAprep Spin Miniprep Kit (Qiagen). In this way pDONR plasmids were constructed that contained *Mp10* and *Mp10* with an N-terminal FLAG tag (FLAG-Mp10). LR Clonase II was used according to the manufacturer's instructions to clone: *Mp10* into the Gateway adapted vector pB7WGF2 (Karimi *et al.*, 2002), which creates an enhanced green fluroscent protein (eGFP)-ORF fusion and has a 35S promoter fragment cloned at the 5′ end of the clonase compatible insertion site; and FLAG-Mp10 into the Gateway adapted vector pB7WG2 (Karimi *et al.*, 2002), which also has a 35S promoter fragment cloned at the 5′ end of the clonase compatible insertion. Positive clones were identified using the attb1 colony and Mp10-GW-R or Mp10-GW-F and attb2 colony primers and GoTaq polymerase (Promega) as described above. Clones were grown overnight at 37^{o}C in LB plus the appropriate antibiotic and plasmid DNA purified using QIAprep Spin Miniprep Kit (Qiagen). Purified plasmid DNA was then transformed into electrocompetent *A. tumefaciens* (GV3101 with pSOUP) for pB7WG2 and pB7WGF2 by electroporation, incubated in SOC media at 30^{o}C, plated on LB plates containing the appropriate antibiotics, and grown at 30^{o}C for 48 hours. Colonies were tested for the presence of the plasmid by amplification using the attB1 colony primer and Mp10-GW-R, and GoTaq polymerase (Promega) as described above. Permanent freezer stocks were made of positive colonies as decribed above and stored at -80^{o}C.

Primer name	Representative EST Genbank identifier	Sequence (5'>3')
Mp10-GW-F*	ES225905	AAAAAGCAGGCTCCATGGCG CCGCAAAAAGATGCTGTG
Mp10-GW-FL	ES225905	AAAAAGCAGGCTCCATGGAC TACAAGGACGACGATGACAA AGCGCCGCAAAAAGATGCTG TG
Mp10-GW-R*	ES225905	AGAAAGCTGGGTCTTAAAATT TGACAACACCTTTTTTC
attb1 adapter	N/A	GGGGACAAGTTTGTACAAAA AAGCA GGCT
attb2 adapter	N/A	GGGGACCACTTTGTACAAGA AAGCTGGGT
pDONR207-FW	N/A	TCGCGTTAACGCTAGCATGG ATCTC
pDONR207-RV	N/A	GTAACATCAGAGATTTTGAGA CAC
attB1 colony	N/A	ACAAGTTTGTACAAAAAAGCA GGC
attB2 colony	N/A	ACCACTTTGTACAAGAAAGCT GGG

Table 2.4 – Primers for cloning of *Mp10* constructs.

*Primers designed by Dr. Jorunn I.B. Bos (DSB, JIC, Norwich UK)

Gateway cloning of *Mp10* truncated versions

Primers were designed to create Mp10 constructs with deletions in the N or C terminus. These primers (shown below) were used to clone truncated versions of *Mp10* into pB7WGF2 (Karimi *et al.*, 2002) using the Gateway system and the method described above.

Primer name	Starting position in Mp10 amino acid sequence of forward primers	Ending position in Mp10 amino acid sequence of reverse primers	Sequence (5'>3')
Mp10-GW-F*	23	N/A	AAAAAGCAGGCTCCATGGCGCCGCAAAAAGATGCTGTG
Mp10-GW-F1	37	N/A	AAAAAGCAGGCTCCATGACCACAAAATACGACCATATT
Mp10-GW-F1T	38	N/A	AAAAAGCAGGCTCCATGACAAAATACGACCATATTGAC
Mp10-GW-F1Y	40	N/A	AAAAAGCAGGCTCCATGTACGACCATATTGACATCGAC
Mp10-GW-F1D	41	N/A	AAAAAGCAGGCTCCATGGACCATATTGACATCGACC
Mp10-GW-F1H	42	N/A	AAAAAGCAGGCTCCATGCATATTGACATCGACCAAG
Mp10-GW-F1I	43	N/A	AAAAAGCAGGCTCCATGATTGACATCGACCAAGTTTTGGG
Mp10-GW-F2	45	N/A	AAAAAGCAGGCTCCATGATCGACCAAGTTTTGGGTTCCAA
Mp10-GW-F3	53	N/A	AAAAAGCAGGCTCCATGAGATTAGTAAACAGCTACGTTC
Mp10-GW-F4	61	N/A	AAAAAGCAGGCTCCATGTGTCTGCTGGACAAGAAACCG
Mp10-GW-F5	92	N/A	AAAAAGCAGGCTCCATGAATGCTACGCAAAAAAATGCTGCT

Table 2.5.

Mp10-GW-R*	N/A	145	AGAAAGCTGGGTCTTAA AATTTGACAACACCTTT TTTC
Mp10-GW-R1	N/A	126	AGAAAGCTGGGTCTTAA TATTCACGCTTAGGGTC CCA
Mp10-GW-R1.5	N/A	122	AGAAAGCTGGGTCTTAA GGGTCCCAT TTGTCAAGAA GCTG
Mp10-GW-R1D	N/A	121	AGAAAGCTGGGTCTTAG TCCCATTTG TCAAGAAGCTG
Mp10-GW-R1W	N/A	120	AGAAAGCTGGGTCTTAC CATTTGTCA AGAAGCTGTT TC
Mp10-GW-R1K	N/A	119	AGAAAGCTGGGTCTTAT TTGTCAAGAAGCTGTTT CC
Mp10-GW-R2	N/A	118	AGAAAGCTGGGTCTTAG TCAAGAAGCTGTTTCCA TTC
Mp10-GW-R4	N/A	91	AGAAAGCTGGGTCTTAA TTGCATTTCACACATTG GGT

Table 2.5 – Primers for cloning of *Mp10* truncated versions.

*Primers designed by Dr. Jorunn I.B. Bos (DSB, JIC, Norwich UK)

Gateway cloning of *MpOS-D1* and *Ag10*

Primers were designed to clone the ORF minus the signal peptide of the *M. persicae* homolog of Olfactory-specific D1 (*MpOS-D1)*. *MpOS-D1* was cloned into pB7WGF2 (Karimi *et al.*, 2002) using the Gateway system, the method described above and the primers shown below. Primers were designed to clone the ORF minus the signal peptide of *Ag10*. *A. gossypii* RNA was extracted and cDNA synthesised in the way described above. *Ag10* was cloned into pB7WGF2 (Karimi *et al.*, 2002) using the Gateway system, the method described above and the primers shown below.

Primer name	Representative EST Genbank identifier	Sequence (5'>3')
MpOS-D1_GW_F	AJ870491	AAAAAGCAGGCTCCATGGAAGAAAAG TACACAACTAAATTCG
MpOS-D1_GW_R		AGAAAGCTGGGTCTTAATGTTTAGCA GCTGCAGCC
Ag10__GW_F	FJ387487	AAAAAGCAGGCTCCATGGCGCCACAA AAAGATGCCGTAG
Ag10_GW_R		AGAAAGCTGGGTCTTAAAATTTAGTAA AACCC

Table 2.6 – Primers for cloning of *MpOS-D1* and *Ag10*.

Plasmids

All plasmids were generated as described above with the exception of the following: AvrPtoB containing vector (Gimenez-Ibanez *et al.*, 2009) was obtained from Dr. John Rathjen (TSL, Norwich, UK). 35s:GFP and Mp10 expressing pCB302-3 vectors, along with the empty vector (EV) control (Bos *et al.*, 2010), 35s-INF1, and pJawohl8-RNAi containing *Mp10* were provided by Dr. Jorunn I.B. Bos (DSB, JIC, Norwich, UK). pJawohl8-RNAi constructs containing sequences for *GFP* and *M. persicae C002* (pJGFP and pJMpC002, (Pitino *et al.*, 2011)) were kindly provided by Marco Pitino (DSB, JIC, Norwich UK). 35s:GFP expressing pB7WG2 vector was donated by Dr. Akiko Sugio (DSB, JIC, Norwich, UK). The binary TRV RNA1 construct, pBINRTA6, and the TRV RNA2 vectors, pTRV:EV and pTRV:NbSerk3 (Chaparro-Garcia *et al.*, 2011) were acquired from Prof. Sophien Kamoun (TSL, Norwich, UK).

Plasmid	Vector	Donor vector	Use in this thesis
35s:Mp3	pCB302-3	N/A	Effector screen assays
35s:Mp4	pCB302-3	N/A	Effector screen assays
35s:Mp5	pCB302-3	N/A	Effector screen assays
35s:Mp6_2	pCB302-3	N/A	Effector screen assays
35s:Mp6_6	pCB302-3	N/A	Effector screen assays
35s:Mp8	pCB302-3	N/A	Effector screen assays
35s:Mp46	pCB302-3	N/A	Effector screen assays
35s:Mp47	pCB302-3	N/A	Effector screen assays
35s:Mp50	pCB302-3	N/A	Effector screen assays
35s:Mp51	pCB302-3	N/A	Effector screen assays
35s:Mp53	pCB302-3	N/A	Effector screen assays
35s:Mp54	pCB302-3	N/A	Effector screen assays
35s:Ap10	pCB302-3	N/A	flg22 ROS burst assay
Mp10 pDONR	pDONR207	N/A	Gateway entry clone
FLAG-Mp10 pDONR	pDONR207	N/A	Gateway entry clone
GFP-Mp10 a.k.a Mp10ΔSP	pB7WGF2	Mp10 pDONR	flg22 ROS burst assay
FLAG-Mp10	pB7WG2	FLAG-Mp10 pDONR	flg22 ROS burst assay
Mp10Δ1-36 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-37 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-39 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-40 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-41 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-42 pDONR	pDONR207	N/A	Gateway entry clone

Table 2.7

Mp10Δ1-44 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-52 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-60 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-91 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ127-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ123-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ122-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ121-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ120-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ119-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10ΔSP Δ92-145 pDONR	pDONR207	N/A	Gateway entry clone
Mp10Δ1-36	pB7WGF2	Mp10Δ1-36 pDONR	flg22 ROS burst assay
Mp10Δ1-37	pB7WGF2	Mp10Δ1-37 pDONR	flg22 ROS burst assay
Mp10Δ1-39	pB7WGF2	Mp10Δ1-39 pDONR	flg22 ROS burst assay
Mp10Δ1-40	pB7WGF2	Mp10Δ1-40 pDONR	flg22 ROS burst assay

Table 2.7

Mp10Δ1-41	pB7WGF2	Mp10Δ1-41 pDONR	flg22 ROS burst assay
Mp10Δ1-42	pB7WGF2	Mp10Δ1-42 pDONR	flg22 ROS burst assay
Mp10Δ1-44	pB7WGF2	Mp10Δ1-44 pDONR	flg22 ROS burst assay
Mp10Δ1-52	pB7WGF2	Mp10Δ1-52 pDONR	flg22 ROS burst assay
Mp10Δ1-60	pB7WGF2	Mp10Δ1-60 pDONR	flg22 ROS burst assay
Mp10Δ1-91	pB7WGF2	Mp10Δ1-91 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ127-145	pB7WGF2	Mp10ΔSP Δ127-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ123-145	pB7WGF2	Mp10ΔSP Δ123-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ122-145	pB7WGF2	Mp10ΔSP Δ122-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ121-145	pB7WGF2	Mp10ΔSP Δ121-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ120-145	pB7WGF2	Mp10ΔSP Δ120-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ119-145	pB7WGF2	Mp10ΔSP Δ119-145 pDONR	flg22 ROS burst assay
Mp10ΔSP Δ92-145	pB7WGF2	Mp10ΔSP Δ92-145 pDONR	flg22 ROS burst assay
MpOS-D1 pDONR	pDONR207	N/A	Gateway entry clone
GFP-MpOS-D1	pB7WGF2	MpOS-D1 pDONR	flg22 ROS burst assay

Table 2.7

Ag10 pDONR	pDONR207	N/A	Gateway entry clone
GFP-Ag10	pB7WGF2	Ag10 pDONR	flg22 ROS burst assay

Table 2.7 – Plasmids in the work described in this thesis.

2.6 qRT-PCR

Quantitative RT-PCR (qRT-PCR) assays were conducted to quantify *M. persicae* gene expression upon feeding from *N. benthamiana* leaves expressing RNAs corresponding to *M. persicae* genes. Samples were composed of the remaining initial first-instar nymphs collected at the end of the RNAi fecundity experiment (see 2.9). RNA was extracted and cDNA synthesised (using 100 ng of RNA) as described above. cDNA from these reactions was diluted 1:6 with dH_2O prior to qRT-PCR. The 20 µl reactions were setup in 96-well white ABgene PCR plates (Thermo Scientific, Loughborough, UK) in a CFX96 Real-Time System with a C1000 Thermal Cycler (Biorad, Hemel Hempstead, UK) using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). All reactions were carried out using the following program: 3 mins at 95°C, followed by 40 cycles of (30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C), followed by melt curve analysis: 30 sec at 50°C, (65°C - 95°C at 0.5°C increments, 5 sec for each).

Reference genes for this study were chosen by selecting candidates in the published aphid literature. These were *L27, β-Tubulin, actin, acetylcholinesterase, EF1α, GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GAPDH*)*, RIBOSOMAL PROTEIN L32* (*RPL32*)*,* and *PROGENY INCREASE TO OVEREXPRESSION1* (*PIntO1*)*.* Using geNORM (Vandesompele *et al.*, 2002) within qbase^{PLUS} software (Biogazelle – www.biogazelle.com), it was established that three reference genes were required for accurate normalisation of the data and that *L-27, β-Tubulin,* and *actin* were the most stable genes across aphids fed on the three different dsRNA expressing *N. benthamiana* leaf disc types.

Primers for *MpC002* were already available (Pitino *et al.*, 2011). Primers for *Mp10* and *MpOS-D1* were designed using Primer-Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and the following conditions: Melting temperature of 60^{o}C, PCR amplicon lengths of 90 to 150 bp, primer sequences with lengths of 18 to 24 nucleotides with an optimum at 21 nucleotides,

and GC contents of 40 to 60%. Primers were tested for efficiency and specificity before continuing.

For analysis of the experimental data mean cycle threshold (Ct) values for each sample/primer pair combination were calculated from three replicate reaction wells. Mean Ct values were then converted to relative expression values using the formula 2-ΔCt (Pfaffl, 2001) such that the sample with the lowest mean Ct value (most concentrated) was assigned a value of 1. The geometric mean of the relative expression values of the three reference genes was calculated to produce a normalisation factor unique to each sample (Vandesompele *et al.*, 2002). Relative expression values for each gene of interest were normalised using the normalisation factor for each sample. The normalised expression values for each gene of interest were then compared between different dsRNA fed aphid samples. Student's *t*-tests were then used to compare the level of gene expression in dsMpC002 or dsMp10 samples compared to dsGFP samples. For display of data, mean expression values were rescaled such that aphids fed on dsGFP leaf discs represents a value of 1. The primer sequences used for both reference (Table 2.8) and target gene (Table 2.9) quantification are listed below.

Gene name	**Primer name**	**Sequence (5'>3')**	**Origin of primer**
L27	L27 F	CCGAAAAGCTGTCATAATGAAGAC	(Pitino *et al.*, 2011)
	L27 R	GGTGAAACCTTGTCTACTGTTACATCTTG	
ßTubulin	ßTubulin F	CCATCTAGTGTCGCTGACCA	(Pitino *et al.*, 2011)
	ßTubulin R	GTTCTTGGCGTCGAACATTT	
Actin	MpActF1	GGTGTCTCACACACAGTGCC	(Bass *et al.*, 2011)
	MpActR1	CGGCGGTGGTGGTGAAGCTG	
Acetylcholinesterase	Mp1aceF4	TAACGTAGTAGTGCCAAAGC	(Bass *et al.*, 2011)
	Mp1aceR3	CACTGTAGAGCCATTAGCTG	
EF1α	Ap EF1α F1	CTGATTGTGCCGTGCTTATTG	(Shakesby *et al.*, 2009)

Table 2.8.

	Ap EF1α R1	TATGGTGGTTCAGTAGAG TCC	
GAPDH	GAPDH_qPCR_F	AGA TGA AGT TGT GTC TTC CGA CTT T	(Shakesby *et al.*, 2009)
	GAPDH_qPCR_R	GAC AAA TTG GTC GTT CAA TGA AAT C	
RPL32		CGT CTT CGG ACT CTG	(Shakesby *et al.*,
	RPL32_qPCR_F	TTG TCA A	2009)
		CAA AGT GAT CGT TAT	
	RPL32_qPCR_R	GAC AAA CTC AA	
PIntO1	qPCR Mp1 F		(Pitino and
		CGGAAGAAGGAAGAAAT	Hogenhout,
		TGAAA	2013)
	qPCR Mp1 R	AGGTCTCCTCCCAATCCA AT	

Table 2.8 – *M. persicae* qRT-PCR reference gene primer sequences.

Gene name	Primer name	Sequence (5'>3')	Origin of primer
MpC002	MpC002 F	ACGATGATGAGGGAGGAGTG	(Pitino *et al.*, 2011)
	MpC002 R	GGGTTGCTAAATGCATCGTT	
Mp10	Mp10_qPCR_F2	GGTCGGAGCGCCGCAAAAAG	This thesis
	Mp10_qPCR_R2	TTGGAACCCAAAACTTGGTCGATGT	
MpOS-D1	OS-D1_qPCR_F2	ACCAACGAAGGCCGAGAATTGAGG	This thesis
	OS-D1_qPCR_R2	GGCGGTCAAACGATCAAAGTCAGT	

Table 2.9 – *M. persicae* qRT-PCR target gene primer sequences.

2.7 Preparation of aphid extracts and saliva collection

M. persicae extracts

Whole extract

Whole *M. persicae* extract was prepared by collecting apterous late instar and adult aphids from the Chinese cabbage stock cage using a moist paintbrush, placing them in a 2 ml Eppendorf tube, freezing in liquid nitrogen, and then grinding to a fine powder using a pre-chilled mortar and pestle. The powder was then transferred to a 50 ml Corning tube (Corning, New York, USA) on ice using a pre-chilled spoon. Sterile, distilled water was added to the ground powder at the volume of 1 ml water per 0.02 g (wet weight) of aphid, and the solution was mixed using a pipette.

Treated extract

M. persicae extract for fractionation by weight and treatment with proteinase K/boiling was prepared using a modified version of the protocol, similar to that of Lapitan and colleagues (Lapitan *et al.*, 2007). Aphids were collected, frozen, ground, and transferred to a Corning tube (Corning) as above. At this point, sterile 0.025 M potassium phosphate buffer (KH_2PO_4, pH 6.8) was added to the powder at the volume of 1 ml buffer per 0.02 g (wet weight) of aphid, and the solution was mixed with a pipette. The extract was then placed in Eppendorf tubes and centrifuged at 13200 rpm for 15 mins at 4^{o}C. The supernatant was then removed and placed in fresh tubes and processed further. Treated *M. persicae* extract was prepared in a similar way to the treatment of saliva in de Vos and Jander 2009, and Schafer *et al.,* 2011. *M. persicae* supernatant was boiled for 10 mins in order to denature the proteins or treated with proteinase K in order to degrade proteins. 500 µl of *M. persicae* supernatant was treated with 1 µl of 100 mg/ml (total 100 µg) proteinase K (Sigma-Aldrich) and incubated at 37^{o}C for 30 mins.

Fractionated extract

M. persicae extract fractionated by weight was prepared in a similar way to the fractionation of saliva in de Vos and Jander, 2009 and Schafer *et al.,* 2011. *M. persicae* supernatant was produced as described above and filtered by centrifuging at 13200 rpm for 15 mins at 4^{o}C using a 10 kDa cutoff-column (Ultracel 10K membrane, Millipore, Carrigtwohill, Co. Cork, Ireland). The fraction that passed through the column was termed less than 10 kDa, and the fraction that remained in the column was termed greater than 10 kDa. The fraction remaining in the column was retrieved by placing the column upside-

down in a fresh centrifuge tube at centrifuging at 1000 x g for 2 mins. The less than 10 kDa fraction was then further filtered by centrifuging at 13200 rpm for 15 mins at 4^{o}C using a 3 kDa cutoff-column (Ultracel 3K membrane, Millipore). The fraction that passed through the column was termed less than 3 kDa, and the fraction that remained in the column was termed 3 to 10 kDa. The fraction remaining in the column was retrieved by placing the column upside-down in a fresh centrifuge tube at centrifuging at 1000 x g for 2 mins. After filtering, all fractions were made back up to their original volume using potassium phosphate buffer.

Extracts of other aphid species

Species other than *M. persicae* were collected and processed as described above in order to obtain the larger than 10 kDa fraction of aphid extract. This extract was then boiled for 10 mins.

***M. persicae* saliva collection**

M. persicae saliva was collected using a Parafilm sachet (e.g. Harmel *et al.,* 2008, de Vos and Jander, 2009). Two 500 ml plastic tumblers (Sainsbury's Supermarkets Ltd, London, UK) had several small holes pierced in them with a hot syringe (Terumo, Egham, Surrey, UK). Approximately 1000 adult *M. persicae* from the Chinese cabbage stock cage, amounting to a weight of 0.2 g (50 adult *M. persicae* weighed 0.01 g), were added to one of the tumblers. The other tumbler served as a no aphid control. A thin layer of Parafilm (Brand GMBH, Wertheim, Germany) was stretched over each tumbler, and 1 ml of sterile, distilled water pipetted onto the Parafilm. A second layer of Parafilm was then stretched over each tumbler (Figure 2.1A). The tumblers were placed underneath a sheet of yellow plastic (Lincoln Polythene Ltd, Lincoln, Lincolnshire, UK) to enhance feeding activity, in a CER with 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod. After 24 hours the saliva/water was collected from both tumblers under sterile conditions. The 3 to 10 kDa fraction of the saliva and control was obtained using centrifugal filters as described above. After filtering, the saliva and control were made back up to their original volume using sterile, distilled water.

2.8 Infiltration of *N. benthamiana* with *A. tumefaciens*

Agrobacterium-mediated transient expression in *N. benthamiana*

A. tumefaciens strain GV3101 was used for all transient expression assays in *N. benthamiana*. Cultures of freezer stocks were grown overnight at 28^{o}C with gentle

shaking in LB media using the appropriate antibiotics. Cultures with an optical density at 600 nm (OD_{600}) of 0.3 were pelleted and resuspended in infiltration buffer (10 mM $MgCl_2$ (Sigma-Aldrich), 10mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich), pH 5.6) with 100 µM acetosyringone (Sigma-Aldrich) freshly added to initiate expression. Cultures were left for at least an hour and then infiltrated into the two youngest, fully expanded leaves of three and a half to four and a half-week old *N. benthamiana* plants using a needless 1 ml syringe (Terumo).

TRV-induced gene silencing in *N. benthamiana*

A. tumefaciens suspensions expressing the binary TRV-RNA1 construct, pBINTRA6, and the TRV-RNA2 vector, pTRV:EV or pTRV:NbSerk3 were mixed in a 1:1 ratio (RNA1:RNA2) in infiltration buffer (final OD_{600} was 0.6). Two to three week old *N. benthamiana* were infiltrated with the *A. tumefaciens* using a 1 ml needless syringe (Terumo). The second youngest fully expanded leaf was collected at two and a half weeks post infiltration and was used for experiments.

2.9 Aphid performance experiments

M. persicae and *N. benthamiana* experiments

Induced resistance assay

M. persicae induced resistance fecundity experiments were conducted in a MLR-351H environmental test chamber (Sanyo) with a 14 h day (18^{o}C) and 10 h night (15^{o}C) photoperiod. Three and a half week old *N. benthamiana* plants had 10 adult *M. persicae* caged to it with a clip cage (Figure 2.1B). These aphids came from the *N. benthamiana* adapted stock colony. Following the addition of aphids the plant was placed in the test chamber. After 24 h, all the adults were removed from the plants (day 0) and the plants returned to the test chamber. The plants were covered by a perforated plastic bag (30 cm by 40 cm, Seal-Packaging Limited) secured by an elastic band in order to stop the aphids escaping. These plants formed an aged population of *M. persicae*. On day 9 four-week old test plants were infiltrated using a needless 1 ml syringe (Terumo) with the three different fractions of aphid extract (diluted 1:10 with potassium phosphate buffer) and a buffer control on the youngest fully expanded leaf. The leaf's central vein and a marker pen line drawn horizontally across the leaf formed four quarters of the leaf and each quarter was infiltrated with one of the four solutions. After 24 h, an aged adult (10 days old) was placed in a clip cage using a moist paintbrush, and one cage placed on each

infiltrated area of each leaf. Plants were placed in the test chamber and left for 10 days. After 10 days (day 20), the number of aphids in each clip cage was counted. Each experiment included four or five leaves per repeat. Each experiment was repeated five times. Leaves where one of the initial adult aphids died were taken out of the analysis.

M. persicae and Agrobacterium-infiltrated *N. benthamiana*

Fecundity assays in 24-well plates

Candidate effector assays

A medium-throughput 24-well assay was designed in collaboration with Dr. Jorunn I.B. Bos and Marco Pitino to test the performance of aphids on *N. benthamiana*. *A. tumefaciens* was used to transiently express candidate effectors in *N. benthamiana* using the method described above. Up to four different constructs were infiltrated onto a *N. benthamiana* leaf and the infiltrated areas marked with permanent marker. One day after infiltration, leaf discs were collected using a circular cork borer (No. 7 – diameter 1.1 cm) from the infiltration sites. Clear, plastic 24-well cell culture plates (Nunc, Roskilde, Denmark) had 1 ml of 1% distilled water agar added to them. The leaf discs were placed upside-down on top of the cooled water agar. Each plate had leaf discs representing six different infiltration sites from six different leaves, and up to four different constructs per plate. During the initial screening of the candidate effector, three plates containing all the candidates, minus Mp51 and Mp54, and vector and GFP control were done at the same time. The plates were arranged with the vector and GFP controls, and then the candidates in numeral order. The four candidates in a plate were infiltrated side-by-side on the same six leaves. Mp51 and Mp54 were later screened along with the vector and GFP controls. For confirmation assays, up to three candidates were compared to the vector control by infiltrating them side-by-side on the same leaf and placing the leaf discs in the same plates. Four first-instar (1-2 days old) *M. persicae* nymphs from the *N. tabacum* stock cage (the *N. benthamiana* stock had not been established at this point) were placed on the leaf disc in each well. The wells in the plate were individually sealed off using cap of a 5 ml BD Falcon round bottomed test tube (BD Falcon, Franklin Lakes, New Jersey, USA) with the top of the cap removed and mesh glued in its place (Figure 2.1C). This allowed the wells to be ventilated without the aphids escaping. Plates (Figure 2.1D) were placed in a CER with 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod. After 6 days, the nymphs were moved to a new 24-well plate with fresh leaf discs infiltrated with the same candidate effector constructs or controls. Another 6 days later, the now adult aphids were again moved to a new 24-well plate with freshly

infiltrated leaf discs. The numbers of surviving adults (initially first-instar nymphs) were counted 6, 12, 14 and 17 days after setting up the first 24-wells plate and the number of newly produced nymphs were counted on day 12, 14 and 17. The newly produced nymphs were removed from the wells during counting. Wells wherein all four aphids that were initially placed on the discs died were taken out of the analyses. To calculate the production of nymphs per adult aphid, we calculated the average number of nymphs produced per adult by combining the average production rates throughout the experiment. These average production rates were obtained by dividing the number of nymphs on day 12 by the number of adults on day 6 (calculated per well), dividing the number of nymphs on day 14 by the number of adults on day 12, and dividing the number of nymphs on day 17 by the number of adults on day 14. To obtain the total average production rate, we calculated the sum of the average production rates for days 12, 14 and 17. The experiments were repeated 3 times to generate 3 independent biological replicates each containing up to 6 leaf discs per construct.

RNAi assays

The same assay design as described above was used to investigate the effect of silencing the candidate effector gene *Mp10*. Single *A. tumefaciens* colonies containing the plasmids pJGFP (negative control), pJMpC002 (positive control) and pJMp10 (created by Dr. Jorunn I.B. Bos) were used to transiently express dsRNA corresponding to *GFP, MpC002* and *Mp10* respectively in *N. benthamiana* using the method described above. All constructs were infiltrated side-by-side on the same leaf, and placed in the same plate. 1.25 ml of agar was used in these assays as it stopped the leaf discs drying out as quickly. The experiment was otherwise carried out as described above, and at the end of the experiment the initial first-instar nymphs were collected for qRT-PCR (see 2.6). Samples were flash frozen and stored at -80^{o}C until processed. The experiment was repeated 6 times to generate 6 independent biological replicates each containing up to 6 leaf discs per construct.

***M. persicae* and *A. thaliana* survival and fecundity assays**

Whole plant assays

M. persicae whole plant survival and fecundity assays were carried out in a similar way to Pitino *et al.,* 2011. Experiments were conducted in a CER with an 8 h day (90 µmol m^{-2} sec^{-1} at 18^{o}C) and 16 h night (16^{o}C) photoperiod. Four-week old plants were potted into one litre round black pots (13 cm diameter, 10 cm tall) containing Scotts Levington F2 compost (Scotts) and were caged in clear plastic tubing (10 cm diameter, 15 cm tall)

(Jetran tubing, Bell Packaging, Luton, UK) capped at the top with white gauze-covered plastic lids. Each plant had five adult *M. persicae* added to it from the Chinese cabbage stock colony and the plants were placed in the experimental CER. After 48 hours, all adults were removed from test plants whilst the nymphs remained, and the plants were returned to the CER (day 0). After a further 72 hours, excess nymphs were removed from the plants leaving five nymphs per plant. The number of new nymphs was counted on day 11 and these nymphs were removed, whilst the adults were counted but remained on the plant. A second nymph count was conducted on day 14, and the number of surviving adults was also counted. The number of nymphs produced per live adult was calculated for each time point and combined. Each experiment included 5 plants per genotype that were randomly assigned to trays that could hold a maximum of 10 plants. Each experiment was repeated three times to create data from three independent biological replicates.

Induced resistance assays

M. persicae induced resistance fecundity assays were carried out using a protocol modified from de Vos and Jander, 2009. Experiments were conducted in a CER with an 8 h day (90 µmol m^{-2} sec^{-1} at 18^{o}C) and 16 h night (16^{o}C) photoperiod. Five-week old Col-0 plants were potted into one litre round black pots (13 cm diameter, 10 cm tall) containing Scotts Levington F2 compost (Scotts) and were caged in clear plastic tubing (10 cm diameter, 15 cm tall) (Jetran tubing, Bell Packaging) capped at the top with white gauze-covered plastic lids. Each plant had 20 adult *M. persicae* added to it from the Chinese cabbage stock colony and the plants were placed in the experimental CER. After 24 hours, all adults were removed from the Col-0 plants whilst the nymphs remained and the plants returned to the CER (day 0). These plants formed an aged population of *M. persicae.* On day 9 five-week old test plants in black plastic pots (base measurement 3.5 cm x 3.5 cm, top measurement 5.5 cm x 5.5 cm, height 5.5 cm) were infiltrated using a needless 1 ml syringe (Terumo) with the test solution or a control on the first fully expanded leaf. *M. persicae* extract was diluted 1:10 with distilled water or potassium phosphate buffer as appropriate. The 3 to 10 kDa fraction of *M. persicae* saliva and the no aphid control were diluted 1:2 with distilled water. The infiltrated leaf was marked by the presence of a white plastic label (1.2 cm wide, 10 cm tall) and the plants put into the experimental CER. After 24 hours, an aged adult (10 days old) was placed in a clip cage using a moist paintbrush, and the cage placed on the infiltrated leaf of each plant. Plant were returned to the experimental CER and left for 10 days. After 10 days (day 20), the number of aphids in each clip cage was counted. Each experiment included 10 plants per condition and/or genotype. Each plant was randomly assigned to trays that could hold a

maximum of 15 plants. Each experiment was repeated at least three times to create data from at least three independent biological replicates. Leaves which had shriveled and died, thus killing all the aphids, were removed from the analysis.

A. pisum performance assays

A. thaliana survival assays

A novel assay was designed to measure *A. pisum* survival on *A. thaliana*. Experiments were conducted in a CER with an 8 h day (90 µmol m^{-2} sec^{-1} at 18^{o}C) and 16 h night (16^{o}C) photoperiod. Three mature *V. faba* plants between three to four weeks old were potted into one litre round black pots (13 cm diameter, 10 cm tall) containing Scotts Levington F2 compost (Scotts). One or two pots were placed in 24 cm x 54 cm x 47 cm cages, and 50 adult *A. pisum* were added to them from the stock population. The cage was placed in a CER with a 14 h day (90 µmol m^{-2} sec^{-1} at 18°C) and a 10 h night (15°C) photoperiod. After 24 hours all adults were removed from the plants whilst the nymphs remained, and the cage returned to the CER (day 0). This cage formed an aged population of *A. pisum*. The population was used for the experiment when the aphids were 10 to 14 days old. Five adult *A. pisum* from the aged population were placed in a clip cage using a moist paintbrush, and the clip cage attached to a seven-week old *A. thaliana* plant in a black plastic pot (base measurement 3.5 cm x 3.5 cm, top measurement 5.5 cm x 5.5 cm, height 5.5 cm). The plants were then put into the experimental CER (day 0). In initial experiments to optimise the experimental design the numbers of aphids remaining alive on days 3 to 7 were counted. In subsequent experiments, the number of adult aphids remaining alive on days 3 and 4 was recorded, and the average of these two readings taken. Each experiment included 5 plants per genotype. Each plant was randomly assigned to trays that could hold a maximum of 15 plants.

2.10 Elicitor assays

ROS burst assays

The ROS burst assays were carried out using the peptide flg22 (QRLSTGSRINSAKDDAAGLQIA) (Felix *et al.*, 1999) (Peptron, Daejeon, South Korea), crab shell chitin (Yaizu Suisankagaku, Shizuoka, Japan) (both provided by the group of Dr. Cyril Zipfel, TSL, Norwich) or extracts of aphids made in the way described above.

For the assays using *A. thaliana* leaf discs one leaf disc was taken from each of the two youngest fully expanded leaves of five-week old Col-0 *A. thaliana* plants using a circular cork borer (No. 1 – diameter 4 mm). The leaf discs were floated on water overnight in 96 well plates (Grenier Bio-One, Stonehouse, Glouchestershire, UK). PAMPs or aphid extract were added in the concentrations stated to a solution containing 20 µg/ml horseradish peroxidise (Sigma) and 21 nM of the luminol derivative 8-amino-5-chloro-7-phenylpyrido [3,4-d] pyridazine-1,4(2H,3H) dione (L-012) (Nishinaka *et al.*, 1993) (Wako, Osaka, Japan). Before the experiment began the water was removed from the wells and 100 µl of this solution was then used in each well in the experiment. Controls of the horseradish peroxidise and L-012 solution with water or phosphate buffer (as appropriate) instead of PAMPs/aphid extract were included. 100 µg of proteinase K (Sigma-Aldrich) or 100 µg of proteinase K boiled for 10 minutes was used to test for its ability to induce a ROS response.

For the assays using *N. benthamiana* leaf discs, discs were taken using a circular cork borer (as above) from either the two youngest fully expanded leaves of three and a half to four and a half week old plants, or leaves that had been infiltrated with *A. tumefaciens* to transiently express genes of interest two days previously (described in 2.8), or from leaves that had been infiltrated with *A. tumefaciens* to knock down expression of a gene of interest two and a half weeks previously (described in 2.8). The method of the assays were the same as described above, except that 34 µg/ml luminol (Sigma) was used instead of L-012 in some assays where genes of interest were transiently expressed. 100 µg of proteinase K (Sigma-Aldrich) was used to test for its ability to induce a ROS response.

Luminescence was captured using either a Photek camera system (Photek, St Leonards on Sea, East Sussex, UK) or a Varioskan Flash multiplate reader (Thermo Scientific, Basingstoke, Hampshire, UK) and the appropriate manufacturer's software. The amount of relative light units (RLUs) shown might differ depending on the light capturing apparatus used. Assays were repeated three times with similar results unless stated.

Ca^{2+} burst assays

All experiments were done using SLJR15 *N. benthamiana* plants. Leaf discs were taken using a circular cork borer (No. 1 – diameter 4 mm) from the two youngest fully expanded leaves of three and a half to four and a half week old plants or from leaves that had been infiltrated with *A. tumefaciens* to transiently express genes of interest two days previously (described in 2.8). The leaf discs were incubated in the dark overnight in 96 well plates (Grenier Bio-One) containing 12.5 µM coelenterazine (Biosynth AG, Staad, Switerland or

LUX Innovate, Edinburgh, UK). Before the experiment the coelenterazine was removed and 100 µl of the solution being assayed (water, buffer, flg22, chitin, whole *M. persicae* extract or fractionated *M. persicae* extract) was added to each well. Luminescence was measured using a Photek camera system (Photek).

2.11 Statistical analysis

Statistical analyses were conducted using Genstat v.11 (VSN International, Hemel Hempstead, UK). Student's *t*-tests were used to analyse the qRT-PCR results. Aphid survival or fecundity assays were analysed by Analysis of Deviance (ANODE) using a Poisson distribution within a generalized linear model (GLM). The only exception was the fecundity results for assays using leaf discs in 24-well plates (effector and RNAi assays). Here, the number of nymphs produced per adult was checked for approximate normal distribution by visualising the residuals and then analysed by Analysis of Variance (ANOVA). The ROS burst assays were analysed by ANOVA except in instances were only two conditions were being compared (e.g. *NbSerk3* VIGS experiments), and then Student's *t*-tests were used. The Ca^{2+} assays in chapter 7 were analysed using Student's *t*-tests.

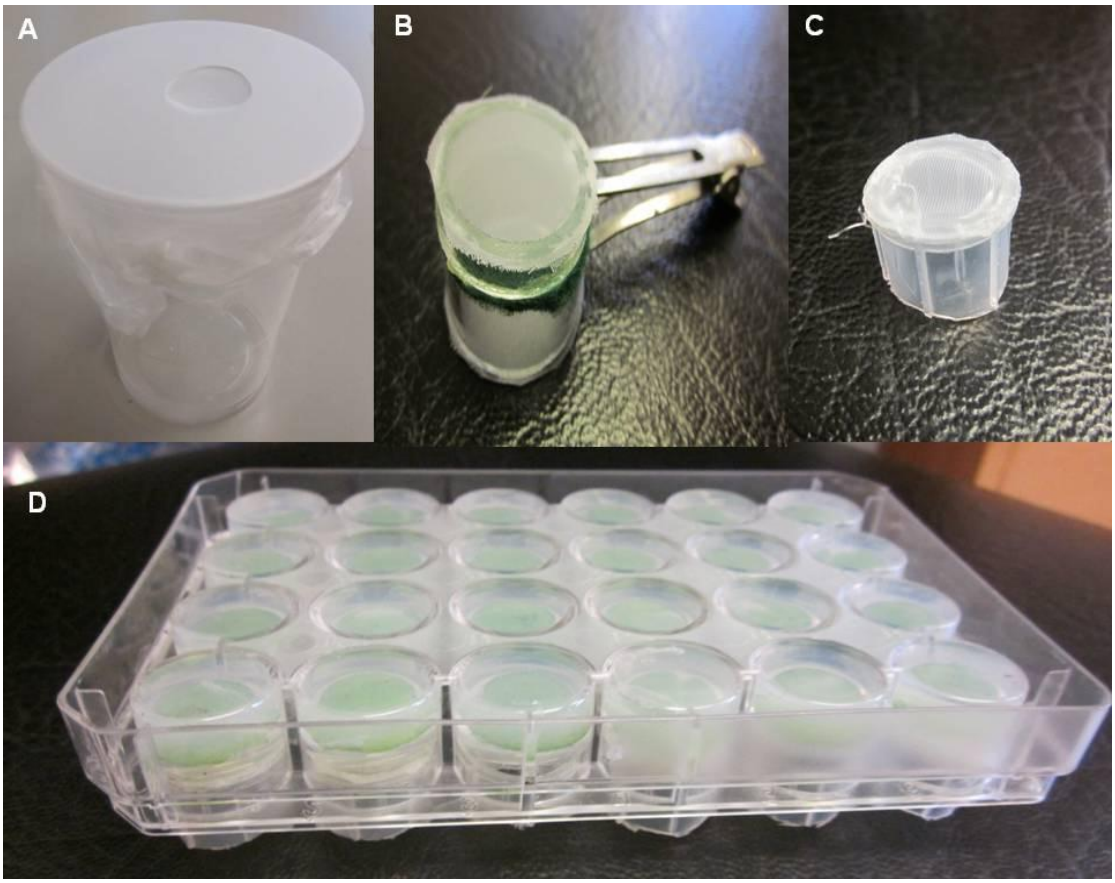

Figure 2.1 – Custom-made equipment used in experiments described in this thesis.

(A) A Parafilm sachet of water was placed over a plastic tumbler to collect saliva from *M. persicae.* (B) Clip cages allowed aphids to be isolated to particular leaves or parts of a leaf on a plant. They were made from two cylinders of plastic tubing with fine mesh glued on the external surface and felt glued on the surfaces in contact with the leaf. The tubing was attached to a hair clip in order to control opening and closing.
(C) Breathable seals for the 24-well plates stopped aphids escaping whilst allowing gas exchange. They were made by removing the top of a cap from a 5 ml BD Falcon round bottomed test tube and then gluing a fine mesh to the opposite end.
(D) 24-well plates were used to conduct fecundity experiments. The wells contain water agar, a *N. benthamiana* leaf disc and aphids. The wells are sealed with mesh covered caps (Figure 2.1C).

Chapter 3 - Identifying *M. persicae* candidate effectors using functional genomics

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Part of this chapter was published in Bos *et al.,* 2010 (Appendix A).

3.1 Introduction

Aphids are major agricultural pests, as they can vector plant viruses and over 250 species of aphid feed on crop plants (Blackman and Eastop, 2000). Aphid damage leads to losses in crop production estimated to be hundreds of millions of dollars each year (Oerke *et al.*, 1994). Aphids feeding behaviour is similar to that of biotrophic plant-pathogens, as it requires long-term, intimate association between aphid and plant below the plant surface. How plants defend themselves against aphid attack, and how aphids modulate plant processes is not well understood. It is also unclear whether plant immunity plays a role in the plant-aphid interaction. We started to investigate these questions by looking for aphid salivary genes that encode proteins that act effectors.

Plant-pathogen interactions involve the triggering of plant defences by elicitors and the disruption of normal plant function by effectors (Jones and Dangl, 2006). It is therefore likely that aphids also trigger plant defences with elicitors and need to deliver effectors inside hosts in order to modulate host defences and cellular processes to allow successful colonization. Plant pathogen effectors modulate plant cell processes in a number of ways. For example effectors can suppress immunity in many ways including suppressing HR (Bos *et al.*, 2006), and mimicking or modulating plant hormones (Hogenhout and Loria, 2008; Sugio *et al.*, 2011). However, plant disease *R* genes produce proteins that confer resistance to a plant by mediating direct or indirect recognition of a pathogen effector (van der Hoorn and Kamoun, 2008). *R* gene products often contain NBS and LRR domains (Jones and Dangl, 2006). The perception of a pathogen's effector by a plant's resistance gene is known as the gene-for-gene hypothesis (Flor, 1971) and many examples of such interactions have been characterized (Dangl and Jones, 2001). Interestingly, *R* genes have been identified that confer resistance to aphids. Two of these genes have been cloned, *Mi-1.2* and *Vat*, and encode NBS-LRR proteins (Hogenhout and Bos, 2011). This suggests overlap in how plants defend themselves against pathogens and aphids, and infers that aphids secrete effectors that are recognised by the *R* gene products, although so far no such effectors have been identified in insects.

A common feature of plant pathogen effectors is modulating host cell processes. Various assays have been developed to identify the functions of effectors from bacterial and eukaryotic filamentous plant pathogens (Huitema *et al.*, 2004; Cunnac *et al.*, 2009). Suppression of PTI, the plants first line of active defence, is an important function of plant pathogen effectors and appears commonly shared among pathogens, and therefore it may also be shared by insect pests. PTI suppression is especially common among type III secretion system (T3SS) effectors of bacterial plant pathogens. For example, the large

majority of *Pseudomonas syringae* pv. tomato DC3000 (PtoDC3000) effectors can suppress PTI responses, including the associated ROS burst (Guo *et al.*, 2009). However, other plant pathogens besides bacteria need to suppress PTI to successfully infect a plant. Effectors from eukaryotic filamentous plant pathogens can suppress PTI, as demonstrated by several effectors from *Hyaloperonospora arabidopsidis* (*Hpa)* that can suppress flg22-triggered ROS burst and callose deposition in *A. thaliana* (Fabro *et al.*, 2011). PTI responses to different PAMPs requires different plant PRRs, for example in *N. benthamiana* flg22 perception requires NbFLS2 and NbBAK1 (Heese *et al.*, 2007) whereas chitin perception requires NbCERK1 but flg22 perception does not (Gimenez-Ibanez *et al.*, 2009). Some effectors target more than one PTI signalling pathway, for example AvrPtoB targets FLS2 and CERK1-dependent PTI (Göhre *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009).

PTI is not the only form of plant defences against pathogens, and so other activities of plant pathogen effectors exist. Plant based expression of pathogen genes can identify the importance of effectors in plant colonization. For example, overexpression of PtoDC3000 effector AvrPtoB enhances virulence in *A. thaliana* (Gimenez-Ibanez *et al.*, 2009). Deletion or knockdown of single effectors can have detrimental effects on pathogen virulence, although this does not always occur due to redundancy in the function of some effectors.

Effectors are subject to diversifying selection because of the co-evolutionary arms race between host and pathogen proteins (Chisholm *et al.*, 2006; McCann *et al.*, 2012) and therefore amino acid polymorphisms amongst homologs of effectors within and between species are of interest. For example Bos and colleagues (Bos *et al.*, 2006) found amino acid changes in the C-terminal of *P. infestans* effector AVR3a affected recognition by the plant resistant gene *R3a*.

Another characteristic of effectors is the production of visible phenotypes upon overexpression in plants. For example, several effectors such as CRINKLER2 (CRN2) and INF1, from the oomycete plant pathogen *P. infestans,* induce cell death upon overexpression *in planta* (Kamoun *et al.*, 1997; Torto *et al.*, 2003); whereas other effectors like AvrB from PtoDC3000 induce chlorosis (Shang *et al.*, 2006). These phenotypes are often linked to recognition by *R* genes. Diverse *R* genes are regulated by several chaperones including SUPRESSOR OF G-TWO ALLELE OF SKP1 (SGT1) (Meldau *et al.*, 2011b). SGT1 is an ubiquitin-ligase associated protein required for plant cell death responses, including those involved in plant resistance (Azevedo *et al.*, 2006). Effectors also produce visible phenotypes in plants by altering plant development pathways, such as the crinkled leaves and green leaf-like flowers produced in *A. thaliana* plants by Aster Yellows phytoplasma strain Witches' Broom (AY-WB) effectors

SECRETED AY-WB PROTEIN11 (SAP11) and SAP54 respectively (MacLean *et al.*, 2011; Sugio *et al.*, 2011).

The responses of plants at a molecular level to aphid feeding, including the discovery of NBS-LRR resistance genes, suggest that aphids secrete effectors to manipulate the host plant defence system. Any effectors must enter the plant through aphid saliva. Efforts have been made to identify aphid salivary effectors. Studies by Ramsey and colleagues (Ramsey *et al.*, 2007), Carolan and colleagues (Carolan *et al.*, 2011) Nicholson and colleagues (Nicholson *et al.*, 2012) and Harmel and colleagues (Harmel *et al.*, 2008) identified many salivary gland proteins using a combination of transcriptomics or proteomics approaches (or both). However, whilst there are many hypotheses as to how some of these proteins may function, there are no functional studies on these proteins as yet. Functional studies were carried out on aphid saliva proteins potentially involved in disrupting plant sieve tube occlusion upon aphid feeding, but the proteins remain unidentified (Will *et al.*, 2007).

Functional data on individual aphid effector candidates is starting to appear, and one example is *C002*. There is evidence that *C002* is secreted by two different aphids who feed on two different hosts (*A. pisum* and *M. persicae*) (Harmel *et al.*, 2008; Mutti *et al.*, 2008) and that it plays a role in the plant-insect interaction in both cases (Mutti *et al.*, 2008; Pitino and Hogenhout, 2013). Homologs of the gene have recently been identified in several aphid species (Ollivier *et al.*, 2010; Cui *et al.*, 2012; Liu *et al.*, 2012b), and appear to be under positive selection (Ollivier *et al.*, 2010; Pitino and Hogenhout, 2013). Taken together these data suggest an important role for C002 in the plant-aphid interaction, and make it the best currently identified candidate for an aphid effector.

We decided to use *M. persicae* to search for candidate aphid effectors, rather than another commonly studied aphid such as *A. pisum*. Whilst *A. pisum* has a completed genome sequence (The International Aphid Genomics Consortium, 2010), the legume host plants that it feeds from have less well developed tools and are more time consuming to transform than some other plant species. In contrast, *M. persicae* has a broad plant host range including the plant species *A. thaliana* and *N. benthamiana.* Numerous tools are available for these two plants and they are easy to transform, allowing much quicker screening of candidate effectors and follow up of initial findings. These plant species are also the subject of much study in the plant-pathogen field, allowing us to compare our findings to a wealth of literature. The *M. persicae*/*A. thaliana*/*N. benthamiana* system will also give our findings the potential to be adapted to crop systems. *M. persicae* is an important aphid species because it is a major pest worldwide and is responsible for transmission of many viruses (Blackman and Eastop, 2000). The aphid can be difficult to manage as it is thought to have developed resistance

to many insecticides (Anstead *et al.*, 2005). Findings in *A. thaliana* could be adapted to Brassica crop plants, and findings in *N. benthamiana* could inform improvements to other Solanaceae such as *S. lycopersicum* or *S. tuberosum*.

In this chapter we use *M. persicae* and *N. benthamiana* as a system to search for candidate aphid effectors. Candidate effectors were identified by bioinformatics. We then functionally screened the identified candidates for common properties of plant-pathogen effectors. Functional screens found three candidates amongst aphid salivary proteins that displayed common effector characteristics, including one candidate that suppressed the flg22-triggered ROS burst. This indicates that aphids possess effectors in their saliva, and that suppression of plant immunity may be necessary for successful plant colonization.

3.2 Results

Identification and allocation of candidate effectors

We hypothesized that aphid effector were likely to be genes expressed in the salivary glands that possessed a secretion signal. In order to identify candidate effector for *M. persicae* a bioinformatics pipeline was performed on publically available salivary gland ESTs (Ramsey *et al.*, 2007) by a postdoctoral researcher in the Hogenhout lab, Dr. Jorunn I.B. Bos (Bos *et al.*, 2010). The pipeline identified 115 ORFs that contained an N-terminal signal peptide and no transmembrane domain, and were therefore likely to be present in the aphid saliva. 46 candidate effectors, including three *M. persicae* ORFs identified by analysing *A. pisum* salivary gland ESTs, were selected for functional analysis on the basis of full length sequences. The candidates were assigned names starting with Mp for *Myzus persicae* followed by a number. The only exception was the *M. persicae* homolog of *A. pisum* C002 (Mutti *et al.*, 2006; Mutti *et al.*, 2008) which we named MpC002. Our approach to test whether these candidates might be effectors was to screen them in functional assays looking for common phenotypes of plant-pathogen effectors. These phenotypes included production of a visible phenotype upon expression *in planta*, suppression of PTI, and effect on performance/reproduction. In order to functionally screen a large number of candidate effectors, the candidates were distributed between Dr. Jorunn I.B. Bos, a PhD student in the Hogenhout lab, Marco Pitino, and myself during the first year of my PhD project. I cloned and screened 12 effector candidates allocated at random (Table 3.1). Most of these candidate genes were specific to aphids. Many had no sequence similarity to proteins with known functions in public depositories, although some of them had similarities to functional domains.

Table 3.1

Name of effector	Representative EST Genbank identifier[1]	Predicted protein size (amino acids)	Putative conserved domain[2]	Homology to known predicted protein[2]	*A. pisum* homolog protein identifier[2]	Range of organsims with homologs[3]	Other aphid species with a similar EST[3]
Mp3*	DW011743	80	Yos1	Immediate early response 3-interacting protein 1-like	LOC100573518	Invertebrates	MA,AP,AK, RP,RM,PS
Mp4	ES226741	145	None	*A. pisum* hypothetical protein	LOC100573202	Aphid	MA, AP, AK, AG
Mp5	EC389841	199	None	*A. pisum* hypothetical protein	LOC100166545	Aphid	MA, AP, AG
Mp6	EC387921	205	None	*A. pisum* hypothetical protein	LOC100569335	Aphid	AG
Mp7	ES226253	138	None	Partner of bursicon-like	LOC100166282	Arthropods	AP, AK
Mp8	ES226080	98	None	*A. pisum* hypothetical protein[4]	LOC100575146[4]	Aphid	AP
Mp46	EE264770	205	PEBP	Phosphatidylethanolamine-binding protein-like precursor	LOC100159619	Insects	AP
Mp47	EE264742	99	None	Uncharacterized *A. pisum* protein precursor	LOC100164831	Aphid	MA, AP, AK, AG, RM
Mp50	EE264652	276	Alpha CA	Carbonic anhydrase 7-like	LOC100574112	Animals	MA, AP, SA, RP, RM
Mp51	EE264632	171	None	*A. pisum* hypothetical protein	LOC100159109	Aphid	AP, AG
Mp53	EE264538	148	None	*A. pisum* protein	LOC100166220	Aphid	AP, AK, AG, RP
Mp54*	EE261990	147	None	*A. pisum* protein precusor	LOC100159485	Aphid	AP, AK, AG, RM, TC

Table 3.1 – Candidate *M. persicae* effector genes screened in this chapter

[1] All ESTs used to design the primers for the candidate effector proteins were submitted to NCBI by Georg Jander as part of Ramsey *et al.*, 2007

[2] Identified using blastp

[3] Identified using tblastn of EST database

Key = MA = *Myzus ascalonicus,* AP = *A. pisum,* AK = *A. kondoi,* AG = *A. gossypii,* RP = *R. padi,* RM = *Rhopalosiphum maidis,* SA = *S. avenae,* TC = *T. citricida,* PS = *Pemphigus atropunctata*

[4] Identified using tblastn of nucleotide collection, as blastp gave no good matches

* Identified through *A. pisum* salivary gland EST analysis

Cloning of 12 candidate effectors reveals Mp6 is polymorphic within *M. persicae* clone O

In order to screen the 12 candidate effector genes in functional assays, I cloned them into a binary vector (pCB302-3 (Xiang *et al.*, 1999)). Eleven of the twelve candidates were cloned, as it was not possible to amplify *Mp7* from *M. persicae* cDNA. Our *M. persicae* colony has been genotyped by Dr. Brian Fenton (The James Hutton Institute, Dundee, UK) as clone O (Fenton *et al.*, 2010) and has been reared on Chinese cabbage (*Brassica rapa*, subspecies *chinensis*) for many years. The ESTs used to identify the candidate effector came from a *M. persicae* clone that has *N. tabacum* as a host and was reared on *Brassica oleracea* (Ramsey *et al.*, 2007), and therefore this aphid may have different effector genes or gene sequences compared to our aphid.

Several bacterial clones for each candidate effector were sequenced. Sequencing of *Mp6* clones revealed three polymorphic sites in the amino acid sequence (Figure 3.1A). These resulted from three nucleotide substitutions; C to A at nucleotide 472, G to A at nucleotide 610 and T to G at nucleotide 614 of EST EC387921. The respective amino acid changes were leucine to isoleucine, glutamic acid to lysine, and valine to glycine (Figure 3.1A). The form of *Mp6* that matched the EST was found in plasmid clone #6 and the new form of *Mp6* was found in plasmid clone #2 and #5. I decided to include both versions of *Mp6* in my functional screen, and designated them *Mp6_6* and *Mp6_2* for the previous and newly identified version respectively. As there is only one complete EST for *Mp6* in NCBI (EC387921) I investigated further whether these polymorphisms were genuine, and whether individual aphids contained one version of the gene or both. cDNA was made from several batches of aphids collected in groups. *Mp6* was amplified by PCR and sequenced. In all cases, peaks for both the nucleotides were present at the polymorphic sites (Figure 3.1B and C). No peaks were found for different nucleotides to the identified polymorphisms. This suggests that the polymorphism is genuine. Repeating the process with cDNA made from single aphids gave the same results, with no aphids

displaying homozygosity for the *Mp6* polymorphisms. This suggests that both versions of *Mp6* are present in our population of *M. persicae* clone O.

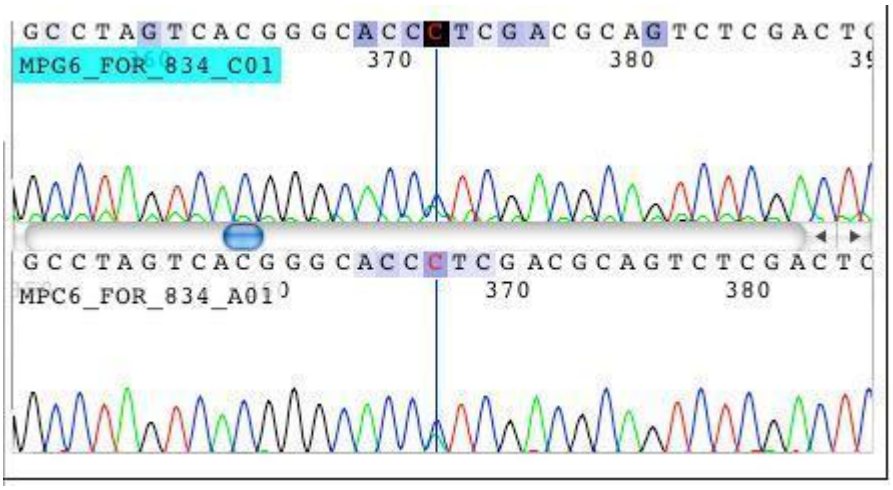
A

```
Mp6_5           MTPVPAEDQNAAASTPSSTASSALDKANSMVTDGAKTAINGVENVGDMFKTLIKKTGSAV 60
Mp6_2           MTPVPAEDQNAAASTPSSTASSALDKANSMVTDGAKTAINGVENVGDMFKTLIKKTGSAV 60
Mp6_6           MTPVPAEDQNAAASTPSSTASSALDKANSMVTDGAKTAINGVENVGDMFKTLIKKTGSAV 60
EC387921        MTPVPAEDQNAAASTPSSTASSALDKANSMVTDGAKTAINGVENVGDMFKTLIKKTGSAV 60
                ************************************************************

Mp6_5           SDLGSEANKSLGEIAKGGGRSTLALGYLFSQLFDGLSATVATGGGYLASGFRNIDDVVGD 120
Mp6_2           SDLGSEANKSLGEIAKGGGRSTLALGYLFSQLFDGLSATVATGGGYLASGFRNIDDVVGD 120
Mp6_6           SDLGSEANKSLGEIAKGGGRSTLALGYLFSQLFDGLSATVATGGGYLASGFRNIDDVVGD 120
EC387921        SDLGSEANKSLGEIAKGGGRSTLALGYLFSQLFDGLSATVATGGGYLASGFRNIDDVVGD 120
                ************************************************************
                        Fig 3.1B                                        Fig 3.1C
Mp6_5           WPVVGLVTGTIDAVSTHVSNAVTEMSENGRNSRQKMFEGLRGHLNRSGSSLPNAENKGTS 180
Mp6_2           WPVVGLVTGTIDAVSTHVSNAVTEMSENGRNSRQKMFEGLRGHLNRSGSSLPNAENKGTS 180
Mp6_6           WPVVGLVTGTLDAVSTHVSNAVTEMSENGRNSRQKMFEGLRGHLNRSGSSLPNAENEVTS 180
EC387921        WPVVGLVTGTLDAVSTHVSNAVTEMSENGRNSRQKMFEGLRGHLNRSGSSLPNAENEVTS 180
                **********:*************************************************: **

Mp6_5           DANSDAVV 188
Mp6_2           DANSDAVV 188
Mp6_6           DANSDAVV 188
EC387921        DANSDAVV 188
                ********
```

B



C

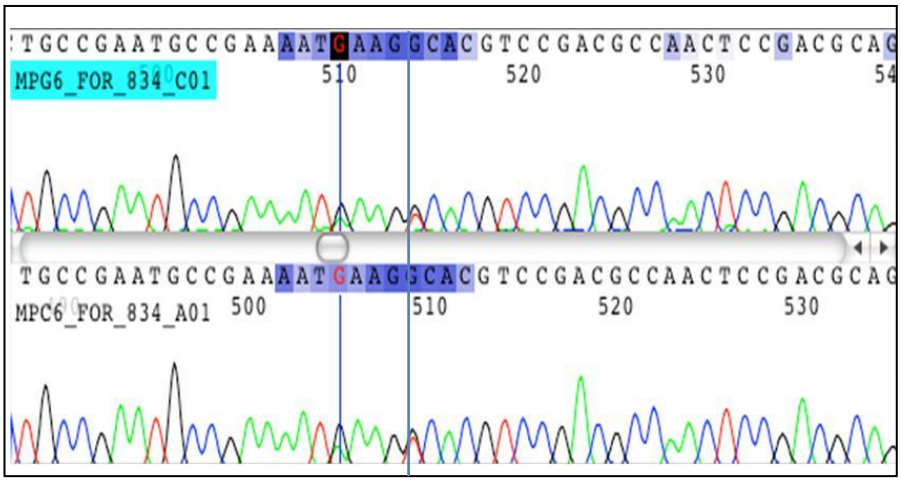


Figure 3.1 – Candidate effector Mp6 is polymorphic at three amino acid residues.
(A) Polymorphisms exist in the Mp6 amino acid sequence at residues 131 (L or I), 177 (K or E) and 178 (G or V). ClustalW alignment of the amino acid sequences for three Mp6 clones plus the original EST (EC387921 from NCBI). Sequences are for the region of the protein after the signal peptide. Green residues denotes the sites of the polymorphisms.
(B) Sequence traces confirm the presence at the nucleotide level of the polymorphism in Mp6 at the amino acid position 131. (C) Sequence traces confirm the presence at the nucleotide level of the polymorphism in Mp6 at the amino acid positions 176 and 177. (B and C) PCR products for Mp6 amplified from cDNA made from groups of *M. persicae* were sequenced and then traces analysed with CodonCode Aligner software. The peak trace shows both bases are present at the polymorphic regions. Products from two different groups of *M. persicae* are shown. The same result was obtained using cDNA made from single aphids.

The 12 candidate effectors do not produce a visible phenotype upon overexpression in *N. benthamiana*.

In order to test whether the 12 candidates produced a visible response such as chlorosis, or a HR from the plant upon overexpression I used *A. tumefaciens* to express the candidates in *N. benthamiana* leaves. As a positive control I used the *P. infestans* elicitin INF1, which produces HR in Solanaceae (Kamoun *et al.*, 1997). None of the 12 candidates produced a visibile phenotype upon transient expression in *N. benthamiana* leaves (Figure 3.2).

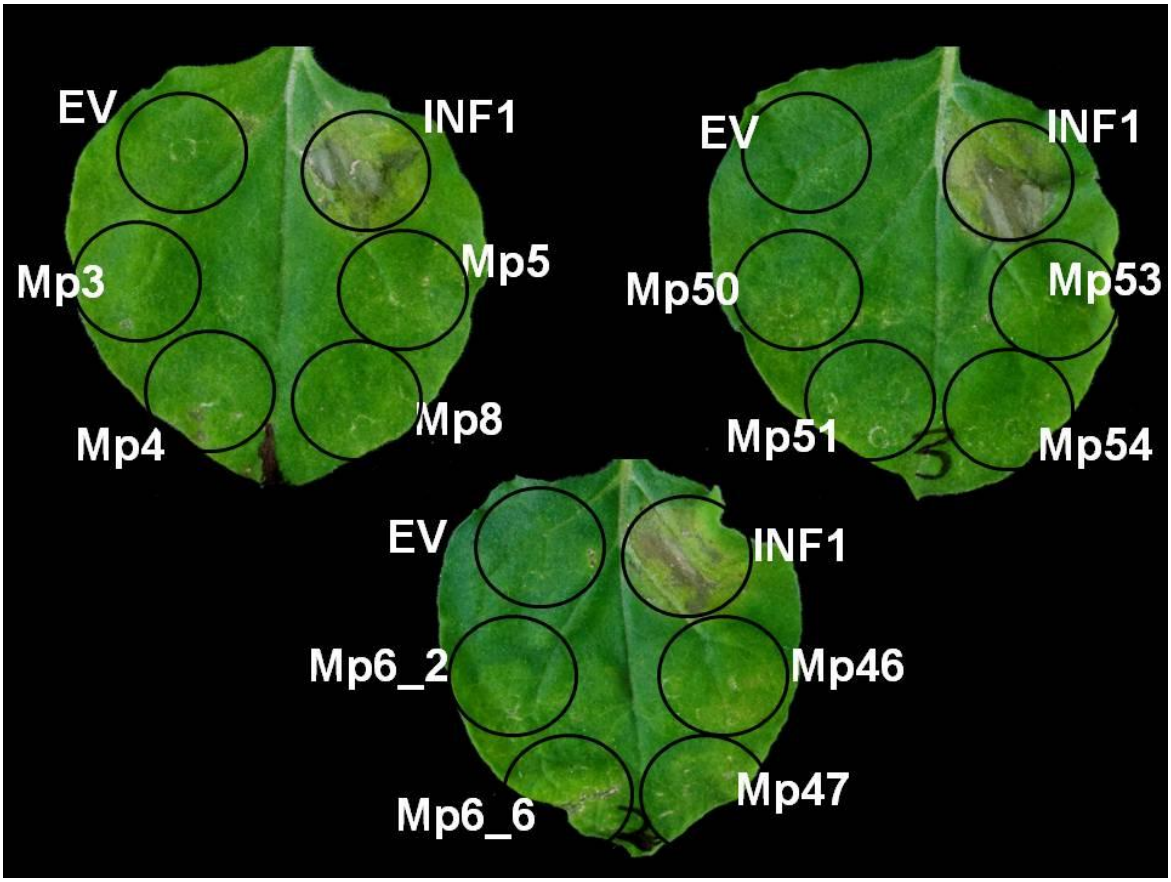


Figure 3.2 – The 12 candidate effectors do not produce a visible phenotype upon overexpression in *N. benthamiana*.
Agrobacterium-mediated transient expression of candidate effector genes, negative control (EV) and positive control (INF1 elicitin) in *N. benthamiana* at five days post infiltration. Experiment repeated three times with similar results, with one representative experiment shown.

The 12 candidate effectors do not suppress the flg22-triggered ROS burst when overexpressed in *N. benthamiana*.

In order to test whether my candidates were able to suppress PTI I investigated if they were able to suppress the ROS burst triggered by application of the PAMP flg22. The candidates were overexpressed in *N. benthamiana* leaves, which were subsequently challenged with flg22. The ROS burst triggered was measured using a luminol based assay (Keppler *et al.*, 1989) to measure hydrogen peroxide production in terms of light emitted. It was decided to screen for suppression of the ROS burst induced by flg22 only, as this PAMP gives a strong and consistent ROS burst response in *N. benthamiana*, which is convenient for use in large screens. As a positive control I used the PtoDC3000 effector AvrPtoB, which suppresses the flg22-triggered ROS burst (Hann and Rathjen, 2007). All the candidates were screened twice, and none of them showed a greatly

decreased response in either assay (Figure 3.3). Mp6_2 was statistically different from Mp4 in both experiments, but neither were different from the EV control.

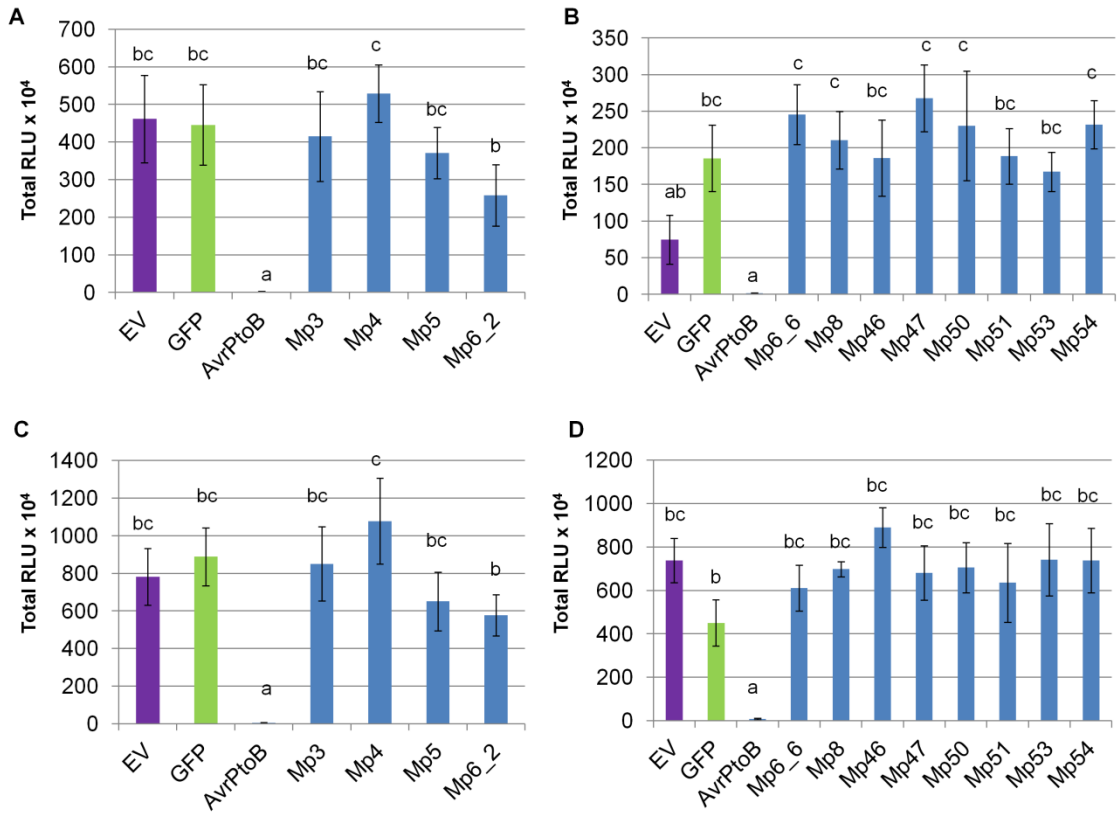


Figure 3.3 – The 12 candidate effectors do not suppress the flg22-triggered ROS burst when overexpressed in *N. benthamiana*.

None of the 12 aphid candidate effectors suppressed the ROS burst triggered by flg22. The ROS burst in response to flg22 was measured using a luminol-based assay in *N. benthamiana* leaf discs expressing the candidate effectors or EV, GFP or AvrPtoB (positive) controls. Results are represented as RLUs and the mean ± standard error (SE) is shown (n = 8 leaf discs per replicate). All the candidates were screened twice against the controls (A and B, C and D). Letters indicate significant differences at $P < 0.05$.

The 12 candidate effectors do not effect aphid reproduction or survival.

Effectors may benefit aphid performance through modulation of plant host processes, and thus lead to an increase in aphid performance. Alternatively, the effectors may be recognised by the plant host leading to effector-triggered immunity, and thus leading to a potential decrease in aphid performance. We developed a medium-throughput 24-well plate assay in order to assess the effect of our candidate effectors on *M. persicae* reproduction and survival. *N. benthamiana* leaf discs were infiltrated with *A. tumefaciens* in order to transiently express the candidate effectors. Infiltrated leaf discs were placed upside down on water agar in a 24-well plate, and four first-instar *M. persicae* nymphs

were added to each well. After 6 days the aphids were moved to plates of fresh leaf discs expressing the candidates. A total of three sequential plates made up an experiment, with four constructs being tested per plate. Survival and reproduction of the initial four nymphs was recorded over the 18 days of the experiment. Due to the large number of constructs to test, an initial screen was conducted with all candidates except Mp51 and Mp54. In this screen only Mp3 and Mp4 were infiltrated on the same *N. benthamiana* leaf as the EV and GFP controls, with the other candidates being on different leaves. Subsequently, Mp51 and Mp54 were tested on the same leaf as EV and GFP, and these results completed the screen. At this stage, candidates were considered interesting if they differed from EV by one SE. In the initial screen, Mp4, Mp50 and Mp53 showed increased reproduction compared to EV, and Mp51 showed decreased reproduction compared to EV (Figure 3.4A). However, in the Mp51 and Mp54 assays, GFP also differed in aphid reproduction from EV by one SE, suggesting that the EV results may have been abnormally high (Figure 3.4A). All candidates except Mp3, Mp6_6, Mp51 and Mp54 showed decreased survival compared to EV, and Mp54 showed increased survival compared to EV (Figure 3.4B). Therefore, the only candidates to show no change in reproduction or survival in the screen were Mp3 and Mp6_6. However, Mp6_6 did show increased reproduction and survival compared to Mp6_2 (Figure 3.4A and B).

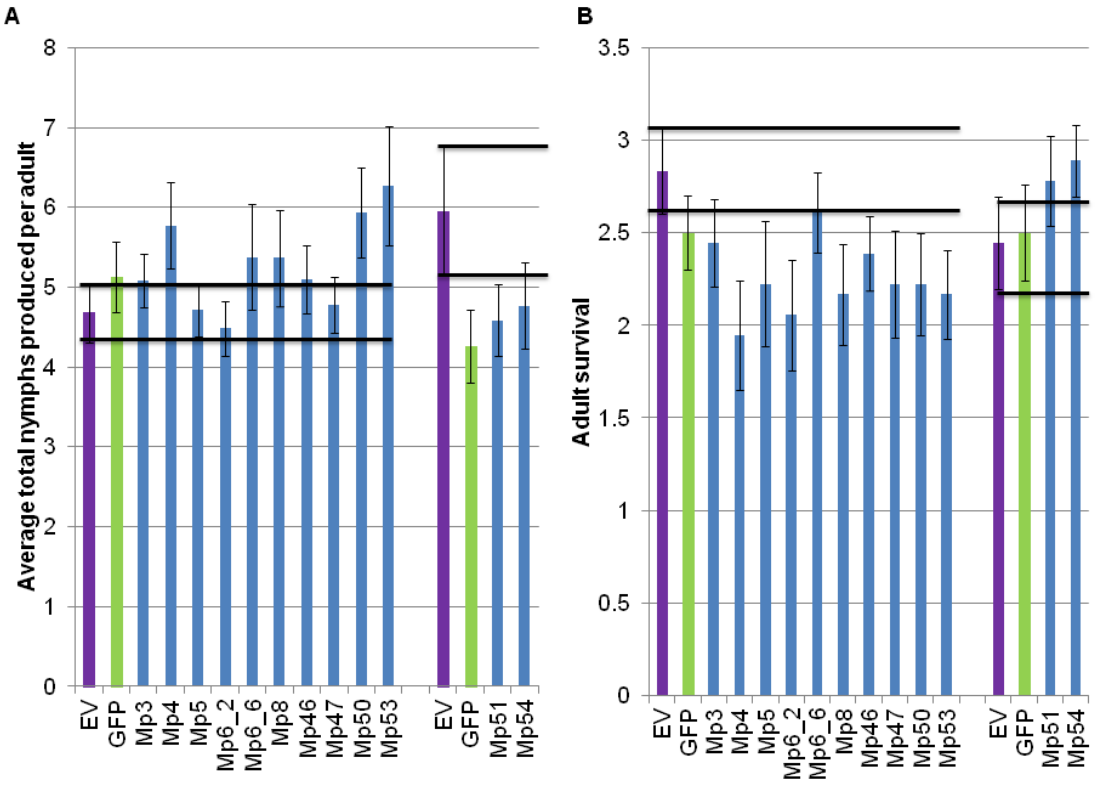


Figure 3.4 – Initial assay revealing that candidate effectors potentially affect aphid reproduction.

(A) Candidate effectors Mp4, Mp50, Mp51 and Mp53 affected aphid reproduction compared to EV by more than 1 SE. (B) Candidate effectors Mp4, Mp5, Mp6_2, Mp8, Mp46, Mp47, Mp50, Mp53 and Mp54 affected aphid survival compared to EV by more than 1 SE. (A and B) *N. benthamiana* leaf discs transiently expressing candidate effectors upon infiltration with *A. tumefaciens* were placed in 24-well plates and four *M. persicae* nymphs were added. Reproduction (A) and survival (B) of these four aphids was measured over an 18 day period. Six wells were used per construct, with a maximum of four constructs per plate. Data shown are means ± SE of three biological replicates with n = 6 per replicate. Total n ≥ 15 leaf discs. Wells where all the aphids died were removed from the analysis. Horizontal lines mark 1 SE of EV to aid comparison.

Thus, all the candidates apart from Mp3 were tested in the 24-well plate assay again for reproduction and surivial, but this time they were all infiltrated on the same leaf as the EV control in order to reduce variation. Mp6_2 and Mp54 showed increased reproduction compared to EV, but this was not statistically significant (ANOVA, Mp6_2: P = 0.129, Mp54: P = 0.165, Figure 3.5A). Mp50 showed decreased survival compared to EV, but this was also not statistically significant (ANODE; $F_{2, 53}$ = 0.887, P = 0.412; Figure 3.5B). Mp5, Mp46 and Mp47 were tested later than the other candidates, and this

is denoted by their seperate graphs (Figure 3.5C and D). None of these three candidates showed a difference in reproduction compared to EV. Mp47 showed increased survival compared to EV, but this was not statistically significant (ANODE; $F_{3, 71} = 1.305$, $P = 0.271$; Figure 3.5D). Of the re-tested candidates, the most promising phenotypes were the increased reproduction with Mp6_2 and Mp54. Further repetition of similar results would lead to statistical significance. However, repeating the 24-well plate assay a further three times for these candidates showed no difference from EV (Figure 3.5E), leading to the conclusion that none of the 12 candidate effectors consistently affect aphid reproduction or survival when produced transiently in *N. benthamiana*.

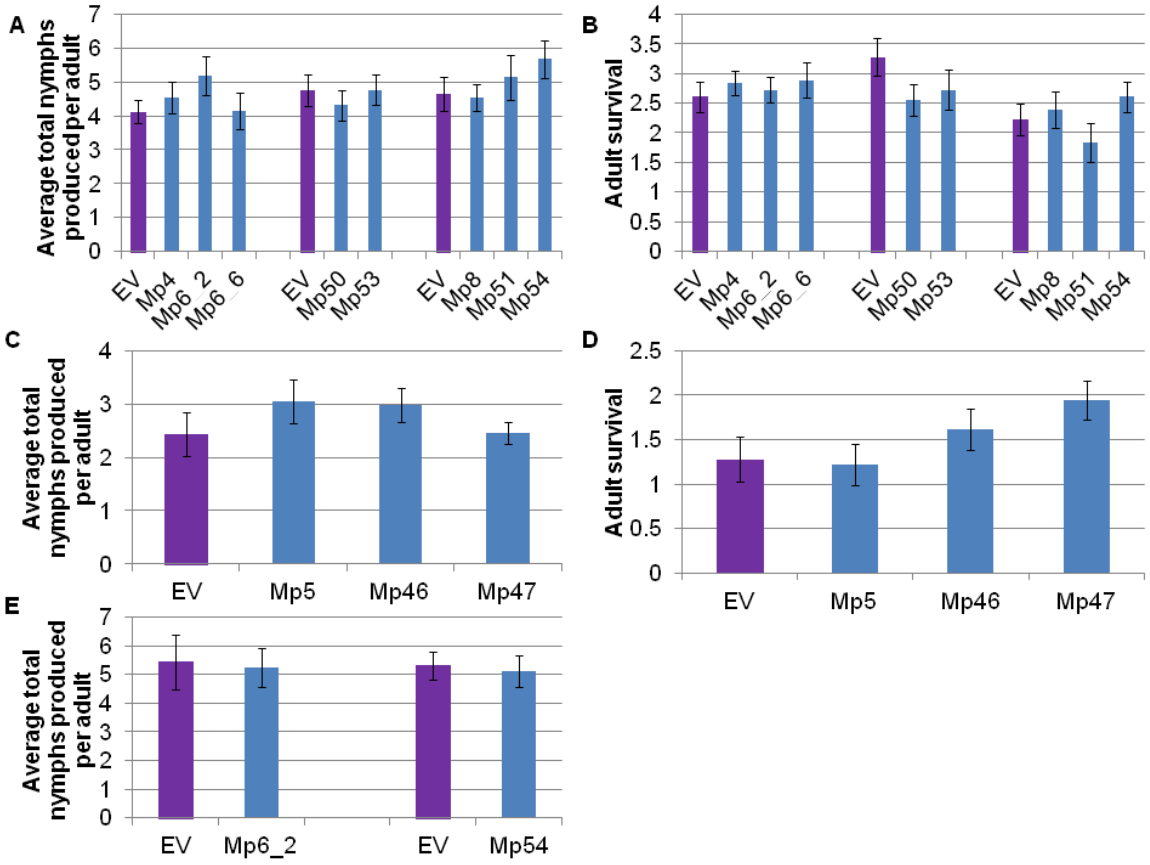


Figure 3.5 – Confirmation assays revealing that candidate aphid effectors did not consistently affect aphid survival and reproduction.

A) Candidate effectors Mp6_2 and Mp54 slightly improved aphid reproduction but differences were not significantly different from the EV control ($P = 0.129$ and $P = 0.165$ respectively). (B) Candidate effector Mp50 slightly decreased aphid survival but differences were not significantly different from the EV control ($P = 0.412$). (C) No candidate effectors caused differences in reproduction from the EV control. (D) Candidate effector Mp47 slightly increased aphid survival but differences were not significantly different from the EV control ($P = 0.114$). (E) Further replicating of Mp6_2 and Mp54 led to no differences in reproduction. (A to E) *N. benthamiana* leaf discs transiently expressing

candidate effectors upon infiltration with *A. tumefaciens* were placed in 24-well plates and four *M. persicae* nymphs were added to each well. Six wells were used per construct, with a maximum of four constructs per plate. Reproduction (A, C and E) and survival (B and D) of these four aphids was measured over an 18 day period. Data shown are means ± SE of three biological replicates with n = 6 per replicate. Total n ≥ 16 (A and B), n ≥ 14 (C and D), n ≥ 12 (E). Wells where all the aphids died were removed from the analysis. Data analysed by ANOVA (A, C and E) and ANODE (B and D).

The screen identified three candidate effectors that showed phenotypes upon overexpression in *N. benthamiana.*

Although none of the 12 candidates I screened showed a common effector phenotype when overexpressed in *N. benthamiana*, the screening of the remaining candidates by Jorunn and Marco identified a total of three effectors that did show phenotypes. Mp10 produced chlorosis in *N. benthamiana* upon transient expression mediated by *A. tumefaciens* (Figure 3.6A) and potato virus X (PVX) (Figure 3.6B). Chlorosis did not appear in leaf discs detached from the plant 24 hours after infiltration. To ascertain whether the chlorosis may be link to activation of an *R* gene we tested whether it was dependent on the presence of chaperone SGT1. SGT1 expression was knocked down using VIGS and leaves from these plants showed greatly reduced instances of chlorosis, similar to the INF1 control (Figure 3.6C and D). Mp10 expression also decreased the flg22-triggered ROS burst in *N. benthamiana* leaf discs (ANOVA; $P \leq 0.043$; Figure 3.6E) but did not affect the chitin-triggered ROS burst (ANOVA; $P > 0.347$ (not significant (n.s.)); Figure 3.6F). Finally, Mp10 caused decreased aphid reproduction in the 24-well plate assay (ANOVA; $P \leq 0.026$; Figure 3.6G). Mp10 has homology to an insect protein of predicted function, OS-D2-like protein, which is a member of the CSP family in aphids.

The two other candidate effectors identified showed phenotypes in the 24-well plate assay. Mp42 decreased aphid reproduction (ANOVA; $P \leq 0.036$; Figure 3.6G) while MpC002 increased aphid reproduction compared to EV (ANOVA; $P \leq 0.038$; Figure 3.6G). Mp42 and MpC002 are aphid specific genes with no homology to proteins of known function. The *A. pisum* homolog of MpC002 contributes to *A. pisum* feeding and survival (Mutti *et al.*, 2006; Mutti *et al.*, 2008).

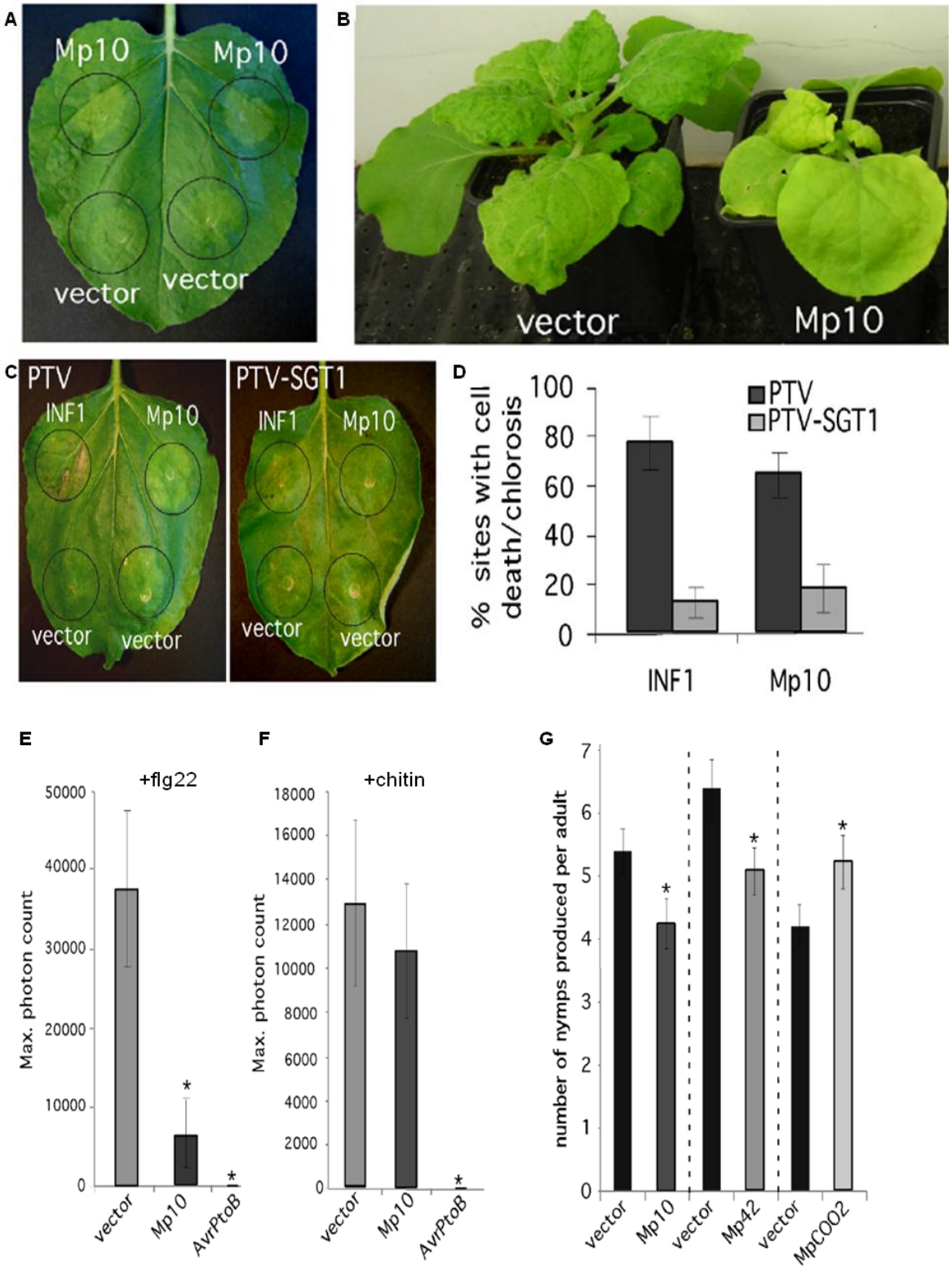


Figure 3.6 – Screening 48 candidate effector genes identified three that showed phenotypes upon overexpression in *N. benthamiana*.

(A) Mp10 induces chlorosis in *N. benthamiana.* Overexpression of Mp10 by infiltration of *N. benthamiana* with *A. tumefaciens* compared to the vector control. Symptoms of chlorosis started to appear from 2 days post infiltration (dpi). Photos were taken 4 dpi.
(B) PVX-based expression of Mp10 in *N. benthamiana* compared to the vector control. Symptoms of chlorosis started to appear from 10 days post wound-inoculation (dpwi). Photos were taken 14 dpwi.
(C) Mp10 chlorosis is SGT1 dependent. Over-expression of INF1 and Mp10 in SGT1-silenced (TRV-SGT1) and control (TRV) *N. benthamiana* plants. Photos were taken 4 dpi.
(D) Percentage of infiltration sites showing either INF1 cell death or Mp10 chlorosis 4 dpi on SGT1-silenced and control *N. benthamiana* plants. The graphs show the averages calculated from 3 replicated experiments, with 8–10 infiltration sites per individual replicate. Error bars indicate SE.
(E) Mp10 suppresses the ROS burst triggered by flg22. The ROS burst in response to flg22 was measured using a luminol-based assay in *N. benthamiana* leaf discs transiently expressing Mp10, the vector or AvrPtoB (positive) controls upon infiltration with *A. tumefaciens*. Results are mean ± SE (n = 8 leaf discs per replicate). The experiment was repeated three times with similar results, with one representative experiment shown. Asterisks indicate statistical significance compared to the vector control ($P \leq 0.043$).
(F) Mp10 does not suppress the ROS burst triggered by chitin. The ROS burst in response to chitin was measured using a luminol-based assay in *N. benthamiana* leaf discs transiently expressing Mp10, the vector or AvrPtoB (positive) controls upon infiltration with *A. tumefaceins*. Results are mean ± SE (n = 8 leaf discs per experiment). The experiment was repeated three times with similar results, with one representative experiment shown. Asterisks indicate statistical significance compared to the vector control ($P \leq 0.028$).
(G) Overexpression of Mp10 and Mp42 reduces aphid nymph reproduction and overexpression of MpC002 increases aphid nymph reproduction. *N. benthamiana* leaf discs transiently expressing candidate effector upon infiltration with *A. tumefaciens* were placed in 24-well plates and four *M. persicae* nymphs were added to each well. Six wells were used per construct. Data shown are means ± SE of three biological replicates with n = 6 per replicate (n = 18 in total). Asterisks indicate statistical significance compared to the vector control based on a one-way ANOVA (Mp10: $P \leq 0.026$, Mp42: $P \leq 0.036$ and MpC002: $P \leq 0.038$).
The experiments in this figure were carried out by Dr. Jorunn I.B. Bos (Mp10 and Mp42) and Marco Pitino (MpC002), whilst I conducted the statistical analysis for these experiments. These data were published in Bos *et al,.* 2010 (Appendix A).

3.3 Discussion

In this chapter I cloned twelve *M. persicae* candidate effectors representing eleven different genes, and screened them in functional assays as part of a larger effort to identify and functionally characterise aphid effectors. I found one candidate effector, Mp6, which possessed two versions which consisted of three polymorphisms in the amino acid sequence. Whilst none of the candidate effectors I screened showed a phenotype in functional assays, the screen as a whole identified three candidate effectors (Mp10, Mp42 and MpC002) that did show phenotypes.

The presence of polymorphisms in the sequence of candidate effector Mp6 is interesting, as changes in effector sequence can be an indicator of positive selection in the plant-pathogen interaction. In the screen as a whole we found four candidates that had polymorphisms in the clones we sequenced (Mp6, Mp17, Mp33 and Mp35). The polymorphism in Mp17, Mp33 and Mp35 were represented in the EST database used for the bioinformatics pipeline, however the polymorphisms in Mp6 were not. This was due to there being only one completed Mp6 EST in the database. A second EST, ES226303, covers the N-terminal part of the sequence up to amino acid 62, but is not long enough to cover the polymorphisms. The differences in the sequence for Mp6 are unlikely to be PCR or sequence mistakes, as they were reproducible from independent groups and individuals of *M. persicae*. Whilst a single polymorphism might be dismissed as having little affect on the function of the protein, three polymorphisms hints at the possibility of differing functions between the two alleles. The polymorphism at position 610 in the nucleotide sequence (C-terminus) resulting in a change from negatively charged glutamic acid to positively charged lysine may be of interest as Bos and colleagues (Bos *et al.*, 2006) found that a change from glutamic acid to lysine in the C-terminal of *P. infestans* effector AVR3a caused avirulence in the interaction with *S. tuberosum*, due to recognition by the plant resistant gene *R3a*. The other two polymorphisms see neutrally charged resides exchanged for other neutrally charged residues, and so are unlikely to have a large affect on the properties of the protein. Whether *Mp6* is present in two copies or is a heterozygous locus within the aphid could be further investigated by doing a southern blot. Mp6 showed no phenotypes in the functional assays, so we currently don't know if and how it works as an effector, but if future research does discover a role for it in plant-aphid interactions then the importance of the polymorphisms can be better studied.

The results of the experiments to test for a visible phenotype in *N. benthamiana* upon expression of the candidates were consistent in the absence of any phenotype. The results of the assays testing for flg22-triggered ROS suppression were slightly more variable. The first four candidates showed a consistent pattern relative to each other and

the controls over the two replicates, with Mp6_2 giving the lowest response apart from the positive control on both occasions. It would be interesting to compare Mp6_2 and Mp6_6 side by side in order to see whether there were any differences in ROS suppression between the two versions. The remaining candidates showed a variable pattern between the two replicates, as did the EV and GFP controls. The discrepancy between the two negative controls in these assays shows the benefit of including them both. These assays may have benefit from more replication, however the current data do not give any strong suggestions that further replication would lead to one of the candidates showing significant suppression of flg22-triggered ROS.

The 24-well leaf disc fecundity assay that we developed was also variable in the results that it gave. Effects seen in the initial screen were not always reproducible when later repeated in more controlled experiments, with the candidates expressed on the same *N. benthamiana* leaves as the controls. However, testing every candidate with the more controlled experiment would have been an unfeasible task and given that the repeatition of phenotypes from the screen was confirmed for three candidates, the screening method was useful in identifying previously unknown effectors.

None of the candidate effectors that I screened with functional assays showed a phenotype of HR or similar, flg22 PTI suppression, or changed reproduction/survival when overexpressed in *N. benthamiana*. There are a number of possible reasons for this. Our functional screens only looked at three possible characteristics of effectors, and so screening other effector functions may have revealed phenotypes in the candidates. For example, our reproduction/survival assay assumed that the presence of more of the candidate effector would affect the plant-aphid interaction. However, some effectors might already be present in sufficient quantities to adequately modulate the plant and so the additional amount provided in our assays made no difference. Screening the effectors with the plant-based RNAi system developed for *M. persicae* (Pitino *et al.*, 2011) may reveal effectors that fall into this category, as the reduced expression of the effectors in the aphid might lead to their being insufficient amounts to manipulate the plant, leading to decreased aphid performance. However, some effectors may be functionally redundant, and so the knockdown of one gene may not affect the aphids' ability to feed on the plant. This approach also has the disadvantage that it does not guarantee functionality as an effector, because knock-down of essential genes can also lead to reduced aphid survival and fecundity. An example of this is the knock-down of RECEPTOR FOR ACTIVATED C KINASE (RACK-1). RACK-1 is an intracellular receptor that binds activated protein kinase C (PKC), an enzyme primarily involved in signal transduction cascades (Seddas *et al.*, 2004) and it is involved in many processes inside the aphid. Silencing this gene leads to lower aphid fecundity (Pitino *et al.*, 2011), yet it is unlikely that is it an effector. A

combination of transgenic expression of effector candidate *in planta,* accompanied by RNAi may be the best approach for discovering candidate effectors.

Alternatively, the localization of an effector in the plant may be important. We transiently expressed the candidates under the 35S promoter, however it unclear how much expression this leads to in the phloem where aphids feed. A recent study showed that candidate effectors MpC002, PIntO1 and PIntO2 (named Mp1 and Mp2 in this study) produced in *A. thaliana* phloem under the *A. thaliana* SUCROSE-PROTON SYMPORTER2 (AtSuc2) promoter increased aphid reproduction (Pitino and Hogenhout, 2013). We only observed the same phenotype in this study for MpCOO2 and not Mp1 or Mp2 produced in *N. benthamiana* under a 35S promoter.

Additionally, *M. persicae* feeds on many host plants, and so it is possible that its effectors are host-specific. Recently, Jaquiéry and colleagues identified a homolog of Mp4 in *A. pisum* as a loci under ecologically divergent selection among *A. pisum* host races (Jaquiéry *et al.*, 2012). Plant host specificity may explain why we found no change in *M. persicae* fecundity in Mp1 and Mp2 expressing *N. benthamiana*, but Pitino and Hogenhout saw an increase in *A. thaliana* (Pitino and Hogenhout, 2013). Host specificity of effectors could be tested by using another model plant species host, such as *A. thaliana*. Similar assays looking for affect on plant defence or the plant-aphid interaction could be carried out on stable transgenics expressing the candidates or RNAi constructs to the candidate.

There may also be technical reasons why none of the effectors I screened produced a phenotype. The objective of screening a large number of candidates led to the necessity of devising feasible and practical assays. We did not check the expression of effector proteins expressed in *N. benthamiana*, as the large number of candidates would make this time-consuming and the nature of the screen was to identify positive candidates rather than functionally characterise each individual candidate in detail. Therefore it is likely that some of the candidates may not have been expressed in the functional assays. In the case of testing the affect of candidate effectors on aphid reproduction/survival, the need for a feasible assay led us to use leaf discs expressing the effectors rather than whole plants. Leaf disc responses may be different from those of whole plants. We saw one example of this with Mp10, which did not show the chlorosis phenotype when the leaf discs were removed from the plants for the 24-well plate assay. If plant responses are different in detached leaf discs then some effectors may not behave as they would in the natural situation.

Whilst none of the candidates that I screen showed phenotypes, the screen as a whole identified three candidates that did. Mp10 showed phenotypes in all of the functional assays carried out. The chlorosis induced by expression of Mp10 in *N.*

benthamiana may be a genuine effector function of this gene. Three *P. syringae* type III effectors have been described that induce chlorosis; AvrB (Shang *et al.*, 2006), HR AND PATHOGENICITY OUTER PROTEIN (HOP) HOPQ-1 (Wroblewski *et al.*, 2009) and HOPG1 (Cunnac *et al.*, 2011). Alternatively, Mp10 chlorosis might be viewed as an artifact due to the artificially high expression level given by Agrobacterium-mediated expression. However, as mentioned above, leaf discs removed from the plant 24 hours after infiltration did not display chlorosis, suggesting that plant factors are involved in the chlorosis. One possible explanation of Mp10 chlorosis is that it results from recognition of Mp10 by the plant, potentially by an NBS-LRR resistance protein leading to ETI. This explanation is supported by the fact that AvrB chlorosis in *N. benthamiana* is thought to be due to weak activation of TARGET OF AVRB OPERATION1 (TAO1), an NBS-LRR protein, and requires the plant-signalling component REQUIRED FOR MLA12 RESISTANCE1 (RAR1) (Eitas *et al.*, 2008). We found that Mp10 chlorosis requires SGT1, which is required for activation of NBS-LRR proteins and plant resistance (Azevedo *et al.*, 2006). However, SGT1 is involved in a series of plant processes including JA response (Meldau *et al.*, 2011a). Therefore, further investigation is needed to determine if Mp10 is indeed triggering ETI. However, Mp10 may be the first aphid effector identified to trigger ETI, even though the identity of the associated *R* gene is unknown.

Mp10 also suppressed the ROS burst triggered by flg22, which suggests that aphids may need to suppress PTI in order to successfully colonize a host, in the same why that plant pathogens do. Other PTI responses to flg22 such as MAPK activation or marker gene induction could be investigated to further confirm the ability of Mp10 to suppress PTI. The role of Mp10 suppression of the flg22 ROS response is still unclear. Aphids are unlikely to possess flagellin, the protein which flg22 represents. Aphids do contain bacterial symbionts, the primary endosymbiont being *Buchnera spp*, but this bacteria species have lost the *fliC* gene to produce flagellin (Shigenobu *et al.*, 2000). One study has shown that aphid saliva induces plant defence responses in *A. thaliana*, but the identity of the elicitor is unclear (De Vos and Jander, 2009). Similar situations to those observed in this chapter have been reported for plant-pathogens; for example the oomycetes *Hpa* can suppress flg22 PTI in *A. thaliana*, although its PAMP complement remains unknown (Fabro *et al.*, 2011). Recent evidence suggests that FLS2, the receptor for flg22, may not be specific to flg22 as it has been reported to mediate plant perception to *Xanthomonas* Ax21 secreted peptides (Danna *et al.*, 2011) and plant CLAVATA3 peptides (CLV3p) (Lee *et al.*, 2011). These findings have proved controversial, as other groups have failed to replicate them (Mueller *et al.*, 2012b; Mueller *et al.*, 2012a; Segonzac *et al.*, 2012). However, the authors of the original articles state that

experimental differences are likely to explain the failure to replicate the results (Danna, 2012; Lee, 2012), and therefore stand by their findings. If FLS2 is the receptor for multiple ligands then this raises the possibility that an unidentified aphid molecule may interact with FLS2 to trigger PTI. A more likely explanation may be that aphids trigger a PTI pathway that involves downstream plant genes that are common to the flg22 and other elicitor-triggered pathway. One example is SERK3/BAK1, a signalling component shared by the flg22 and other PAMP pathways. Any aphid effector that targeted the PTI pathway below the PRR would impair the response of other PAMPs that trigger the same pathway, and this may be what we see with Mp10 suppression of flg22-triggered ROS burst. Until we have a better understanding of the presences of elicitors in aphids and the role of PTI in plant-aphid interactions then the function of Mp10 in PTI suppression will remain unclear. We are currently unable to rule out the possibility that flg22-triggered ROS burst suppression by Mp10 is a result of the chlorosis induced upon Mp10 overexpression. However, the ROS burst triggered by chitin was not suppressed by Mp10, suggesting that Mp10 is specifically suppressing flg22 ROS burst and not ROS bursts in general.

Suppression of flg22-triggered ROS burst would potentially be beneficial for *M. persicae* interacting with *N. benthamiana*, yet overexpression of Mp10 led to a reduction in reproduction in the 24-well plate assay. This apparent contradiction could be explained if Mp10 activates ETI due to recognition by an NBS-LRR resistance protein, as postulated above. If this is the case, then the resistance induced by ETI would obscure us from seeing any beneficial effect of aphid elicitor-triggered immunity suppression on aphid performance. It would also be expected that *M. persicae* may possess another effector which suppresses Mp10-induced ETI, thus allowing Mp10 PTI suppression to benefit the aphid.

The 24-well plate leaf disc assay identified two other candidate effectors in addition to Mp10. Mp42 showed decreased reproduction compared to the vector control, and this may also be because it activates ETI or another plant defence pathway. Mp42 shares no homology with proteins of known function. Mp42 has currently not been shown to have an effector function but it is very different at the amino acid level to its closest homolog in *A. pisum* (appendix A (Bos *et al.*, 2010)), and therefore it is more likely to be a recognized effector than a conserved elicitor. The finding that aphids reproduced better on plants that produce MpC002 suggested for the first time that C002 is an effector that functions in the plant, modulating plant processes to apparently benefit aphid colonization. Since we published this result the Hogenhout lab has subsequently provided further evidence for this by showing that MpCOO2 also functions to modulate plant processes in *A. thaliana* (Pitino and Hogenhout, 2013). How MpC002 manipulates the host remains unclear, although we know from this work that it does not suppress flg22-

triggered ROS burst. Silencing of MpC002 leads to decreased aphid reproduction on *N. benthamiana* and *A. thaliana* (Pitino *et al.*, 2011); further suggesting it has an important role in the success of the aphid. Whilst C002 shares no homology with a protein of known function, the *A. pisum* homolog has also been shown to be important for aphid feeding, as silencing it leads to decreased aphid performance and death when feeding on plants (Mutti *et al.*, 2006; Mutti *et al.*, 2008). Whether *A. pisum* perform better on plants expressing ApC002 has not yet been determined. These results from two different aphid species suggest C002 may play an important role in plant-aphid interactions.

Whilst Mp42 and MpC002 show no homology to other proteins, Mp10 shows homology to OS-D2, a member of a family of predicted CSPs. CSPs are thought to bind small molecules, such as fatty acids, and for some members of this protein family there is evidence that they bind to pheromones (Jacquin-Joly *et al.*, 2001; Briand *et al.*, 2002).

The functional genomics approach adopted in this chapter has proved valuable in providing an alternative approach that complements effector searches based on homolog and mass spectrometry analyses, such as (Carolan *et al.*, 2011; Nicholson *et al.*, 2012). Homology based searches identify proteins already somewhat functionally described, whereas mass spectrometry approaches identify proteins in saliva gathered from diet, which may not reveal all proteins such as those specifically secreted during plant feeding. In this study two candidate effectors (MpCOO2 and Mp42) with no known protein homology have been identified, and these would have been overlooked if we had adopted an approach based on homology alone. Given that most plant-pathogen effectors do not possess homology to proteins of known function based on amino acid sequence, it is important that functional genomics approaches are adopted as part of a range of approaches to identify and verify aphid candidate effectors. Additionally we identified a protein of known homology with an unexpected function. We would not have been likely to predict that a CSP such as Mp10 would suppress the flg22-triggered ROS burst. In future other functional assays of the aphid proteins identified in this and other studies may reveal more candidate effectors and aspects of their function.

Chapter 4 - The discovery of aphid elicitors perceived by *N. benthamiana*

Contributors: David C. Prince, Cyril Zipfel and Saskia A. Hogenhout.

4.1 Introduction

Plants need to defend themselves from attack by pathogenic organisms. The first level of active defence is recognition of conserved elicitors by specific membrane receptors termed PRRs. Recognition of elicitors, such as PAMPs, by PRRs plays a key role in plant immunity (Boller and Felix, 2009; Monaghan and Zipfel, 2012). This recognition is highly sensitive and specific and leads to a series of signalling cascades that change the biology of the host and lead to restricted pathogen growth (Boller and Felix, 2009). Whilst many elicitors have been identified, there is little data on what elicitors of plant defence aphids contain. We previously identified a *M. persicae* effector, Mp10, which suppressed the ROS burst triggered by the PAMP flg22 in *N. benthamiana* (chapter 3). There are no data on what elicitors of plant defence in *N. benthamiana* aphids contain. Based on the effector function of Mp10 we hypothesised that aphids contain molecules that trigger plant immune responses in *N. benthamiana* (chapter 3). We also hypothesised that such responses would require SERK3/BAK1, a signalling component shared between the flg22 and other elicitor perception signalling pathways.

Whilst the signalling events in elicitor-triggered immunity are subject to much study, our current knowledge relies heavily on reports using the model plant species *A. thaliana* (Nguyen *et al.*, 2010; Segonzac *et al.*, 2011). Perception of an elicitor upon binding to a PRR leads to various downstream signalling including a Ca^{2+} burst, ROS burst, and MAPK cascades, which in turn contribute to later outputs including changes in gene expression, and callose deposition (Boller and Felix, 2009). A recent study on the hierarchy of these signalling events during flg22 and chitin-triggered signalling in *N. benthamiana* found that the Ca^{2+} burst is upstream of two parallel signalling cascades, one leading to MAPK activation and then gene expression, the other leading to ROS production (Segonzac *et al.*, 2011). This study used VIGS to investigate gene function, as knockout lines for *N. benthamiana* are not available. VIGS is commonly used to study the function of genes in solanaceous plants. Other VIGS studies of genes involved in elicitor-triggered immunity *N. benthamiana* have identified roles for a variety of genes including those encoding proteins with functions in hormone signalling, protein stability and cell wall biosynthesis (Chakravarthy *et al.*, 2010), as well as G proteins (Zhang *et al.*, 2012a). The recent release of a draft of the *N. benthamiana* genome (http://solgenomics.net/) (Bombarely *et al.*, 2012) will help future studies into the genes involved in elicitor responses.

Different response kinetics have been observed in *N. benthamiana* challenged with different elicitors. For example, the Ca^{2+} burst triggered in *N. benthamiana* upon

chitin challenge occurs almost immediately and returns to normal levels of intracellular Ca^{2+} in around 10 minutes. In contrast, the Ca^{2+} burst triggered by flg22 is delayed by five minutes and takes nearly 30 minutes to return to normal levels (Segonzac *et al.*, 2011). ROS bursts triggered by elicitors are usually measured up to the first 60 minutes, as the bursts triggered by the commonly used elicitors flg22 and chitin have returned to normal levels by this timepoint (e.g. (Segonzac *et al.*, 2011)). However, some elicitors trigger ROS bursts over longer time periods. For example, the *P. infestans* elicitin INF1 has a ROS burst in *N. benthamiana* that is much delayed compared to flg22, starting around 30 minutes after challenge. The duration of the burst is also much longer when compared to flg22, and does not show the same single peak kinetic (Chaparro-Garcia *et al.*, 2011). How plants interpret different signatures of signalling molecules such as Ca^{2+} and ROS and mount appropriate responses to them is not well understood.

In the previous chapter, flg22 was used to investigate PTI suppression phenotypes in candidate aphid effectors. FLS2 is a LRR-RLK and is the PRR that recognises flg22 (Gómez-Gómez and Boller, 2000). In *A. thaliana*, the binding of flg22 to FLS2 leads to complex formation between FLS2, BAK1 and BIK1, triggering rapid phosphorylation events (Lu *et al.*, 2010, Zhang, 2010 #431), and downstream signalling responses. BAK1 is a member of the SERK family. In *A. thaliana* there are five members of this family, of which BAK1 is SERK3 (Albrecht *et al.*, 2008), and BAK1 plays a role in the signalling following perception of many elicitors (Shan *et al.*, 2008; Krol *et al.*, 2010).

Homologs of *Serk3/Bak1* have been identified in solanaceous species, with two homologs being reported for *S. lycopersicum* and *N. benthamiana* (Chaparro-Garcia *et al.*, 2011; Mantelin *et al.*, 2011) and one homolog being reported for wild potato (*Solanum phureja*) and a wild tobacco species (*N. attenuata*) (Chaparro-Garcia *et al.*, 2011; Yang *et al.*, 2011). In *N. benthamiana*, experiments silencing *Serk3/Bak1* have provided data to show that it is involved in signalling following recognition of flg22, cold shock protein (csp22), INF1 and unidentified *Hpa* elicitors (Heese *et al.*, 2007). Another study found that silencing of *NbSerk3* leads to enhanced susceptibility to the adapted oomycetes *P. infestans* but not to non-adapted *Phytophthora mirabilis* (Chaparro-Garcia *et al.*, 2011). Interestingly, the SERK3/BAK1 homolog in *N. attenuata* is thought not to play a role in the perception of caterpillar elicitors (Yang *et al.*, 2011). Whilst the current data show some functional similarities between the BAK1 homologs in *A. thaliana* and *N. benthamiana*, the full extent of the role of BAK1 in *N. benthamiana* signalling responses is still unclear.

Substantial research has been conducted on *N. attenuata* defences triggered by elicitors of chewing herbivores, such as lepidopteran larvae (caterpillars). Several caterpillar elicitors have been characterised including FACs and GOX (Alborn *et al.*, 1997; Musser *et al.*, 2005; Diezel *et al.*, 2009). The plant responses observed to insect

elicitors include Ca^{2+}, and ROS bursts as well as MAPK activation (e.g (Maffei *et al.*, 2006; Wu *et al.*, 2007; Schafer *et al.*, 2011)). Such responses are shared with other elicitor perception pathways including those of flg22. The model of plant perception of insect elicitors proposed by Wu and Baldwin (Wu and Baldwin, 2010) bears a striking resemblance to plant perception of bacteria and fungal elicitors. There are still many components of the model where little is known, especially the receptors at the plant membrane involved in elicitor perception and binding. LecRK1 is a recently identified RLK in *N. attenuata* and is involved in plant defence responses against *M. sexta* (Gilardoni *et al.*, 2011). It is unclear if this protein is acting as a receptor to elicitors from the insect, and the authors currently hypothesize that it acts downstream of the signalling cascade upon insect elicitor perception (Gilardoni *et al.*, 2011).

No examples of aphid elicitors perceived by *N. benthamiana* have currently been characterised. The best example to date of an aphid salivary elicitor of plant defence was found in *M. persicae-A. thaliana* interactions. Applying the saliva of *M. persicae* to *A. thaliana* plants leads to an induced resistance against the aphid, shown by a reduction in aphid performance (De Vos and Jander, 2009). Fractionation by molecular weight, heat and proteinase K treatment revealed that the elicitors are likely to be a heat-sensitive peptides/proteins between 3 and 10 kDa.

In this chapter we investigated if aphids possess elicitors that are perceived by *N. benthamiana.* We opted to use *M. persicae* whole extracts for convenience, as this is significantly faster than preparing extracts from aphid mouthparts or saliva. We used VIGS to investigate the role of SERK3/BAK1 in the ROS response to *M. persicae* elicitors.

4.2 Results

Whole *M. persicae* extract elicits immune responses in *N. benthamiana.*

We wished to investigate if components of *M. persicae* were able to trigger immune responses in *N. benthamiana*, such as Ca^{2+} and ROS bursts (Segonzac *et al.*, 2011). We used the SLJR15 line of *N. benthamiana* (Segonzac *et al.*, 2011) stably expressing the reporter protein aequorin within the cytoplasm (Knight *et al.*, 1993) to monitor Ca^{2+} responses to *M. persicae* elicitors. In the presence of the luminophore coelenterazine, aequorin emits blue light in a dose-dependent manner upon binding Ca^{2+} (Knight *et al.*, 1993). Whole *M. persicae* extracts elicited a double-peaked trace, with a sharp first peak between one and four minutes and a second peak between four and 30 minutes (Figure 4.1A). This is in contrast to flg22, which produced one peak between four and 30 minutes (Figure 4.1A).

To assess if whole *M. persicae* extract was able to elicit a ROS burst we used a luminol based assay as previously described (chapters 2 and 3). In the first hour of the experiment we saw a small ROS burst in the aphid extract treated leaf discs (Figure 4.1B). In subsequent hours we saw a second, multi-peaked ROS burst that was different to the one seen from flg22 (Figure 4.1C). Taken together, these results suggest that *M. persicae* elicitors trigger immune responses in *N. benthamiana* that are similar and different to those of the PAMP flg22.

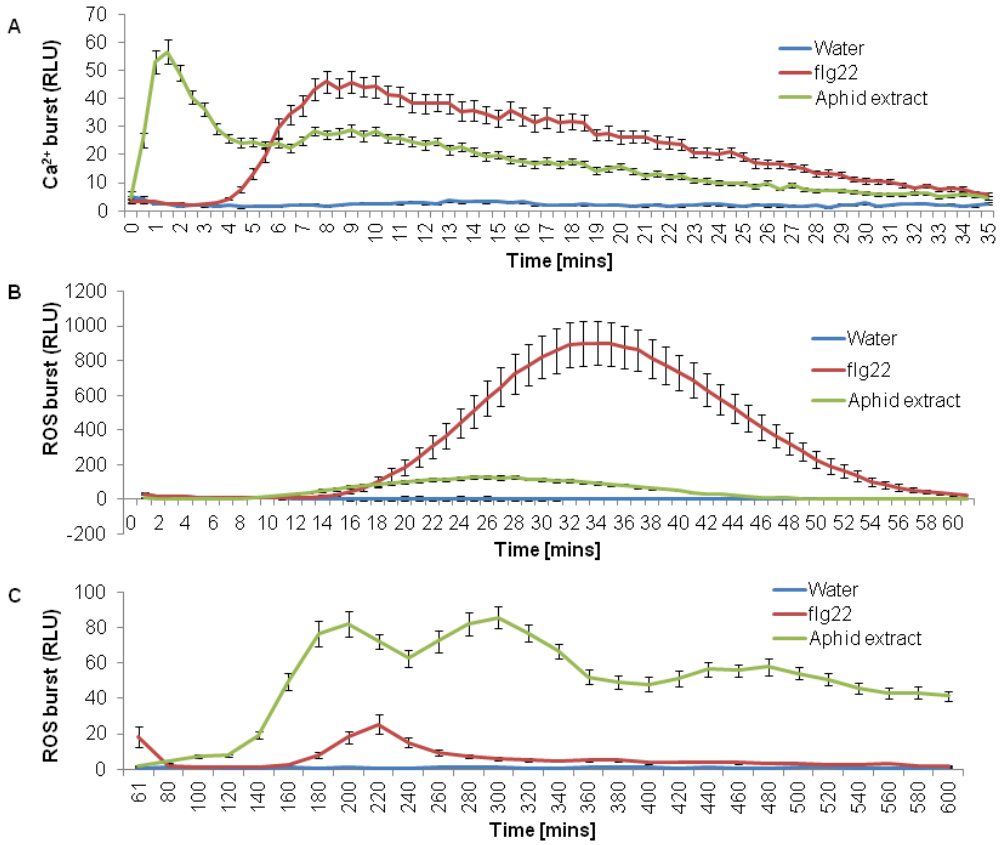


Figure 4.1 - Whole *M. persicae* extract elicits immune responses in *N. benthamiana*.
(A) Whole *M. persicae* extract elicits a double-peaked Ca^{2+} burst. Ca^{2+} burst was measured in the *N. benthamiana* SLJR15 line over 35 minutes after elicitation by water, 20 nM flg22 or whole aphid extract. Results are mean ± SE (n = 32 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown.
(B) Whole *M. persicae* extract elicits a ROS burst in the first hour. (C) Whole *M. persicae* extract elicits a multi-peaked ROS burst between 61 and 600 minutes. ROS bursts were measured between 0 and 60 minutes (B) and 61 to 600 minutes (C) in *N. benthamiana* leaf discs after elicitation by water, 5 nM flg22 or whole aphid extract. Results are mean ± SE (n = 32 leaf discs). This experiment was repeated three times with similar results, with one representative experiment shown.

Investigation of the proteinaceous properties of the aphid elicitors.

To investigate the chemical properties of the *M. persicae* elicitors we applied various treatments to the whole aphid extract and then tested if the elicitors were still able to trigger a ROS burst. We first removed the insoluble molecules from the extract by centrifuging the extract and then transfering the supernatant to a fresh tube. We boiled the aphid extract supernatant in order to denature proteins and therefore assess the role of secondary and tertiary structure in the elicitation of defence responses. We also proteinase K treated the aphid extract supernatant in order to digest and therefore

degrade proteins. Boiling of the aphid extract supernatant led to a significant increase in the magnitude of the ROS burst triggered in the first hour (ANOVA; $P < 0.05$, Figure 4.2A), and a significant decrease in the later ROS burst. However, the extract still triggered a later ROS response when compared to background levels (ANOVA; $P < 0.05$, Figure 4.2B).

Initially we tested the ability of proteinase K to elicit a ROS burst in *N. benthamiana*, and found that no ROS burst was produced (Student's *t*-test; $P > 0.05$ (n.s.) Figure 4.2C and D). This result indicated that the proteinase K was a suitable enzyme to digest the aphid extract with. It also provided evidence that the presence of protein alone is not sufficient to trigger a ROS burst in *N. benthamiana*. We then investigated the effect of proteinase K treatment of *M. persicae* extract on the ROS bursts. The ROS burst in the first hour triggered by proteinase K-treated supernatant was not stastistically different to that elicited by buffer or untreated supernatant (ANOVA; $P > 0.05$ (n.s.), Figure 4.2A), although any significant difference between the untreated supernatant and the proteinase K-treated supernatant may have been masked by the magnitude of the ROS burst from the boiled aphid extract. The ROS burst triggered by proteinase K-treated aphid extract in subsequent hours was significantly less than the untreated supernatant and similar to that of the boiled treatment (ANOVA; $P < 0.05$, Figure 2B). These data suggest that the elicitor(s) that trigger the ROS burst in the first hour are may be proteinaceous in nature, because boiling of the extracts may denature proteins exposing epitopes that then can be recognized by the plant receptors. Boiling of EF-Tu also improves recognition of the PAMP by EFR (Kunze *et al.*, 2004). The elicitors of the ROS burst in subsequent hours may be a mix of proteins and non-proteins, as disrupting the proteins with boiling and proteinase-K treatments reduced the ROS burst levels, however treatments did not completely eliminate the induction of ROS bursts. Thus, the eliciting aphid extracts are of complex nature, although we cannot exlude the possibility that the proteinase-K treatment may not have digested all the proteins in the aphid extracts.

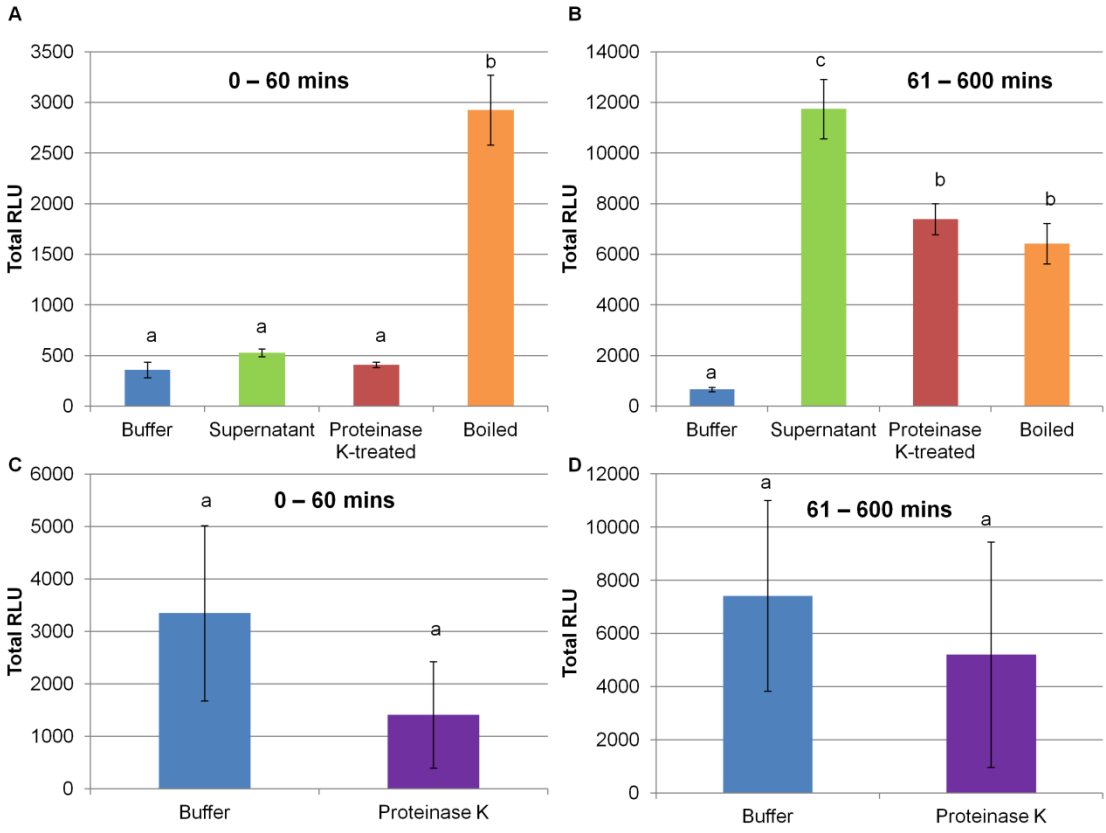


Figure 4.2 – Investigation of the proteinaceous properties of aphid elicitors.
(A) Boiling the supernatant of whole *M. persicae* extract increases the ROS burst in the first hour. (B) Boiling and proteinase K treatment of whole *M. persicae* extract reduces the ROS burst between 61 and 600 minutes, but does not return it to the baseline level of buffer alone. The ROS bursts between 0 and 60 minutes (A) and 61 to 600 minutes (B) were measured in *N. benthamiana* leaf discs after elicitation by buffer and aphid extracts. Results are mean ± SE (n = 24 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.
(C) Proteinase K does not trigger a ROS burst during the first hour. (D) Proteinase K does not trigger a ROS burst between 61 and 600 minutes. The ROS bursts between 0 and 60 minutes (C) and 61 to 600 minutes (D) were measured in *N. benthamiana* leaf discs after elicitation by buffer or 100 µg proteinase K. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

The ROS bursts to whole *M. persicae* extract are independent of NbSERK3.
We wished to examine if NbSERK3 is involved in the perception of aphid elicitors, becuase SERK3/BAK1 is involved in the perception of several proteinaceous elicitors in *N. benthamiana* (Heese *et al.*, 2007). Plant expression of *NbSerk3* was knocked down by VIGS using the constructs *TRV:SERK3*, which targets both variants of *NbSerk3* in *N.*

benthamiana (Chaparro-Garcia *et al.*, 2011), and *TRV:EV*. Leaf discs were taken from leaves infiltrated with the VIGS constructs upon which the leaf discs were exposed to whole *M. persicae* extracts and analysed for a ROS response. No decrease in ROS response in *TRV:SERK3* leaves was seen in the first hour (Student's *t*-test; $P > 0.05$ (n.s.), Figure 4.3A) or subsequent hours (Student's *t*-test; $P > 0.05$ (n.s.), Figure 4.3B). Surprisingly, *TRV:SERK3* leaves produced significantly more ROS in one experiment out of three in the first hour (Student's *t*-test; $P < 0.05$, Figure 4.3A) and two experiments out of three in subsequent hours (Student's *t*-test; $P < 0.01$, Figure 4.3B). To check if *NbSerk3* expression was downregulated in the VIGS-treated leaves we conducted parallel ROS experiments with flg22, which triggers a reduced ROS burst compared to control leaves in leaves with reduced *NbSerk3* expression levels (Heese *et al.*, 2007). All three experiments showed a significant reduction in flg22 ROS response in the *TRV:SERK3* leaves compared to the control leaves (Student's *t*-test; $P = 0.001$, Figure 4.3C), confirming that the expression of *NbSerk3* was knocked down. These data show that the ROS bursts elicited by whole *M. persicae* extracts are not dependent on SERK3, and that SERK3 may even negatively regulate the ROS bursts to aphid extracts.

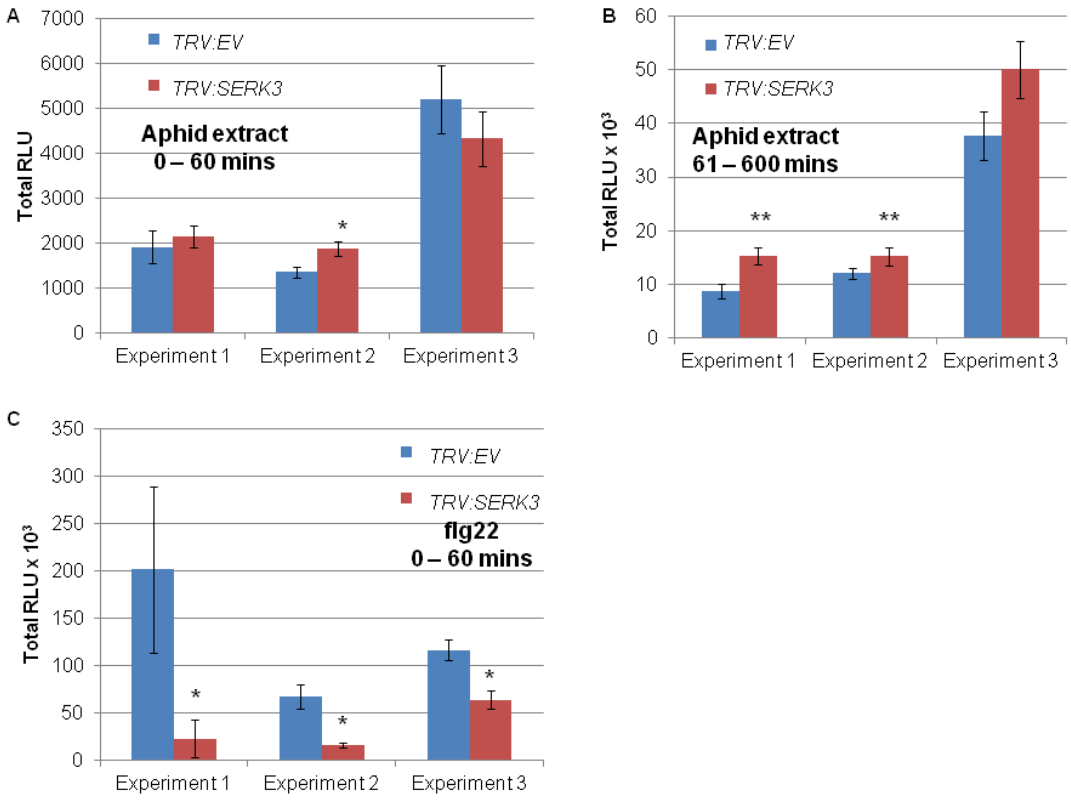


Figure 4.3 - The ROS bursts to whole *M. persicae* extract are independent of NbSERK3.

(A) Knocking down *NbSerk3* expression levels leads to no change in the ROS burst in *N. benthamiana* leaves triggered by whole *M. persicae* extract over the first hour. (B) Knocking down *NbSerk3* expression levels leads to an increase in the ROS burst in *N. benthamiana* leaves triggered by whole *M. persicae* extract between 61 and 600 minutes. The ROS bursts were measured in *N. benthamiana* leaves infiltrated with *TRV:EV* or *TRV:SERK3* and elicited with whole *M. persicae* extract. Results are mean ± SE (n = 12 leaf discs per replicate). Three independent experiments are shown. (Student's *t*-test; $*P < 0.05$, $**P < 0.01$).
(C) Knocking down *NbSerk3* expression levels showed a reduced ROS burst in *N. benthamiana* leaves triggered by flg22. The ROS burst was measured in *N. benthamiana* leaves infiltrated with *TRV:EV* or *TRV:SERK3* and elicited with 50 nM flg22. Results are mean ± SE (n = 12 leaf discs per replicate). The leaf discs were harvested at the same time and from the same leaves as those used in (A). (Student's *t*-test; $*P = 0.001$).

Distinct *N. benthamiana* Ca^{2+} and ROS responses to aphid extracts of different molecular weights.

Next we assessed if the eliciting fractions may be separated by molecular weight. Hence we fractionated extracts by weight into <3 kDa, 3 to 10 kDa and >10 kDa fractions using centrifugal filters. We tested each fraction's ability to trigger ROS bursts. We found that in the first hour the >10 kDa fraction triggered a large ROS burst, in contrast to the other fractions (ANOVA; $P < 0.05$, Figure 4.4A). In the subsequent hours the >10 kDa fraction elicited a large ROS response as well (ANOVA; $P < 0.05$, Figure 4.4B), whilst the <3 kDa fraction gave a small ROS burst that was not significantly different from the amount of background ROS of the buffer alone (ANOVA; $P > 0.05$, Figure 4.4B). No significant ROS burst was triggered by the 3 to 10 kDa fractions in early and later time points (ANOVA; $P > 0.05$ (n.s.) Figure 4.4A and B).

We then tested if the <3 kDa fraction and the >10 kDa fraction produced a Ca^{2+} burst in addition to the ROS burst. The <3 kDa fraction produced a double peak burst with a sharp first peak lasting between one and four minutes and a second peak between six and 30 minutes (Figure 4.4C). This is similar to the peaks given by the whole extract (Figure 4.1A). The >10 kDa fraction produced a single peak starting in the first minute, reaching its maximum around 11 minutes, and finishing by 30 minutes (Figure 4.4C). This peak is similar in shape, although not size, to that produced by flg22 (Figure 4.4C).

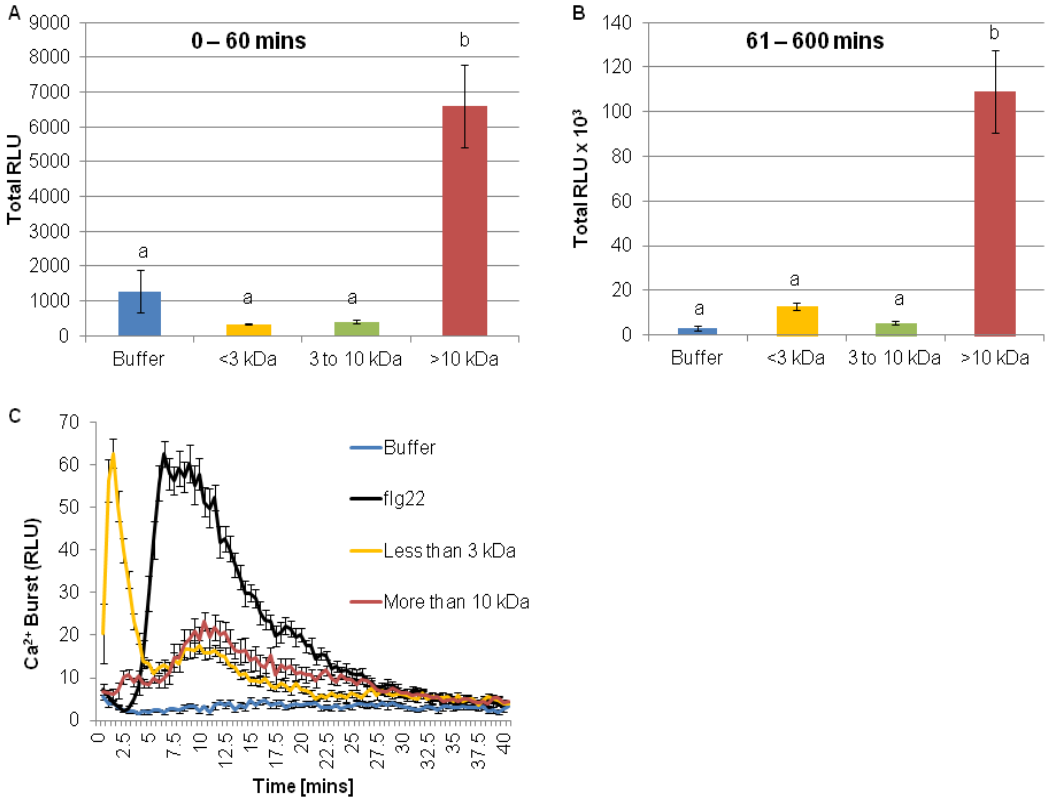


Figure 4.4 - Distinct *N. benthamiana* Ca^{2+} and ROS responses to aphid extracts of different molecular weight.

(A) The ROS burst in the first hour is caused by elicitors larger than 10 kDa. (B) The multi-peaked ROS burst between 61 and 600 minutes is caused by elicitors larger than 10 kDa. The ROS bursts between 0 and 60 minutes (A) and 61 to 600 minutes (B) were measured in *N. benthamiana* leaf discs after elicitation by buffer or fractionated *M.* persicae extracts. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

(C) <3 kDa and >10 kDa *M. persicae* extracts produce distinct Ca^{2+} bursts. The Ca^{2+} bursts were measured in the *N. benthamiana* SLJR15 line over 40 minutes after elicitation by buffer, 5 nM flg22, <3 kDa aphid extract, or >10 kDa aphid extract. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown.

M. persicae produces less progeny on *N. benthamiana* leaves pretreated with aphid extract.

Pre-treatments of plants with elicitors such as PAMPs induce plant defence against pathogens leading to decreased pathogen colonization. To test if any of the fractions of *M. persicae* extract were capable of triggering induced resistance, leading to reduced aphid colonization on *N. benthamiana*, we designed an aphid performance assay by

adapting the method of De Vos and Jander (De Vos and Jander, 2009). *N. benthamiana* leaves were infiltrated with buffer and the three fractionated extracts, so that all four treatments were present on the same leaf at one leaf area per treatment. Aged adult *M. persicae* were than caged to each of the infiltrated areas and total aphids produced per cage measured after 10 days. Aphids on leaf areas pretreated with the three fractionated aphid extracts produced significantly less aphids than the buffer control, with the 3 to 10 kDa fraction treated leaf areas producing the least number of aphids (ANODE; $F_{3,59}$ = 12.14, $P < 0.001$; Fig. 4.5). This result is surprising, as the 3 to 10 kDa fraction does not elicit a detectable ROS burst (Fig. 4.4A and B). In summary, these results indicate that the responses induced by *M. persicae* fractions in *N. benthamiana* leaves result in reduced aphid colonization.

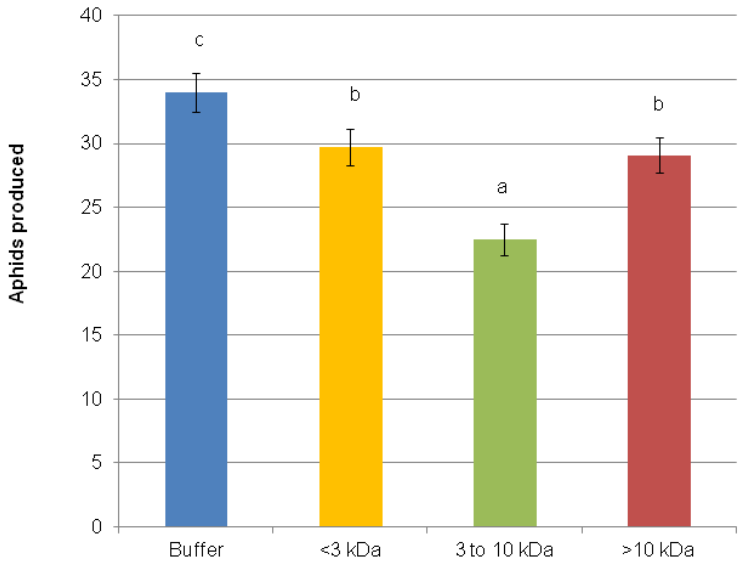


Figure 4.5 – *M. persicae* produces less progeny on *N. benthamiana* leaves pretreated with aphid extract.

Aphid fecundity on *N. benthamiana* leaves pretreated with buffer and fractionated extracts was measured over a 10 day period. Data shown are total aphids produced on the different treated area of the same leaf with means ± SE of five biological replicates with n = 1-4 leaves per replicate. Different letters above the columns indicate significant differences in aphid numbers (n = 15, $F_{3, 59}$ = 12.14 ($P < 0.001$) (t probabilities calculated within GLM)).

The >10 kDa elicitor fraction of multiple aphid species trigger a ROS burst in *N. benthamiana* leaves.

We wished to assess if the elicitor fractions of multiple aphid species trigger defence responses such as a ROS burst in *N. benthamiana* leaves. The most robust elicitor phenotype was the production of a ROS burst over the first hour that was enhanced by boiling of the extracts (Fig 4.2A). Similar ROS burst kinetics to boiled >10 kDa fraction of *M. persicae* was obtained (Fig 4.6A). Extracts from three different aphid species were tested, along with buffer and *M. persicae* controls. The ROS bursts of *A. pisum* and *B. brassicae* varied in amplitude but were similar in kinetics to that of *M. persicae.* However, the ROS burst to *R. padi* extract was always lower than the ROS bursts to the extracts of the other aphid species.

The >10 kDa elicitor extract of *M. rosae* was also prepared and tested for ROS burst. Two separate experiments showed that it was capable of eliciting a ROS burst similar to that of *M. persicae* (Fig. 4.6B), although the amplitude of the response was not as large. These data suggest that the >10 kDa fractions of multiple aphid species trigger ROS bursts in *N. benthamiana* leaves with similar kinetics.

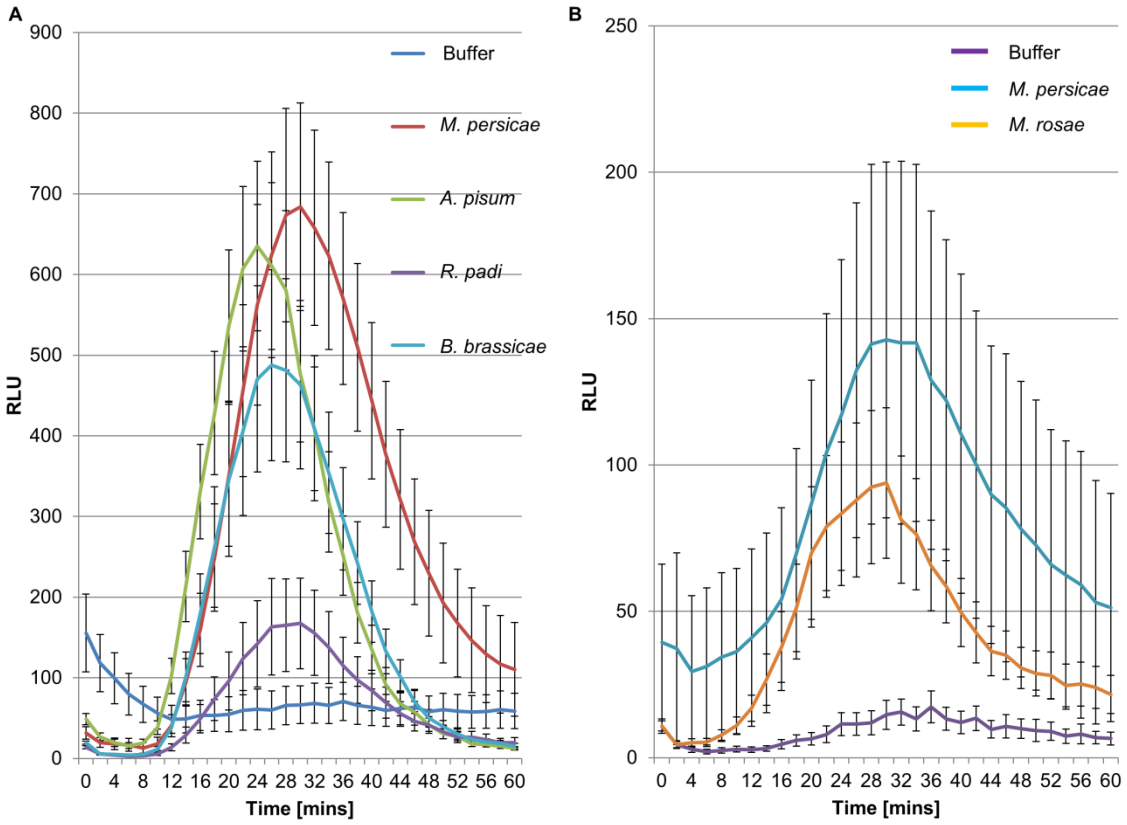


Figure 4.6 – The >10 kDa elicitor fraction of multiple aphid species trigger a ROS burst in *N. benthamiana* leaves.

(A) Boiled >10 kDa extract from diverse aphids triggers a ROS burst. The ROS burst was measured between 0 and 60 minutes in *N. benthamiana* leaf discs after elicitation by buffer or boiled >10 kDa extracts. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown.
(B) Boiled >10 kDa extract from *M. rosae* gives a ROS burst. The ROS burst was measured between 0 and 60 minutes in *N. benthamiana* leaf discs after elicitation by buffer or boiled >10 kDa extract. Results are mean ± SE (n = 16 per replicate). This experiment was repeated two times with similar results, with one representative experiment shown.

4.3 Discussion

The data provide the first evidence that *M. persicae* contains a range of molecules that elicit plant immunity responses in *N. benthamiana*. Fractions of different molecular weights give response signatures in the assays used. It is unlikely that SERK3/BAK1 is involved in most of these responses. Elicitor fractions of a diverse range of aphids induce similar responses in *N. benthamiana* suggesting that there is conservation of elicitors among the aphids. Therefore, aphid elicitors share this characteristic with elicitors from microbial pathogens.

We chose to use whole aphids to search for *M. persicae* elicitors of plant immunity, rather than aphid saliva alone. Elicitors from chewing insects are often identified from their oral secretions, and a previous study on aphid elicitors had used aphid saliva (De Vos and Jander, 2009). However, the advantage of our choice to use the whole aphid was that it gave us large quantities of extract to work with, containing every possible molecule the aphid makes. The practicalities of collecting saliva would have given us smaller quantities, which would have been more variable in composition and missing many molecules produced by the aphid. Moreover, the saliva potentially contains proteins from aphid body parts other than the salivary glands (Carolan *et al.*, 2011), including proteins from bacterial symbionts that live in the aphid abdomen (personal observation). Therefore any aphid protein secreted out of the cell has the potential to be present in the saliva and thus elicit plant defence responses. Whole *M. persicae* extract produced a similar ROS response to that of extract with the insoluble parts of the aphid removed (supernatant), suggesting that the insoluble parts were not contributing significantly to the responses seen with the whole extract. Further evidence was found for this upon fractionation of the extract, as the combined Ca^{2+} and ROS burst of the <3 kDa and >10 kDa fraction mimic that of the whole extract.

Whole *M. persicae* extract elicits both Ca^{2+} and ROS bursts. The ROS bursts are unlikely to be aspecific responses to protein, as they were not triggered by proteinase K, nor by 100 µg bovine serum albumin (BSA) in preliminary experiments. The Ca^{2+} and ROS bursts share characteristics with those of the PAMP flg22, but are not identical to them. *M. persicae* can trigger a fast Ca^{2+} burst in addition to one similar to flg22, and produces a multi-peaked later ROS burst in addition to one similar to flg22. The *P. infestans* elicitor INF1 also produces a late, multi-peaked ROS burst in *N. benthamiana*, however it does not also produce one with the same timing as flg22 (Chaparro-Garcia *et al.*, 2011). The INF1 ROS burst is associated with cell death (Heese *et al.*, 2007), but no similar cell death was observed in the induced defence assays. The production of a Ca^{2+} and ROS burst by aphid extract in *N. benthamiana* agrees with a transcriptional study of

plant responses to aphid attack on *A. thaliana* (Kusnierczyk *et al.*, 2008). During PTI signalling in *N. benthamiana* a Ca^{2+} burst precedes a ROS burst (Segonzac *et al.*, 2011) and the relative timings of the Ca^{2+} and ROS bursts triggered by *M. persicae* extracts suggest the same hierarchy may be present in signalling to aphid elicitors. The similarity in hierarchy and kinetics in Ca^{2+} and ROS responses strongly suggest that the molecules in *M. persicae* are triggering PTI signalling pathways. Further evidence could be added to this by investigating MAPK activation or marker gene induction in response to the extract, as these signalling events rely on the Ca^{2+} burst but are independent of the ROS burst (Segonzac *et al.*, 2011).

The Ca^{2+} assays conducted in this chapter were done without normalising the Ca^{2+} measurements after the assay by discharging the remaining aequroin at the end of the experiment, as is commonly done in the literature (e.g. (Ordenes *et al.*, 2012)). The literature does however contain examples of experiments where the discharge was not used for calibration (e.g. (Segonzac *et al.*, 2011). I do not think that this methological difference invalidates the conclusions I have drawn from the data. The objective of the experiment was to test the ability of elicitors to trigger Ca^{2+} bursts in *N. benthamiana* and observe the kinetics of the burst, rather than to make an accurate comment on the concentration of Ca^{2+} that was involved in the bursts. The experiments were conducted with the same line of transgenic plants stably expressing aequroin and samples from several leaves were present in each treatment in the experiment. Furthermore, the amount of leaf tissue was standardized by leaf age and disc size. Taken together, it is highly unlikely that the levels of aequorin were significantly different between different treatments in the experiments. Even in the unlikely event that the levels of aequorin were different, it would not affect the conclusion that a Ca^{2+} burst repeatably occurred.

The chemical properties of the elicitors suggest that the ROS burst produced in the first hour, which is similar to that of flg22, is likely to be produced by a protein or proteins. The properties of these proteins are similar to that of the PAMP EF-Tu (Kunze *et al.*, 2004), as degrading these proteins with proteinase K leads to a loss of the ROS response, whereas denaturing the protein by boiling leads to a large increase in the response. The later ROS burst is decreased upon boiling or proteinase K treatment of the extract, but not abolished, suggesting that they are in part due to proteinaceous elicitors and in part due to other molecules. It is possible that some carbohydrates may be soluble in the phosphate buffer used, and may by triggering the later responses. Whilst the later responses look atypical of those produced by chitin (e.g. (Segonzac *et al.*, 2011)), this could be further investigated through silencing of the Lys-M RLK *NbCerk1*, which is necessary for chitin-induced ROS production in *N. benthamiana* (Gimenez-Ibanez *et al.*, 2009). In *A. thaliana*, AtCERK1 has been shown to bind chitin directly (Iizasa *et al.*, 2010;

Petutschnig *et al.*, 2010) however it is unclear whether this happens in *N. benthamiana*. It is also unclear how homologs of other Lys-M RLKs and RLPs identified in plant perception of carbohydrate elicitors in *A. thaliana*, such as LYM1, LYM3 (Willmann *et al.*, 2011) and LYK4 (Wan *et al.*, 2012) function in *N. benthamiana*, and whether these genes would also need to be silenced in order to assay the presence or absence of carbohydrate elicitors in aphid extract.

Our efforts to define the elicitors by molecular weight as well as chemical properties have identified three fractions with different responses in the plant, the <3 kDa, 3 to 10 kDa and >10 kDa fractions. The protein composition of these fractions could have been compared visually using SDS-PAGE gels stained with coomassie or silver staining. This would have given us an indication of the level of complexity of the different protein fractions, and whether any protein bands were specific to a given fraction. It would have also provided confirmation of the selectivity of the fractionation columns used. However, SDS-PAGE gels would have only shown the most abundant proteins in the complex aphid extracts. Currently identified elicitor plant receptors can detect small amounts of elicitors and therefore the protein does not necessarily have to be present in quantities visible on a gel in order to trigger a response. This was the case for de Vos and Jander (De Vos and Jander, 2009), who reported that they could find no detectable protein bands on gels from their aphid saliva elicitor. Hence, SDS-PAGE gels may prove inconclusive in identifying the elicitors and therefore would need to be complemented with other approaches.

The <3 kDa fraction elicits a Ca^{2+} burst that is initially very fast, followed by a lower, longer one. This is followed by a later, reduced ROS burst. This fraction also induces defence against the aphid. It is possible that this fraction may contain small fragments of chitin or other soluble glycans small enough to pass through the filtration columns. The initial Ca^{2+} burst is reminiscent of that triggered by chitin (Segonzac *et al.*, 2011), although it does not lead to a similar ROS burst to chitin. The ROS burst that is triggered occurs later, and may be responsible for the remaining late ROS burst after the proteinase K and boiling treatments. This is also consistent with the <3 kDa fraction containing molecules other than peptides. The chemical properties of the <3 kDa fraction could be examined independently of the whole extract to ascertain if this is the case. It is unlikely that NbSERK3/BAK1 is involved in the perception of this elicitor, as the later ROS was generally higher in leaves with knocked down *NbSerk3* expression. The hypothesis that the <3 kDa fraction may contain chitin could be tested through examining the responses following the silencing of *NbCerk1* with VIGS. Further characterization of the elicitor is necessary before it is possible to comment on whether it is conserved between aphid species.

The 3 to 10 kDa fraction triggered induced resistance leading to significantly fewer aphids on *N. benthamiana* compared to the other two fractions, and the buffer control. The molecular weight and defence-inducing properties of this elicitor match those of the *M. persicae* saliva elicitor perceived by *A. thaliana* (De Vos and Jander, 2009). The elicitor of induced resistance in *A. thaliana* was sensitive to heat and proteinase K treatment, and was not perceived through known defence signalling pathways JA, SA, ET and PAD4 (De Vos and Jander, 2009). The lack of a ROS burst produced by this elicitor means we can not currently tell from the data in this chapter whether boiling and proteinase K treatment cause loss of the elicitor activities, but analysis of other defence responses could be conducted in order to investigate this further. The lack of a ROS burst means it is also not possible to tell from our current data whether NbSERK3/BAK1 is involved in the perception of this elicitor. This is harder to test by measuring aphid fecundity after pre-treatment with the elicitor, as silencing by VIGS is transient and patchy, although the protocol may be able to be modified to accommodate this. An alternative negative control, such as GFP or beta-glucuronidase (GUS) RNA expressing constructs, would be needed because empty vector silencing constructs have adverse effects on aphid performance in VIGS experiments in *S. lycopersicum* (Wu *et al.*, 2011). In addition, the ROS pathway in PTI *N. benthamiana* is parallel to the MAPK and gene expression pathways (Segonzac *et al.*, 2011), which could be investigated in response to the 3 to 10 kDa fraction. Thus, it is possible that the 3 to 10 kDa fraction triggers a Ca^{2+} burst leading to downstream activation of MAPKs and no ROS burst. This needs to be tested. Lipase activity from the desert locust *Schistocerca gregaria* has been reported to activate MAPKs in *A. thaliana* but not induce a ROS burst (Schafer *et al.*, 2011), although the method used to measure the ROS burst in that study was not very sensitive. If the same elicitor is detected by *N. benthamiana* and *A. thaliana*, then receptors and defence pathways that detect this elicitor may be conserved between these two plant species.

The >10 kDa elicitor has characteristics that best match those of elicitors from microbial pathogens. The Ca^{2+} burst is a single peak with similar kinetics to that of flg22, as is the initial ROS burst. Infiltration into *N. benthamiana* leaves triggered induced resistance. It is likely that the early responses seen in whole aphid extract are due to this fraction of the extract. Therefore, it may be predicted that the >10 kDa fraction increases in activity when it is boiled, suggesting a protein, and its perception by the plant does not require NbSERK3/BAK1. The elicitation activities of the >10 kDa fractions of several aphid species are similar suggesting the presence of conserved elicitor(s) among the aphids. Interestingly, the response of the fraction from the monocot feeding aphid *R. padi* was lower than that of the other aphids, which feed on dicot plants. More aphids that feed on monocots will need to be tested before it is clear whether this is a repeatable

phenomenon. The >10 kDa fraction also causes a significant late ROS burst. It is unclear whether this is from the same protein(s) that cause(s) the initial burst, or whether other elicitors are present. Treatment of this fraction alone with boiling and proteinase K would clarify what contribution the >10 kDa fraction made to the late ROS burst.

The identity of the >10 kDa protein elicitor is still to be resolved. A protein larger than 10 kDa from *S. gregaria* triggered defence responses in *A. thaliana*, including a Ca^{2+} burst, and was identified as a lipase (Schafer *et al.*, 2011). However, the elicitor identified from *M. persicae* is unlikely to be a lipase, as the defence eliciting activity of the lipase was dependent on its enzyme ability, and hence lost upon boiling. Boiling of the *M. persicae* elicitor increased its activity, suggesting a non-enzyme protein. The triggering of plant defences by another enzyme, GOX, can not be ruled out, although it once again seems unlikely that a denatured enzyme would be able to elicit the responses. PTI-like responses in *A. thaliana*, including a ROS burst, were triggered by eggs from *P. brassicae* (Little *et al.*, 2007). Eggs from distant insect species showed similar responses, and therefore acted analogously to elicitors such as PAMPs (Bruessow *et al.*, 2010). However, the elicitors in these studies seem to be lipids rather than proteins (Little *et al.*, 2007; Bruessow *et al.*, 2010) and the aphids used in our study did not contain eggs because they are maintained in conditions in which aphids reproduce in a parthenogenic viviparous fashion without an egg-laying phase. It is possible that proteins from the aphid endosymbionts may be causing the responses seen. If so, then the conservation of the responses across different species suggests that the obligate symbiont *B.aphidicola* is the likely candidate. As suggested above, the identity of the elicitor could be further investigated using SDS-PAGE to visualise and separate out the proteins in the extract. A similar approach to this was used by by Kunze and colleagues to identify EF-Tu (Kunze *et al.*, 2004), where they separated out bacterial proteins on SDS-PAGE, isolated the weight of protein responsible for the phenotype, and then identified using mass spectrometry.

In summary, we have further dissected how aphid elicitors induce defences in *N. benthamiana.* We identified a number of eliciting activities from the aphid, including the 3-10 kDa fraction that also induces defence responses in *A. thaliana* (De Vos and Jander, 2009), and the >10 kDa fractions that appear conserved among aphids in elicitation properties of *N. benthamiana*.

Chapter 5 – BAK1 is involved in *A. thaliana* perception of *M. persicae* elicitors.

Contributors: David C. Prince, Claire Drurey, Simon Lloyd, Cyril Zipfel and Saskia A. Hogenhout.

5.1 Introduction

We previously identified several *M. persicae* fractions with eliciting activities in *N. benthamiana* (chapter 4). One of these eliciting activities was derived from fractions of the same molecular weight, 3 to 10 kDa, as a previously reported *M. persicae* fraction that activated an unknown defence pathway in *A. thaliana* (De Vos and Jander, 2009). We also previously found an aphid effector that suppressed the ROS burst triggered by the PAMP flg22 (chapter 3). The flg22-triggered signalling pathway requires the LRR-RLK BAK1 (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). NbSERK3/BAK1 is unlikely to be involved in the signalling pathways triggered by *M. persicae* elicitors in *N. benthamiana* (chapter 4), although we have not tested this for the 3 to 10 kDa fraction. We wanted to take advantage of the well-developed tools available for *A. thaliana* and the greater understanding of its PTI pathway compared to *N. benthamiana*. We hypothesised that *M. persicae* contained molecules that would trigger plant immune responses in *A. thaliana* involving AtBAK1.

The first active layer of the immune system is a mechanism to recognize non-self or modified-self through the perception of elicitors such as PAMPs (e.g. bacterial flagellin (Felix *et al.*, 1999)), DAMPs (e.g. Atpep1 (Huffaker *et al.*, 2006)) and HAMPs (e.g. β-glucosidase (Mattiacci *et al.*, 1995)). Elicitors are perceived through PRRs at the plant cell plasma membrane, and this recognition plays a key role in immunity (Boller and Felix, 2009; Monaghan and Zipfel, 2012). Perception of an elicitor by a PRR leads to a series of downstream signalling including a Ca^{2+} burst, ROS burst, and MAPK cascades, which in turn contribute to later outputs including changes in gene expression, and callose deposition (Boller and Felix, 2009) and result in induced resistance against the pathogen that produced the elicitors. In response to this plant immunity, pathogens secrete many effector proteins to evade and/or suppress PTI (Dodds and Rathjen, 2010). Some of these effectors may be recognized by plant *R* genes, leading to ETI (Jones and Dangl, 2006).

Several elicitors and their respective PRR(s) have been identified in *A*. *thaliana*. All the PRRs identified so far are RLKs/RLPs, which share the property of possessing an ectodomain that binds the ligand. The identified elicitors and plant receptors are flg22 and FLS2 (Felix *et al.*, 1999; Gómez-Gómez and Boller, 2000), elf18 and EFR (Kunze *et al.*, 2004; Zipfel *et al.*, 2006), PGN and LYM1, LYM3 and CERK1 (Gust *et al.*, 2007; Willmann *et al.*, 2011), chitin and CERK1 (Ramonell *et al.*, 2002; Miya *et al.*, 2007; Wan *et al.*, 2008; Iizasa *et al.*, 2010; Petutschnig *et al.*, 2010), Atpep peptides and PEPR1 and PEPR2 (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010), and OGs and WAK1 (Moscatiello *et al.*, 2006; Brutus *et al.*, 2010).

The perception of several elicitors by *A. thaliana* involves the LRR-RLK BAK1. BAK1 is a member of the SERK family and is also named SERK3 (Albrecht *et al.*, 2008). BAK1 has been shown to interact with the LRR-RLKs PRRs FLS2 and EFR *in vivo* (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Roux *et al.*, 2011) and PEPR1 and PEPR2 in yeast two-hybrid assays (Postel *et al.*, 2010). Importantly, BAK1 acts as a regulator for the recognition of these elicitors and therefore BAK1 mutants are compromised in responsiveness to the elicitors. Besides its role in elicitor-triggered signalling, BAK1 is also involved in regulating BR responses (Li *et al.*, 2002; Nam and Li, 2002), light signalling (Whippo and Hangarter, 2005) and cell death (Kemmerling *et al.*, 2007). Other members of the SERK family also play a role in these responses, for example BKK1/SERK4 plays a role in elicitor-triggered signalling, BR responses and cell death regulation in *A. thaliana* (He *et al.*, 2007; Roux *et al.*, 2011).

The role of BAK1 in regulating several independent plant signalling pathways means that null mutants of *A. thaliana* have deficiencies in all these pathways, making it difficult to understand the role of BAK1 in only one of the pathways. Recently, an additional mutant of *Bak1* was identified, *bak1-5*, that is compromised in elicitor-triggered immune signalling but not in BR responses or cell death control (Schwessinger *et al.*, 2011). *bak1-5* is an ethyl methane sulphonate (EMS) mutant with a substitution in a conserved cysteine within the cytoplasmic kinase domain (Schwessinger *et al.*, 2011). This mutant makes it possible to study the role of BAK1 in elicitor-immune signalling independent of other responses.

In a similar way to pathogens, insects possess elicitors of plant defence. Much research has been conducted on the elicitors of chewing insects perceived by *N. attenuata,* but comparatively little is known about insect elicitors perceived by *A. thaliana* or the plant genes involved in perception. Schmelz and colleagues took phytohormone production as a proxy for elicitor recognition by the plant (Schmelz *et al.*, 2009). They found that *A. thaliana* responded with ET and JA production to the grasshopper elicitor caeliferin A16:0 but it did not produce measurable phytohormome responses to the other insect elicitors (FACs and inceptin) (Schmelz *et al.*, 2009). Schafer and colleagues identified a lipase activitiy in chewing herbivore saliva that triggered *A. thaliana* responses associated with elicitor perception including a Ca^{2+} burst and MAPK activation (Schafer *et al.*, 2011). Elicitors, possibly lipid in nature, are present in *P. brassicae* eggs and trigger responses including a ROS burst (Little *et al.*, 2007; Bruessow *et al.*, 2010). These plant responses appear to be common to eggs from distant insect species, suggesting a conserved elicitor (Bruessow *et al.*, 2010). The plant responses to the elicitor are reduced in mutants of *LecRK-I.8* (Gouhier-Darimont *et al.*, 2013), although it is unclear what part this RLK plays in the elicitor-triggered signalling response.

De Vos and Jander (De Vos and Jander, 2009) identified an elicitor activity in the saliva of *M. persicae*. The elicitor induced plant defences in *A. thaliana* that led to decreased aphid reproduction when feeding on plants pre-treated with the saliva. Transcriptional studies identified a number of plant genes induced by the saliva elicitor, including the elicitor-triggered immune signalling marker gene *Pad3*. The identity of the saliva elicitor remains unknown but it has properties of a protein or peptide between 3 and 10 kDa, which loses its eliciting activity upon heating or proteinase K treatment. *A. thaliana* mutants in plant defence and signalling pathways (JA, SA, ET and *Pad4*) were tested for their role in the elicitor perception but all showed the same decreased aphid reproduction upon pre-treatment with saliva, suggesting that these pathways are not solely involved in the triggered defences.

Insect elicitors that trigger plant immune responses in *A. thaliana* are not well characterised, nor are the plant genes involved in their perception. To investigate if aphids contain elicitors of plant immunity that are perceived by *A. thaliana* we looked for plant immune responses to different preparations of *M. persicae*. We used the whole aphid in order to discover the largest possible range of molecules. We also used *A. thaliana* mutants of known components of elicitor-triggered signalling to investigate which plant genes were involved in the perception of the elicitors.

5.2 Results

Whole *M. persicae* extract elicits immune responses in *A. thaliana*.

We wishes to investigate if components of *M. persicae* were able to trigger immune responses in *A. thaliana*, such as a ROS burst, callose deposition or induced resistance (Boller and Felix, 2009). Whole *M. persicae* extract was used in order to allow for the presence of all possible elicitors of immune responses, as saliva composition varies depending on the medium used to collect it (Cherqui and Tjallingii, 2000; Cooper *et al.*, 2010), and proteins are present in aphid saliva that may not be produced by the salivary gland (Carolan *et al.*, 2011). To test if whole *M. persicae* extract was able to elicit a ROS burst we used a luminol based assay (Keppler *et al.*, 1989) to measure hydrogen peroxide production in terms of light emitted. Whilst there was no obvious difference between the whole *M. persicae* extract and the water control during the first hour of the experiment (Figure 5.1A), we noticed a reproducible peak in the whole *M. persicae* extract treatment starting between the first and second hours and finishing around the tenth hour (Figure 5.1B). This ROS burst is delayed compared to those seen with some other elicitors of immune responses, such as flg22 (Figure 5.1A), but nonetheless provides evidence that the plant perceives the whole *M. persicae* extract.

To examine if whole *M. persicae* extract was able to cause callose deposition we infiltrated the supernatant of the extract into *A. thaliana* leaves, stained the leaves with aniline blue, and compared the callose deposition levels to those of buffer (negative control) and flg22 (positive control). The supernatant of aphid extract was used because it was less viscous, and therefore easier to infiltrate. The supernatant of aphid extract produced significantly more callose depositions than the buffer control, although not as many as the flg22 control (ANODE; $F_{2,103} = 2039.93$, $P < 0.001$; Figure 5.1C). This result provides additional evidence that whole *M. persicae* extract can elicit immune responses in *A. thaliana*.

To ascertain if whole *M. persicae* extract was able to trigger induced resistance against *M. persicae* in *A. thaliana* we infiltrated the leaves with whole *M. persicae* extract, or a water control, and then measured aphid performance on the leaves over 10 days. Aphids living on leaves treated with whole *M. persicae* extract produced significantly fewer progeny than those on control leaves (ANODE; $F_{1, 59} = 8.129$, $P = 0.004$; Figure 5.1D), indicating that induced resistance against the aphid had been triggered in leaves treated with whole *M. persicae* extract. Taken together, these results show that *M. persicae* contains elicitors of *A. thaliana* immune responses, and that these responses lead to a defence in this plant species that reduces aphid colonization.

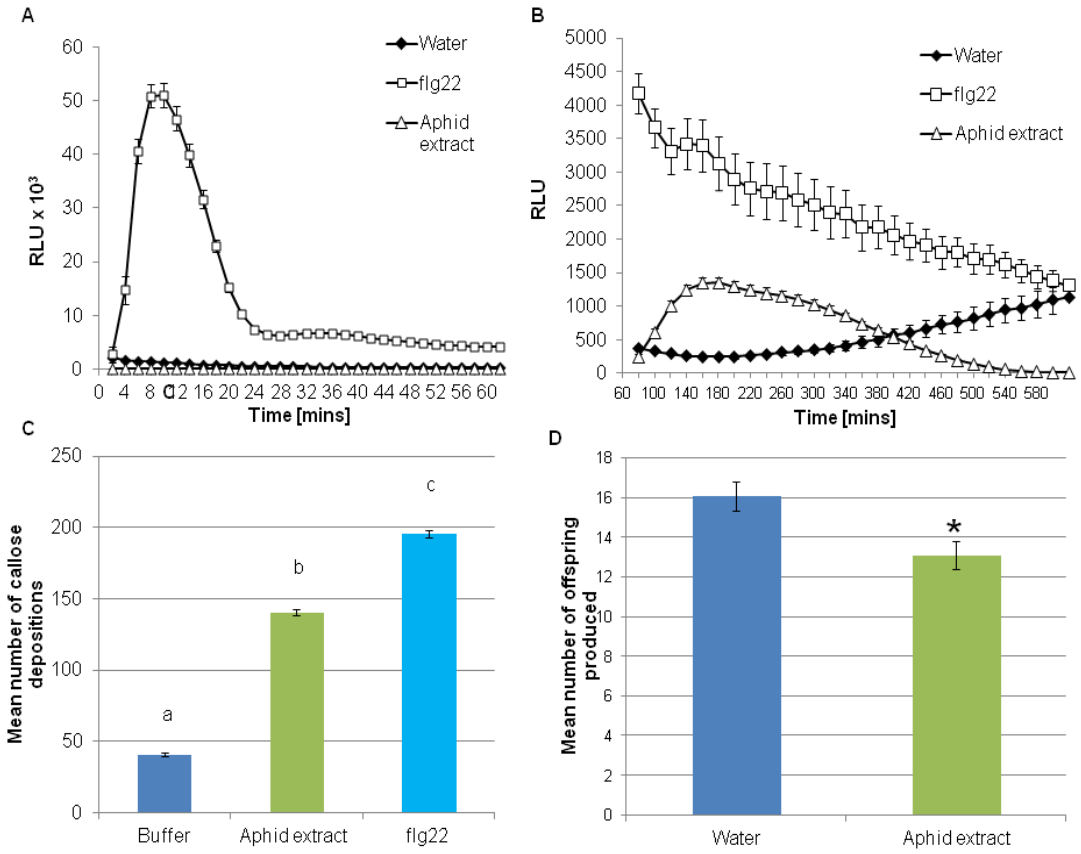


Figure 5.1 - Whole *M. persicae* extract elicits immune responses in *A. thaliana*.

(A) Whole aphid extract does not cause a ROS burst in the first hour. (B) Whole aphid extract causes a ROS burst between one and ten hours. The ROS burst was measured between 0 and 60 minutes (A) and 61 and 600 minutes (B) in Col-0 leaf discs after elicitation by water, 12.5 nM flg22 or whole aphid extract. Results are mean ± SE (n = 32 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown.

(C) Whole aphid extract supernatant causes callose deposition. Water, 100 nM flg22 or supernatant of whole aphid extract was infiltrated in Col-0 leaves. Leaves were collected after 24 hours, stained with aniline blue, and callose visualised using fluorescence microscopy with a ultraviolet (UV) filter. Data shown are mean callose depositions produced upon each treatment with means ± SE of three biological replicates with n = 12 leaf discs per replicate. Letters indicate significant differences in treatment (n = 36, $F_{2,103}$ = 2039.93 (P < 0.001) (t probabilities calculated within GLM)). (Experiment conducted by Claire Drurey).

(D) Whole aphid extract triggers induced resistance in *A. thaliana*. Aphid fecundity on water or aphid extract infiltrated *A. thaliana* leaves over a 10 day period. Data shown are total aphids produced on each treatment with means ± SE of three biological replicates with n = 10 per replicate. Asterisk indicate significant differences in treatment compared to water (n = 30, $F_{1,59}$ = 8.129 (*P = 0.004) (t probabilities calculated within GLM)).

***A. thaliana* defence responses to *M. persicae* extract fractions of different molecule weight.**

Having found that whole *M. persicae* extract elicits immune responses in *A. thaliana* we wanted to determine what molecular weight the elicitor(s) were. In order to do this we fractionated the supernatant of the extract by weight into <3 kDa, 3 to 10 kDa and >10 kDa fractions using centrifugal filters. We tested each fraction's ability to trigger a ROS burst and found that only the 3 to 10 kDa fraction triggered a significant ROS burst when compared to the buffer control (ANOVA; $P < 0.001$, Figure 5.2A). We also tested the ability of each fraction to trigger induced resistance against the aphid. Treatments with both the 3 to 10 kDa and >10 kDa fractions caused a significant decrease in the number of progeny compared to control leaves (ANODE; $F_{3, 237} = 6.051$, $P < 0.002$; Figure 5.2B). Together these results suggest the presence of elicitors in the 3 to 10 kDa and >10 kDa aphid extracts. The identification of a *M. persicae* elicitor in the 3 and 10 kDa fraction agrees with a previous study (de Vos and Jander 2009).

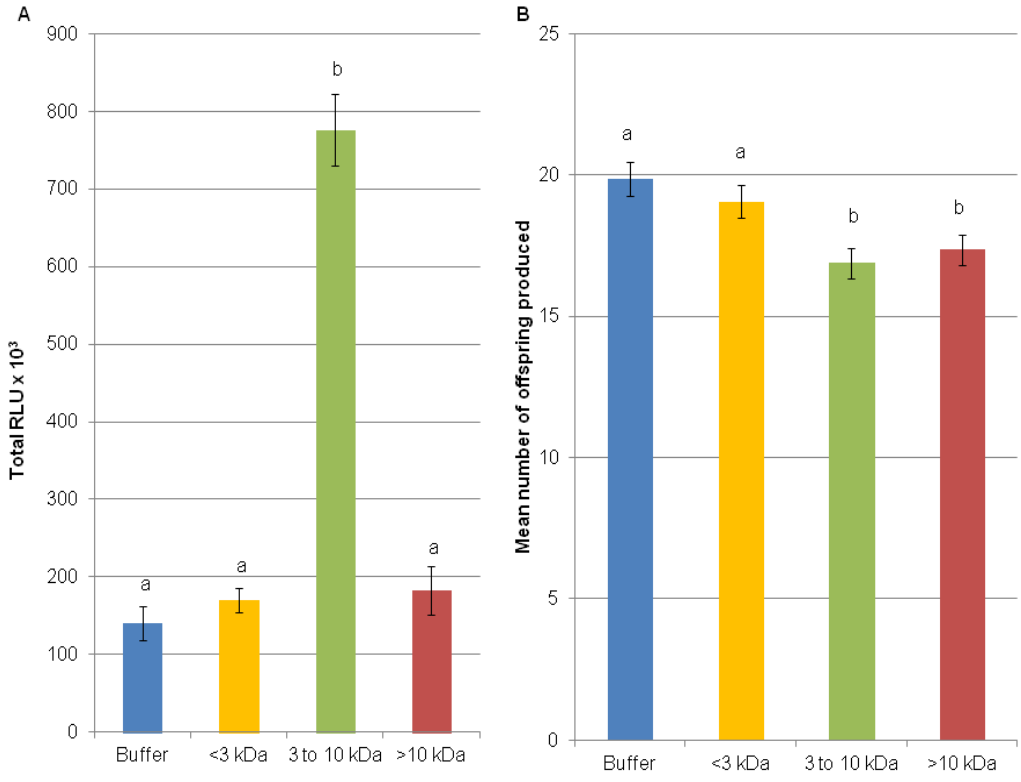


Figure 5.2 – *A. thaliana* defence responses to *M. persicae* extract fractions of different molecule weight.

(A) The 3 to 10 kDa aphid extract fraction induces a ROS burst. The total ROS production (represented as RLUs) was measured over an 800 minute period in Col-0 leaf discs upon

elicitation by buffer or fractionated aphid extract. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.001$. (B) Pretreatment of *A. thaliana* with elicitors between 3 and 10 kDa, and larger than 10 kDa inhibits *M. persicae* reproduction. Aphid offspring were assessed after a 10 day period on *A. thaliana* leaves pretreated with buffer or fractionated aphid extracts. Data shown are total aphids produced on each treatment with means ± SE of six biological replicates with n = 10 per replicate. Letters indicate significant differences in treatment (n = 60, $F_{3,237} = 6.051$ ($P < 0.002$) (t probabilities calculated within GLM)).

***M. persicae* elicitor activities are lost upon boiling and proteinase K treatments.**

To determine the chemical properties of the *M. persicae* elicitors we applied treatments to the supernatant of the whole aphid extract and then tested whether the elicitors were still able to trigger plant immune responses. We boiled the aphid extract in order to denature proteins and therefore assess the role of secondary and tertiary structure in the elicitation of defence responses. We also proteinase K treated the aphid extract in order to digest and therefore degrade proteins. Boiling of the aphid extract supernatant led to a loss in the ability to trigger a ROS burst (Figure 5.3A). Induced resistance to the aphid was not triggered after boiling or proteinase K treatment of the aphid extract supernatant (Figure 5.3B). The effect of proteinase K treatment on the ROS bursts triggered by *M. persicae* extract could not be tested as proteinase K causes a ROS burst similar in size and kinetic to *M. persicae* extract in the absence of *M. persicae* extract ($P < 0.05$, Figure 5.3C and D). This ROS burst is dependent of the enzyme activity of proteinase K, as it is lost upon boiling of the enzyme (Figure 5.3C and D). The loss of immune triggering properties after boiling and proteinase K treatment suggests the *M. persicae* elicitors are proteinaceous in nature, rather than carbohydrate based. In addition, whilst the proteinase K elicits a ROS burst, this ROS burst did not lead to a decrease in *M. persicae* progeny on *A. thaliana*, indicating that the responses instigated to aphid elicitors are specific.

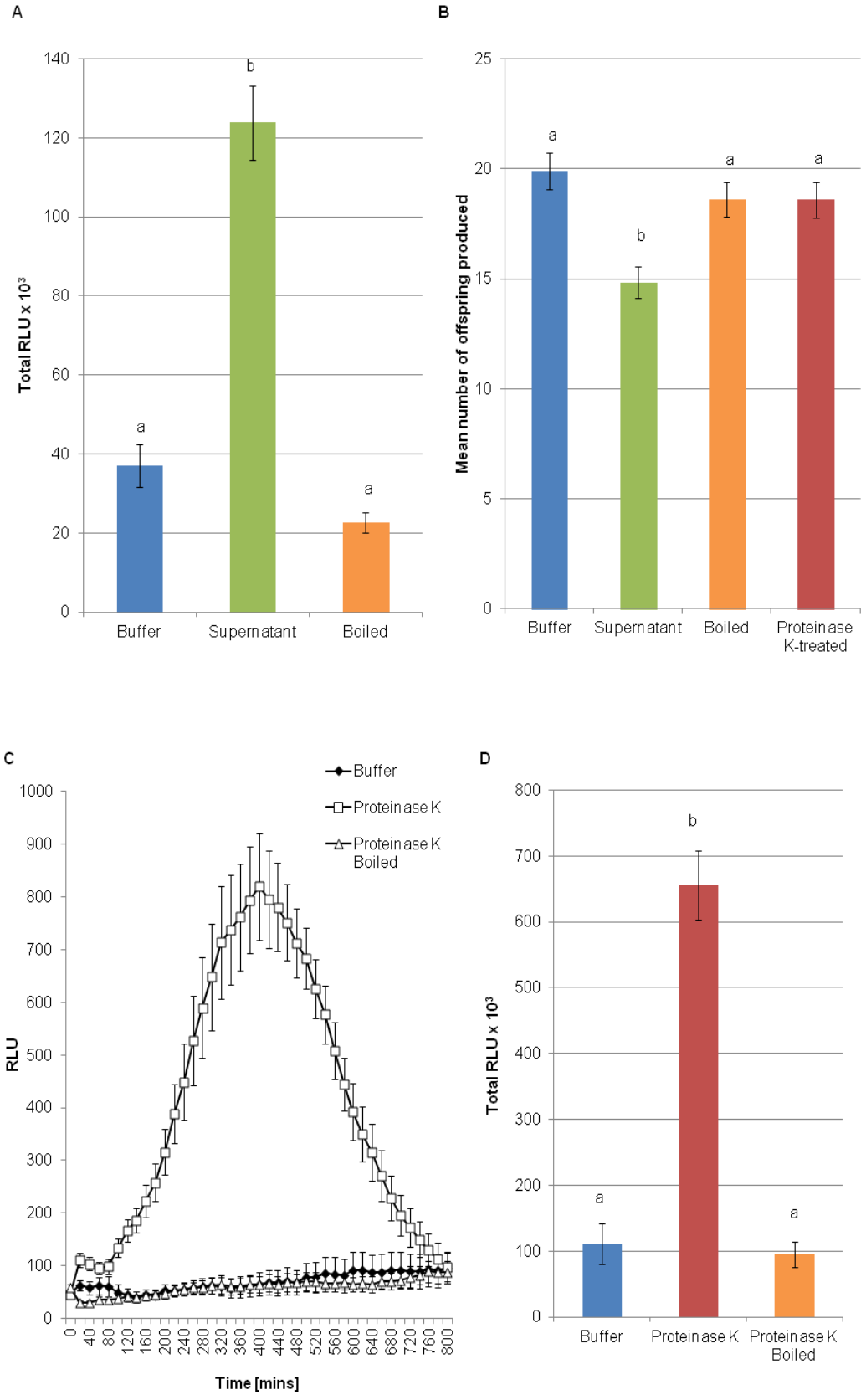


Figure 5.3 - *M. persicae* elicitor activities are lost upon boiling and proteinase K treatments.

(A) Boiling of aphid extract leads to loss of ROS eliciting activity. The total ROS production (represented as RLUs) was measured over a 600 minute period in Col-0 leaf discs after elicitation by buffer or treated aphid extract. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P = 0.01$.
(B) Pretreatment of *A. thaliana* with elicitors treated with proteinase K or boiling does not inhibits *M. persicae* reproduction. Aphid offspring were assessed after a 10 day period on *A. thaliana* leaves pretreated with buffer or treated aphid extracts. Data shown are total aphids produced on each treatment with means ± SE of three biological replicates with n = 10 leaves per replicate. Letters indicate significant differences in treatment (n = 30, $F_{3,119} = 7.688$ ($P < 0.001$) (t probabilities calculated within GLM)).
(C and D) The ROS burst was measured over an 800 minute period in Col-0 leaf discs after elicitation by buffer, 100 µg proteinase K or 100 µg boiled proteinase K. Results shown as RLU over time (C) and total RLU (D). Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

M. persicae colonization ability is not significantly affected on *A. thaliana bak1-5* mutant.

BAK1 is involved in the signal transduction of many elicitors of plant immunity (Monaghan and Zipfel, 2012), as well as regulating BR responses (Li *et al.*, 2002; Nam and Li, 2002), light signalling (Whippo and Hangarter, 2005) and cell death (Kemmerling *et al.*, 2007). Null mutants of BAK1 are compromised in all of these areas. We investigated the response of *M. persicae* to *bak1-5*, a null mutant of BAK1 (*bak1-4* (He *et al.*, 2007)), and a null mutant of the closely related BKK1 (He *et al.*, 2007), which is involved in PTI, BR and cell death responses (He *et al.*, 2007; Roux *et al.*, 2011). We tested the response of aphids to these plants by comparing the numbers of nymphs produced over a 14 day period. Aphid numbers were similar on *bak1-5* plants compared to Col-0, but were reduced on *bak1-4* and *bkk1* plants. (ANODE; $F_{3, 59} = 3.998$, $P = 0.008$; Figure 5.4). These results suggest that impairment in BR signalling or cell death control leads to a decrease in aphid performance. This pleiotropic affect of the null mutants may interfere with investigation of the aphid elicitor, so we continued our investigation using *bak1-5* alone.

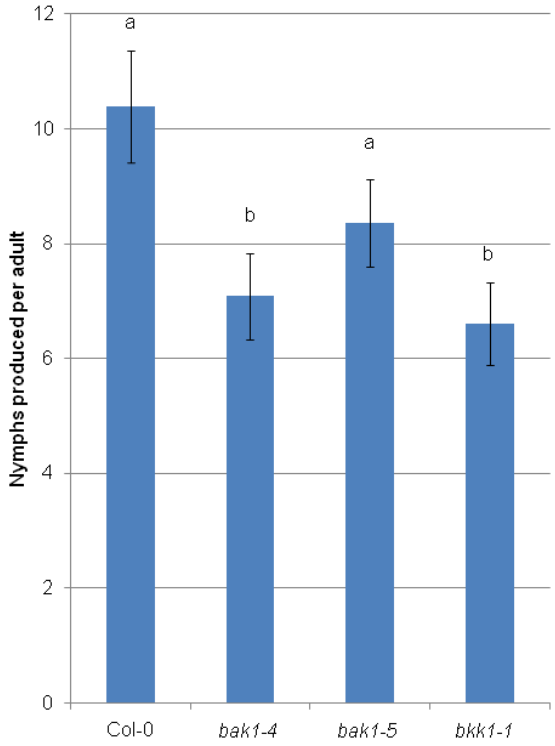


Figure 5.4 - *M. persicae* colonization ability is not significantly affected on *A. thaliana bak1-5* mutant.

The number of nymphs produced by aphid nymphs born on Col-0, *bak1-4, bak1-5* and *bkk1-1* plants was counted over 14 days. Data shown are aphids produced per adult on each treatment with means ± SE of three biological replicates with n = 5 per replicate. Letters indicate significant differences in treatment (n = 15, $F_{3,59}$ = 3.998 (P = 0.008) (t probabilities calculated within GLM)). There was no difference in aphid survival amongst the plants (data not shown).

A. thaliana responses to the 3 to 10 kDa aphid extract fraction are BAK1 dependent.

We concentrated on how *A. thaliana* perceives the 3 to 10 kDa eliciting fraction of *M. persicae*, as this fraction has similar properties to an elicitor identified in a previous study (De Vos and Jander, 2009). To investigate if BAK1 was involved in the perception of the 3 to 10 kDa elicitor we used the *bak1-5* mutant. The *A. thaliana bak1-5* mutant gave a significantly reduced ROS burst in response to the 3 to 10 kDa fraction compared to Col-0 (Student's *t*-test, $P < 0.035$, Figure 5.5A). Pretreatment of *bak1-5* with the 3 to 10 kDa fraction failed to inhibit *M. persicae* reproduction (ANODE; $F_{1, 53}$ = 0.043, P = 0.835; Figure 5.5B), whilst the extract was still able to inhibit *M. persicae* reproduction in Col-0 (ANODE; $F_{1, 53}$ = 8.065, P = 0.005; Figure 5.5B). Both of these results show that *bak1-5* is deficient in the triggering of immune responses to the 3 to 10 kDa elicitor. Therefore

BAK1 is involved in the signalling pathway triggered by an elicitor in the 3 to 10 kDa fraction of the *M. persicae* extract.

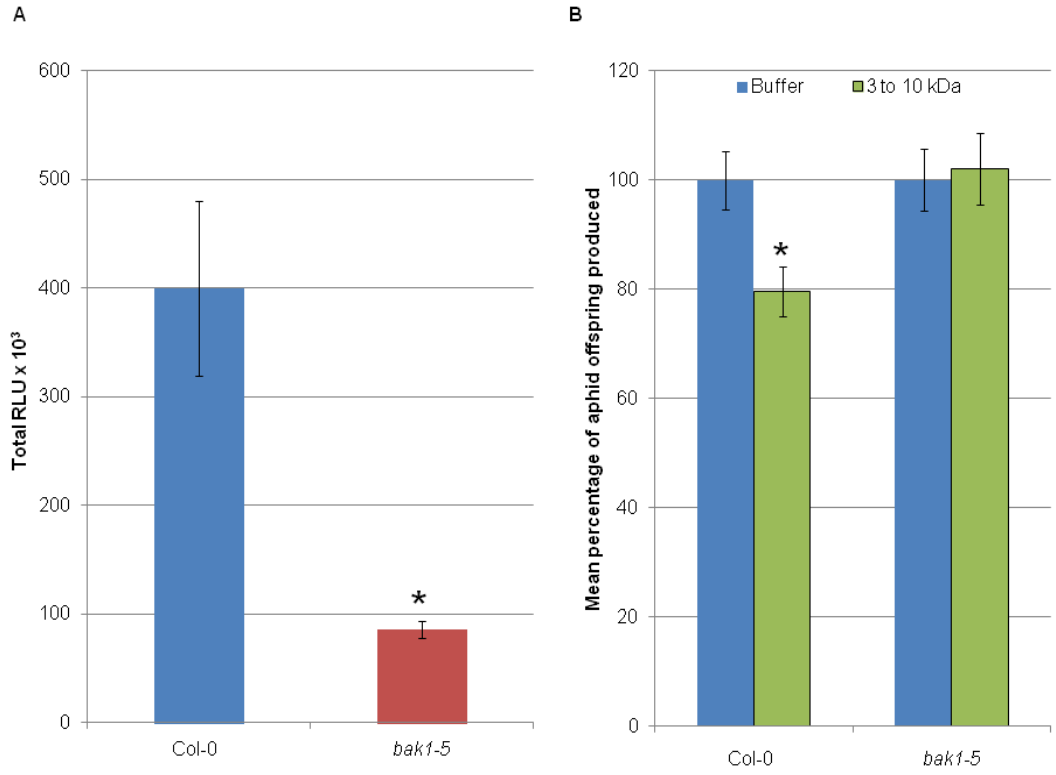


Figure 5.5 - *A. thaliana* responses to the 3 to 10 kDa aphid extract fraction are BAK1 dependent.

(A) The ROS bursts of the 3 to 10 kDa fraction of aphid extract are significantly decreased in *bak1-5* compared to Col-0. The total ROS production (represented as RLUs) was measured over an 800 minute period in Col-0 and *bak1-5* leaf discs after elicitation by the 3 to 10 kDa fraction of aphid extract. Results are mean ± SE (n = 8 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. (Student's *t*-test **P* = 0.035).

(B) BAK1 is involved in inhibition of *M. persicae* reproduction on *A. thaliana*. Aphid offspring were assessed after a 10 day period on *A. thaliana* leaves pretreated with buffer or the 3 to 10 kDa fraction of aphid extract. Data is normalised so that buffer control is set to 100% for both plant lines. Data shown are total aphids produced on each treatment with means ± SE of three biological replicates with n = 10 plants per replicate. Asterisk indicate significant differences in treatment compared to buffer (Col-0 n ≥ 28, $F_{1,56}$ = 8.065 (**P* = 0.005). *bak1-5* n ≥ 25, $F_{1,53}$ = 0.043 (*P* = 0.835) (t probabilities calculated within GLM)).

Induced resistance to the 3 to 10 kDa saliva fraction is BAK1 dependent

The elicitor we have identified in *M. persicae* extract is identical in molecular weight and biochemical properties to an elicitor previously identified in *M. persicae* saliva (De Vos and Jander, 2009). Both the elicitor we identified in aphid extract and the elicitor De Vos and Jander identified in saliva inhibit *M. persicae* reproduction when *A. thaliana* is pretreated with them. Therefore, we wished to examine if the induced resistance from the elicitor in saliva also requires BAK1. Saliva was collected from sterile water sandwiched between two layers of thinly stretched Parafilm and exposed to *M. persicae* for 24 hours. The 3 to 10 kDa fraction of the saliva was then isolated using centrifugal filters. We included a control consisting of sterile water treated in the same way but not exposed to aphids. *A. thaliana* leaves were infiltrated with *M. persicae* extract, or the control, and aphid performance was measured over 10 days. Aphids on Col-0 leaves treated with 3 to 10 kDa fractions of *M. persicae* saliva produced significantly fewer progeny than those on leaves treated with sterile water (ANODE; $F_{1, 59} = 12.224$, $P < 0.001$; Figure 5.6), indicating that the saliva fraction was capable of triggering induced resistance in *A. thaliana*. Pretreatment of *bak1-5* plants with the 3 to 10 kDa fraction of aphid saliva led to fewer progeny being produced compared to the water control but this difference was not significant (ANODE; $F_{1, 59} = 3.773$, $P = 0.052$; Figure 5.6). This result indicates that BAK1 is potentially involved in the *A. thaliana* signalling pathway triggered by the 3 to 10 kDa fraction of *M. persicae* saliva and whole aphid extract, indicating that saliva and whole aphid extract are likely to contain similar elicitors that trigger defence responses in a BAK1-dependent manner.

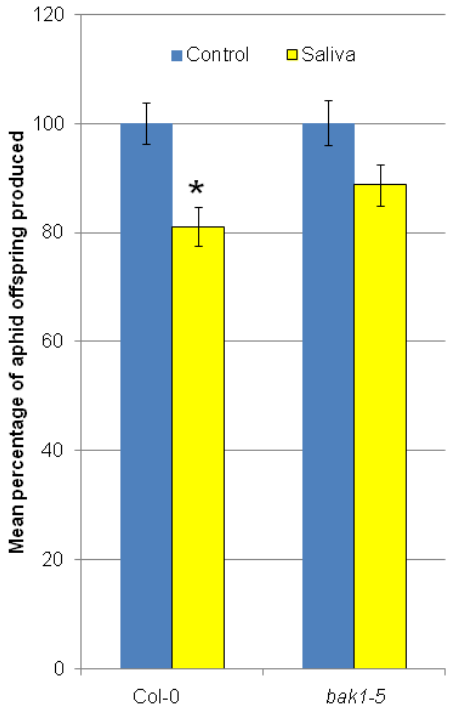


Figure 5.6 – Induced resistance to the 3 to 10 kDa saliva fraction is BAK1 dependent. Aphid offspring were assessed after a 10 day period on *A. thaliana* leaves pretreated with the 3 to 10 kDa fraction of *M. persicae* saliva or a control. Data is normalised so that buffer control is set to 100% for both plant lines. Data shown are total aphids produced on each treatment with means ± SE of three biological replicates with n = 10 plants per replicate. Asterisk indicate significant differences in treatment compared to control (Col-0 $F_{1,59}$ = 12.224 (*P < 0.001). *bak1-5* $F_{1,59}$ = 3.773 (P = 0.052) (t probabilities calculated within GLM)).

A. pisum survives better on *A. thaliana bak1-5* mutants compared to Col-0 wildtype plants

We hypothesised that *bak1-5 A. thaliana* plants would make better hosts for non-adapted aphids than Col-0, due to having compromised elicitor perception signalling and therefore a reduced induction of defences against the aphid. To test this hypothesis we looked at adult survival of *A. pisum*, an aphid that specialises on legumes and does not survive on *A. thaliana*. We caged the aphids onto individual *A. thaliana* leaves to prevent *A. pisum* escaping. We found that adult *A. pisum* survival dropped to 50% between three and four days after being caged onto Col-0 *A. thaliana* leaves (Figure 5.7A). We compared *A. pisum* survival on Col-0 and *bak1-5* plants at this time point by averaging the numbers of adult alive in each cage on days three and four of the experiment. *A. pisum* on *bak1-5* plants survived significantly better at this time point (ANODE; $F_{1, 59}$ = 5.028, P = 0.025;

Figure 5.7B) although they could not permanently colonise the plant. This suggests that plant immunity plays a role in the inability of *A. pisum* to successfully colonize *A. thaliana*, although other factors are also involved.

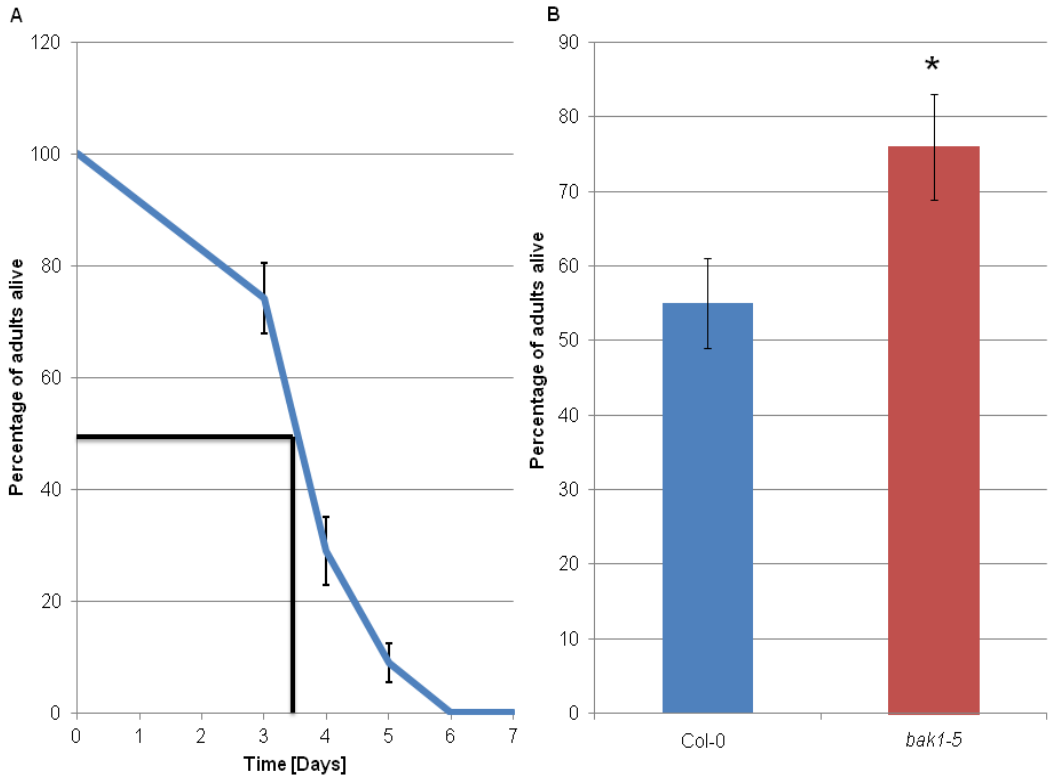


Figure 5.7 - *A. pisum* survives better on *A. thaliana bak1-5* mutants compared to Col-0 wildtype plants.

(A) *A. pisum* survival on *A. thaliana* reaches 50% between days 3 and 4. Five adult *A. pisum* were caged to a leaf of a Col-0 *A. thaliana* plant and the number of live adults measured on days three to seven. Data shown are percentages of aphids alive at a given time point with means ± SE of four biological replicates with n = 5 plants per replicate.

(B) *A. pisum* survives better on *A. thaliana bak1-5* mutants compared to wildtype Col-0 plants. Five adult *A. pisum* were caged to a leaf of a Col-0 or *bak1-5 A. thaliana* plant and the number of live adults on days three and four were averaged. Data shown are percentage of aphids alive with means ± SE of six biological replicates with n = 5 plants per replicate. Asterisk indicate significant differences in aphid survival (n = 30, $F_{1,59} = 5.028$ (*$P = 0.025$) (t probabilities calculated within GLM)).

Known BAK1-interacting PRRs are not required for the ROS response to the 3 to 10 kDa *M. persicae* extract

The extracellular LRR domain of BAK1 is small, consisting of only five repeats (Chinchilla *et al.*, 2009), making BAK1 an unlikely candidate to be the receptor for the 3 to 10 kDa

elicitor. BAK1-mediated perception of the *M. persicae* elicitor is therefore likely to require a PRR, in the same way that flg22 perception requires the PRR FLS2. Four *A. thaliana* PRRs involved in plant immunity are known to interact with BAK1 (FLS2, EFR, PEPR1 and PEPR2) (Monaghan and Zipfel, 2012). To test whether any of these were the receptor for *M. persicae* elicitor(s) we conducted ROS burst experiments with the 3 to 10 kDa fraction of *M. persicae* extract on *A. thaliana* mutants. We found that mutants in the PAMP PRRs FLS2, EFR, or both genes showed a wildtype ROS response to the *M. persicae* elicitor (Figure 5.8A). Surprisingly, the triple mutant of *fls2 efr cerk1* showed a trend of producing more ROS than Col-0 in response to aphid extract, although the difference was not significant (Figure 5.8A). Mutants in DAMP PRRs PEPR1 and PEPR2, or both genes showed a wildtype ROS response to *M. persicae* elicitor as well (Figure 5.8B). If any of these genes were the PRR for the *M. persicae* elicitor we would expect to see a decrease in the ROS response, similar to that of *bak1-5*. Therefore, these data suggest that an unknown BAK1-interacting PRR is involved in the perception of elicitor(s) in the *M. persicae* 3 to 10 kDa fraction.

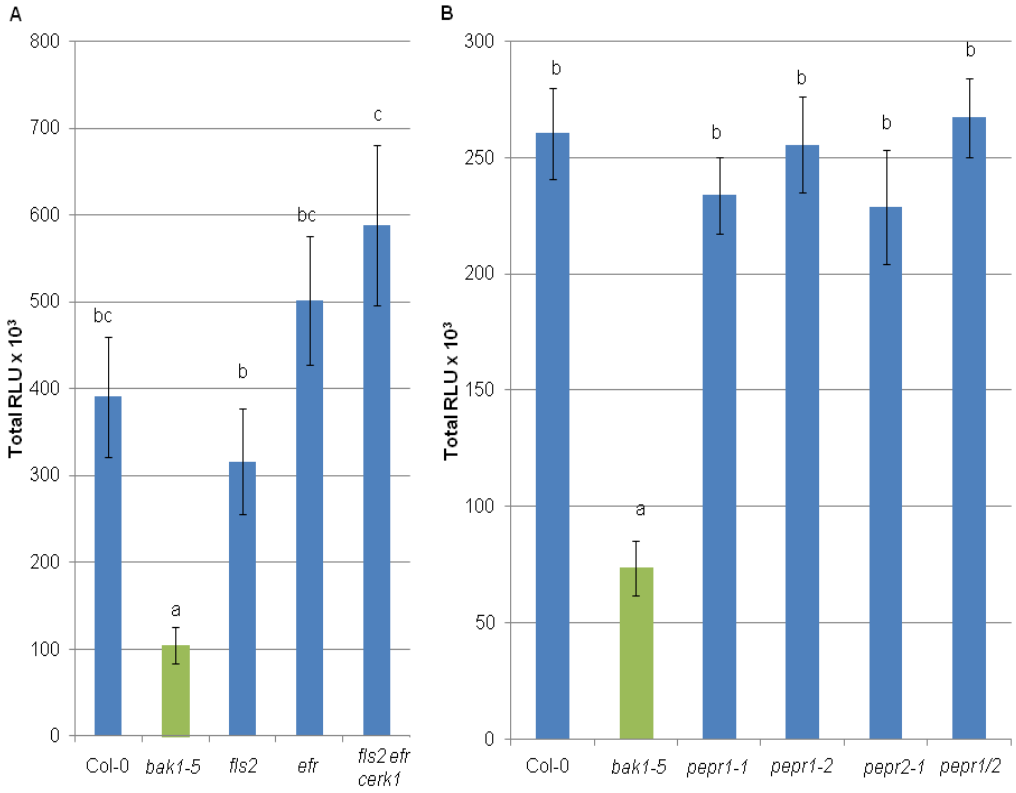


Figure 5.8 - Known BAK1-interacting PRRs are not required for the ROS response to the 3 to 10 kDa *M. persicae* extract.

(A) The ROS response triggered by the 3 to 10 kDa fraction does not require the PAMP PRRs FLS2 or EFR. The total ROS production (represented as RLUs) was measured over an 800

minute period in Col-0 or mutant leaf discs after elicitation by the 3 to 10 kDa fraction of aphid extract. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

(B) The ROS response triggered by the 3 to 10 kDa fraction does not require the DAMP PRRs PEPR1 or PEPR2. The total ROS production (represented as RLUs) was measured over an 800 minute period in Col-0 or mutant leaf discs after elicitation by the 3 to 10 kDa fraction of aphid extract. Results are mean ± SE (n = 16 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

5.3 Discussion

Plants need to process and respond to externally and internally produced signals. Plant are alerted to the presence of herbivourous insects and plant pathogens by pathogen/pest elicitors. Here, we show evidence that insects can trigger plant immunity leading to the initiation of a specific defence response. We found that molecules in the aphid *M. persicae* are capable of eliciting plant immune responses similar to PTI that include a ROS burst, callose deposition and induced resistance to aphids in *A. thaliana*. A heat-sensitive protein/peptide fraction of 3 to 10 kDa in weight elicits these responses. For the first time we demonstrate that LRR-RLK BAK1 is involved in the initial perception of an insect by a plant. Finally, we demonstrate that the *A. thaliana bak1-5* mutant is compromised in induced resistance to *M. persicae* saliva and a non-adapted aphid species.

The advantages of using the whole aphid to search for *M. persicae* elicitors were discussed in chapter 4. In this chapter we examined both the 3 to 10 kDa fractions of whole aphid and saliva. We found that these two fractions have similar elicitors of *A. thaliana* induced resistance to *M. persicae.* Therefore, use of the whole aphid extract is justified at least for the 3 to 10 kDa fraction. Whole *M. persicae* extract produced similar immune responses in *A. thaliana* to that of extract with the insoluble parts of the aphid removed (supernatant), suggesting that the insoluble parts of the aphid were not contributing significantly to the responses seen with the whole extract.

Whole *M. persicae* extract elicits plant signalling and defence responses similar to PTI such as a ROS burst, callose deposition and induced resistance against *M. persicae*. Marker genes of elicitor-triggered immunity such as *Flg22-induced receptor-like kinase1* (*Frk1), Pad3* and *Cytochrome p450, family 81, subfamily f, polypeptide 2 (Cyp81f2)* are also triggered by *M. persicae* elicitors (Claire Drurey, unpublished). Other elicitor-triggered responses such as activation of MAP kinases could also be investigated. The elicitor-triggered responses are likely to be induced by the specific proteins in aphid extracts, as opposed to being triggered by a generic plant response to a large amount of protein. Indeed, preliminary experiments conducted with 100 µg BSA did not show a ROS response. Proteinase K produced a ROS burst that was similar to the 3 to 10 kDa elicitor that was lost upon boiling, but proteinase K treatment of aphid extract did not result in induced resistance to the aphid. However, in order to completely rule out generic protein responses are occurring in *A. thaliana* more accurate quantification of the levels of protein in the extract and more comprehensive controls could be used.

Whilst responses similar to PTI are triggered there are some differences. Most noticeable is the difference in timing and duration of the ROS burst. The production of

ROS by a plant in response to herbivory has been reported for several plant species (Leitner *et al.*, 2005; Maffei *et al.*, 2006; Diezel *et al.*, 2009) including at aphid feeding sites in *A. thaliana* (Kusnierczyk *et al.*, 2008). A recent study on a grasshopper elicitor of plant defences in *A. thaliana* found signalling activities in common with PTI, such as MAPK activation and a Ca^{2+} burst, however they did not find ROS production triggered by the elicitor (Schafer *et al.*, 2011). This may have been due to the use of 3,3'-Diaminobenzidine (DAB) staining for hydrogen peroxide detection rather than more sensitive luminescence-based assays. Also, elicitor induced ROS may have been masked by the ROS induced upon wounding in their assays. The ROS burst triggered by flg22 is a sharp peak, which starts very soon after addition of the PAMP and finishes within 30 minutes. In contrast, the ROS burst triggered by *M. persicae* extract occurs much later; starting more than an hour after addition of the extract. Its duration is also much longer compared to flg22, as the burst takes nearly 9 hours to reach base level again. *P. infestans* elicitin INF1 triggers a ROS burst in *N. benthamiana* that is also much longer than that of flg22 (Chaparro-Garcia *et al.*, 2011). It is not yet clear if and how the strength, timing and duration of ROS bursts affect downstream defence responses in plants.

Callose is deposited in plant cell walls as part of PTI (Gómez-Gómez *et al.*, 1999) and is commonly used as a marker for induction of plant immunity (Luna *et al.*, 2010). Recently, callose deposition and induced defence were used to validate bacterial elicitors identified through bioinformatics (McCann *et al.*, 2012). We observed both callose deposition and induced resistance in *A. thaliana* in response to the supernatant of whole *M. persicae* extract, providing further evidence that *M. persicae* molecules are capable of triggering plant immunity. Observing these responses to the supernatant reduces the likelihood that the responses were due to carbohydrate motifs in the aphid exoskeleton, as insoluble molecules had been removed. Callose deposition occurs in plants that are resistant (e.g. (Villada *et al.*, 2009)) and susceptible to aphids, including in *A. thaliana* exposed to *M. persicae* (Kusnierczyk *et al.*, 2008). How callose deposition affects plant defence to aphids may be further investigated with mutants in callose synthesis.

We saw a 20-25% reduction in *M. persicae* reproduction in our experiments when induced resistance was triggered. This reduction is within the 20 to 85% range cited by Walters and colleagues as being typical in induced resistance (Walters *et al.*, 2013). The previous study of aphid elicitors of *A. thaliana* by de Vos and Jander reported reductions in reproduction between 20-40% (De Vos and Jander, 2009), and therefore our experiments showed smaller reductions in reproduction. This may be due to the different experimental conditions used for the experiments, as we conducted experiments in CERs with a shorter day length (8 hours vs. 16 hours) and a slightly cooler temperature (18^{o}C

vs. 19^{o}C). Our conditions are likely to have led to a slower overall reproductive rate for the aphids, and therefore the differences between different plant conditions are likely to be smaller as a result. The different aphid clones used in our experiment compared to de Vos and Jander may also vary in reproductive rate or susceptibility to the plant defences induced by the elicitor.

As previously discussed in chapter 4, the assays with fractionated or treated aphid extract, and fractionated saliva, may have benefitted from being conducted in conjunction with stained protein gels.

Fractionation of the *M. persicae* extract revealed an elicitor activity between 3 and 10 kDa. This fraction triggered a ROS burst and induced resistance. No ROS burst was observed upon boiling of *M. persicae* extract, and boiled and proteinase K-treated extracts were unable to trigger induced resistance, suggesting that elicitors in the fraction are likely to be comprised of small, heat-sensitive proteins or peptides. De Vos and Jander (De Vos and Jander, 2009) also identified elicitor(s) between 3 and 10 kDa in molecular weight that trigger induced resistance to aphids and that lose activity upon boiling and proteinase K treatment. They found the elicitor in saliva of aphids fed on a diet of sucrose and amino acids, whereas we collected saliva of aphids fed on water. Aphids secrete a variety of salivary proteins the composition of which is likely to be different depending on the artificial diet they are fed on. Overall, aphids secrete fewer proteins into water compared to more complex diets (Cherqui and Tjallingii, 2000; Cooper *et al.*, 2010). The presence of the induced resistance triggering activity in saliva collected from water suggests that the elicitor is a protein or peptide secreted in the initial stages of feeding behaviour, and therefore may have an important function. In line with the concept that PAMPs/MAMPs are conserved proteins with essential functions to pathogens/microbes, we hypothesise that the aphid elicitor will be essential for aphid feeding and therefore may be present in the saliva during the aphid initial feeding stages. Whilst a ROS burst was not identified in *N. benthamiana* to the 3 to 10 kDa fraction, induced resistance is nonetheless triggered to the fraction in this plant species (chapter 4). As our clone of *M. persicae* can effectively colonize *A. thaliana* and *N. benthamiana*, it would be interesting to investigate if a 3 to 10 kDa fraction of aphids maintained on *N. benthamiana* can trigger ROS bursts and other defence responses in *A. thaliana*. This should elucidate if elicitors are differently present depending on the host plant of the aphid. It would also be interesting to investigate the presence of the 3 to 10 kDa elicitors in aphid species other than *M. persicae*.

The identity of the elicitor(s) in the 3 to 10 kDa aphid fraction has not been identified. The ROS burst triggered by *M. persicae* extract is delayed in timing and is longer in duration compared to that of PAMPs. This may suggest that the elicitor needs to

interact with the plant before it triggers the ROS burst. The heat-sensitive nature of the elicitor activity may denote an enzyme that damages the plant and releases plant peptides, which are then perceived by the plant. However, few enzymes smaller than 10 kDa have been identified. Running of the 3 to 10 kDa protein fraction on an SDS-PAGE gel would indicate if any proteins larger than 10 kDa are present in the fraction. Aphids do have large gene families encoding short peptides however. Ollivier and colleagues (Ollivier *et al.*, 2012) identified a family of short peptides ("sp" family) 38 or 39 residues in length by mining the *A. pisum* genome and ESTs from other aphids. The "sp" family has multiple copies present within all eight aphid species investigated (23 copies in *M. persicae)*, and shares no similarity with known sequences outside of aphids. Whilst little is known about this family of peptides and whether it is present in the saliva, the estimated molecular weight, the potential specificity of the peptides to aphids and the conservation between aphid species make them candidates for elicitors or effectors. The ability of this peptide family to elicit immune responses in *A. thaliana* could be investigated using synthetic peptides.

In addition to the 3 to 10 kDa elicitor activity, we also identified an elicitor activity in the larger than 10 kDa fraction, which triggered induced resistance in *A. thaliana* but no significant amount of ROS production. Whilst ROS production is a well characterised part of PTI signalling, its function in PTI is unclear (Mersmann *et al.*, 2010). The lack of significant ROS from the larger than 10 kDa fraction suggests that ROS production is not necessary to induce defence to the aphid. In agreement with this is the fact that proteinase K elicits a ROS burst in *A. thaliana* similar to that of *M. persicae* extract, yet proteinase K-treated aphid extract does not induce defence to *M. persicae*. However, it has been reported that *A. thaliana* mutants in the NADPH-oxidase RbohD, which is required for PTI triggered ROS generation, are more susceptible to *M. persicae* (Miller *et al.*, 2009), suggesting that ROS production does lead to *M. persicae* defence in *A. thaliana*. The most likely explanation for these apparent contrasting findings are that aphid elicitors trigger different defence pathways in plants, some of which involve ROS and others that do not. All these pathways together contribute to effective defence against the aphid, and therefore the mutation of one of these pathways makes the plant more susceptible to the aphid but not fully susceptible. We are yet to identify the source of the ROS burst produced by *M. persicae* extract, and therefore it may not be dependent on RbohD, and is instead generated by another NADPH-oxidase. In addition, apoplastic peroxidases of plants have also shown to be involved in PAMP triggered ROS bursts in *A. thaliana* (Daudi *et al.*, 2012), and therefore may also be the source of the ROS burst triggered by *M. persicae* fractions. Alternatively, the different *M. persicae* clones used in this study and that of Miller and colleagues may vary in sensitivity to ROS induced

defence. Further study is needed to discover how *M. persicae* triggers *A. thaliana* ROS bursts and the role of these bursts in plant defences to aphids.

The identity of the larger than 10 kDa elicitor remains unclear. It is most likely a protein, as proteinase K treatment led to a loss of induced resistance. It may also possess an enzymatic function, as boiling led to a loss of induced resistance also. Enzymes larger than 10 kDa have been identified to act as elicitors in other insect herbivores; these include lipases and GOX from chewing insects (Musser *et al.*, 2005; Schafer *et al.*, 2011). *M. persicae* saliva contains GOX peptides and GOX activity (Harmel *et al.*, 2008) and so this is one potential candidate for further study.

The role of BAK1 in plant-insect interactions has been little studied, and no work has previously been carried out on its role in plant-aphid interactions. We found that the BAK1 null mutant *bak1-4* significantly decreased *M. persicae* performance. *M. persicae* fecundity on *A. thaliana* plants is measured using different aphid clones and protocols in different research groups, and therefore quantative comparison of changes in reproduction between our results and those published by other groups is difficult. BAK1 null mutants are misregulated in several signalling pathways. The affect on aphid performance is likely to be due to either misregulated BR responses or a lack of cell death containment in *bak1-4*, as BKK1 is also involved in both these processes (He *et al.*, 2007) and *bkk1-1* showed the same decrease in aphid performance. The *bak1-5* plants do not show misregulation of cell death or BR responses, and are only deficient in plant immune signalling (Roux *et al.*, 2011; Schwessinger *et al.*, 2011). Whilst *M. persicae* fecundity was slightly reduced in *bak1-5* it was not to the same extent as *bak1-4* or *bkk1-1,* suggesting that deficiencies in immune signalling do not solely cause the decreases in aphid performance seen in the *bak1-4* or *bkk1-1* mutants. Therefore *bak1-5* allowed us to study the role of immune signalling in plant-aphid interactions with a reduced level of the pleiotrophic affects associated with BAK1 null mutants, which may have otherwise masked results in our assays, especially the aphid induced resistance assays.

For bacterial pathogens such as *P. syringae*, lack of elicitor perception by the plant, and its associated immunity, leads to increased colonization of the plant as represented by increased colony forming units per squared centimetre (e.g. (Roux *et al.*, 2011)). However, we found that *M. persicae* reproduction was similar, if not slightly lower, on *bak1-5* plants compared to Col-0. One reason why a mutation to *bak1* may give no increase in aphid performance under normal conditions may be that defence triggered by aphid elicitors is usually localised, and aphids are able to move to other parts of the plant to avoid it (De Vos and Jander, 2009). In our induced resistance assays we prevented the aphids ability to escape, because the whole leaf had been induced and the aphids were confined to leaf cages on the induced leaf. Therefore, a more likely explanation for the

observed results is that *M. persicae* is very effective at suppressing elicitor-triggered responses in such a manner that the absence of a fully functional BAK1 does not make a substantial difference to aphid performance; the aphids will continue to produce the effectors required for elicitor-triggered immunity suppression pathways involving BAK1. We previously found a candidate *M. persicae* effector, Mp10, which can suppress the flg22-triggered ROS burst in *N. benthamiana* (chapter 3). Recently, three *M. persicae* effectors, C002, PIntO1 and PIntO2, were shown to increase *M. persicae* fecundity when expressed in *A. thaliana*, whereas the *A. pisum* homologs of these effectors did not increase *M. persicae* fecundity (Pitino and Hogenhout, 2013). This provides further evidence of the ability of *M. persicae* to manipulate plant defences.

The *bak1-5* plants produce a significantly decreased ROS burst and no induced resistance to the 3 to 10 kDa elicitor from *M. persicae* extract. Therefore BAK1 must be involved in the aphid elicitor triggered signalling pathway. *M. persicae* performance is the same on *bak1-5* plants with and without pretreatment with the 3 to 10 kDa fracion of whole aphids. This difference was less pronounced upon pretreatment with the 3 to 10 kDa fraction of saliva. This apparent discrepancy possibly suggests that the elicitor content may be different between the 3 to 10 kDa fractions of whole aphid extract and saliva, potentially leading to more BAK1-independent triggered induced resistance of saliva versus whole aphid extract. Plants are exposed to both aphid saliva and aphid mouth parts during feeding, and elicitors present in the latter are mostly absent from the saliva collected on artificial diets. Therefore, investigating the plant responses to the 3 to 10 kDa fraction of aphid saliva plus aphid mouth parts may provide helpful information in understanding the BAK1-dependent and independent responses to aphid elicitors.

Survival of *A. pisum*, which does not use *A. thaliana* as a host, significantly increased on *bak1-5 A. thaliana* mutants compared to wild type Col-0, although these aphids did not successfully reproduce on the mutant plants. We found at least two *M. persicae* fractions eliciting *A. thaliana* immune responses (i.e. 3 to 10 kDa and >10 kDa), and have evidence for the involvement of BAK1 in the perception of one of the elicitors. As mentioned above, BAK1-independent elicitor-triggered immunity pathways may be involved in responses to aphid elicitors. In addition, *A. pisum* proteins may trigger ETI. *R* genes to aphids have been identified in several plant species (Dogimont *et al.*, 2010), including biotype-specific resistance to *A. pisum* in *M. truncatula* (Stewart *et al.*, 2009). Micro-array or RNA sequencing experiments between Col-0 and *bak1-5* plants challenged with *A. pisum* would provide more information on the defence response pathways that are induced upon exposure to aphid elicitors. Examining the reproduction on *bak1-5* plants of a *M. persicae* clone that does not perform well on *A. thaliana* may also give further insight on the role of immunity on plant-aphid interactions.

Perception of the 3 to 10 kDa elicitor by *A. thaliana* is likely to require a novel PRR, as *A. thaliana* mutants in the known BAK1-interacting PRRs (FLS2, EFR, PEPR1 and PEPR2) showed no reduction in the ROS burst triggered by the elicitor. The interpretation of the increased ROS burst in *fls2 efr cerk1* to the 3 to 10 kDa elicitor is difficult. The *fls2* mutant does not give an increased ROS burst but the *efr* mutant gives a small increase in ROS burst, and preliminary data shows that mutation of *cerk1* alone also gives an increase in the ROS burst. This suggests that the effect of combining the mutants gives the overall increase in ROS burst. Interactions between different elicitor signalling pathways are not well characterised and therefore how the mutation of PRRs contribute to an increased ROS response is another pathway may be an interesting subject for future study, once the aphid-triggered pathway is better characterised.

Identification of the PRR may reveal more about the properties and identity of the aphid elicitor, such as if the PRR is more likely to recognise an aphid protein or a plant derived protein. Strategies to identify PRRs in the past have included forward genetics followed by map-based cloning (Gómez-Gómez and Boller, 2000), targeted reverse genetics (Zipfel *et al.*, 2006), chemical crosslinking of the ligand and biochemical purification of the receptor (Yamaguchi *et al.*, 2006), and production of chimeras between PRR domains (Brutus *et al.*, 2010).

In summary, we show evidence that *M. persicae* molecules elicit plant immunity responses. We identified at least two *M. persicae* elicitor activities. This study is the first demonstration of the involvement of the LRR-RLK BAK1 in plant perception of insect elicitors. As known BAK1-interacting PRRs are unlikely to be involved in aphid elicitor perception, it is probable that the interaction between BAK1 and an unidentified PRR triggers aphid elicitor-triggered immunity.

Chapter 6 – A targeted reverse genetic screen did not identify the aphid elicitor receptor in *A. thaliana*

Contributors: David C. Prince, Lena Stransfeld, Cyril Zipfel and Saskia A. Hogenhout.

6.1 Introduction

We previously identified a BAK1 mutant, *bak1-5*, that is deficient in perception of a *M. persicae* elicitor that triggers *A. thaliana* immunity (chapter 5). BAK1 interacts with several RLKs to positively regulate plant immunity (Monaghan and Zipfel, 2012). We previously tested mutants of known BAK1-interacting RLKs and found that none gave a decreased immune response, indicating that they were not the receptor for the aphid elicitor (chapter 5). Therefore, the next step is to identify the *A. thaliana* receptor that perceives the aphid elicitors. We hypothesise that this receptor was a non-RD RLK.

Many plant RLKs are located at the cell membrane and often consist of a ligand-binding extracellular domain, a transmembrane segment and a cytoplasmic domain. RLKs are often involved in the perception of extracellular molecules and modulate signals in the cell to orchestrate an appropriate response. The extracellular domains amongst plant RLKs are as divergent as the molecules or other plant genes that they interact with and have been used to classify them into subfamilies (Shiu and Bleecker, 2001). The cytoplasmic domain possesses a conserved serine/threonine kinase domain. The binding of the extracellular domain to its ligand leads to complex formation with other membrane-associated proteins, including RLKs, and the activation of the cytoplasmic kinase domain, that is the first step of the intracellular signalling cascade. There are over 600 predicted RLKs in the *A. thaliana* genome (Shiu *et al.*, 2004), although only a small number of these have been functionally characterised.

Kinases, including RLKs, can be classified as RD or non-RD based on the amino acid preceding aspartate in subdomain VIb of the kinase domain. RD kinases generally need auto-phosphorylation of the activation loop for full kinase activity, whereas non-RD kinases don't and are activated in a different manner (Nolen *et al.*, 2004). So far, non-RD RLKs for which functions are known are associated with innate immunity in both the plant and animal kingdoms (Dardick and Ronald, 2006).

RLKs are involved in a wide range of plant processes apart from immunity including development (De Smet *et al.*, 2009) and response to abiotic stress (Ouyang *et al.*, 2010). The largest subfamily of RLKs is the LRR family, which itself is divided into more than 13 subfamilies (Shiu and Bleecker, 2001). Less than fifteen percent of this family consisting of more than 200 members have been characterised (Gou *et al.*, 2010). PAMP and DAMP PRRs are amongst the genes that have been characterised. LRR-RLK subfamily XII includes the non-RD PAMP PRRs FLS2, EFR (Segonzac and Zipfel, 2011) whilst the role of other genes in this subfamily are unknown. The DAMP PRRs PEPR1 and PEPR2 belong to LRR-RLK subfamily XI (Yamaguchi *et al.*, 2010). This is a large subfamily of which members have diverse functions. This includes RECOGNITION OF

COLLETOTRICHUM HIGGINSIANUM1 (RCH1) that is involved in resistance to the hemibiotrophic fungus genus *Colletotrichum* (Narusaka *et al.*, 2004), although the mechanism of resistance is unknown. Other members of the family tend to function in development and differentiation. CLAVATA1 (CLV1), and BARELY ANY MERISTEM1, 2 and 3 (BAM1, BAM2 and BAM3) are involved in proliferation and maintenance of dividing cells in meristems upon perception of the CLE (CLV3/EMBRYO SURROUNDING REGION (ESR)) peptides (Clark *et al.*, 1997; DeYoung *et al.*, 2006; Guo *et al.*, 2010). Floral organ abscission regulation involves HAESA and HAESA-LIKE2 (HSL2), which may be perceiving signalling peptides of the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and the IDA-LIKE (IDL) families (Cho *et al.*, 2008; Stenvik *et al.*, 2008). PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR (PXY/TDR) is a receptor for tracheary element differentiation inhibitory factor (TDIF) and is involved in maintenance of polarity during plant vascular-tissue development (Fisher and Turner, 2007; Hirakawa *et al.*, 2008). GASSHO1 (GSO1) and GSO2 are required for formation of the epidermal surface during embryogenesis (Tsuwamoto *et al.*, 2008) and HAIKU2 (IKU2) regulates seed size (Luo *et al.*, 2005) although the ligands are still to be discovered. Despite being well studied, there are still nearly a dozen members of LRR-RLK subfamily XI with unknown functions.

We know little about the plant genes that perceive insect elicitors. Elicitors produced by other organisms are perceived by plant RLKs, and therefore it is likely that insect elicitors are too. Two RLKs, LecRK1 in *N. attenuata* and LecRK-I.8 in *A. thaliana* (Gilardoni *et al.*, 2011; Gouhier-Darimont *et al.*, 2013), play a role in the plant responses to different insect elicitors, although it is currently unclear if they physically interact with the elicitor itself or are involved in downstream signalling responses.

A number of PRRs involved in *A. thaliana* immunity to microbial pathogens have been identified (Monaghan and Zipfel, 2012). Strategies to identify PRRs in the past have included forward genetics followed by map-based cloning (FLS2) (Gómez-Gómez and Boller, 2000), targeted reverse genetics using candidates selected by critieria such as upregulation upon elicitor treatment or similarity to known PRRs (EFR, CERK1, PEPR2, LYM1 and LYM3) (Zipfel *et al.*, 2006; Miya *et al.*, 2007; Yamaguchi *et al.*, 2010; Willmann *et al.*, 2011), chemical crosslinking of the ligand and biochemical purification of the receptor (PEPR1*)* (Yamaguchi *et al.*, 2006), and production of chimeras between PRR domains (WAK1) (Brutus *et al.*, 2010).

We had previously found that BAK1 was involved in aphid elicitor perception, but that known BAK1-interacting kinases were not (chapter 5). We hypothesise that another RLK is necessary to perceive the aphid elicitor. As a first step to characterising the RLK of the 3 to 10 kDa aphid elicitor, we adopted a targeted reverse genetics approach similar

to the one used by Danna and colleagues (Danna *et al.*, 2011), where they generated a collection of *A. thaliana* T-DNA insertion lines for non-RD IRAKs. This collection was screened for responses to the aphid elicitor in an attempt to identify the aphid elicitor receptor.

6.2 Results

First round of screening identified three receptor candidates

We hypothesise that the 3 to 10 kDa *M. persicae* elicitor is perceived by a non-RD IRAK in *A. thaliana*. To test this we obtained a collection of T-DNA insertion mutants in the non-RD IRAKs in *A. thaliana* from Prof. Frederick Ausubel (Danna *et al.*, 2011). Homozygous T-DNA insertion generates loss-of-function mutants (Alonso *et al.*, 2003). Dr. Cyril Zipfel's lab found that some of the plant lines were not homozygous for the T-DNA insertion and therefore generated homozygous mutants for 43 of the 47 genes. Some genes in the collection were represented by more than one independent T-DNA insertion line, giving a total of 52 mutant lines. A table of gene identities for the mutant lines can be found in chapter 2. The ROS responses to the 3 to 10 kDa fraction of *M. persicae* extract were tested for the homozygous mutants in high-throughput screening assays. Eight leaf discs of Col-0, *bak1-5* and up to 10 non-RD IRAK mutants were tested in the same 96 well plate so that the amounts of ROS generated could be compared. The first screen of the mutants identified three lines that gave statistically significantly lower ROS responses than Col-0; lines 1, 10 and 43 (Figure 6.1). Several other lines showed decreased responses compared to Col-0 that were not statistically significant (lines 2, 11, 31, 39, 53, 59, 72 and 73). Lines 41 showed an increased response compared to Col-0, which was surprising as the T-DNA was inserted in the same gene as line 43, which showed a significant decrease.

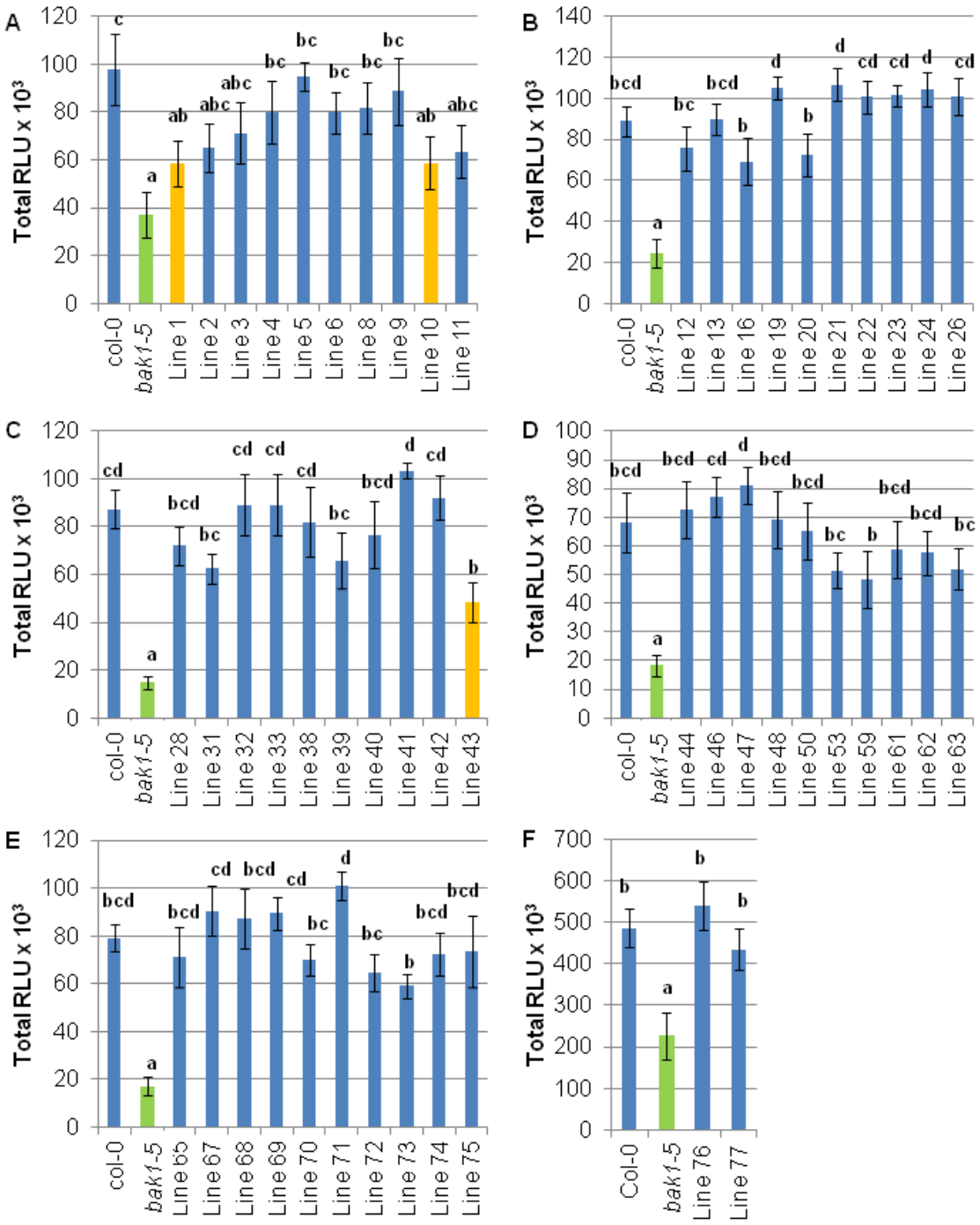


Figure 6.1 – First round of screening identified three receptor candidates.

Mutants in non-RD IRAKs were screened for reduced ROS responses. The total ROS production (represented as RLUs) was measured over an 800 minute period in leaf discs of Col-0, *bak1*-5 (green bars) and non-RD IRAK mutants after elicitation by the 3 to 10 kDa fraction of *M. persicae* extract. Results are mean ± SE (n = 8 leaf disc). Letters indicate significant differences at $P < 0.05$. Orange bars correspond to mutants with significantly lower responses than Col-0.

Receptor candidates identified in the first screen were not confirmed in the second screen.

In order to identify additional lines and confirm the already identified receptor candidates, we screened the mutants a second time. Although the ROS responses to aphid elicitor was decreased in several lines when compared to Col-0 (lines 6, 24, 38 and 43), none of them were significantly lower (Figure 6.2). Several lines also showed increased ROS bursts (lines 2, 40, 41, 50, 67, 68, 69, 70, 71, 72, 74), whilst decreased ROS responses were observed in some of these lines in the previous assay. Lines 1 and 10, which showed decreased ROS responses in the previous assay, did not show decreased responses in this second assay (Figure 6.2A). Only the trend in the decreased ROS response of line 43 was confirmed between assays, although it was not statistically significant in this second experiment (Figure 6.2C). Both this screen and the previous one confirmed our observation from chapter 5 that mutants in the BAK1-interacting non-RD IRAKs EFR (line 76) and FLS2 (line 77) are not deficient in *M. persicae* elicitor-triggered ROS bursts (Figure 6.1F and 6.2F).

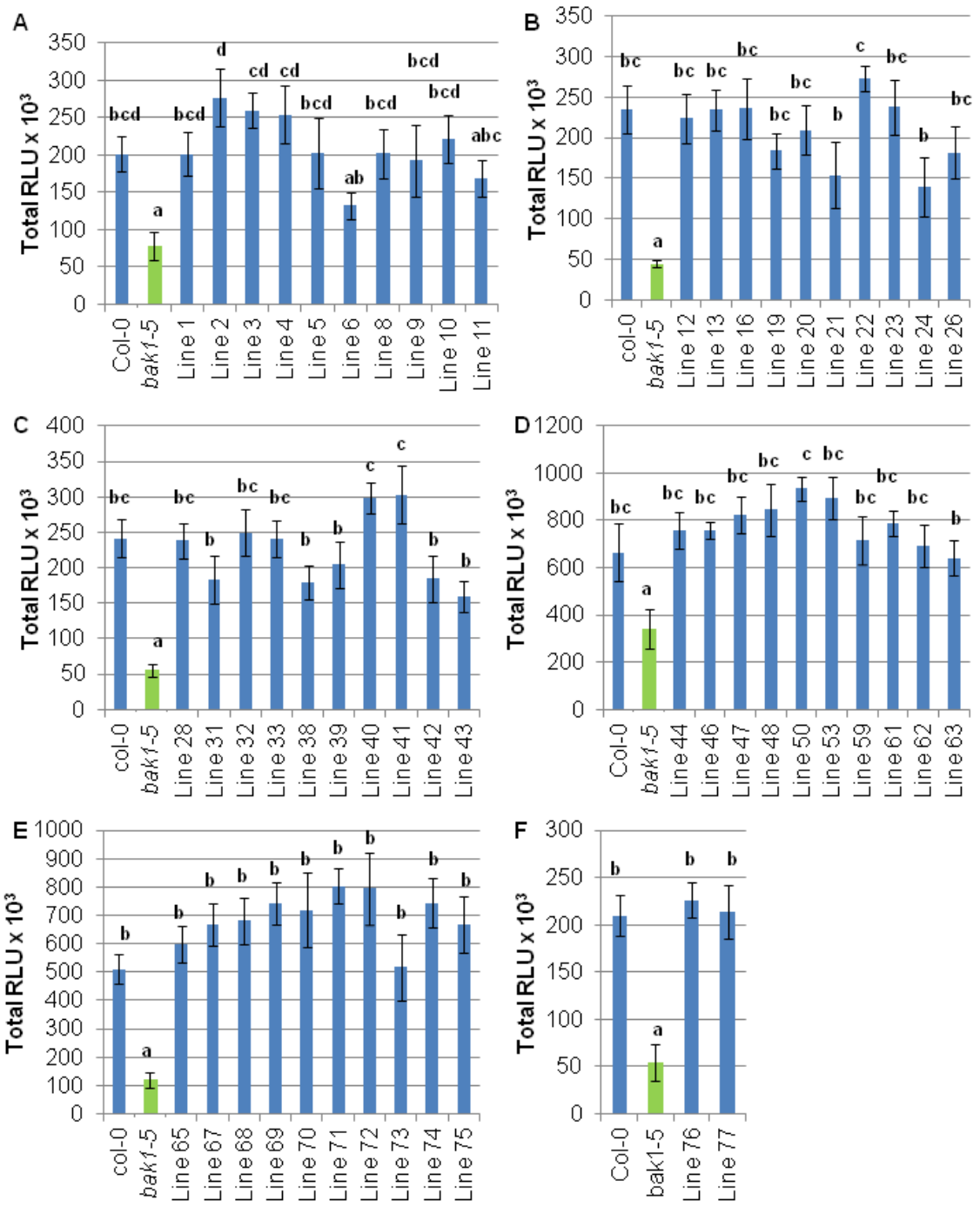


Figure 6.2 – Receptor candidates identified in the first screen were not confirmed in the second screen.

Mutants in non-RD IRAKs were screened for reduced ROS response. The total ROS production (represented as RLUs) was measured over an 800 minute period in leaf discs of Col-0, *bak1*-5 (green bars) and non-RD IRAKs mutant after elicitation by the 3 to 10 kDa fraction of *M. persicae*

extract. Results are mean ± SE (n = 8 leaf discs). Letters indicate significant differences at $P < 0.05$.

Third round of screening did not confirm the receptor candidates identified in the first two screens

We chose to continue studying lines 1, 10 and 43 that significantly differed in ROS responses compared to Col-0 in the first screen. Given the variability in responses between the two rounds of screening we tested each line three times with increased replication compared to the screen (n = 16 compared to n = 8). These lines were tested alongside Col-0, *bak1-5,* and line 33 as controls. Line 33 was one of several lines that responded in a very similar way to Col-0 in both the screens. Lines 1, 10 and 43 contain T-DNA insertion in genes that were represented by second independent T-DNA insertion lines in the mutant collection, and so the other line for each candidate was also included in the assay. Lines 1, 9 and 41 contain T-DNA insertions in exons of At4g26540, At5g05160 and At5g51770 respectively, in which the exons encode the predicted protein kinase domains of the protein. Lines 11, 10 and 43 contain insertions in exons of At4g26540, At5g05160 and At5g51770 respectively, in which the exons correspond to the predicted LRR domain of the protein. In this third experiment, it was revealed that the ROS response to aphid extract of line 1 was inconsistent; in one of the three replicates line 1 significantly differed from Col-0 but not from Line 33 (Figure 6.3A) whilst in the other two replicates no significant differences were found (Figure 6.3B). There were no decreased ROS responses observed in lines 10 (Figure 6.3C) and line 43 (Figure 6.3D) at all. Thus, the results in this third experiment did not confirm those of the two screens.

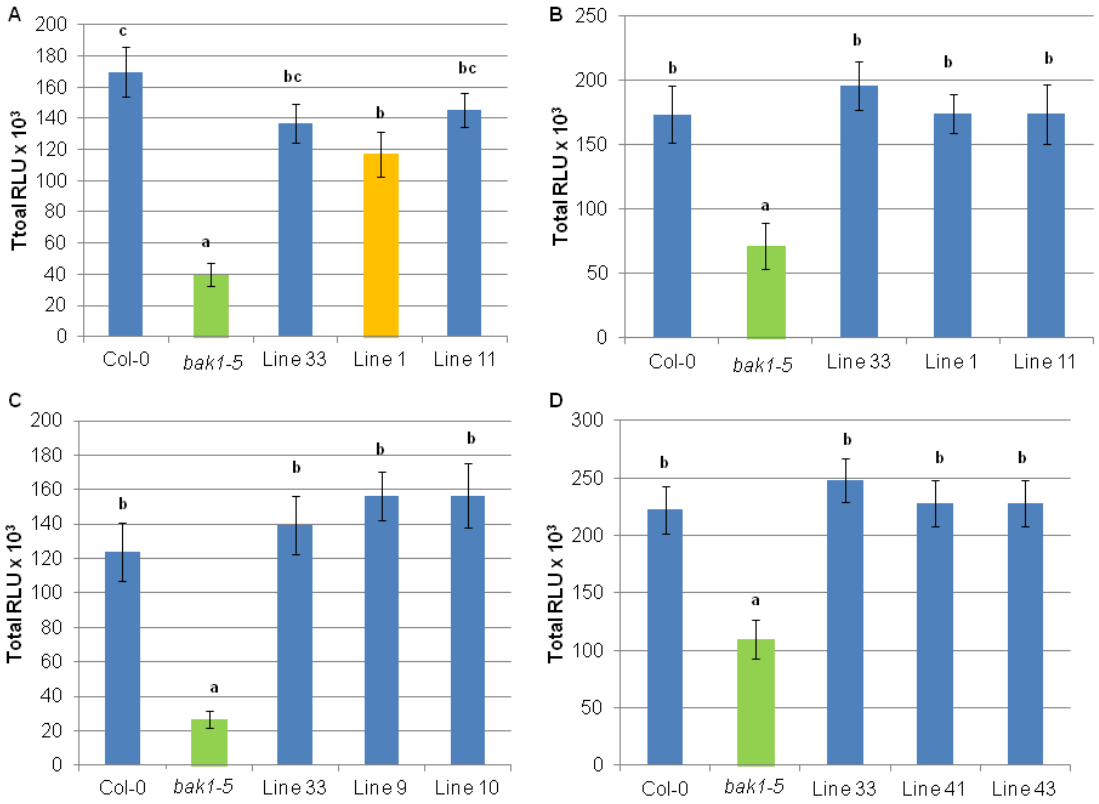


Figure 6.3 – Third round of screening did not confirm the receptor candidates identified in the first two screens.

A significant reduction in ROS response was observed in line 1 on one occasion (A) but not on two other occasions (one assay shown) (B). Lines 9 and 43 showed no reduction in ROS response (C and D). (A-D) Lines 1, 10 and 43 from the first two screens were re-tested for ROS responses along with lines 9, 11 and 41 (which have T-DNA insertions in the same genes), and Col-0, *bak1-5* and line 33 controls. The total ROS production (represented as RLUs) was measured over an 800 minute period in leaf discs of Col-0, *bak1*-5 (green bars) and non-RD IRAK mutants after elicitation by the 3 to 10 kDa fraction of *M. persicae* extract. Results are mean ± SE (n = 16 leaf discs per replicate). Letters indicate significant differences at $P < 0.05$. (C and D) These experiments were repeated three times with similar results, with one representative experiment shown.

The *M. persicae* elicitor may be perceived by a pair of receptors

To try and understand why line 1 showed an inconsistent level of ROS response to the 3 to 10 kDa fraction of *M. persicae* we looked at the identity of the gene containing the T-DNA insertion. Line 1 has a T-DNA insertion in At4g26540. At4g26540 has been identified as a member of LRR-RLK subfamily XI (Hirakawa *et al.*, 2008; Yamaguchi *et al.*, 2010). At4g26540 is closely related to another member of the family, At5g56040, and both genes are similar at the amino acid level (Hirakawa *et al.*, 2008; Yamaguchi *et al.*, 2010) (Figure 6.4A). Line 22 contains a T-DNA insertion in the predicted LRR region of

AT5G56040, and showed a ROS response comparable to Col-0 in both of the screens (Figure 6.1B and 6.2B). There are several examples of redundancy in function amongst related members of LRR-RLK subfamily XI. For example complete loss of response to AtPep1 is seen in a mutant of both PEPR1 and PEPR2 but not in single mutants (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). Thus At4g26540 and At5g56040 may both be required for sensing the aphid elicitor.

To provide further evidence for this hypothesis we examined the expression patterns of both genes using the Arabidopsis electronic fluorescent pictograph (eFP) browser (Winter *et al.*, 2007). If both genes are required for perception of the aphid elicitors then it would be expected that they have similar expression profiles in leaves. Both genes are expressed in the leaves, as well as in other tissues (Figure 6.4B and C). The expression levels of At5g56040 in cauline leaves and stamen is higher than that of At4g26540.

A double mutant for At4g26540 and At5g56040 was identified at NASC (N31396). However, the seed received did not germinate therefore preventing us from examining this mutants responses to aphid extracts

A

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At5g56040      MPRN-PRFCFFLFLLFHSSLFFSIPCFSIDEQGLALLSWKSQLNISGDALSSWKASESNP
At4g26540      MPPNIYRLSFF----SSLLCFFFIPCFSLDQQGQALLSWKSQLNISGDAFSSWHVADTSP
               ** *  *:.**         ** *****:*:** ***************:***:.::::.*

At5g56040      CQWVGIKCNERGQVSEIQLQVMDFQGPLPATNLRQIKSLTLLSLTSVNLTGSIPKELGDL
At4g26540      CNWVGVKCNRRGEVSEIQLKGMDLQGSLPVTSLRSLKSLTSLTLSSLNLTGVIPKEIGDF
               *:***:***.**:******: **:** **.*.**.:**** *:*:*:**** ****:**:

At5g56040      SELEVLDLADNSLSGEIPVDIFKLKKLKILSLNTNNLEGVIPSELGNLVNLIELTLFDNK
At4g26540      TELELLDLSDNSLSGDIPVEIFRLKKLKTLSLNTNNLEGHIPMEIGNLSGLVELMLFDNK
               :***:***:******:***:**:***** ********** ** *:***  *:** *****

At5g56040      LAGEIPRTIGELKNLEIFRAGGNKNLRGELPWEIGNCESLVTLGLAETSLSGRLPASIGN
At4g26540      LSGEIPRSIGELKNLQVLRAGGNKNLRGELPWEIGNCENLVMLGLAETSLSGKLPASIGN
               *:*****:*******:::*********************.** **********:*******

At5g56040      LKKVQTIALYTSLLSGPIPDEIGNCTELQNLYLYQNSISGSIPVSMGRLKKLQSLLLWQN
At4g26540      LKRVQTIAIYTSLLSGPIPDEIGYCTELQNLYLYQNSISGSIPTTIGGLKKLQSLLLWQN
               **:*****:************** ******************.::* ************

At5g56040      NLVGKIPTELGTCPELFLVDLSENLLTGNIPRSFGNLPNLQELQLSVNQLSGTIPEELAN
At4g26540      NLVGKIPTELGNCPELWLIDFSENLLTGTIPRSFGKLENLQELQLSVNQISGTIPEELTN
               ***********.****:*:*:*******.******:* ***********:********:*

At5g56040      CTKLTHLEIDNNQISGEIPPLIGKLTSLTMFFAWQNQLTGIIPESLSQCQELQAIDLSYN
At4g26540      CTKLTHLEIDNNLITGEIPSLMSNLRSLTMFFAWQNKLTGNIPQSLSQCRELQAIDLSYN
               ************ *:**** *:.:* **********:*** **:*****:**********

At5g56040      NLSGSIPNGIFEIRNLTKLLLLSNYLSGFIPPDIGNCTNLYRLRLNGNRLAGNIPAEIGN
At4g26540      SLSGSIPKEIFGLRNLTKLLLLSNDLSGFIPPDIGNCTNLYRLRLNGNRLAGSIPSEIGN
               .******: ** :*********** *************************.**:****
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At5g56040      LKNLNFIDISENRLIGNIPPEISGCTSLEFVDLHSNGLTGGLPG-TLPKSLQFIDLSDNS
At4g26540      LKNLNFVDISENRLVGSIPPAISGCESLEFLDLHTNSLSGSLLGTTLPKSLKFIDFSDNA
               ******:*******:*.*** **** ****:***:*.*:*.* * ******:***:***:

At5g56040      LTGSLPTGIGSLTELTKLNLAKNRFSGEIPREISSCRSLQLLNLGDNGFTGEIPNELGRI
At4g26540      LSSTLPPGIGLLTELTKLNLAKNRLSGEIPREISTCRSLQLLNLGENDFSGEIPDELGQI
               *:.:** *** ************:*********:**********:* *:****:***:*

At5g56040      PSLAISLNLSCNHFTGEIPSRFSSLTNLGTLDVSHNKLAGNLNVLADLQNLVSLNISFNE
At4g26540      PSLAISLNLSCNRFVGEIPSRFSDLKNLGVLDVSHNQLTGNLNVLTDLQNLVSLNISYND
               ************:*.********.*.***.******:*:******:***********:*:

At5g56040      FSGELPNTLFFRKLPLSVLESNKGLFISTRPENG--IQTRHRSAVKVTMSILVAASVVLV
At4g26540      FSGDLPNTPFFRRLPLSDLASNRGLYISNAISTRPDPTTRNSSVVRLTILILVVVTAVLV
               ***:**** ***:**** * **:**:**.  ..     **. *.*::*: ***..:.***

At5g56040      LMAVYTLVKAQRIT--GKQEELDSWEVTLYQKLDFSIDDIVKNLTSANVIGTGSSGVVYR
At4g26540      LMAVYTLVRARAAGKQLLGEEIDSWEVTLYQKLDFSIDDIVKNLTSANVIGTGSSGVVYR
               ********:*:        **:**************************************

At5g56040      VTIPSGETLAVKKMWSKEENRAFNSEINTLGSIRHRNIIRLLGWCSNRNLKLLFYDYLPN
At4g26540      ITIPSGESLAVKKMWSKEESGAFNSEIKTLGSIRHRNIVRLLGWCSNRNLKLLFYDYLPN
               :******:***********. ******:**********:**********************

At5g56040      GSLSSLLHGAGKGSGGADWEARYDVVLGVAHALAYLHHDCLPPILHGDVKAMNVLLGSRF
At4g26540      GSLSSRLHGAGKG-GCVDWEARYDVVLGVAHALAYLHHDCLPTIIHGDVKAMNVLLGPHF
               ***** ******* * .************************ *:************ :*

At5g56040      ESYLADFGLAKIVSGEGVTDGDSSKLSNRPPLAGSYGYMAPEHASMQHITEKSDVYSYGV
At4g26540      EPYLADFGLARTISGYPNTGIDLAKPTNRPPMAGSYGYMAPEHASMQRITEKSDVYSYGV
               * ********: :**   *  * :* :****:***************:************

At5g56040      VLLEVLTGKHPLDPDLPGGAHLVQWVRDHLAGKKDPREILDPRLRGRADPIMHEMLQTLA
At4g26540      VLLEVLTGKHPLDPDLPGGAHLVKWVRDHLAEKKDPSRLLDPRLDGRTDSIMHEMLQTLA
               ************************:******* **** .:***** **:* **********

At5g56040      VSFLCVSNKASDRPMMKDIVAMLKEIRQFDMDRSESDMIKGGKCEKWQPQPLPPEKIVST
At4g26540      VAFLCVSNKANERPLMKDVVAMLTEIRHIDVGRSETEKIKAGGCGSKEPQQFMSNEKIIN
               *:********.:**:***:****.***::*: ***:: **.* * . :** :  :: : .

At5g56040      PRGSSNCSFAYSDESV
At4g26540      SHGSSNCSFAFSDDSV
                :********:**:**
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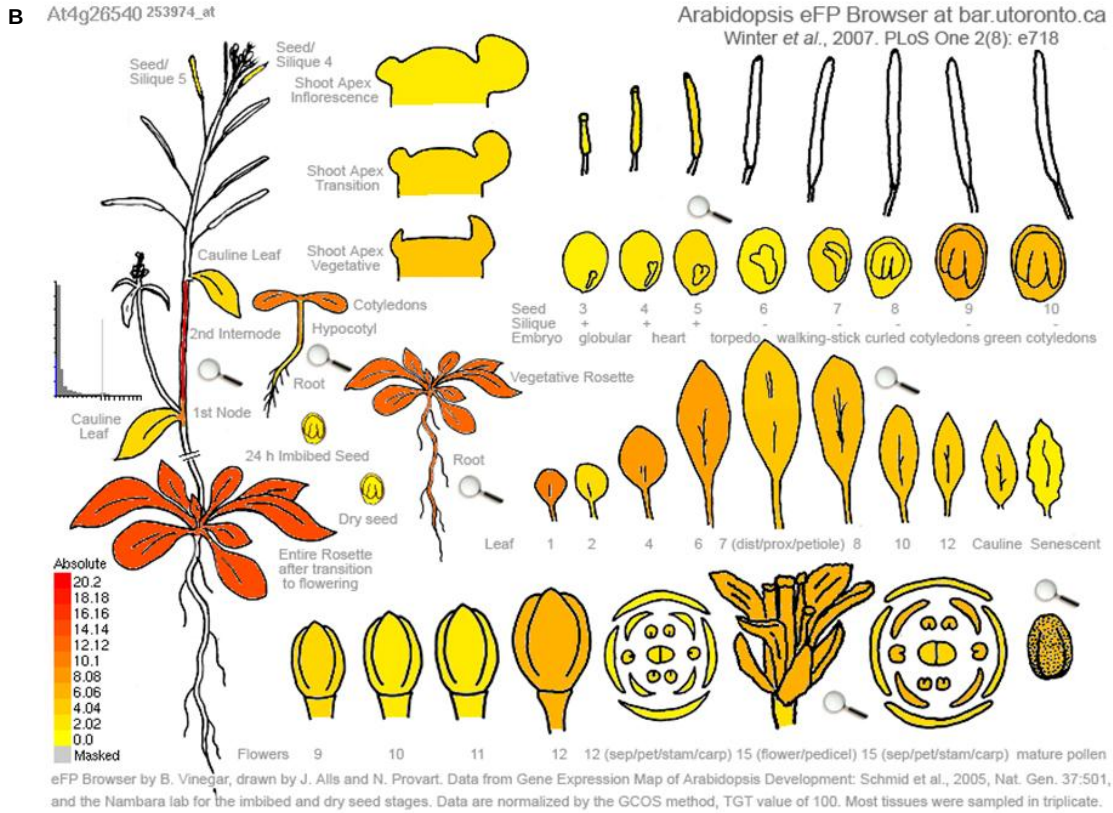

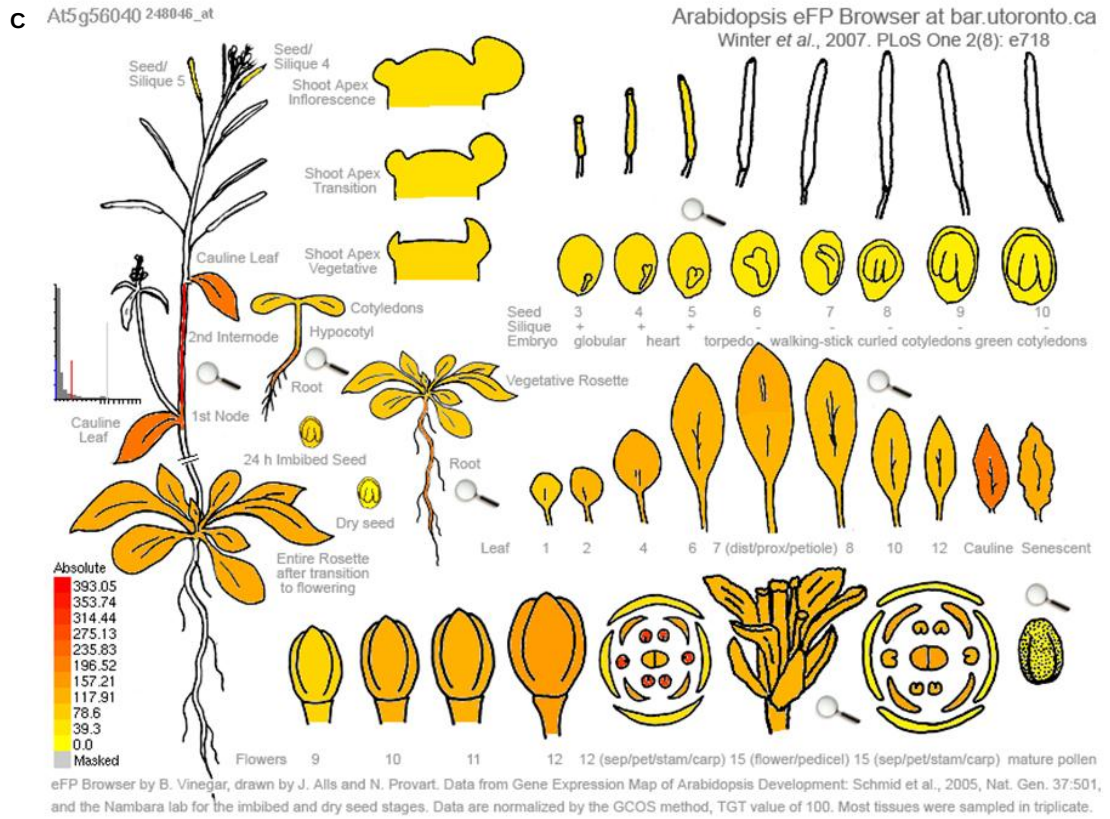

Figure 6.4 – The *M. persicae* elicitor may be perceived by a pair of receptors.

(A) At4g26540 and At5g56040 share a large degree of similarity at the amino acid level. Amino acid sequences were aligned using Clustal Omega.
(B) At4g26540 is expressed primarily in the leaves, but also in other tissues. (C) At5g56040 is expressed in leaves and other tissues. (B and C) Images taken from Arabidopsis eFP Browser at bar.utoronto.ca (Winter *et al.,* 2007).

Homologs of At5g56040 and At4g26540 are present in diverse plant species, but few plant species have homologs of both RLKs

We wished to look for the presence of the two RLKs encoded by At5g56040 and At4g26540 in other plant species in order to learn more about their evolutionary history. Homologs were found across plant groups and in basal plants such as liverworts and club-mosses (Table 6.1). However, most plant species only contain one homolog of this gene, as BLAST searches with both the *A. thaliana* genes gave the same result (Table 6.1). Only three species outside the *Arabidopsis* genus were found to contain homologs of both genes suggesting that they have duplicated recently in evolutionary time. The duplication in the club-moss *Selaginella moellendorffii* may have occurred independently.

Plant species	Species group	At4g26540 homolog	At5g56040 homolog
Arabidopsis lyrata subsp. lyrata	Eudicots	**XP_002867561**	**XP_002864427**
Vitis vinifera	Eudicots	XP_002267870	XP_002267870
Populus trichocarpa	Eudicots	**XP_002299054**	**XP_002330548**
G. max	Eudicots	**XP_003545087**	**XP_003551393**
Ricinus communis	Eudicots	XP_002532173	XP_002532173
M. truncatula	Eudicots	XP_003600412	XP_003600412
*N. benthamiana**	Eudicots	Niben.v0.3.Scf25202020 25184 597396 14987223-,...,15639093	Niben.v0.3.Scf25202020 25184 597396 14987223-,...,15639093
*S. lycopersicum***	Eudicots	AC210371	AC210371
S. bicolor	Monocot	XP_002445956	XP_002445956
O. sativa Indica Group	Monocot	EAZ07465	EAZ07465
O. sativa Japonica Group	Monocot	BAD10022	BAD10022
Triticum aestivum	Monocots	CAJ19346	CAJ19346
Brachypodium distachyon	Monocot	XP_003572332	XP_003572332
B. sylvaticum	Monocot	CAJ26360	CAJ26360
H. vulgare subsp. *Vulgare*	Monocot	BAK02741	BAK02741
Pinus sylvestris	Conifers	CAC20842	CAC20842
Marchantia polymorpha	Liverworts	No hit	BAF79936
S. moellendorffii	Club-mosses	**XP_002992868**	**XP_002969057**

Table 6.1 – Homologs of At5g56040 and At4g26540 are present in diverse plant species, but few plant species have homologs of both RLKs.

Blastp was used to identify homologs of At4g26540 and At5g56040 amongst the NCBI non-redundant protein sequences database. Top significant hit is shown for each species. Plant species that gave different results for the two *A. thaliana* genes are shown in bold. * *N. benthamiana* homolog found using tblastn of the draft genome at solgenomics.net. ** *S. lycopersicum* homolog found using tblastn of NCBI nucleotide collection database.

6.3 Discussion

Our targeted reverse genetics approach did not conclusively show that any of the non-RD IRAKs tested were involved in recognition of the aphid elicitor. There are several possible reasons why this is the case, such as the approach used, the presence of multiple elicitors in the 3 to 10 kDa fraction, redundancy amongst more than one non-RD RLK, or the involvement of RD RLKs.

The results of the two rounds of screening of RLK mutants were variable, with some mutants giving ROS responses lower than Col-0 in one round and higher than Col-0 in the next. Several mutant lines were more extensively tested in a third round of screening and produced more consistent results. Had sufficient time and seed been available the initial screen would have been more informative if conducted with a larger number of leaf discs per mutant, and performed three or four times rather than two. The use of the 3 to 10 kDa fraction of *M. persicae* rather than an identified, purified elicitor may also have introduced variation. If multiple elicitors of plant defence are present in this aphid fraction then their relative concentration may vary between each extract made, thereby varying the stimulation of different elicitor-triggered signalling pathways. However, I am confident that if the approach taken was capable of identifying the elicitor receptor then it was not overlooked because of the variation. We would have observed a loss of ROS response if one of the non-RD IRAKs was the sole receptor for the aphid elicitor, and none of the mutant lines showed a loss of ROS response in any of the experiments.

Whilst our approach did not produce conclusive results, the phenotypes we had previously identified for the *M. persicae* elicitor made a targeted reverse genetic screen the best starting point for the search for the elicitor receptor. Previous forward genetic screens to identify PTI signalling components have used the seedling growth inhibition phenotype (Gómez-Gómez and Boller, 2000; Nekrasov *et al.*, 2009; Schwessinger *et al.*, 2011) because it is high throughput. We have not yet tested whether the *M. persicae* elicitor causes a similar inhibition in seedling growth. High throughput assays are more difficult to develop for decreased ROS burst and induced resistance phenotypes. However, a screen of approximately 50 mutant plant lines for ROS response was feasible in the time available. Our targeted reverse genetic approach differed from those previously published (Zipfel *et al.*, 2006; Yamaguchi *et al.*, 2010; Danna *et al.*, 2011; Willmann *et al.*, 2011) as we have not yet identified one specific elicitor in aphids. The 3 to 10 kDa fraction of *M. persicae* extract may contain several elicitors, as discussed in chapter 5. Therefore, multiple RLKs, including non-RD IRAKs, may be involved in the

perception of aphid elicitors and this would not have been identified in a screen with *A. thaliana* mutants in single RLKs.

Therefore, one reason why the targeted reverse genetics approach we have adopted in this chapter did not produce conclusive results may be due to functional redundancy between plant RLKs. This point is highlighted by the identity of one of the receptor candidates identified in the first round of screening. Line 1 represents an insertion in At4g26540, a member of LRR-RLK subfamily XI. This subfamily is composed of groups of related RLKs with redundant functions, perceiving groups of related plant peptides. Therefore, phenotypes are often seen only when all the redundant members of the RLKs are disfunctional (e.g. (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010)). We were unable to examine the phenotype of the double mutant of At4g26540 and the closely related At5g56040, which would have provided valuable information on whether these RLKs function redundantly as the *M. persicae* elicitor receptors. Further efforts will be made to generate or obtain the double mutant so that it can be tested. BLAST searches suggested that most plant species have one copy of a homolog of these genes rather than two distinct copies, and so studying other plant species might shed further light on whether the receptors are involved in aphid elicitor perception. However, whether homologs in other plant species have similar functions to the RLKs we identified from *A. thaliana* genes is unclear.

The LRR-RLKs encoded by At4g26540 and At5g56040 may not be *M. persicae* elicitor receptors. The hypothesis that these genes are the receptors rests on inconsistent data. There are several other possibilities. Firstly, the *M. persicae* elicitor receptor may be one of the four non-RD IRAKs not tested in the genetic screen. These genes were not screened because we either did not receive the seed or did not identify homozygous insertion mutants in time. Secondly, the *M. persicae* elicitor receptor may be a RD IRAK. BAK1 regulates the signalling pathways of the RD IRAK DAMP receptors PEPR1 and PEPR2 (Roux *et al.*, 2011), and so it is possible that the phenotype we see in *bak1-5* is due to interactions with an RD IRAK. We currently have no strong evidence as to whether the *M. persicae* elicitor is acting on plant material to produce plant-derived elicitors that could be considered DAMPs, but all DAMP receptors identified so far are RD kinases (Dardick *et al.*, 2012). Investigating all RD kinases would increase the number of candidate RLKs to nearly 600 (Shiu *et al.*, 2004), which would be too many to easily conduct a reverse genetic screen on. Finally, the receptor may not be an IRAK at all, but a RLP. BAK1 is a positive regulator of LRR-RLPs as well as LRR-RLKs (Monaghan and Zipfel, 2012), although the RLP examples come from *S. lycopersicum* and not *A. thaliana*. BAK1 is involved in the signalling of the fungal PAMP ethylene-inducing xylanase (Eix) by its receptors Eix1 and Eix2 in *S. lycopersicum*, which are RLPs (Bar *et al.*, 2010). BAK1 is

also involved in regulating signalling from the RLP immune receptor Ve1 in *S. lycopersicum* (Fradin *et al.*, 2009; Fradin *et al.*, 2011). It is unclear how SERK3/BAK1 function compare in Solanaceae and *A. thaliana*, but these examples raise the possibility that a RLP may be involved.

Approaches complementary to the targeted reverse genetic screen could be adopted to identify receptors involved in aphid elicitor perception. For instance, an approach similar to Roux and colleagues (Roux *et al.*, 2011) could be adopted; immunoprecipitation of tagged BAK1 in *A. thaliana* after elicitation with the *M. persicae* elicitor, followed by mass spectrometry to identify associated proteins, may identify the BAK1-interacting RLK or RLP. Alternatively, further purification of the elicitor and characterisation of the plant responses it induces may lead to the discovery of a phenotype that would allow a forward genetics approach to be adopted.

In summary, our targeted reverse genetics screen of non-RD IRAKs did not generate consistent results indicating that aphid elicitor recognition by *A. thaliana* may not rely on a single non-RD IRAK. Whether single or complexes of non-RD IRAKs, RD IRAKs or other plant receptors perceive aphid elicitors remains unclear. The Hogenhout lab is pursing methods for identifying the aphid elicitor and its receptor(s).

Chapter 7 – Investigating the role of effector Mp10 in interactions between *N. benthamiana* and aphids

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7.1 Introduction

Diverse plant pathogens such as bacteria, fungi, oomycetes, and nematodes secrete proteins and other molecules to different cellular compartments of their hosts where they act as effectors by disrupting normal host function. In this thesis evidence is provided that aphids also secrete effectors. We previously identified several candidate effectors from *M. persicae* (chapter 3; Bos *et al.,* 2010). One effector, Mp10, is a CSP and showed several common effector phenotypes upon overexpression in *N. benthamiana*, including suppression of the ROS burst triggered by the PAMP flg22 (chapter 3). We also identified *M. persicae* elicitors of plant immunity in *N. benthamiana* (chapters 3 and 4). Suppression of elicitor-triggered immunity is a key part of successful colonization of a host by a pathogen (Jones and Dangl, 2006). We therefore hypothesised that Mp10 is an aphid effector that contributes to successful plant-aphid interactions by suppressing the plant immune response triggered by aphid elicitors. The next step is to investigate how Mp10 may suppress plant immunity.

Mp10 is a member of the CSP family, and is also referred to as OS-D2 (Jacobs *et al.*, 2005). CSPs are small water-soluble proteins abundantly secreted into the lymph of insect chemosensory sensilla and are thought to bind small molecules, such as fatty acids (Pelosi *et al.*, 2006). CSPs share some features with OBPs but share no sequence homology with them and are a distinct class of protein (Wanner *et al.*, 2004), with very different 3D protein structures (Liu *et al.*, 2012a). CSPs have a conserved motif of four cysteines ($CX_6CX_{18}CX_2C$) (Maleszka and Stange, 1997) which form two disulphide bridges, and three conserved sections (motifs) of amino acids (Wanner *et al.*, 2004). Whilst the name of CSPs suggests a role in chemoperception, there is little evidence that CSPs are purely involved in olfaction (Sanchez-Gracia *et al.*, 2009; Liu *et al.*, 2012a), although some CSPs may have a role in host searching and recognition (e.g. (Gu *et al.*, 2012; Liu *et al.*, 2012a)). Some insect CSPs are thought to be involved in olfaction and gustation - several CSPs have been specifically found in chemosensory organs and are predicted to function in chemoperception (Nagnan-Le Meillour *et al.*, 2000; Monteforti *et al.*, 2002; Jacobs *et al.*, 2005). However, functions for some members of this large protein family have been identified in diverse processes such insect as development (Stathopoulos *et al.*, 2002) and leg regeneration (Nomura *et al.*, 1992), suggesting CSPs may have divergent functions. This is further supported by gene expression studies, which show that some CSPs are specifically expressed in antenna (Calvello *et al.*, 2005) or mouthparts (Maleszka and Stange, 1997), whereas others are expressed throughout the insect (Zhou *et al.*, 2004).

CSPs in aphids were first identified by Jacobs and colleagues (Jacobs *et al.*, 2005), who found two different CSPs (OS-D1 and OS-D2) in *M. viciae* that were conserved amongst several aphid species. Xu and colleagues (Xu *et al.*, 2009) used ESTs to predict CSPs in 54 insect species, including four aphid species, and predicted ten CSPs for *A. pisum*, four CSPs for *A. gossypii*, five CSPs for *M. persicae*, and no CSPs for *T. citricida*. These numbers are an under estimate of the real number of CSPs each aphid possess, as analysis of the *A. pisum* genome revealed 13 CSPs (Zhou *et al.*, 2010) and a *T. citricida* CSP had previously been identified by Jacobs and colleagues (Jacobs *et al.*, 2005). CSPs in aphids are not well studied and so the role(s) in aphid biology of the two CSPs currently identified for *M. persicae*, OS-D1 and Mp10 (OS-D2) are unclear. Ghanim and colleagues (Ghanim *et al.*, 2006) found *Mp10* expression was higher in alates than apterous aphids. They also found *Mp10* was ubiquitously expressed across the aphid body, with significantly higher expression in legs and antennae. We previously found *Mp10* was highly expressed in the head and salivary glands, but not in the gut (Appendix A (Bos *et al.*, 2010)). These observations suggest Mp10 is not involved solely in antennal chemoperception. In the aphid species *M. viciae, OS-D1* and *OS-D2* were found to have similar expression patterns across antennae, heads minus antennae, legs and the whole body (Jacobs *et al.*, 2005). However, protein for OS-D1 was not detected in the head, but only the antennae and legs, whereas protein for OS-D2 was detected in the heads minus the antennae (Jacobs *et al.*, 2005). OS-D2 is unlikely to bind twenty-eight different compounds known to elicit an electrophysiological response in electroantennograms or in single olfactory neurone preparations (Table 7.1), whilst a similar study could not be conducted for OS-D1 (Jacobs *et al.*, 2005). This raises further questions as to what OS-D2's role in the aphid might be. The conservation of the CSP sequences across several aphid species led Jacobs and colleagues to conclude that the gene may have an important function.

Until recently, the role of individual *M. persicae* genes in the aphid-plant interaction has not been studied. The work in chapter 3 was one of the first examples of expressing aphid genes *in planta* in order to study their role in the interaction. The Hogenhout lab recently developed plant-mediated RNAi for *M. persicae* that allows knock down of specific aphid genes by expressing dsRNA to these genes in the plant (Pitino *et al.*, 2011). This technology facilitates analysis of the role of effectors in aphid-plant interactions.

Compounds used for binding assays	
(*E*)-2-hexenal	Chrysanthenone
1-octen-3-ol	Methylfurfiral
2-phenylethanol	Linalool
4-pentenyl isothiocyanate	Verbenol
α-pinene	Humulene
Methyl salicylate	Thujone
EBF	Benzaldehyde
(*E,E*)-α-farnesene	Carvone
1*R*,4a*S*,7*S*,7a*R*-nepetalactol	6-methyl-5-hepten-2-one
(4a*R*,7*S*,7a*S*)-nepetalactol	Camphor
(4a*S*,7*S*, 7a*R*)-nepetalactone	(1*S*)-(+)-fenchone
(*Z*)-jasmone	(1*R*)-(-)-fenchone
(*Z*)-3-hexenol	(*Z*)-3-hexenyl acetate
(E)-caryophyllene	(-)-(1*R*,5*S*)-myrtenal

Table 7.1 – Compounds identified as being unlikely to bind OS-D2 in Jacobs *et al.,* 2005

Plants recognise conserved molecules of pathogens such as flagellin (Felix *et al.*, 1999), which elicit an immune response. Elicitors are perceived by transmembrane PRRs at the plant cell membrane leading to various downstream signalling events including a Ca^{2+} burst, ROS burst, and MAPK cascades, which in turn contribute to later events, including changes in gene expression, and callose deposition (Boller and Felix, 2009). In *N. benthamiana* it was recently shown that the Ca^{2+} burst is upstream of two parallel signalling cascades, one leading to MAPK activation and then gene expression, the other leading to ROS production (Segonzac *et al.*, 2011). We previously found that whole *M. persicae* extracts trigger readouts of plant immunity in *N. benthamiana*, such as Ca^{2+} bursts and ROS bursts (chapter 4). Fractionation of the whole extract identified that the readouts were primarily caused by the more than 10 kDa fraction and to a lesser extent by the less than 3 kDa fraction. The more than 10 kDa elicitor was present in several aphid species (chapter 4).

Pathogens secrete many effector proteins to evade and/or suppress PTI (Dodds and Rathjen, 2010). Suppression of PTI is important for successful colonization of host plants (Jones and Dangl, 2006). Many different steps in the PTI signalling pathway are targeted by bacterial effectors. For example, the PtoDC3000 effector AvrPtoB acts at the plasma membrane to target the PRRs FLS2 and CERK1 for degradation (Göhre *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). Bacterial pathogens also target other signalling

components of the pathway, such as using extracellular polysaccharides (EPS) to chelate apoplastic Ca^{2+} and prevent the Ca^{2+} burst in PTI signalling (Aslam *et al.*, 2008), and targeting MAPKs through the effectors HopF2, HopAl1 and AvrB (Cui *et al.*, 2010; Wang *et al.*, 2010; Zhang *et al.*, 2012b).

To investigate if Mp10 has a function in aphid-plant interactions, we used plant-mediated RNAi to knock down the expression of *Mp10* and examined the ability of Mp10 to suppress the plant immune response triggered by aphid elicitors. We then investigated where in the elicitor-triggered signalling cascade Mp10 acts and which parts of the protein were necessary for the suppression phenotype. Finally, we investigated if Mp10 homologs of other aphids have similar properties as *M. persicae* Mp10.

7.2 Results

Knocking down expression of *Mp10* in *M. persicae* by *N. benthamiana*-mediated RNAi may associate with reduced aphid fecundity.

To further investigate if Mp10 has an important function in aphids (Jacobs *et al.*, 2005), we used plant-mediated RNAi technology for knocking down Mp10 expression. This technology was previously used for the successful knock-down of *MpC002* expression leading to reduced *M. persicae* fecundity (Pitino *et al.*, 2011). We used *N. benthamiana* leaf disc assays that involves *A. tumefaciens*-mediated transient expression of constructs encoding aphid dsRNAs in *N. benthamiana* leaves. These leaf discs assays have been described previously (chapters 2 and 3). Leaf discs expressing dsRNAs corresponding to *GFP* and *MpC002* were used as controls. Aphids were transferred to fresh leaf disc plates every six days to ensure constant exposure to the dsRNAs. *M. persicae* survival and reproduction on the leaf discs were recorded over a 17 day period, at which time the aphids were collected for RNA extractions and analysis of gene expression levels by qRT-PCRs. The gene expression level of *MpOS-D1*, encoding a CSP similar to Mp10, was also investigated to test the specificity of the *Mp10* silencing construct. Three of the six biological replicates of the assay showed downregulation of the intended target gene (*MpC002* or *Mp10*), whereas the other three replicates did not. The possible reasons for this difference are explained in the discussion. In the replicates showing reduced gene expression the expression of the intended target gene was reduced by an average of 40-50% compared to the dsGFP control, and non-target genes were also sometimes reduced but not significantly or to the same degree as the target gene (Figure 7.1A). Expression of dsMpC002 in *N. benthamiana* led to significant downregulation of *MpC002* (Student's *t*-test, $P = 0.006$), but not *Mp10* in *M. persicae* (Student's *t*-test, $P = 0.067$) (Figure 7.1A). Conversely, dsMp10 expression in *N. benthamiana* led to significant downregulation of *Mp10* (Student's *t*-test, $P = 0.008$), but not *MpC002* in *M. persicae* (Student's *t*-test, $P = 0.061$) (Figure 7.1A). Neither dsMpC002 nor dsMp10 expression in *N. benthamiana* led to significant downregulation of *MpOS-D1* in the aphids (Student's *t*-test, dsMpC002 $P = 0.187$, dsMp10 $P = 0.077$) (Figure 7.1A).

M. persicae feeding from leaf discs expressing dsMp10 produced less progeny (nymphs) than aphids fed on leaf discs expressing dsGFP in the six biological replicates (ANOVA, $P < 0.05$) (Figure 7.1B). Nymph production by aphids fed on leaf discs expressing dsMpC002 was also significantly lower than the dsGFP control (ANOVA, $P < 0.05$) (Figure 7.1B). These results suggest that downregulation of *Mp10* may be associated with a decrease in aphid fecundity, and therefore imply the possibility that

Mp10 has an essential function in aphids.

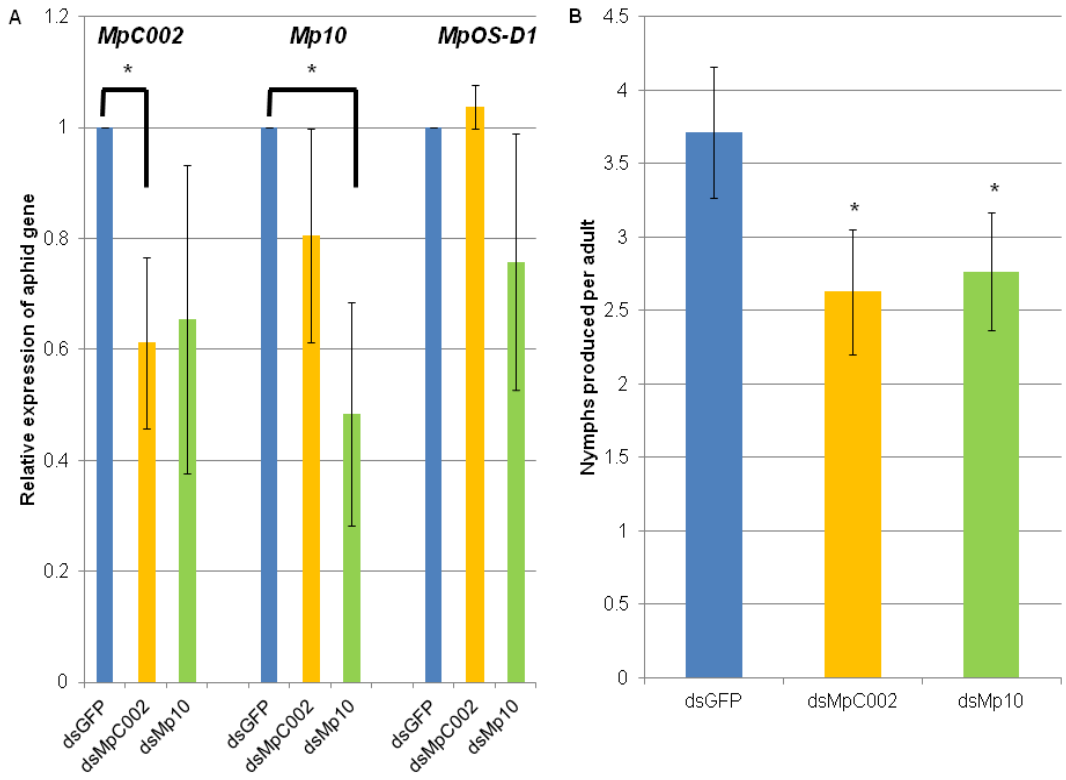


Figure 7.1 – Knocking down expression of *Mp10* in *M. persicae* by *N. benthamiana*-mediated RNAi may associate with reduced aphid fecundity.

(A) *Mp10* expression is down-regulated in aphids fed on *N. benthamiana* leaves transiently expressing dsMp10 RNAs, whilst *MpOS-D1* expression is uneffected. Aphids fed on transgenic *N. benthamiana* leaf discs for 17 days were harvested and analyzed for downregulation of *Mp10, MpOS-D1* and *MpCOO2* by qRT-PCR. Aphids fed on *N. benthamiana* leaf discs expressing dsMpC002 were used as positive controls. Data shown are means ± SE of three biological replicated with n = 3 per replicate. Asterisk indicate significant differences in treatments compared to dsGFP (Student's *t*-test, n = 3, $P < 0.05$). Three of the six biological replicates in which knock down of *M. persicae Mp10* and *MpC002* was observed are shown. The other three biological replicates did not show significant silencing of target genes.

(B) *M. persicae* feeding from dsMp10 leaf discs are less fecund. Nymphs produced by aphids feeding on *N. benthamiana* leaf discs expressing aphid gene specific dsRNAs were analysed and compared to control aphids exposed to dsGFP. Data shown are average number of nymphs produced per adult aphid with means ± SE of six biological replicates with n = 3–6 leaf discs per replicate. Asterisks indicate significant differences in treatments compared to dsGFP (ANOVA, n ≥ 28, $P < 0.05$). All the replicates are shown.

Mp10 suppresses ROS bursts triggered by *M. persicae* elicitors in *N. benthamiana*

We previously found that Mp10 suppresses the flg22-triggered ROS burst in *N. benthamiana* (chapter 3). We also found that whole *M. persicae* extracts trigger ROS bursts in *N. benthamiana* and that aphid fractions of less than 3 kDa and larger than 10 kDa are major contributors to the ROS bursts (chapter 4). The next step is to determine if Mp10 suppresses the ROS burst triggered in *N. benthamiana* by aphid elicitors. We used a luminol based assay as previously described. Mp10 was able to suppress the ROS burst triggered by whole *M. persicae* extract (Student's *t*-test, $P < 0.001$) to the same degree as the AvrPtoB control (Student's *t*-test, $P < 0.001$) (Figure 7.2A). Mp10 also suppressed the ROS burst triggered by the less than 3 kDa (Student's *t*-test, $P < 0.015$) (Figure 7.2B) and the more than 10 kDa fractions (Student's *t*-test, $P < 0.036$) (Figure 7.2C), although the suppression activitiers of AvrPtoB were stronger in both cases (Student's *t*-test, $P < 0.001$) (Figure 7.2B and C). These data show that Mp10 is capable of suppressing the ROS burst triggered by *M. persicae* elicitors. Thus, Mp10 may have a function in plant-aphid interactions.

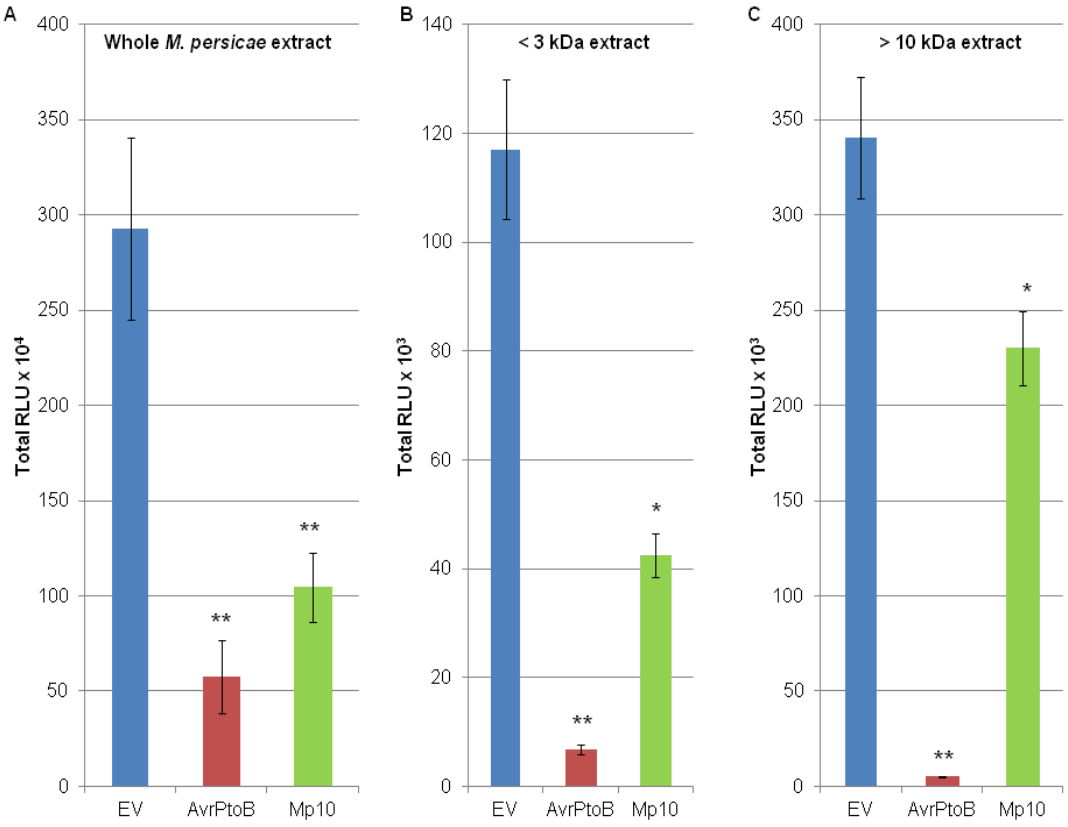


Figure 7.2 – Mp10 suppresses ROS bursts triggered by *M. persicae* elicitors in *N. benthamiana*.

Mp10 can suppress the ROS burst triggered by whole *M. persicae* extract (A), less than 3 kDa fraction of *M. periscae* (B) and more than 10 kDa fraction of *M. persicae* (C) in *N. benthamiana*.

(A-C) The ROS bursts were measured using a luminol-based assay in *N. benthamiana* leaf discs transiently expressing Mp10, EV or AvrPtoB (positive control). Results are mean ± SE (n = 24 leaf discs per replicate). These experiments were repeated three times with similar results, with one representative experiment shown. Asterisks indicate significant differences in treatment compared to EV (Student's *t*-test, n = 24, **P* < 0.05, ***P* < 0.001).

Mp10 suppresses PAMP-triggered Ca^{2+} bursts in *N. benthamiana*

We wanted to better understand how Mp10 suppresses elicitor-triggered ROS bursts in *N. benthamiana*. Mp10 suppresses flg22-triggered but not chitin-triggered ROS bursts in *N. benthamiana* (chapter 3). The flg22-triggered ROS bursts in *N. benthamiana* require BAK1/SERK3 (Heese *et al.*, 2007), whilst chitin-triggered ROS bursts require CERK1 in *N. benthamiana* (Segonzac *et al.*, 2011). We found that the *M. persicae* extract-elicited ROS burst of *N. benthamiana* does not require BAK1/SERK3 (chapter 4). Nonetheless, Mp10 suppresses *M. persicae* extract-elicited ROS bursts in *N. benthamiana* (above) suggesting that Mp10 may block signalling upstream of ROS induction. In *N. benthamiana* a Ca^{2+} burst occurs upstream of ROS bursts in PTI signalling (Segonzac *et al.*, 2011), and so we hypothesised that Mp10 may suppress PAMP-triggered Ca^{2+} bursts. To test this we transiently expressed Mp10, EV or AvrPtoB (positive control) in the SLJR15 line of *N. benthamiana* (Segonzac *et al.*, 2011) using *A. tumefaciens*-mediated transient assays. We then measured the Ca^{2+} burst, emitted as blue light, after elicitation by the PAMPs flg22 and chitin. We decided not to use fractions of *M. persicae* extract as we did not have experimental data linking the observed Ca^{2+} and ROS bursts. We found that Mp10 suppresses the Ca^{2+} burst triggered by flg22 (Student's *t*-test, $P < 0.023$) (Figure 7.3A), providing evidence that Mp10 is acting upstream of ROS triggered by the aphid elicitor(s). Surprisingly, Mp10 also suppressed the Ca^{2+} burst triggered by chitin (Student's *t*-test, $P < 0.007$) (Figure 7.3B), even though it does not suppress the chitin-triggered ROS burst. These data provide evidence that Mp10 suppresses the Ca^{2+} burst triggered by multiple PAMPs, suggesting that it is acting near the top of the PTI signalling cascade.

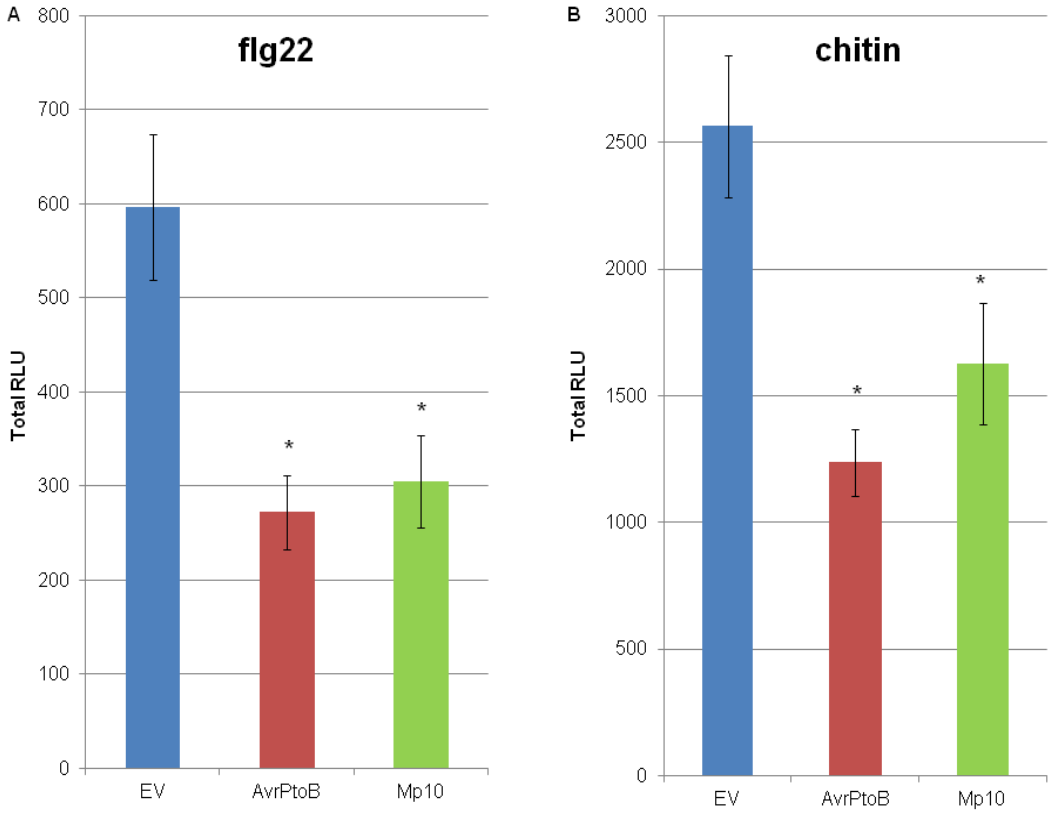


Figure 7.3 – Mp10 suppresses PAMP-triggered Ca^{2+} bursts in *N. benthamiana.*
Mp10 suppresses the Ca^{2+} burst triggered by flg22 (A) and chitin (B). (A and B) Ca^{2+} bursts were measured in the *N. benthamiana* SLJR15 line transiently expressing Mp10, EV or AvrPtoB (positive control) after elicitation by 100 nM flg22 (A) or 2 mg/ml chitin (B). Results shown are mean ± SE (n = 21 leaf discs per replicate). These experiments were repeated three times with similar results, with one representative experiment shown. Asterisks indicate significant differences in treatment compared to EV (Student's *t*-test, n = 21, $P < 0.05$)

N-terminal tagged Mp10 suppresses flg22-triggered ROS burst in *N. benthamiana.*
In order to assess which sections of Mp10 were important for its ability to suppress plant immune signalling, we wanted to use tagged proteins. Therefore, we first tested if N-terminal tagged Mp10 suppresses flg22-triggered ROS burst in *N. benthamiana*. *N. benthamiana* leaves transiently expressing FLAG-Mp10, GFP-Mp10 or GFP upon infiltration with *A. tumefaciens* were tested for ROS burst triggered by flg22 using a luminol based assay as previously described. We found that both tagged versions of Mp10 suppress the flg22-triggered ROS burst (ANOVA, $P < 0.05$). We decided to continue with the GFP tag as we would have the option to visualise the localisation of constructs in the future.

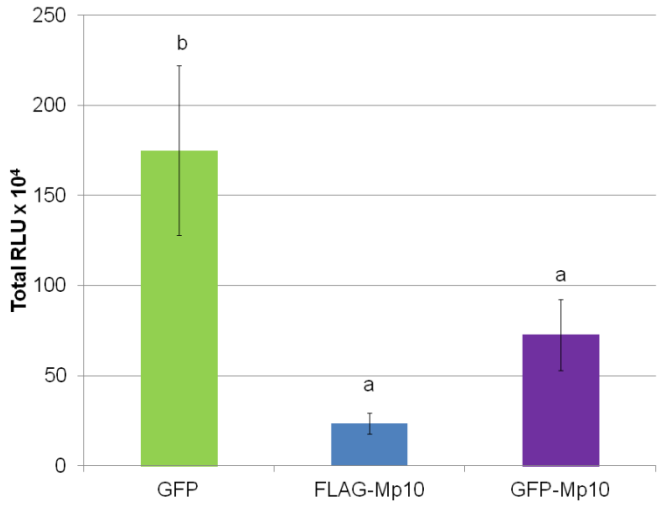


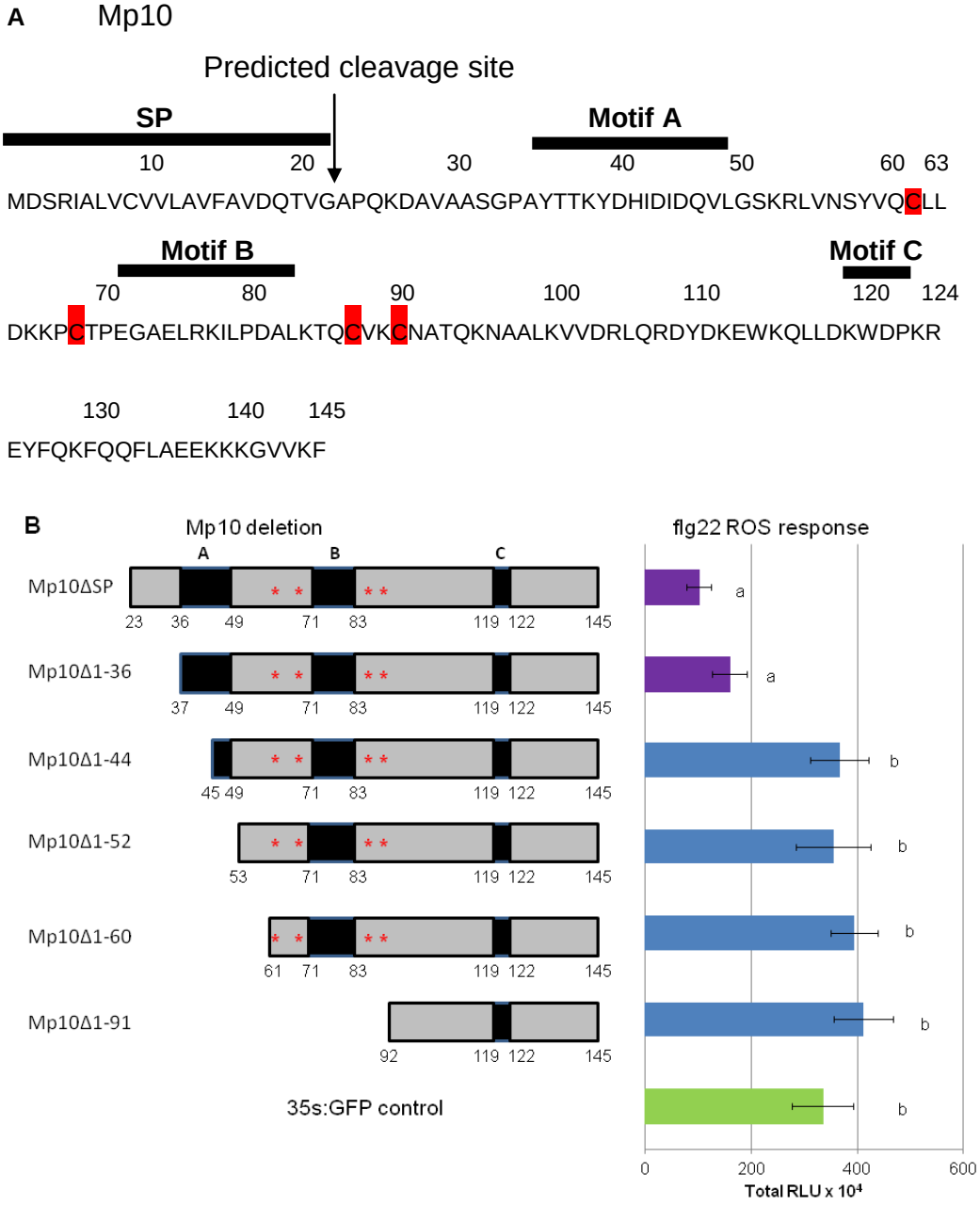
Figure 7.4 – N-terminal tagged Mp10 suppresses flg22-triggered ROS burst in *N. benthamiana*.

The ROS burst triggered by 200 nM flg22 was measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing Mp10 with N-terminal FLAG (FLAG-Mp10) or GFP (GFP-Mp10) tags, or GFP controls. Results shown are mean ± SE (n = 8 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

The N and C termini of Mp10 are likely to be required for suppressing flg22-triggered ROS burst in *N. benthamiana*.

Mp10 is a CSP (chapter 3) that contain four conserved cysteine residues and three conserved motifs (A, B and C) (Wanner *et al.*, 2004) (Figure 7.5A). In order to determine which sections of Mp10 are important for its ability to suppress plant immune signalling we created constructs containing GFP tagged versions of Mp10 with deletions in the N or C terminus. *A. tumefaciens* strains containing these constructs were then infiltrated into *N. benthamiana* along with GFP-Mp10 and GFP controls, and the ROS response triggered by flg22 was measured using a luminol based assay as previously described. Flg22 was used for triggering ROS bursts because of its greater reproducibility and the shorter duration of the ROS burst compared to aphid extract. Mp10 mutants that lack the first 22 amino acids at the N-terminus were unable to suppress flg22-triggered ROS bursts (ANOVA, $P < 0.05$) (Figure 7.5B). In addition, Mp10 mutants lacking the 27 amino acids at the C-terminus were unable to suppress flg22-triggered ROS bursts (ANOVA, $P < 0.05$) (Figure 7.5C). Taken together these data indicate both the N and C termini of Mp10 are involved in the immune signalling. The 22 and 27 amino acid regions at Mp10

N and C-termini do not contain any of the four cysteines indicating that the cysteines are not sufficient for Mp10 suppression activity



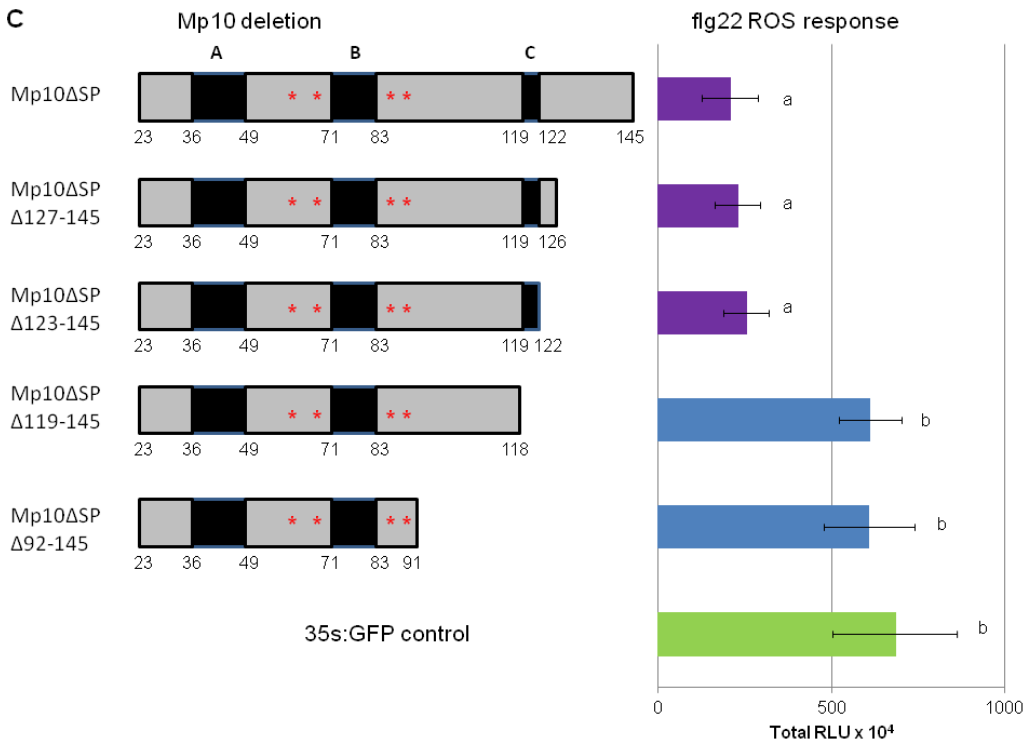


Figure 7.5 – The N and C termini of Mp10 are likely to be required for suppressing flg22-triggered ROS burst in *N. benthamiana*.

(A) Amino acid sequence of Mp10. Motifs as defined in Wanner *et al*., 2004. C indicate conserved cysteines. SP = signal peptide.

(B) The N terminus of Mp10 is required for suppression of flg22 ROS burst. (C) The C terminus of Mp10 is required for suppression of flg22 ROS burst. (B and C) The ROS burst triggered by 200 nM flg22 was measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing GFP tagged Mp10 deletion constructs, GFP-Mp10 (positive control), or GFP control. Results shown are mean ± SE (n = 8 leaf discs per replicate). Purple bars denote constructs that caused significant suppression of ROS bursts. The green bars denote the GFP control. These experiments were repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$. Red asterisks denote the position of conserved cysteines.

Removal of Tyr (40) and Trp (120) from Mp10 disrupts its flg22-triggered ROS suppression phenotype

In order to determine which specific amino acids were involved in the ability of Mp10 to suppress plant immune signalling we created constructs containing GFP tagged version of Mp10 with deletions in the N terminus between residues 37 and 45 and between residues 118 and 122 in the C terminus (Figure 7.6A). *A. tumefaceins* straining containing these constructs were then infiltrated into *N. benthamiana* along with GFP-Mp10 and GFP controls, and the ROS response triggered by flg22 was measured using a luminol

based assay as previously described. We found that removing the first 18 amino acids from the N terminus of the mature Mp10 sequence resulted in loss of the flg22-triggered ROS burst suppression (ANOVA, $P < 0.05$) (Figure 7.6B), and thus the tyrosine at position 40 was necessary for the ROS burst suppression phenotype. Removing the final 26 amino acids from the C terminus of Mp10 also resulted in loss of the flg22-triggered ROS burst suppression (ANOVA, $P < 0.05$) (Figure 7.6C), and thus the tryptophan at position 120 was necessary for the ROS burst suppression phenotype.

A

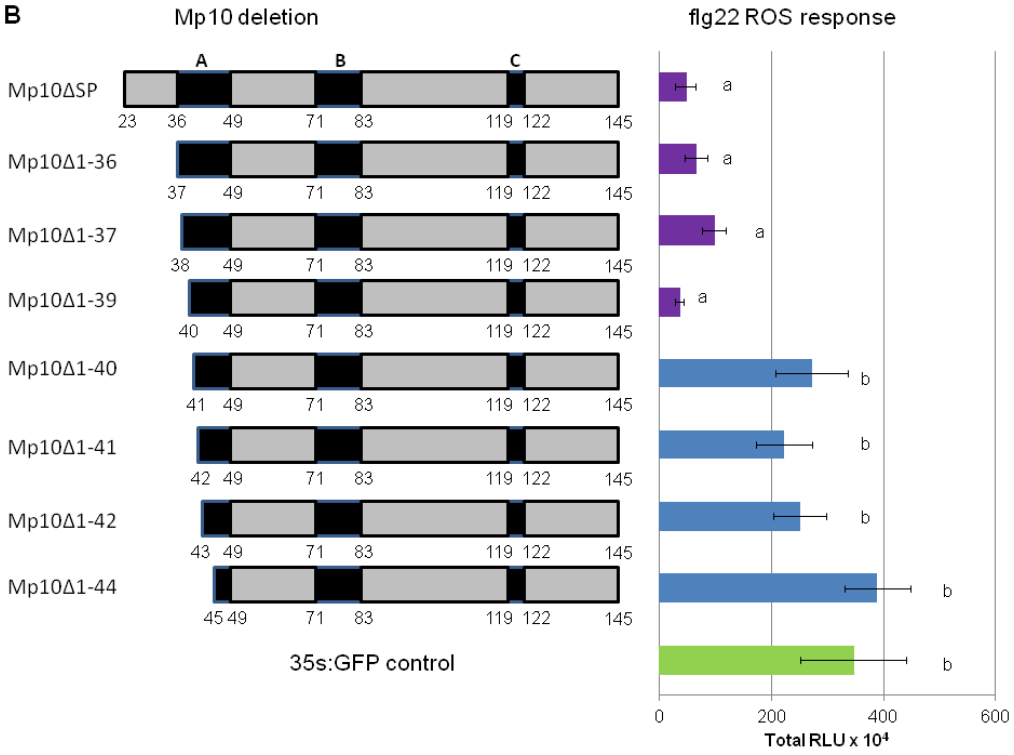
Motif A

Y	T	T	K	Y	D	H	I	D
36	37	38	39	40	41	42	43	44

I	D	Q	V	L
45	46	47	48	49

Motif C

K	W	D	P
119	120	121	122



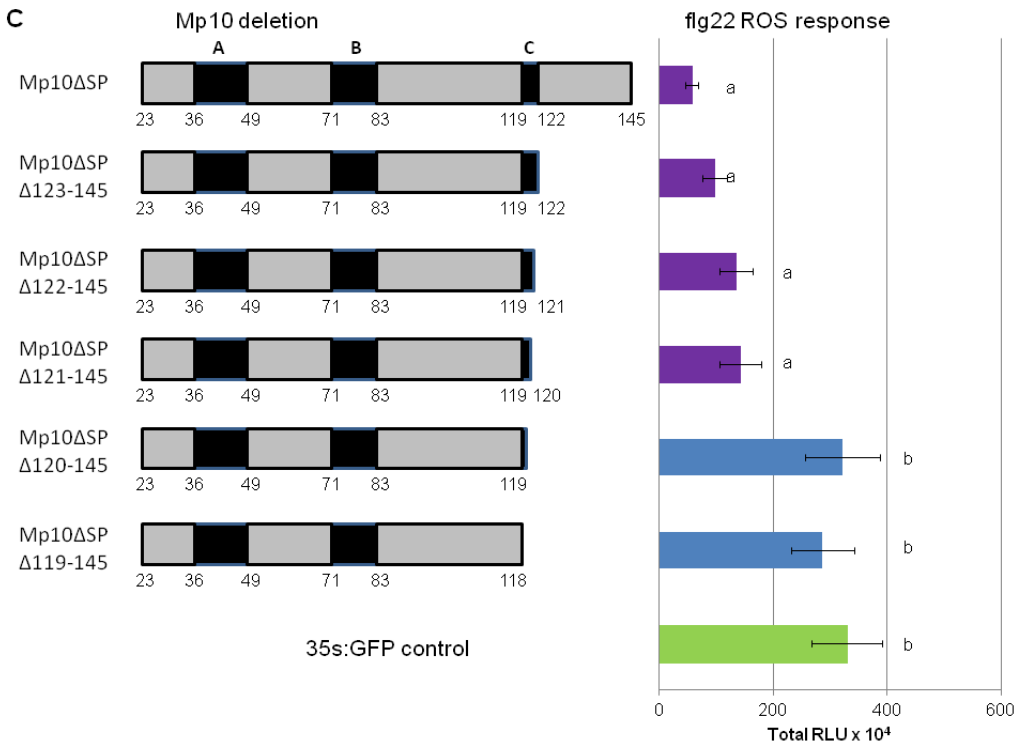


Figure 7.6 – Removal of Tyr (40) and Trp (120) from Mp10 disrupts its flg22-triggered ROS suppression phenotype.

(A) Amino acid sequence of Mp10 motifs A and C, with residue number. Motifs as defined in Wanner *et al*., 2004.

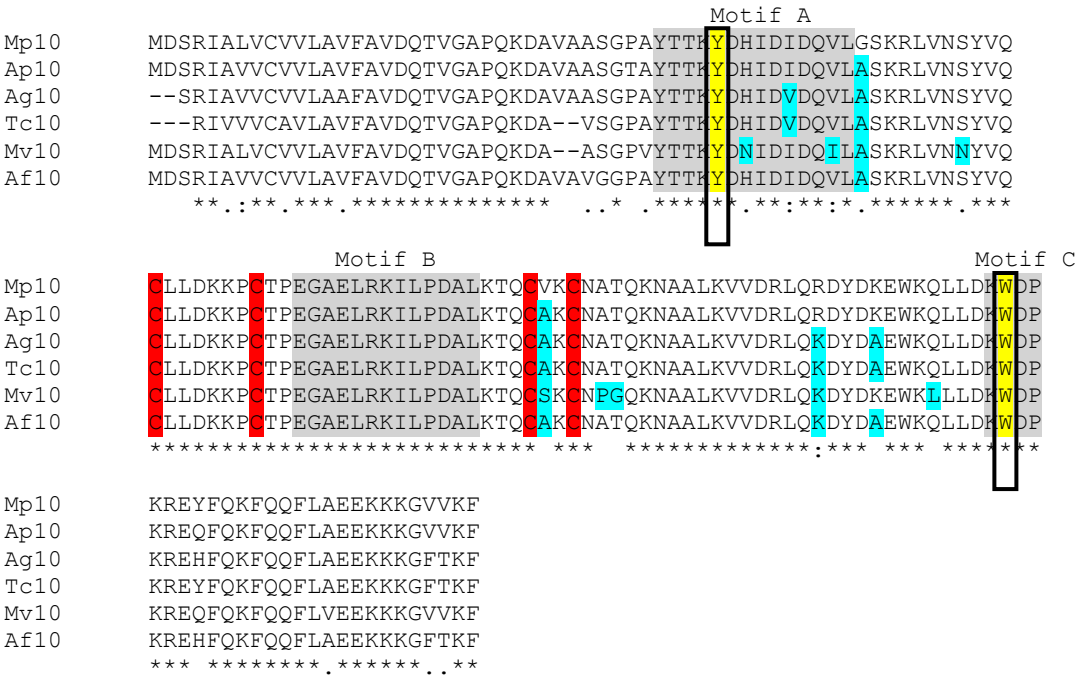
(B) Removal of the 40th amino acid residue of Mp10 removes the flg22-triggered ROS suppression phenotype. (C) Removal of the 120th amino acid residue of Mp10 removes the flg22-triggered ROS suppression phenotype. (B and C) The ROS burst triggered by 200 nM flg22 was measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing GFP tagged Mp10 deletion constructs, GFP-Mp10 (positive control), or GFP control. Results shown are mean ± SE (n = 8 leaf discs per replicate). Purple bars denote constructs that caused significant suppression. Green bars denote the GFP control. These experiments were repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

Mp10 homologs in other aphid species suppress flg22-triggered ROS in *N. benthamiana*

We wished to investigate if homologs of Mp10 in other aphid species possessed the same immune signalling suppression phenotype as *M. persicae* Mp10. Mp10 homologs in other aphids are very similar in amino acid sequence to Mp10 (Jacobs *et al.*, 2005), although several amino acid sequence differences exist within the part of the sequence involved in immune signalling suppression (Figure 7.7A). We cloned the homologs from *A. pisum* (*Ap10*) and *A. gossypii* (*Ag10*) and tested their ability to suppress the flg22-

triggered ROS burst in *N. benthamiana* using a luminol based assay and infiltration with *A. tumefaciens* as previously described. Ap10 contains two amino acid sequence differences in this area, and Ag10 contains the two amino acid sequence differences of Ap10 plus three additional ones. Ap10 expressed in the pCB302-3 vector suppressed the flg22-triggered ROS burst in *N. benthamiana* to the same level as Mp10 (ANOVA, $P < 0.05$) (Figure 7.7B). Ag10 with an N-terminus GFP tag also suppressed the flg22-triggered ROS burst in *N. benthamiana* to the same level as Mp10 (ANOVA, $P < 0.05$) (Figure 7.7C). These data suggest that Mp10 homologs in other species are capable of suppressing immune signalling in *N. benthamiana*.

A



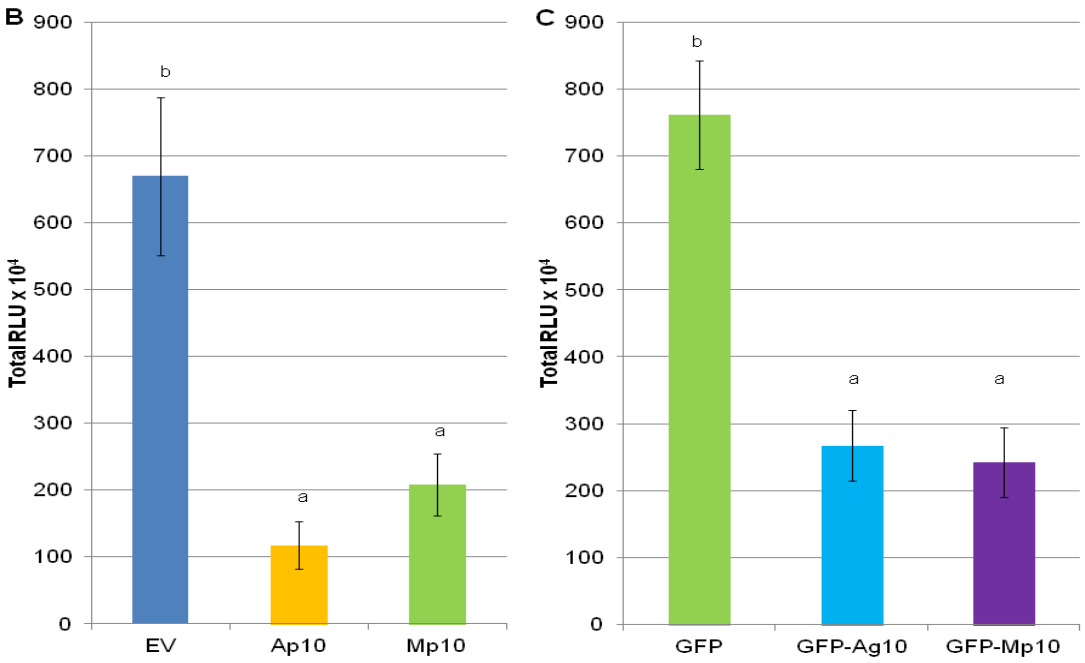


Figure 7.7 – Mp10 homologs in other aphid species suppress flg22-triggered ROS in *N. benthamiana*.

(A) Mp10 homologs are conserved in other aphid species. Clustal Omega alignment of amino acid sequences of *M. persicae* Mp10 and Mp10 homologs of various aphid species. Grey residues denote conserved motifs. Red residues denote conserved cysteines. Yellow residues denote the 40th and 120th residues of the *M. persicae* Mp10 sequence, necessary for immune signalling suppression. Blue residues denote differences from *M. persicae* Mp10 sequence within the part of the sequence necessary for immune signalling suppression. Motifs as defined in Wanner *et al.,* 2004. Ap = *A. pisum*, Ag = *A. gossypii*, Tc = *T. citricida,* Mv = *M. viciae*, and Af = *A. fabae*. Accession numbers are Ap10 NP_001119652, Ag10 ACJ64044, Tc10 CAJ01481, Mv10 CAG25435, and Af10 CAG25440.

(B) Ap10 suppresses flg22-triggered ROS bursts. The ROS bursts triggered by 100 nM flg22 were measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing Ap10, Mp10 or EV control. Results shown are mean ± SE (n = 8 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

(C) Ag10 suppresses flg22-triggered ROS bursts. The ROS bursts triggered by 200 nM flg22 were measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing GFP-tagged Ag10, Mp10 or GFP control. Results shown are mean ± SE (n = 8 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

MpOS-D1 may not suppress flg22-triggered ROS in *N. benthamiana*

Whilst Mp10 homologs between aphid species are similar in amino acid sequence another CSP within *M. persicae*, MpOS-D1, is less similar. MpOS-D1 contains amino acid

sequence differences in the conserved motifs as well as the rest of the sequence. These differences include substitutions at the Tyr (40) and Trp (120) residues identified in Mp10 (Figure 7.8A). To test if MpOS-D1 was functionally redundant with Mp10 we cloned *MpOS-D1* and tested its ability to suppress the flg22-triggered ROS burst in *N. benthamiana* using a luminol based assay and infiltration with *A. tumefaciens* as previously described. MpOS-D1 was not able to suppress the flg22-triggered ROS burst (ANOVA, $P > 0.05$ (n.s.)) (Figure 7.8B) suggesting that it may not share the same function *in planta* as Mp10. However, the level of protein expression produced by the MpOS-D1 construct needs to be assessed in order to confirm this. The inability to suppress flg22-triggered ROS burst result also demonstrated that the pattern of conserved cysteines and other conserved residues between Mp10 and MpOS-D1 may not be sufficient to confer immune signalling suppression ability in *N. benthamiana*.

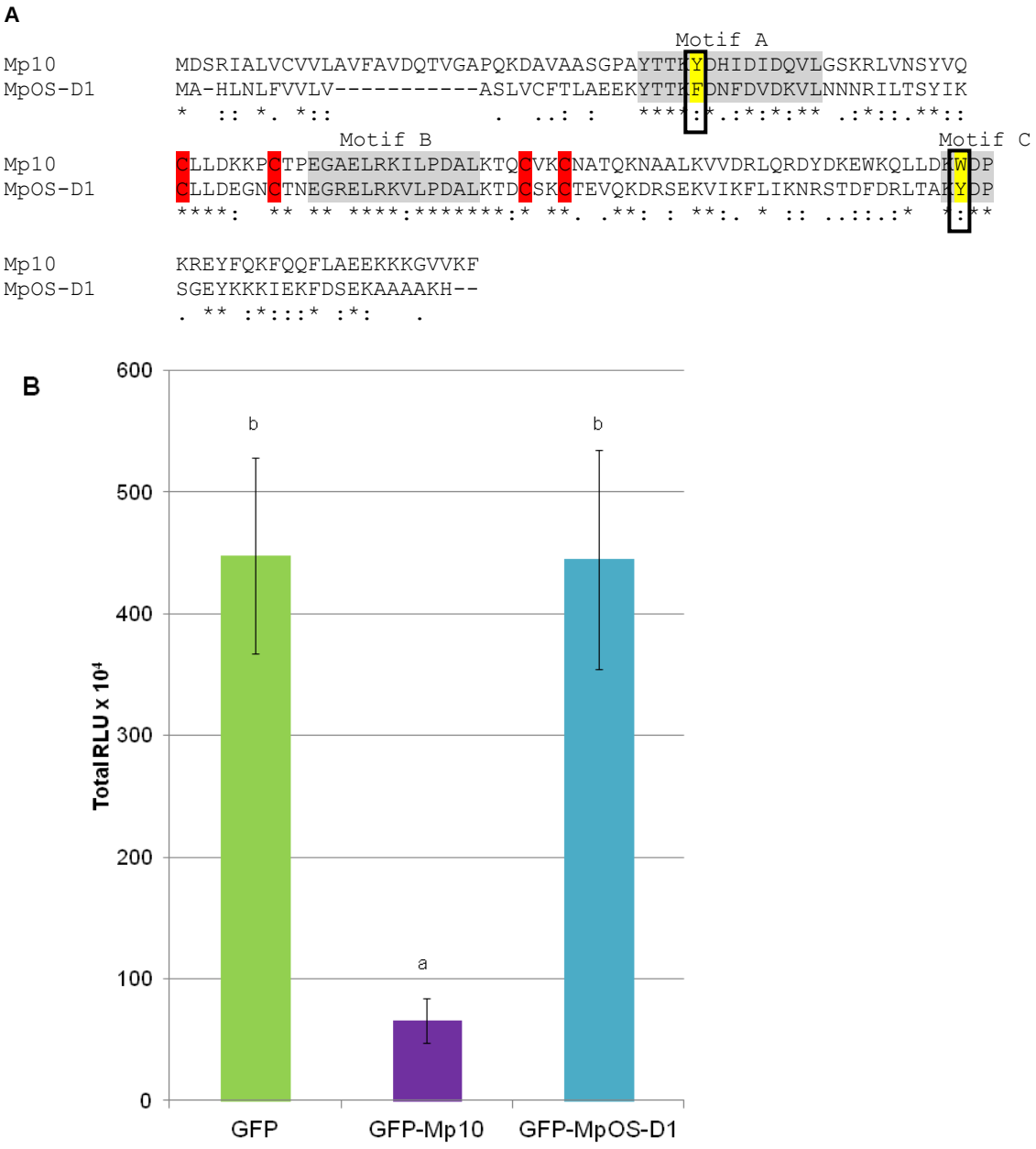


Figure 7.8 – MpOS-D1 may not suppress flg22-triggered ROS in *N. benthamiana*.

(A) Mp10 and MpOS-D1 differ in amino acid sequence. Clustal Omega alignment of amino acid sequences of Mp10 and OS-D1. Grey residues denote conserved motifs. Red residues denote conserved cysteines. Yellow residues denote the position of the 40th and 120th residues of the Mp10 sequence, necessary for immune signalling suppression.

(B) MpOS-D1 does not suppress flg22-triggered ROS burst. The ROS burst triggered by 200 nM flg22 was measured using a luminol-based assay in *N. benthamiana* leaves transiently expressing GFP-tagged MpOS-D1, Mp10 or GFP control. Results shown are mean ± SE (n = 8 leaf discs per replicate). This experiment was repeated three times with similar results, with one representative experiment shown. Letters indicate significant differences at $P < 0.05$.

7.3 Discussion

We investigated the possibility that the CSP Mp10 is involved in suppressing plant immunity to aphids. We found that *Mp10* may be an important gene for aphids, as knocking down gene expression may be associated with a decrease in aphid performance on *N. benthamiana*. We also showed that Mp10 is capable of suppressing the ROS burst triggered by aphid elicitors and that the suppression might occur at or above the Ca^{2+} burst in immune signalling. In addition, we found that motifs conserved amongst CSPs were necessary for this. Immunity suppression was seen for Mp10 homologs of other aphids, but potentially not for the related CSP OS-D1 of *M. persicae*, suggesting a specific conserved function for Mp10.

Attempting to knock down *Mp10* gene expression through RNAi was associated with a decrease in aphid fecundity. This result provides initial evidence that *Mp10* is an important gene for the aphid, supporting the hypothesis of Jacobs and colleagues (Jacobs *et al.*, 2005). It is possible that the decrease in Mp10 in the saliva of the aphid leads to higher levels of plant defence, which leads to decreased aphid performance. However, *Mp10* is expressed in areas other than the salivary glands, such as the legs (Jacobs *et al.*, 2005; Ghanim *et al.*, 2006), and possibly aphid tissues that may affect aphid development. Decreased levels of Mp10 in these parts of the aphid may have led to reduced fecundity. Use of the electrical penetration graph (EPG) system on aphids with knocked down *Mp10* expression may provide information about whether feeding behaviour was affected by decreased levels of Mp10. Normal feeding behaviour in these aphids would advocate that Mp10 was not involved in the plant-aphid interaction, whereas abnormal feeding behaviour would suggest either that Mp10 is involved in the plant-aphid interaction, or in a very fundamental internal aphid process that instigates a decreased ability to feed.

Three of the six replicates of the RNAi assay showed silencing of the intended target gene of *MpC002* or *Mp10*. This data is at odds with that previously published by the Hogenhout lab which showed that the RNAi assay using *N. benthamiana* leaf discs led to significant gene expression knockdown of *MpC002* and decreased aphid fecundity (Pitino *et al.*, 2011), although in that study qRT-PCR was only conducted with three of the six replicates of the experiment rather than all six. There may be several reasons why the aphids I conducted qRT-PCR on did not show knockdown of the intended target. Firstly, *Mp10* expression levels were only analysed in aphids alive at the end of the experiment. It may be that some of these aphids were alive because they did not have significant levels of gene knockdown, and that aphids with high levels of gene knockdown had already died. Secondly, aphids numbers collected at the end of the experiment were

small, and therefore the concentrations of RNA extracted were low, potentially affecting the results. Thirdly, RNAi of *M. persicae* genes has been effective for genes expressed predominately in the salivary glands and gut (Pitino *et al.*, 2011). *Mp10* is also expressed in legs and antennae (Jacobs *et al.*, 2005; Ghanim *et al.*, 2006). Data do not exist as to whether it is possible to silence aphid genes expressed in the antennae or legs, although genes have been silenced in the antennae of other insects such as the light brown apple moth (*Epiphyas postvittana*) and Southern house mosquito (*Culex quinquefasciatus*) (Turner *et al.*, 2006; Pelletier *et al.*, 2010). Dissection of *Mp10* silenced aphids could be carried out to determine whether all tissues are silenced to the same degree. Fourthly, I did not have enough time to test the production of siRNAs by the Mp10 construct. This could be done by a siRNA northern blot or qRT-PCR. However, the production of siRNAs has already been shown for my control constructs (Pitino *et al.*, 2011), and yet there was not significant knockdown of *MpC002* expression in half the replicates. Checking expression of siRNAs for each leaf disc during the experiment would be impractical as the assay is already time consuming.

The replicates of the experiment that did show reduction in gene expression of the intended target gene also showed a trend of reduction, albeit not significant, in non-target genes. This may suggest that the constructs knockdown the expression of non-targets. This possibility was investigated for *MpC002* and found to be unlikely based on computer program predictions (Alex Coleman, personal communication) but has not be investigated for *Mp10*. Alternatively, there may be affects on the expression of other aphid genes from the down regulation of certain genes that are separate from the ingestion of siRNAs. This is yet to be studied for *M. persicae*, but could be investigated by conducting microarrays on aphids fed siRNAs.

Stable transgenic expression of RNAi constructs leads to larger decreases in aphid gene expression than transient expression (Pitino *et al.*, 2011). To further investigate the importance of Mp10 in aphids we started the production of stable transgenic *A. thaliana* plants expressing dsMp10. We also started to investigate if the loss of fecundity of aphids with reduced Mp10 expression can be restored to that of aphids on wild type *A. thaliana* plants, and so *bak1-5* plants producing dsGFP and dsMp10 are being generated. However, there was insufficient time to conduct assays with these plant lines.

Mp10 is capable of suppressing the ROS bursts triggered by whole and fractionated *M. persicae* extract, although its suppression of the <3 kDa fraction ROS burst was more pronounced than the suppression of the >10 kDa fraction ROS burst. We previously showed that the fractionated *M. persicae* extract was able to evoke an induced resistance response in *N. benthamiana* to *M. persicae* (chapter 4). However, it is not

possible to test whether Mp10 can suppress this induced resistance because expression of Mp10 in *N. benthamiana* leads to decreased aphid fecundity as well, possibly because Mp10 triggers ETI (chapter 3). Given that *M. persicae* effectively colonises *A. thaliana* and *N. benthamiana* and if Mp10 is a genuine effector, it is likely that another aphid effector suppresses Mp10-mediated ETI. The role of Mp10 in suppressing plant immunity may be investigated in greater detail upon characterisation of plant proteins that mediate Mp10-mediated ETI.

Mp10 was previously found to suppress ROS bursts in *N. benthamiana* triggered by flg22 but not by chitin (chapter 3). These two PAMP signalling pathways require different genes, with the flg22 pathway requiring FLS2 and BAK1 (Heese *et al.*, 2007) and chitin pathway requiring CERK1 (Gimenez-Ibanez *et al.*, 2009). ROS bursts triggered by *M. persicae* elicitors was found to not involve BAK1 (and hence FLS2, which is dependent on BAK1 for signalling) (chapter 4). It seems unlikely that plant perception of *M. persicae* elicitors requires CERK1, given the properties of the elicitors (chapter 4) and that Mp10 can suppress the ROS burst triggered by the elicitors but not chitin. Therefore it is most likely that in *N. benthamiana* the *M. persicae* elicitors are perceived by a BAK1 and CERK1 independent signalling pathway, which Mp10 is blocking.

The significant decrease in ROS burst in *N. benthamiana* leaves expressing the bacterial effector AvrPtoB implies that the *M. persicae* elicitors are perceived by plant RLKs. AvrPtoB targets the RLKs FLS2, BAK1 and CERK1 in order to suppress plant immune signalling (Göhre *et al.*, 2008; Shan *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). Whilst we know that *M. persicae* elicitor perception in *N. benthamiana* does not involve BAK1 (and therefore FLS2 also) we have also not ruled out the possibility that CERK1 is involved in, although it seems unlikely (as discussed in the previous paragraph and in chapter 4). It is possible that AvrPtoB targets other membrane RLKs in addition to FLS2, BAK1 and CERK1, and that one of these unidentified RLKs is involved in *M. persicae* elicitor perception.

Mp10 suppression of chitin triggered Ca^{2+} burst is surprising given that Mp10 suppresses chitin triggered ROS (chapter 3), and chitin triggered ROS requires the chitin triggered Ca^{2+} burst in *N. benthamiana* (Segonzac *et al.*, 2011). A possible explanation is that a certain threshold of Ca^{2+} is necessary to trigger the ROS burst. Mp10 is slightly less effective at suppressing Ca^{2+} induction than AvrPtoB and the slightly higher Ca^{2+} levels in the Mp10 treatment may be sufficient to trigger a ROS burst.

As previously discussed in chapter 4, the Ca^{2+} assays conducted in this chapter were also done without normalising the Ca^{2+} measurements after the assay by discharging the remaining aequorin. As before, I do not think that this invalidates the conclusions I have drawn from the data, as stable transgenic lines and appropriate

controls were used to produce a repeatable result. There is a possibility that *Mp10* expression in the plant may alter the expression of some of the plant genes but I think it unlikely that it would decrease the expression of the aequorin gene under a 35S promoter. However, these assays could be repeated in order to remove any doubt about their conclusions.

If Mp10 is indeed suppressing multiple immune signalling pathways then it is either targeting multiple genes in different pathways, or a common point in multiple pathways. The fact that Mp10 is suppressing the Ca^{2+} burst triggered by flg22 and chitin favours the explanation that Mp10 is acting at the same point in multiple pathways, which is at or above the Ca^{2+} burst. If so, then it is logical that Mp10 also suppresses defences triggered by *M. persicae* elicitors. Mp10 would then be suppressing the Ca^{2+} burst of (potentially) three different plant immune signalling pathways, further supporting the likelihood that it is acting at a common point in the three pathways. Mp10 may indiscriminately block Ca^{2+} channels in *N. benthamiana* leaves, although because CSPs are thought to bind small molecules it seems unlikely. To further investigate this possibility the Ca^{2+} assay could be repeated with an abiotic stress that induces a Ca^{2+} burst such as extracellular sodium or low temperature (Dodd *et al.*, 2010).

We were interested to know which region(s) of the Mp10 protein were necessary for the immunity suppression phenotype. The initial assays to assess the affect of tagging Mp10 would have benefitted from an untagged Mp10 control, and from varying the terminal to which the tag was attached. However, the addition of an N-terminal GFP tag to Mp10 reduced flg22-triggered ROS bursts sufficiently. Hence, more experiments were thought unnecessary.

I did not have sufficient time left to check if all Mp10 deletion mutants were produced *in planta* using western blots. In instances where the Mp10 mutant does suppress flg22-triggered ROS bursts we can assume that sufficient functional protein is being produced *in planta* otherwise no suppression of ROS bursts would have been observed. However, in instances where a construct does not suppress flg22-triggered ROS bursts there is the possibility that the Mp10 mutant may not have been expressed or produces unstable protein. One of the disadvantages of the structure-function approach taken is that certain truncation may enhance instability of Mp10. Upon analyses of the expression levels of the various Mp10 mutants, this structure-function analysis forms a solid basis for further investigations of the relationships between Mp10 structure and function.

Targeted deletion of the N and C termini of Mp10 revealed that removing the first 40 or last 26 amino acids led to a loss of immune signalling suppression. These deletions corresponded to large disruption of conserved motifs A and C, and particularly the

removal of tyrosine (40) and tryptophan (120) residues, suggesting that the characteristics of being a CSP were important for either Mp10's function in immune signalling or its stability as a protein. The role of motif B was not investigated. Future experiments may include the disruption of motif B whilst leaving A and C intact. In addition, motifs A and C should be disrupted with point mutations rather than deletions to confirm the involvement of residues 40 and 120 in immune signalling suppression.

If the relevant constructs express stable protein then the loss of the Mp10 phenotype upon removal of Tyr-40 and Trp-120 residues is interesting when compared to what is known about the involvement of these amino acids in CSP structures. Aromatic residues such as tyrosine and tryptophan are overrepresented at protein binding sites, as they can form hydrogen bonds and cation-π interactions (Dougherty, 2007). This may suggest that the residues we have identified are involved in binding Mp10 ligands. Structural studies of a CSP from the cabbage moth *Mamestra brassicae* have identified a potential role for a C-terminal tyrosine and an N-terminal tryptophan in closing the hydrophobic channel of the CSP (Mosbah *et al.*, 2003). However, the residues in Mp10 that correspond to the *M. brassicae* residues are Tyr-58 and Tyr-126, not the residues we identified. The Mp10 immune signalling suppression phenotype is lost before Tyr-58 is removed, and remains after Tyr-126 is removed. The presence of tryptophan at position 120 in Mp10 is conserved amongst aphid species (Jacobs *et al.*, 2005), but is very rare amongst insects in general; of the 71 CSPs aligned by Wanner and colleagues (Wanner *et al.*, 2004) only one contained a tryptophan at the corresponding residue. The residue found at this position in most insect CSPs is a tyrosine, as it is in aphid OS-D1. Tyrosine, like tryptophan, is an aromatic amino acid and so the substitution would be predicted to not have a large affect on the properties protein. However, this could be investigated by producing an Mp10 construct with Trp-120 changed to Tyr-120.

Mp10 homologs were identified in other aphid species (Jacobs *et al.*, 2005), and at least the homologs from *A. pisum* and *A. gossypii* can suppress plant immune signalling. If the true function of Mp10 is in suppressing plant immunity, then other diverse aphid species such as *A. pisum* and *A. gossypii* may also suppress elicitor-triggered plant immunity in *N. benthamiana*. Yet, *M. persicae* but not *A. pisum* or *A. gossypii* survives on *N. benthamiana*, suggesting that suppression of elicitor-triggered plant immunity is not sufficient for colonization. It is likely that Ap10 and Ag10 trigger the same defence response (possibly ETI) in *N. benthamiana* as Mp10 does, and therefore it may be that suppression of ETI is required for aphids to effectively colonise plants. However, aphids are likely to produce a cocktail of effectors each of which contributes a little towards generating a compatible interaction of the aphid with its plant host.

Conserved CSP motifs at the N and C-termini of Mp10 may need to be largely intact to suppress plant immunity. There are likely to be more than five CSPs in *M. persicae* (Xu *et al.*, 2009), and therefore functional redundancy amongst these proteins is a possibility. However, it is possible that not all *M. persicae* CSPs possess the same ability to suppress plant immunity, as exemplified by MpOS-D1. MpOS-D1 differs from Mp10 in amino acid sequence whilst largely retaining the conserved features of a CSP, yet MpOS-D1 does not appear to suppress flg22-triggered ROS bursts. MpOS-D1 also contains amino acids polymorphisms in the tyrosine and tryptophan residues identified as being potentially important for the Mp10 suppression phenotype. A future experiment may be to replace these amino acid residues in the MpOS-D1 sequence with the tyrosine and tryptophan residues present in Mp10 and examine if these changes generate a protein that can suppress flg22-triggered ROS bursts. Protein of a homolog in *M. viciae* to OS-D1 is not detected in aphid heads minus antennae, whereas the protein of the Mp10 homolog was (Jacobs *et al.*, 2005), suggesting they may have different functions. The data suggest that the phenotypes of Mp10 may be specific to its sequence and are not associated with it belonging to the CSP family. Other CSPs in *M. persicae* could be identified and tested to provide further evidence for this conclusion.

The data in this chapter suggests that Mp10 may be acting at the plant plasma membrane. Confocal microscopy using the GFP tagged Mp10 would provide further evidence for this. CSPs are thought to be involved in a wide range of insect processes. They generally bind other compounds, mostly short to medium chain fatty acids and their derivatives (Wanner *et al.*, 2004). Based on the mechanisms of plant immune suppression described for effectors from plant pathogens it is tempting to speculate that Mp10 chelates Ca^{2+} perhaps in the same way as EPS (Aslam *et al.*, 2008), because Ca^{2+} binding proteins in saliva have been found to play a role in plant-aphid interactions (Will *et al.*, 2007). However, Mp10 contains no well characterised Ca^{2+} binding motifs such as EF-hands. Fungal pathogens release effectors that bind the fungal PAMP chitin to prevent its perception by plant receptors (de Jonge *et al.*, 2010; Mentlak *et al.*, 2012). It is reasonable to suggest that aphids may have evolved an effector that functions in a similar way to neutralise its elicitors by binding them, but such a function for Mp10 does not explain why flg22 perception is affected in the plant. Plant pathogen effectors also often bind to plant proteins. CSPs are not thought to interact with proteins, but if Mp10 was acting in this way it would either need to target several RLKs, or shared points of the signalling pathway such as Ca^{2+} channels. Alternatively, CSPs may act in a similar way to OBPs, which are believed to transport hydrophobic signalling molecules to chemoreceptors and then release them (Sanchez-Gracia *et al.*, 2009; Fan *et al.*, 2011). It is possible that Mp10 is binding to plant hydrophobic signalling molecules and delivering

them to their respective receptor, thereby disrupting normal plant signalling homeostasis. One candidate for such Mp10 interactors would be oxylipins, a large family of fatty acids that are important signalling molecules and protective compounds in plant response to biotic stress (Blée, 2002; Prost *et al.*, 2005). Oxylipins have been implicated in plant responses that both deter aphids (Hegde *et al.*, 2012) and facilitate their colonization of the plant (Nalam *et al.*, 2012). Disruption of normal oxylipin signalling may repress immune signalling and induce defence in *N. benthamiana* to *M. persicae*. The ability of Mp10 to bind plant signalling molecules could be tested in a similar way to that used by Jacobs and colleagues (Jacobs *et al.*, 2005).

In contrast to CSPs, the biology of the immune signalling pathway is better understood, although much of our knowledge comes from *A. thaliana* rather than *N. benthamiana*. However, it is helpful to consider how Mp10 may be functioning *in planta*. For Mp10 to act at the plasma membrane and be consistent with the data in this chapter there are several plant genes whose function it could be interfering with. Mp10 could somehow disrupt plant kinase function, so that the RLKs involved in flg22, chitin and aphid elicitor perception (FLS2, BAK1, CERK1 and unknown respectively) are unable to signal. A good candidate for a class of kinases to disrupt would be the PBL kinases, as the PBL kinase BIK1 has been shown to play a role in flg22 and chitin signalling in *A. thaliana* (Zhang *et al.*, 2010). A BIK1 ortholog was recently identified in *N. benthamiana* (Bombarely *et al.*, 2012), although its function in PTI signalling remains to be examined. Ca^{2+} changes in response to PAMP perception is one of the earliest responses in *A. thaliana* (Jeworutzki *et al.*, 2010), and is upstream of other PAMP triggered responses in *N. benthamiana* (Segonzac *et al.*, 2011). However, our current knowledge of the genes involved and the regulation of the PAMP-triggered Ca^{2+} burst is limited (Ranf *et al.*, 2008; Kudla *et al.*, 2010). In *A. thaliana*, the Ca^{2+} ATPase AUTOINHIBITED CA2+-ATPASE8 (ACA8) has been shown to play a role in PAMP responses and interacts with FLS2 and other RLKs (Frei dit Frey *et al.*, 2012). Few Ca^{2+} channels have been identified for *N. benthamiana*, although one channel CA2+-ATPASE1 (NbCA1) has been found to play a role in immune programmed cell death (Zhu *et al.*, 2010). Another possibility to consider is that Mp10 may alter the expression of PRRs or regulators of PRRs in *N. benthamiana*. The decreased presence of PRRs or their regulators might lead to decreased responses upon elicitor perception. If this is the case then the phenotypes we have seen so far suggest that Mp10 targets the transcription of multiple PRRs. The transcript and protein levels of candidates for Mp10 targetting such as FLS2, BAK1 and CERK1 could be tested in *N. benthamiana*.

One question that remains open is whether the phenotypes of Mp10 are really those of an effector, or an elicitor, or whether they are an artefact caused by aspecific

interaction in plants. From the sequence conservation of Mp10 homologs and the potential effects of gene knockdown Mp10 may have an essential function in *M. persicae*. However, more evidence is needed that Mp10 functions in plants rather than in aphids. Presence of Mp10 in aphid saliva and hence secretion into the plant has not yet been shown, although the expression of *Mp10* in the salivary glands (Bos *et al.*, 2010) is a good indication that it is likely to be present in the saliva. But the presence of *Mp10* expression in legs and antennae (Ghanim *et al.*, 2006) is consistent with a putative role in chemoperception, since legs and antennae have chemosensory functions in aphids (Jacobs *et al.*, 2005). If Mp10 is involved in chemoperception then its ligand is still unknown, and is unlikely to be one of twenty-eight different compounds known to elicit an electrophysiological response in electroantennograms or in single olfactory neurone preparations (Jacobs *et al.*, 2005). One possible explanation for the incoherency in expression profile and potential function of Mp10 stems from the findings that insect CSPs probably evolve through the 'birth-and-death' model (Sanchez-Gracia *et al.*, 2009, Vieira, 2011 #359). Tandem gene duplication leads to divergence in sequence and function, and eventual loss from the genome (Nei and Rooney, 2005). It is possible that Mp10 is derived from a gene duplication of a CSP involved in chemoperception, and then evolved to be secreted in the saliva and suppress plant immune signalling. Thus its expression patterns may still resemble those of other CSPs, but Mp10 has gained a different function. OS-D1 and OS-D2 (Mp10) homologs in *M. viciae* differ in protein localisation from each other, with OS-D2 protein being present in heads without antennae in addition to the antennae and legs (Jacobs *et al.*, 2005). This suggests different functions for the two CSPs, and fits with the above hypothesis that Mp10 may have diversified in function from other CSPs.

The sequence conservation of Mp10 homologs and the potential importance of Mp10 to *M. persicae* fecundity raise the possibility that Mp10 may be an elicitor. This hypothesis would suggest that if Mp10 has an important function then it is not in plant immune signalling suppression but in another part of the plant-aphid interaction, or even in processes within the aphid. Therefore, the elicitor-triggered signalling suppression that we see may be a result of overstimulation of the pathway by the elicitor. For instance, Mp10 expression *in planta* may cause a lengthy signalling response that shares components with PTI signalling, and thus the homeostasis of these components is disrupted, resulting in a decreased response to further stimulation by any elicitor. In this model the decrease in *M. persicae* fecundity seen upon expression of Mp10 in *N. benthamiana* would be due to elicitor-triggered immunity, rather than ETI as suggested in chapter 3. More data is needed on the plant genes involved in Mp10 phenotypes to further comment.

A pessimistic view to hold would be that Mp10 is toxic to the plant in some way, maybe through blocking Ca^{2+} channels, and therefore all the phenotypes stem from this artefact. In this scenario the chlorosis would be due to the plant's homeostasis being disrupted, as would the Ca^{2+} and ROS burst suppression phenotypes and the defence against the aphid. Further research into the interaction of Mp10 with the plant would help to elucidate whether Mp10 phenotypes are artefacts or not. In particular, isolation and characterisation of plant proteins that interact with Mp10 will give further information. For example, if an NBS-LRR protein was identified to interact with Mp10 then further experiments might link the chlorosis and aphid defence phenotypes to the activation of NBS-LRR resistance gene.

If the phenotypes seen upon expression of Mp10 *in planta* are an artefact, then what is the role of Mp10 in the aphid salivary gland and possibly saliva? Mp10 may truly be a chemosensory protein, as its name suggests. It is conceivable that Mp10 is secreted into the plant during probing in order to bind a plant molecule. Mp10 complexed with the plant molecule would then be ingested and transferred to gustatory receptors in the stylet. In this way Mp10 would be providing the aphid with information about the identity and condition of the plant. Whether Mp10 is ultimately shown to be an effector or not, this work has added to our understanding of aphid CSPs and identified areas for further research.

In summary, we have shown that Mp10, a CSP, has the potential to be important for *M. persicae* fecundity, and that it is capable of disrupting several elicitor signalling pathways in *N. benthamiana*. Mp10 requires largely intact conserved CSP motifs to achieve this phenotype, either for function or protein stability. Mp10 homologs in other aphids share the ability to disrupt plant immune signalling, but a related *M. persicae* CSP may not. Further understanding of how CSPs function will aid efforts to elucidate how Mp10 functions in *N. benthamiana* and whether it is a genuine effector.

Chapter 8 – General discussion

8.1 Summary of research

This study took the starting point that plant immunity was important for plant-aphid interactions and that plant-aphid interactions followed the 'zig-zag model' of plant pathogen-plant interactions (Jones and Dangl, 2006) (chapter 1). We subsequently investigated if the aphid *M. persicae* possesses effectors that modulate plant processes (chapter 3) and elicitors that trigger plant immunity (chapters 4 and 5). Having found both *M. persicae* effectors and elicitors, we attempted to characterise the plant genes involved in elicitor perception in two different plant species (chapters 4,5 and 6) as well as the mode of action of one effector, Mp10 (chapter 7). Together these data provide evidence in favour of the 'zig-zag model', as we show for the first time that plant immunity plays a role in aphid-plant interactions and that aphids possess effectors that suppress plant immunity.

Several studies have concluded that the watery saliva of an aphid contains proteins with potential roles in modulating plant processes (e.g. (Carolan *et al.*, 2009; Will *et al.*, 2009; Nicholson *et al.*, 2012)). One study found that aphid salivary proteins bind Ca^{2+}, potentially leading to prevention of sieve cell occlusion (Will *et al.*, 2007). Another study showed that the abundant salivary protein C002 plays a role in aphid interactions with plants (Mutti *et al.*, 2008). Studies described in this thesis attempted to functionally characterise aphid effectors. One of these effectors, Mp10, suppresses the ROS burst triggered by the PAMP flg22 (chapter 3). *Mp10* expression in *N. benthamiana* also induced chlorosis in the leaves that was dependent on SGT1. *M. persicae* fecundity on *N. benthamiana* decreased on leaves expressing *Mp10* and a second candidate effector *Mp42*. The expression of *MpC002* in *N. benthamiana* led to an increase in *M. persicae* fecundity. Thus, data in chapter 3 provided evidence for the first time that *M. persicae* effectors function in plants and are capable of modulating plant processes, and provide further evidence that *M. persicae* proteins trigger plant defences.

Our finding that Mp10 suppresses flg22-triggered ROS in *N. benthamiana* (chapter 3) led us to investigate in more detail what elicitors of plant immunity in *N. benthamiana M. persicae* possesses (chapter 4). Whole *M. persicae* extract triggers Ca^{2+} and ROS bursts in this plant species. These responses are likely due to a mix of proteins and other molecules in the aphid, as boiling the elicitors or treating them with proteinase K reduced but did not extinguish their eliciting capability. The ROS burst triggered by the extract did not decrease in *Serk3/Bak1* silenced *N. benthamiana* plants, indicating that RLK SERK3/BAK1 was not involved in the triggering of plant immunity to the aphid elicitors in this species. The ROS and Ca^{2+} bursts were mainly triggered by proteins less than 3 kDa and more than 10 kDa in weight, but not by those between 3 and 10 kDa.

Treatment of *N. benthamiana* leaves with three different sets of proteins, i.e. those less than 3 kDa, between 3 and 10 kDa, and greater than 10 kDa, triggered induced resistance against *M. persicae* in *N. benthamiana*. The larger than 10 kDa protein(s) of diverse aphid species triggered immune responses. Therefore, taken together, data in chapter 4 provides evidence that aphids possess elicitors that trigger immune responses in *N. benthamiana* (Figure 8.1A).

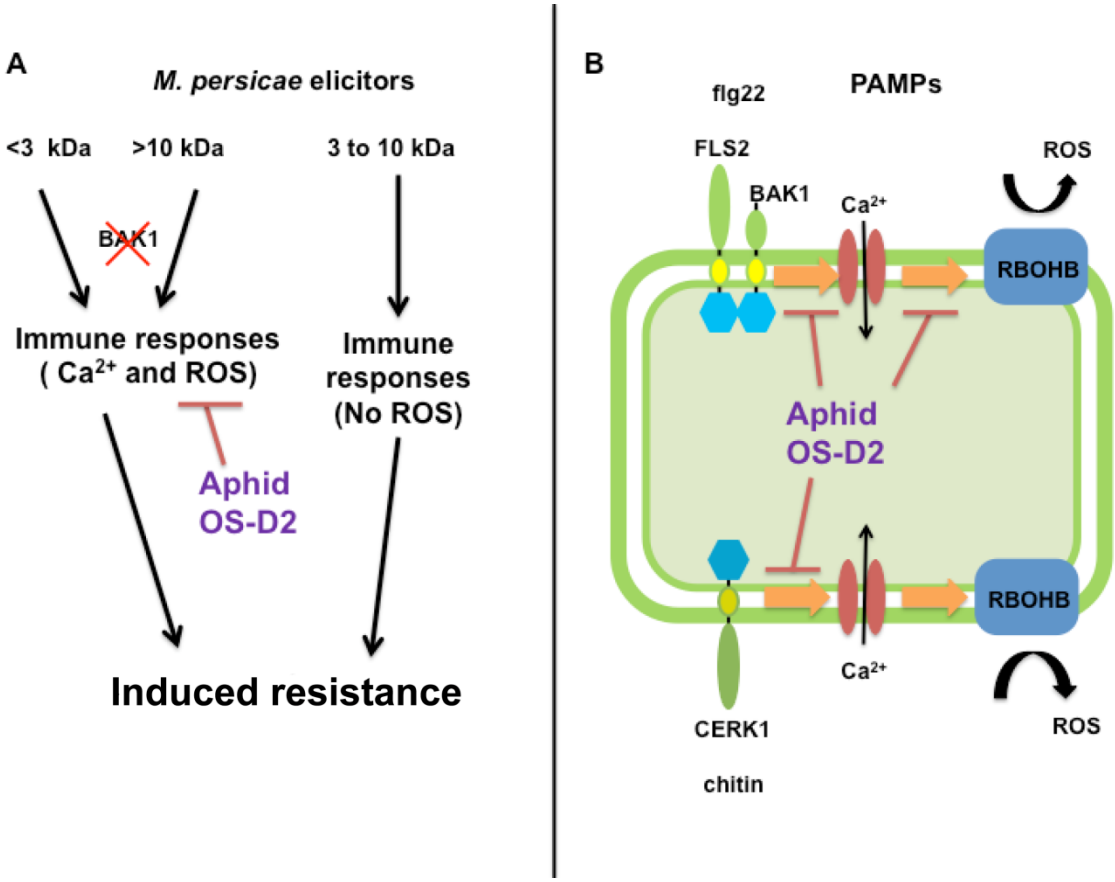


Figure 8.1 – *M. persicae* triggers and suppresses defence pathways in *N. benthamiana*.
A) *M. persicae* triggers plant immunity and induced resistance in *N. benthamiana* (chapter 4). The <3 kDa and >10 kDa fractions trigger Ca^{2+} and ROS bursts, whereas the 3 to 10 kDa fraction does not. The elicitors in the <3 kDa and >10 kDa fractions are likely to be perceived by receptors that do not require the LRR-RLK BAK1. Aphid effector Mp10 (OS-D2) suppresses the ROS burst triggered by *M. persicae* extracts. B) OS-D2 suppresses the Ca^{2+} burst triggered by the PAMPs chitin and flg22 (chapter 7). OS-D2 and its homologs in other aphids suppress flg22-triggered ROS burst but not chitin-triggered ROS burst (chapter 3 and chapter 7). How OS-D2 is acting to suppress elicitor triggered ROS (A and B) is unknown, and this may occur by interfering with the level of PRR protein present at the membrane by methods including disrupting transcription of plant genes in the nucleus.

Having found that aphids induce *N. benthamiana* immune responses (chapter 4), we wished to assess if *M. persicae* elicitors also trigger immunity in *A. thaliana* (chapter 5). Whole *M. persicae* extract triggered a late ROS burst, the deposition of callose, and induced defence in *A. thaliana*. The 3 to 10 kDa *M. persicae* fraction triggered a late ROS burst and induced resistance against aphids, and the larger than 10 kDa fraction also triggered induced resistance, but did not trigger a ROS burst. Unlike *N. benthamiana*, no ROS burst or induced resistance was observed to the *M. persicae* <3 kDa fraction in *A. thaliana*. Plant responses to the 3 to 10 kDa and >10 kDa fractions appear to be sensitive to boiling and proteinase K treatments. Moreover, the ROS burst and induced resistance response to the 3 to 10 kDa whole aphid and saliva fractions did not occur in the *A. thaliana bak1*-5 mutant impaired in BAK1 signalling. In addition, *A. pisum* survived better on the *bak1-5* mutant compared to wild type *A. thaliana*, further demonstrating a role for BAK1 in aphid-plant interactions. CERK1 and the BAK1-dependent RLKs FLS2, EFR1 and PEPR1 and PEPR2 are not required for the 3 to 10 kDa induced ROS burst. Taken together, the data in chapter 5 provides evidence that *A. thaliana* launches an immune response to *M. persicae* (Figure 8.2), and that *A. thaliana* and *N. benthamiana* perceive *M. persicae* differently (Figure 8.1A and Figure 8.2).

To dissect the BAK1-dependent *A. thaliana* immune signalling further, we made use of a collection of *A. thaliana* mutants in individual non-RD IRAKs (Danna *et al.*, 2011) (chapter 6). The mutant collection was screened for a reduction in ROS response to the *M. persicae* 3 to 10 kDa fraction, however none of the mutants showed a consistent reduction in the response. Therefore, the receptor involved in the perception of the 3 to 10 kDa elicitor remains unknown.

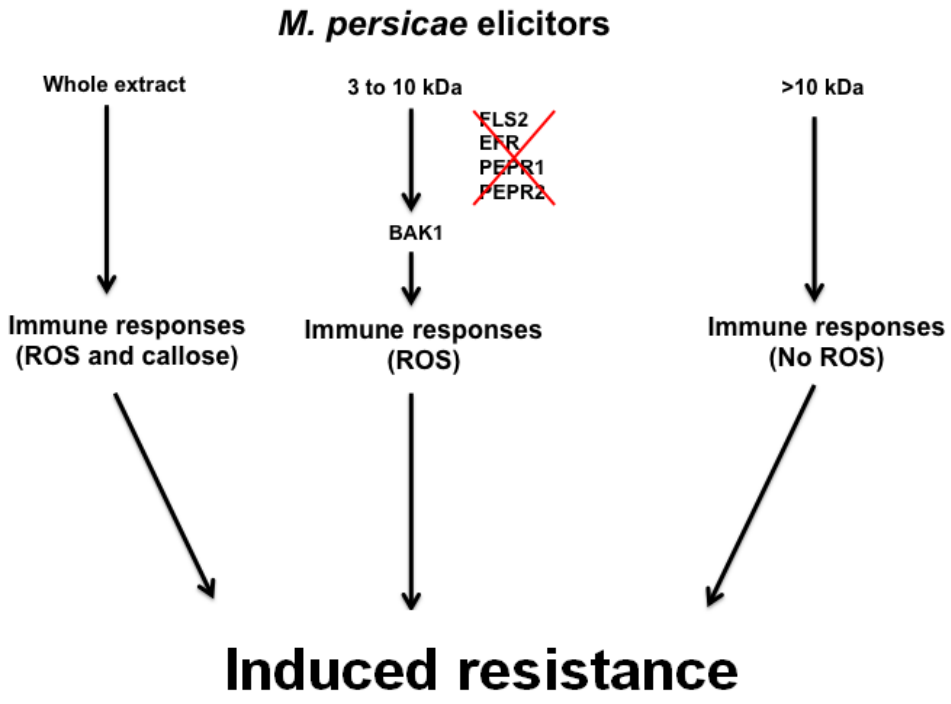


Figure 8.2 – *M. persicae* triggers immunity in *A. thaliana*.
M. persicae triggers plant immunity and induced defence in *A. thaliana* (chapter 5). The 3 to 10 kDa fraction triggers a ROS burst, whereas the >10 kDa fraction does not. Plant immune responses to the 3 to 10 kDa elicitor require BAK1, and probably an unknown PRR (chapter 5).

Having previously found that aphids trigger plant immunity in *N. benthamiana* and *A. thaliana* (chapters 4 and 5), and that Mp10 suppresses flg22-triggered immunity (chapter 3), we further investigated the role of Mp10 in plant-aphid interactions (chapter 7) (Figure 8.1A and B). Mp10 is capable of suppressing the ROS burst triggered in *N. benthamiana* by whole *M. persicae* extract, as well as the ROS burst triggered by the <3 kDa and >10 kDa fractions identified in chapter 4. In addition, Mp10 is capable of suppressing the Ca^{2+} burst triggered in *N. benthamiana* by the PAMPs flg22 and chitin. Mp10 is also known as OS-D2, and homologs in *A. pisum* (Ap10) and *A. gossypii* (Ag10) are largely conserved in amino acid sequence. Ap10 and Ag10 suppress the flg22-triggered ROS burst as well. Thus, data in chapter 7 provides evidence that *M. persicae* and at least two other aphid species have proteins that suppress elicitor-triggered ROS bursts in *N. benthamiana*.

In summary, *M. persicae* (and potentially aphids in general) possess elicitors that that elicit the first active layer of plant immunity in plants (Figure 8.3A and B). Aphids also produce effectors, such as Mp10, that suppresses this immunity (Figure 8.3B). Classical NBS-LRR resistance genes (*Mi* and *Vat*; Figure 8.3) that give resistance to aphids have

been identified in two plant species. Generally, these NBS-LRR proteins induce plant immunity and resistance upon (in)direct recognition of pathogen effectors. Our findings that plants induce a SGT1-dependent chlorosis response to Mp10 suggests that plants may also recognize aphid effectors (Figure 8.3B). Therefore, data presented in all five experimental chapters in this thesis follows the ‘zig-zag model’ of plant pathogen-plant interactions (Figure 8.3)

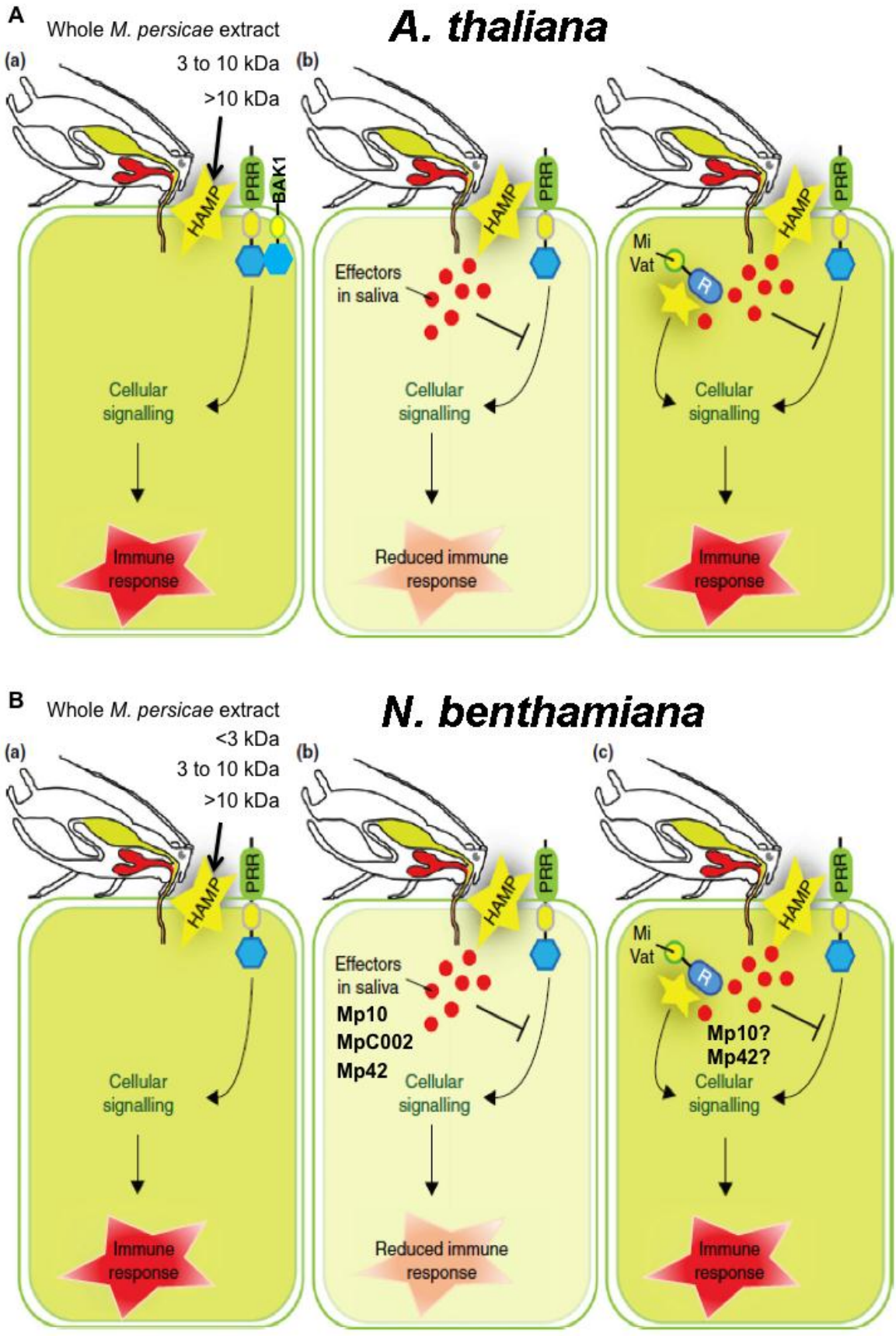


Figure 8.3 – Signalling events in aphid-exposed plant cells.

A) *A. thaliana* (a) Whole *M. persicae* extract, as well as the 3 to 10 kDa fraction and the >10 kDa fraction of it, are capable of eliciting immune responses, and the immune responses to the 3 to 10 kDa elicitor requires the LRR-RLK BAK1 (chapter 5). (b and c) *M. persicae* effectors that suppress immune responses in or are recongized by *A. thaliana* were not investigated in the thesis.
B) *N. benthamiana* (a) Whole *M. persicae* extract, as well as various fractions of it, are capable of eliciting immune responses in plants (chapter 4). (b) The *M. persicae* proteins Mp10, MpC002 and Mp42 are likely to act as effectors with Mp10 suppressing immune responses to the PAMP flg22 and aphid extracts (chapters 3 and 7). (c) Mp10, and possibly Mp42, may be recognised by plant resistant genes (chapter 3). Image adapted from (Hogenhout and Bos, 2011) and used with the permission of the publisher (Elsevier).

8.2 Perception of insect elicitors by hosts

Aphid elicitors triggered induced resistance responses in both *A. thaliana* and *N. benthamiana* in which some of the fractions of *M. persicae* were able to trigger ROS bursts in these plant species and others were not (chapters 4 and 5) (compare Figures 8.1 and 8.2). ROS production by a plant in response to herbivory has been reported for several plant species (Leitner *et al.*, 2005; Maffei *et al.*, 2006; Diezel *et al.*, 2009) including at aphid feeding sites in *A. thaliana* (Kusnierczyk *et al.*, 2008). However, we found that fractions that did not trigger ROS bursts nevertheless evoked induced resistance responses to aphids, indicating that these fractions triggered plant immunity independently of ROS. We also found that ROS bursts in *A. thaliana* and *N. benthamiana* were triggered by different fractions of the aphid. For instance, the >10 kDa fraction of *M. persicae* triggered a ROS burst in *N. benthamiana* but not in *A. thaliana*. Furthermore, *A. thaliana* responded to the 3 to 10 kDa fraction with a ROS burst, whereas no such ROS burst was observed in *N. benthamiana*. Yet, both of these fractions trigger induced resistance responses in both plant species, indicating that induced resistance may not always be preceded by a ROS burst. The differential responses to various fractions within and between plant species suggest that aphid elicitors induce multiple plant immunity pathways involving multiple plant surface receptors and downstream pathways. The existence of multiple pathways is also supported by our data showing the differential perception of various aphid fractions and the involvement of BAK1 in *A. thaliana* but not in *N. benthamiana* in elicitor perception.

A. thaliana and *N. benthamiana* respond differentially to *M. persicae* elicitor fractions. *N. benthamiana* recognizes the <3 kDa fraction of *M. persicae* while *A. thaliana* does not. Secondly, the >10 kDa fraction triggers a ROS burst in *N. benthamiana* but not in *A. thaliana*. Moreover, boiling of this fraction eliminated the induced response of *A.*

thaliana, but not the ROS response of *N. benthamiana*. Finally, the 3 to 10 kDa fraction induces defence in both plant species, but only in *A. thaliana* does it trigger a ROS burst. These data suggest the presence of different PRRs and downstream signalling pathways in the two plant species.

We found evidence to support this hypothesis in the differential requirement of BAK1 for elicitor perception in *A. thaliana* and *N. benthamiana*. BAK1 is involved in *A. thaliana* perception of *M. persicae* 3 to 10 kDa fraction. We found no involvement of SERK3/BAK1 in *N. benthamiana* perception of the <3 and >10 kDa *M. persicae* elicitors, although we can not comment on its involvement in perception of the 3 to 10 kDa fraction.

Whilst elicitors from microbial pathogens of plants have been much studied, little is known about how plants perceive insect elicitors. However, our findings in *N. benthamiana* are in agreement with those of Yang and colleagues (Yang *et al.*, 2011), who found that SERK3/BAK1 in *N. attenuata* is not involved in the perception of elicitors of chewing herbivores. The RLKs recently identified to play a role in insect elicitor perception are likely to play a role downstream in the elicitor-triggered signalling pathway (Gilardoni *et al.*, 2011; Gouhier-Darimont *et al.*, 2013), and therefore our data for the role of BAK1 in *A. thaliana* membrane elicitor perception represents a step forward in understanding how plants perceive insects.

Little is also known about how animals perceive elicitors from blood feeding insects. Our results suggest that plants have plasma membrane receptors to perceive insect elicitors in similar ways to microbe elicitors. As animals also possess plasma membrane receptors for microbe elicitors it is possible that they too possess similar receptors for insect elicitors. It seems unlikely that animals would be able to perceive elicitors specific to aphids as aphids feed only on plants, but if the aphid elicitors are identified as being conserved amongst all insects then it is possible that animals may also be able to perceive them.

8.3 Herbivore elicitors of plant immunity

8.3.1 Evidence for multiple elicitors in aphids

From data discussed in section 8.2 it has become clear that aphids trigger multiple plant immunity pathways likely involving multiple RLKs and different downstream pathways. Our data also show that aphids have multiple elicitors. First, *N. benthamiana* perceives elicitors in three different fractions of *M. persicae* (chapter 4) suggesting the presence of at least three elicitors, as we can not rule out that a fraction contains more than one elicitor. Furthermore, *A. thaliana* perceives two fractions of *M. persicae*, suggesting two or

more elicitors (chapter 5). Below is a discussion of putative elicitors that may be present in aphids based on our results and elicitors already identified in the plant-insect and plant-pathogen field.

8.3.2 Chitin

Aphid chitin would be a strong candidate for an aphid elicitor, as plants respond to chitin from fungi as a PAMP (Kombrink *et al.*, 2011), and chitin from arthropods such as crab chitin is used experimentally to mimic this response (e.g. chapters 3 and 7). However, we have found no positive conclusive evidence to suggest that chitin from aphids is perceived as an elicitor. Preliminary experiments not included in this thesis agree with informally reported results from other groups (Prof. Gary Stacey, TSL seminar 2012) that the chitin receptor in *A. thaliana*, CERK1, does not appear to be involved in perception of insect chitin. Whilst chitin synthesis in fungi and insects share common biosynthetic machineries (Merzendorfer, 2011), there are also differences that may explain why plants perceive one type of chitin but not the other. For instance, insects only have two chitin synthase genes whereas fungi contain a number of functionally diverse chitin synthases (Merzendorfer, 2011). Recent research suggests that aphids are likely to only possess one chitin synthase gene, as only one copy is present in the *A. pisum* genome and *A. glycines* transcriptome (Bansal *et al.*, 2012). *A. thaliana* responds to chitin oligomers in a size-dependent manner, with octamers eliciting strong responses (Zhang *et al.*, 2002). Octamers of chitin are soluble, but larger oligomers are not and hence may not be perceived by the plant. Therefore, it may be the case that aphid chitin is structurally different to that of fungi, containing either shorter chitin oligomers that have less eliciting ability or longer chitin oligomers that are not soluble.

8.3.3 GOX

M. persicae saliva has been found to abundantly contain GOX peptides and also has measurable GOX activity (Harmel *et al.*, 2008). GOX has been identified as a lepidopteran elicitor of plant defence (Musser *et al.*, 2005), and is therefore a candidate for an aphid elicitor of plant defences. GOX is approximately 82 kDa in molecular weight in the lepidopteran insect *H. zea* (Eichenseer *et al.*, 1999) and is therefore most likely present in the >10 kDa fractions of aphid extracts. GOX induces a ROS response in plants (Jabs *et al.*, 1997) and as such is used as a positive control in PTI assays (Thorsten Nürnberger, personal communication). We did not observe a ROS response of the >10 kDa fraction in *A. thaliana*. Moreover, boiling increased the activity of this fraction in *N. benthamiana* and did not eliminate it as would be expected given that GOX is an

enzyme. Therefore, our data indicates that GOX is unlikely to be an elicitor in aphid extracts although we cannot exclude the possibility of having more than one elicitor in the >10 kDa fraction. Moreover, GOX has also been shown to suppress plant defences against the caterpillar *H. zea* in *N. tabacum* (Musser *et al.*, 2002). If GOX also suppresses plant responses to aphids, then this enzyme may be responsible for the reduction in eliciting activity of non-boiled versus boiled >10 kDa aphid extract in *N. benthamiana*.

8.3.4 Small peptides

The aphid peptides identified by Ollivier and colleagues (Ollivier *et al.*, 2012) are also candidates for elicitors, and we have already speculated about whether they may be included in the 3 to 10 kDa aphid fraction (chapter 5). An alternative identity for them could be in a role analogous to the CLE peptides of nematodes, which manipulate plant development to the benefit of the parasite (Mitchum *et al.*, 2012). The aphid peptides may manipulate plant physiology and development in another way that is advantageous to their particular lifestyle, such as diverting sugars to the site of feeding.

8.3.5 Bacterial symbiont proteins – EF-Tu

An additional source of aphid-based candidate elicitors could be the bacterial endosymbionts that aphids possess, especially the obligate symbiont *B. aphidicola*. *B. aphidicola* play an important role in nutritional aphid physiology, including the production of essential amino acids; and depriving aphids of *B. aphidicola* leads to retarded growth and sterility (Douglas, 1989). Given that *B. aphidicola* produces many proteins for aphids, it is possible that some of these are present in aphid saliva and trigger immune responses. Indeed, *B. aphidicola* GroEL (symbionin) was detected in aphid saliva by Western blot hybridization (Filichkin *et al.*, 1997) and by proteomics analysis (Jorunn Bos, David Prince, and Saskia Hogenhout, unpublished results). Analysis of the *B. aphidicola* genome revealed that this symbiont lost the gene for the well-characterised PAMP flagellin (Shigenobu *et al.*, 2000). *B. aphidicola* does however possess a gene for the brassica specific PAMP EF-Tu, and this gene acquires nonsynonymous mutations at significantly higher rates in *B. aphidicola* than *E. coli* (Brynnel *et al.*, 1998). Analysis of the 18 amino acids that form the minimum active epitope of EF-Tu (elf18, (Kunze *et al.*, 2004)) shows substitutions in the *B. aphidicola* sequence at residues 6, 8 and 12 compared to the *E.coli* sequence (Figure 8.4). Kunze and colleagues mutated individual residues in elf18 peptides and found that mutations in residues 2, 4, 5 and 7 lowered the eliciting activity of the peptide (Kunze *et al.*, 2004). A minimal peptide with N-terminal acetylation and a sequence comprising acetyl-xKxKFxRxxxxxxxxx appears to be required

for full activity as elicitor in *A. thaliana* (Kunze *et al.*, 2004). The EF-Tu of *Buchnera* spp. retains this motif suggesting that *B. aphidicola* EF-Tu is capable of eliciting defence responses in *A. thaliana*. EF-Tu is approximately 43 kDa in molecular weight and is therefore most likely present in the >10 kDa aphid fraction. The EF-Tu RLK is EFR, which is BAK1 dependent. Given that *A. thaliana efr* mutant does not show an altered phenotype in response to 3 to 10 kDa aphid extract, and that the >10 kDa aphid fraction does not generate a ROS response, it is unlikely that *A. thaliana* perceives *B. aphidocola* EF-Tu. Moreover, EF-Tu can not be a candidate for the responses triggered by a larger than 10 kDa protein in *N. benthamiana,* as the EF-Tu receptor EFR is brassica specific (Zipfel *et al.*, 2006).

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EFTU_BUCAP      SKEKFQRVKPHINVGTIG
EFTU_BUCAI      SKEKFQRLKPHINVGTIG
EFTU_BUCBP      SKEKFKRSKPHINVGTIG
EFTU_BUCA5      SKEKFQRLKPHINVGTIG
EFTU_BUCAT      SKEKFQRLKPHINVGTIG
EFTU_BUCCC      SKEKFNRSKPHINVGTIG
elf18           SKEKFERTKPHVNVGTIG
                *****:* ***:******
```

Figure 8.4 – The elf18 sequence of *Buchnera* spp. compared to that of *E. coli*.
Alignment of elf18 and the 18 amino acids corresponding to elf18 from the six reviewed *Buchnera* spp. EF-Tu sequences in UniProt that possess a complete N-terminal region. elf18 sequence taken from Kunze *et al.,* 2004. BUCAP = *B. aphidicola* subsp. *S. graminum* (strain Sg), accession number O31298. BUCAI = *B. aphidicola* subsp. *A. pisum* (strain APS), accession number O31297. BUCBP = *B. aphidicola* subsp*. Baizongia pistaciae (*strain Bp), accession number P59506. BUCA5 = *B. aphidicola* subsp. *A. pisum* (strain 5A), accession number B8D9U9. BUCAT = *B. aphidicola* subsp. *A. pisum* (strain Tuc7), accession number B8D851. BUCCC = *B. aphidicola* subsp. *Cinara cedri* (strain Cc), accession number Q057A2.

8.3.6 Bacterial symbiont proteins – GroEL

GroEL from *B. aphidicola* is also a candidate elicitor of plant defence responses as it is detected in aphid saliva (Filichkin *et al.*, 1997) (Jorunn Bos, David Prince, and Saskia Hogenhout, unpublished results). The *B. aphidicola* – aphid symbiosis is an estimated 250 million years old (Baumann *et al.*, 1995). Moreover, GroEL is conserved among bacteria and is produced in abundance by *B. aphidicola.* Hence, GroEL has many of the attributes of an elicitor. GroEL seems to be associated with the triggering of autoimmune diseases in humans, thus indicating that it can be perceived by eukaryotic host cells

(Argueta *et al.*, 2006), although GroEL has not yet been identified as a PAMP from well studied plant pathogenic bacteria. GroEL is approximately 60 kDa in molecular weight and hence is likely present in the >10 kDa aphid fraction, which triggers immune responses in both *A. thaliana* and *N. benthamiana*. Hence, GroEL is a putative elicitor in aphid-plant interactions. If GroEL is an elicitor then the receptor that perceives it may function independently of BAK1 in these plant species.

8.3.7 DAMPs

DAMPs are plant-derived elicitors that are released upon damage of plant cells most likely by pathogen/pest enzymes or other components. It has been proposed that aphid saliva creates OGs by digesting cell wall material (Will and van Bel, 2008). OGs act as a DAMP, are perceived through the receptor WAK1 (Brutus *et al.*, 2010), and trigger defence responses similar to those of PAMPs (Denoux *et al.*, 2008). Lethality and redundancy amongst members of the WAK family makes the involvement of OGs as DAMPs in aphid-plant interactions hard to investigate (Brutus *et al.*, 2010).

Aphid saliva may also contain enzymes that generate peptide DAMPs such as pep1 in *A. thaliana* (Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010). The *A. thaliana* ROS burst in response to the 3 to 10 kDa aphid elicitor fraction occurs more than an hour after application and disappears upon protease K treatment and boiling, thus the eliciting activity of this fraction may be indirect through the release of DAMPs. So far several DAMP receptors have been identified and the activities of two these receptors, PEPR1 and PEPR2, depend on BAK1 (Postel *et al.*, 2010). However, while BAK1 is involved in perception of the 3 to 10 kDa aphid elicitor fraction, PEPR1 and PEPR2 are not (chapter 5).

8.3.8 Elicitors must be available to plant receptors in order to be perceived

By using the whole aphid for our studies we have identified moelcules that trigger plant immunity (chapters 4 and 5), but the challenge remains as to whether these molecules are available to be perceived by plant cell surface receptors during aphid feeding. For this to be the case we would expect the elicitors to be present in aphid saliva, as this is the interface of the plant-aphid interaction. We showed that 3 to 10 kDa elicitor(s) perceived by *A. thaliana* are present in *M. persicae* saliva (chapter 5). Another possibility is that elicitors are present in the aphid mouthparts (stylets) themselves. However, this seems less likely given that the stylets are surrounded by a layer of sheath saliva, which is secreted by the salivary glands and solidifies (gels) upon secretion. Determining the

identity of the elicitors will be a step forward in this process, as more detailed studies of their role in aphid physiology can then be carried out.

8.4 Aphid effectors modulate plant processes

8.4.1 Aphids have effectors that suppress plant defences and modulate plant processes

We have established that plants probably launch multiple immune responses to aphids. However, aphids can still colonize plants successfully, and therefore it is likely that the aphids produce effectors that disrupt normal plant function through the suppression of these immune responses and possibly modulating other plant processes. In chapters 3 and 7 of this thesis we provide evidence of such effectors, including Mp10, Mp42 and MpC002 and these effectors in addition to how they relate to other insect effectors are discussed below.

8.4.2 Aphids have an effector that suppresses elicitor-triggered immunity

We initially identified Mp10 as an effector that suppressed flg22 but not chitin-triggered ROS in *N. benthamiana* (chapter 3). We subsequently observed that Mp10 can suppress the Ca^{2+} burst triggered in *N. benthamiana* by flg22 and chitin (chapter 7). In addition, we found that Mp10 can suppress the ROS response to *M. persicae* elicitors in *N. benthamiana* (chapter 7). These data suggest that Mp10 suppression activity acts early in elicitor-triggered signalling pathways. Mp10 is suppressing plant immunity responses to elicitors whose perception is BAK1 dependent (flg22) and BAK1-independent (chitin and *M. persicae* elicitors), suggesting that it targets either a common signalling step in these pathways or multiple plant proteins with similar characteristics (such as RLKs). Mp10 suppression of plant immunity in species other than *N. benthamiana* is still to be explored, and stable transgenic *A. thaliana* inducibly producing Mp10 are currently being generated as part of this. If Mp10 is also able to suppress flg22-triggered immunity in *A. thaliana* then we would predict it would also suppress the immunity triggered by the 3 to 10 kDa aphid elicitor, as both signalling pathways share BAK1.

The presence of one of the *M. persicae* elicitors in several diverse aphids, and the ability of Mp10 homologs from diverse aphid species to suppress immunity, suggests that effector-mediated suppression of elicitor-triggered plant immunity may be a common part of plant-aphid interactions. No effectors suppressing early signalling events in elicitor-triggered immunity have so far been identified for other insects, thus making Mp10 unique amongst insect effectors.

Blood feeding insects within the Diptera express the D7 family of saliva proteins, which are related to OBPs (Calvo *et al.*, 2006). These proteins function in the suppression of host defences by binding to amines such as serotonin, histamine and norepinephrine, and thus scavenging them. This is anticipated to inhibit, platelet-aggregating, and pain-inducement (Calvo *et al.*, 2006). Therefore, if Mp10 was binding signalling molecules in the plant then an interesting parallel may exist between Mp10 and effectors in blood feeding insects.

8.4.3 Aphids have effectors that improve aphid reproduction.

Aphid effector C002 had previously been identified as being important for aphids because its knockdown led to decreased aphid performance (Mutti *et al.*, 2006; Mutti *et al.*, 2008; Pitino *et al.*, 2011). Here we showed for the first time that the effector plays a role in modulating the plant to make it a better aphid host, as expression of *MpC002* in *N. benthamiana* led to increased *M. persicae* reproduction (chapter 3). The C002-mediated promotion of aphid reproduction has recently been confirmed in another *M. persicae* plant host, *A. thaliana* (Pitino and Hogenhout, 2013), strengthening the case for C002's role as a beneficial effector in plant-aphid interactions. C002 activity appears to depend on the plant host and aphid species, because *M. persicae* reproduction is improved on transgenic *A. thaliana* plants that express *M. persicae* C002, but not on those expressing *A. pisum* C002 (Pitino and Hogenhout, 2013). This is consistent with *A. thaliana* being a host for *M. persicae* but not for *A. pisum*. Future studies should reveal how C002 promotes aphid reproduction in specific plant hosts, although we have revealed that it does not suppress the flg22 ROS in *N. benthamiana* (chapter 3). Without knowledge of the mode of action of C002 it is difficult to compare it to other insect effectors acting in plants or animals.

8.4.4 Aphid effectors trigger plant defence responses

Expression of the effectors Mp10 and Mp42 in *N. benthamiana* led to decreased aphid performance. NBS-LRR *R* genes that confer resistance to specific aphid biotypes have been cloned (Smith and Clement, 2012). NBS-LRRs (in)directly interacts with recognized effectors of a number of bacterial and fungal pathogens leading to resistance (ETI) (Jones and Dangl, 2006), and therefore it is likely that NBS-LRRs also (in)directly interact with aphid effectors. SGT1 is required for NBS-LRR function, including the *S. lycopersicum Mi-1* resistance gene that confers resistance to *M. euphorbiae* (Bhattarai *et al.*, 2007). Therefore, it is interesting that Mp10 induces a chlorosis response that is dependent on SGT1 (chapter 3) indicating that Mp10 may be (in)directly recognized by a

NBS-LRR protein leading to a chlorosis response and reduced aphid performance. However, more evidence of the involvement of NBS-LRRs is needed, as SGT1 is involved in a number of plant processes, including coronatine signalling and *P. syringae* symptom development in *S. lycopersicum* and *A. thaliana* (Ishiga *et al.*, 2011; Uppalapati *et al.*, 2011) and wounding- and herbivory-induced JA accumulation and *N. attenuata* defence to a lepidopteran herbivore (Meldau *et al.*, 2011a). Host plant resistance has been reported for chewing herbivore insects; however none of the identified genes have been NBS-LRR proteins (Smith and Clement, 2012). Instead, host plant resistance to chewing herbivores often involves toxic metabolite production induced upon JA signalling involving SGT1 (Meldau *et al.*, 2011a). Therefore there is no evidence to currently suggest that chewing insects produce effectors that are recognized by the plant in the same way that microbial pathogens and aphids do. However, plant resistance mechanisms to chewing and sucking herbivores are likely to be different, because sucking insects such as aphids require a long-term interaction with plants and as such have more in common with biotrophic pathogens. Future investigations of the involvement of NBS-LRRs in the Mp10-mediated chlorosis response may include yeast two-hybrid analysis and co-immunoprecipitation assays, as they may reveal plant proteins that interact with Mp10. We suggest that Mp10 targets may be small molecules, such as lipids, and therefore the yeast two-hybrid and co-immunoprecipitation experiments may reveal plant proteins involved in the Mp10-mediated chlorosis reaction and/or ETI response. We revealed that knocking down *Mp10* expression may have an effect on aphid reproduction and that Mp10 is conserved among aphids, and therefore Mp10 may be important for aphid-plant interactions. As such, plants may have evolved to interact with this protein to induce ETI. If so, aphids may suppress ETI in compatible plant-aphid interactions.

Research into aspects of ETI in animal immunity is just beginning and so it will be interesting to see whether insects that feed on animals trigger and suppress ETI in ways analogous to phloem feeding insects of plants.

8.5 Potential future application of this study - engineering crops more resistant to aphids

8.5.1 The need for long-term aphid control strategies

Aphids are a threat to crop-plant production, whether it is grown in our own gardens and allotments, or commercially on farms. In order to deal with aphids in domestic settings the Royal Horticultural Society recommends keeping aphids “in check by squashing them by

hand" (Spence, 2009). Bad infestations are to be dealt with "using a suitable pesticide" (Spence, 2009). The impracticality of farmers squashing every aphid on their crop plants leaves spraying crops with pesticides as the predominant aphid control strategy in agriculture. However, in a situation analogous to bacteria and antibiotics, aphids have developed resistance to many insecticides. Targeted use of pesticides, informed by data such as that collected by the Rothamsted Insect Survey (http://www.rothamsted.ac.uk/insect-survey/), can aid in maintaining the efficacy of existing pesticides in the short term. However, in the long term new solutions to crop protection need to be found. Improved understanding of plant defence responses to aphids and how aphids may have evolved strategies to suppress these responses may unravel novel and benign pest control strategies, such as the development of aphid-resistant crop varieties using conventional strategies and biotechnology. It may also allow the development of new chemicals or other agents that target aphid proteins required for aphid-plant interactions.

8.5.2 Transferring novel PRRs between plant species can increase plant resistance

To date, efforts to protect crops have predominantly focused on the detection and characterization of *R* genes encoding NBS-LRRs to multiple pathogens and pests. NBS-LRR-mediated resistance is based on the recognition of specific effectors. These effectors may mutate or get deleted leading to new aphid biotypes that can overcome *R* gene (NBS-LRR)-mediated resistance. The research in this thesis may lead to a novel approach for crop protection strategies. Firstly, the confirmed identification of PRRs to aphid elicitors would open the possibility of transferring them to crop species that do not possess them. This may enable the plant to induce different plant defence pathways that may not be recognized by aphid effectors leading to enhanced plant resistance that aphids cannot easily adapt to, as the aphid will have to evolve new effectors that can suppress these pathways. It is more difficult to generate new effectors than to mutate or delete existing effectors, and so transfer of PRRs is likely to confer more durable crop resistance compared to the stacking of *R* genes. The effectiveness of this strategy was shown by the transfer of the Brassicaceae specific PRR EFR to the solanaceous plants *N. benthamiana* and *S. lycopersicum*, which led to increased resistance to a range of phytopathogenic bacteria (Lacombe *et al.*, 2010). The potential utility of the PRR for each *M. persicae* elicitor is discussed below.

8.5.2.1 The PRR to the less than 3 kDa elicitor may have a role in aphid resistance

Of the *M. persicae* elicitors discovered in the study, the less than 3 kDa fraction triggered immune responses in *N. benthamiana* (chapter 4) but not *A. thaliana* (chapter 5). It is also possible that it is perceived by a RLK, as AvrPtoB suppressed the plant response to 3 kDa eliciting fraction (chapter 7). However, whether this elicitor is present in all aphids, or just *M. persicae*, still needs to be established. If the elicitor is present in all aphids then the transferring of the novel *N. benthamiana* RLK to *A. thaliana* and other brassicas may enhance defence to crucifer specialist aphids such as *B. brassicae*, because these aphid species are unlikely to have evolved effectors that suppress elicitor-triggered immunity specifically in the non-host plant *N. benthamiana*. As *M. persicae* survives on both *A. thaliana* and *N. benthamiana* the transfer of an RLK between the two may have little affect on *M. persicae* performance. However, given the diversity of RLKs and downstream signalling pathways, it is possible that the introduction of new RLKs will generate new connections between pathways that *M. persicae* may not be able to suppress. One caveat may be that all aphids have effectors, such as Mp10, which appears to suppress PTI upstream. Therefore it is important to study the specificity and mechanism of Mp10 action to determine which pathways this effector may not suppress.

8.5.2.2 The PRR(s) to the 3 to 10 kDa elicitor may be conserved

The results of our targeted reverse genetics approach in chapter 6 suggest that there may be more than one *A. thaliana* PRR for the 3 to 10 kDa elicitor. *N. benthamiana* already responds to a *M. persicae* elicitor of this molecular weight, and few aphids beside *M. persicae* colonize *N. benthamiana*, so transfer of the *A. thaliana* PRR(s) to *N. benthamiana* would have little utility. Given that the distantly related plants *A. thaliana* and *N. benthamiana* respond to (potentially) the same elicitor, perception of this elicitor may be common to higher plants in the same way that flg22 perception through FLS2 orthologs is proposed to be common to higher plants (Boller and Felix, 2009). However, if a plant species is found that does not respond to the 3 to 10 kDa fraction of aphids then transferring the PRR(s) to it may enhance aphid resistance.

8.5.2.3 The number of elicitors larger than 10 kDa is still to be determined

The utility of the larger than 10 kDa elicitor in future crop protection strategies is still unclear. Both *A. thaliana* and *N. benthamiana* give immune responses to the elicitor(s) (chapters 4 and 5), although it is unclear whether it is the same elicitor in each case. The elicitor that *N. benthamiana* responds to is present in several aphid species and has the properties of a protein recognized by an epitope (chapter 4), whereas boiling and proteinase K treatment inactives the elicitor in *A. thaliana* (chapter 5). Whether *A. thaliana*

responds to the larger than 10 kDa fraction from aphid species other than *M. persicae* requires further investigation. If both plants respond to different elicitors then the potential for using PRRs is greater than if they both respond to the same elicitor.

8.5.2.4 Chimeras of PRRs present an increased number of options for resistance

Chimeras of extracellular binding domains and intracellular signalling domains from different PRRs also have potential for enhancing crop resistance (De Lorenzo *et al.*, 2011). The more PRRs that are discovered to aphid elicitors, the more potential there is for different combinations of extracellular and intracellular domains in chimeras involving the PRRs.

8.5.3 Priming plant defences can increase plant resistance

The second possibility for crop protection presented by this study is the use of aphid elicitors of plant defence to prime plant defences. Pretreating plants with elicitors, such as the harpins that are produced by several Gram-negative plant pathogenic bacteria, leads to an induction of defence to *M. persicae* (Dong *et al.*, 2004). We have shown that two *M. persicae* elicitors induce aphid defence in two distantly related plant species (chapters 4 and 5). However, there are several contributing factors that may frustrate the utility of this solution for the generation of durable crop resistance to aphids. First, the levels of induced resistance in our experiments were not large, varying between a 20 to 40% reduction in aphid reproduction levels. This is probably because aphids produce effectors that effectively suppress plant defences even after these defences have already been induced. The identification of elicitors that induce various defence pathways that are potentially not suppressed by aphids may enable a further reduction in aphid reproduction levels. Secondly, the eliciting activity of the 3 to 10 kDa fraction is sensitive to heat (chapter 5) and is also diminished after several days storage at -80^{o}C (personal observation). These attributes would make it complicated to store this fraction for long periods of time and use them in certain environments. Fortunately, the larger than 10 kDa elicitor of *N. benthamiana* is more robust, as heating does not decrease its ability to trigger immune responses (chapter 4). A final potential problem for the use of elicitors is that the priming plant defences often counteracts plant growth and yield. For example, the application of PAMPs to seedlings leads to growth inhibition (Gómez-Gómez *et al.*, 1999) and therefore treatment of young crop plants in this manner may not be beneficial. Thus, there may be a trade-off between using priming to induce plant defences and the yield produced.

8.5.4 RNAi of aphid genes decreases aphid performance

The Hogenhout lab has recently shown that RNAi-mediated silencing downregulates aphid gene expression resulting in measurable phenotypes such as a reduction in aphid reproduction levels (Pitino *et al.*, 2011). The dsRNAs corresponding to aphid genes are produced transiently or stably in plants and are acquired by the aphids leading to a reduction in the expression of the aphid genes (Pitino *et al.*, 2011). This allows the use of RNAi-mediated crop protection strategies against aphids, as has been shown for other insects, including chewing herbivores (Price and Gatehouse, 2008) (e.g. (Baum *et al.*, 2007)). Aphid effectors may be suitable candidate target genes for RNAi-based crop protection against aphids. This is because effectors generally have low sequence similarities between species or between insect genera or families (chapter 3) providing opportunities to develop crops that are resistant to specific species, insect genera or families. For example, effector C002 is divergent in sequence amongst aphid species (Ollivier *et al.*, 2010; Pitino and Hogenhout, 2013) thereby facilitating the design of constructs that enable control of single aphid species. Demonstrating silencing specificity is important for obtaining licenses to commercialize transgenic crop variety.

Whilst knocking down gene expression of effectors *MpC002*, *PIntO2* and possibly *Mp10* by plant mediated RNAi decreased *M. persicae* fecundity (Pitino *et al.*, 2011; Pitino and Hogenhout, 2013) (chapter 7), the level of reduction was low. This is expected given that aphids have multiple effectors each suppressing one specific reaction in a defence pathway or modulating a specific plant process. Nonetheless, our data on Mp10 suggest that there may be key effectors in aphids that may be the aphid Achilles' heel. The sequence of Mp10 is conserved amongst aphid species (Jacobs *et al.*, 2005) and therefore it may be a suitable target gene for RNAi using transgenic crops that would give rise to increased resistance to multiple aphid species. It is also possible to target multiple aphid genes for silencing.

Another area that needs further investigation is how PRR-mediated resistance and silencing of effector genes affect virus transmission by aphids. This is important, because *R* gene-mediated resistance may increase virus transmission, especially potyviruses, because of more probing by the insect. Consistent with this observation is that the *R* genes to sap-feeding insects cloned and functionally characterized so far confer resistance at the phloem-feeding stage. PRR-mediated resistance may solve this problem as immunity may act early at the aphid probing stage, thus stopping the aphids before they reach the phloem and transmitting or acquiring phloem-associated plant viruses. Similarly, some aphid effectors may act early at the probing stage as well. It is also

possible to target aphid genes that are involved in virus transmission for RNAi, and therefore reduce the damage that aphid-vectored viruses cause to crops.

8.5.5 Resistance genes against aphids can be further exploited

Plant resistance to aphids has been used in crop protection for many years (Dogimont *et al.*, 2010). It is possible that Mp10 and its homologs in other aphids are activating ETI in *N. benthamiana* (chapter 3). We have also suggested that suppression of the ETI from Mp10 may be caused by another aphid effector. This system offers the potential of identifying a new aphid resistance gene through the further study of Mp10-plant interactions, and identifying another important aphid effector that may be an RNAi target. *A. thaliana* in particular is an unexplored resource for *R* genes to aphids, as its genome contains around 150 NBS-LRR genes (Meyers *et al.*, 2003). However, the durability of plant resistance genes in agriculture is variable, with some genes lasting for many years and other being broken almost immediately (Dogimont *et al.*, 2010).

8.7.6 Combining multiple approaches offers the best hope of long-term resistance

The most effective use of knowledge about the aphid-plant immunity interaction to benefit crop protection is likely to be to combine approaches based on elicitors, RNAi of effectors and/or *R* genes. The reduction in aphid fecundity seen with both eliciting induced defences and RNAi of effectors may not be large, but it is conceivable that combining both approaches would have larger effects. It may also be feasible to synergise the affects of these approaches, such as combining increased plant perception of elicitors through PRR transfer with decreased ability of the aphid to suppress elicitor triggered immunity through Mp10 RNAi. Stacking of complementary aphid protection traits has the potential to increase the durability of crop resistance, as it is harder for multiple defences to be overcome simultaneously.

List of abbreviations

ACA8	Autoinhibited Ca^{2+} ATPase8
ANODE	Analysis of deviance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Avr	Avirulence
AY-WB	Aster yellows phytoplasma strain witches' broom
BAK1	BR insensitive1-associated receptor kinase
BAM	Barely any meristem
BIK1	*Botrytis*-induced kinase1
BKK1	BAK1-like
BLAST	Basic local alignment search tool
bp	Base pair
Bph14	Brown planthopper14
BR	Brassinosteriod
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cDNA	Complementary DNA
CDPK	Ca^{2+}-dependent protein kinase
CER	Controlled environment room
CERK1	Chitin elicitor receptor kinase1
CLE	CLV3/ESR
CLV	Clavata
CNF1	Cytotoxic necrotizing factor 1
Col-0	Columbia
CRN2	Crinkler2
Ct	Cycle threshold
CYP81F2	Cytochrome p450, family 81, subfamily f, polypeptide 2
DAB	3,3'-Diaminobenzidine
DAMP	Damage-associated molecular pattern
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
dpwi	Days post wound inoculation

DSB	Disease and Stress Biology
dsRNA	Double stranded RNA
EBF	(*E*)-β-farnesene
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factor
eFP	Electronic fluorescent pictograph
EFR	EF-Tu receptor
eGFP	Enhanced GFP
Eix	Ethylene-inducing xylanase
EMS	Ethyl methane sulphonate
EPG	Electrical penetration graph
EPS	Extracellular polysaccharides
ESR	Embryo surrounding region
EST	Expressed sequence tag
ET	Ethylene
EtBr	Ethidium bromide
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
FAC	Fatty acid-amino acid conjugates
FLS2	Flagellin-sensitive2
FRK1	Flg22-induced receptor-like kinase1
GFP	Green fluorescent protein
GLD	Glucose dehydrogenase
GLM	Generalised linear model
GMC	Glucose-methanol-choline
GOX	Glucose oxidase
GSO	Gassho
GUS	Beta-glucuronidase
HAMP	Herbivory-associated molecular pattern
HOP	HR and pathogenicity outer protein
Hpa	*Hyaloperonospora arabidopsidis*
HR	Hypersensitive cell death response
HSL2	Haesa-like2
IDA	Inflorescence deficient in abscission
IDL	IDA-like

IKU2	Haiku2
IMD	Immune deficiency
ISR	Induced systemic resistance
JA	Jasmonic acid
JIC	John Innes Centre
LecRK1	Lectin receptor kinase1
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
LYK	Lys-M RLK4
LYM	Lys-M domain-containing glycosylphospatidylinositol-anchored protein
Lys-M	Lysine motif
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MES	2-(*N*-morpholino)ethanesulfonic acid
MPL1	*Myzus persicae* induced lipase1
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NASC	Nottingham Arabidopsis stock centre
NBS	Nucleotide-binding site
NCBI	National Centre for Biotechnology Information
NLR	Nucleotide-binding LRR
NLRC4	NLR family CARD domain-containing 4
n.s.	Not significant
OBP	Odorant-binding protein
OD	Optical density
OG	Oligogalacturonide
PAD	Phytoalexin deficient
PAMP	Pathogen-associated molecular pattern
PBL	PBS1-like
PBS1	AvrPphB susceptible1
PCR	Polymerase chain reaction
PEPR	Pep1 receptor
PGN	Peptidoglycan
PKC	Protein kinase C
PRR	Pattern recognition receptor

PTI	PAMP-triggered immunity
PtoDC3000	*Pseudomonas syringae* pv. tomato DC3000
PVC	Polyvinyl chloride
PVX	Potato virus X
PXY	Phloem intercalated with xylem
Rac2	Ras-related C3 botulinum toxin substrate 2
RACK-1	Receptor for activated C kinase
RAR1	Required for MLA12 resistance1
RBOHD	Respiratory burst oxidase homologue D
RCH1	Recognition of *Colletotrichum* higginsianum1
RD	Arginine-asparate
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLU	Relative light unit
RNA	Ribonucleic acid
RNAi	RNA interference
RPS4	Resistance to *P. syringae* 4
SA	Salicylic acid
SAP	Secreted AY-WB protein
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SERK	Somatic embryogenesis receptor-like kinase
SGT1	Supressor of G-two allele of skp1
SIPK	SA-induced protein kinase
siRNA	Small interfering RNA
Suc2	Sucrose-proton symporter2
T-DNA	Transfer-DNA
T3SS	Type III secretion system
TAO1	Target of AvrB operation1
TDIF	Tracheary element differentiation inhibitory factor
TDR	TDIF receptor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TPS11	Trehalose phosphate synthase11

TSL	The Sainsbury laboratory
UV	Ultraviolet
Vat	Virus aphid transmission
VIGS	Virus induced gene silencing
WAK	Wall-associated kinase
WAK1	Cell wall-associated kinase1
WIPK	Wound-induced protein kinase
XA21	*Xanthomonas* resistance 21

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Appendix A – Bos *et al.*, 2010

A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid)

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Abbreviations: Ap, *Acyrthosiphon pisum*; CSPs, chemosensory proteins; dpi, days post inoculation; ER, endoplasmic reticulum; ESTs, expressed sequence tags; ETI, effector-triggered immunity; EV, empty vector; GFP, green fluorescent protein; LRR, leucine rich repeat; Mp, *Myzus persicae*; NBS, nucleotide binding site; ORFs, open reading frames; OS-D2, olfactory segment D2; PAMP, Pathogen-Associated Molecular Pattern; PTI, PAMP-triggered immunity, PVX, *Potato virus X*; ROS, reactive oxygen species; T3SS, type III secretion system; TRV, *Tobacco rattle virus*; UTRs, untranslated regions; VIGS, virus-induced gene silencing.

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Running title: *M. persicae* candidate effectors

Abstract

Aphids are amongst the most devastating sap-feeding insects of plants. Like most plant parasites, aphids require intimate associations with their host plants to gain access to nutrients. Aphid feeding induces responses such as clogging of phloem sieve elements and callose formation, which are suppressed by unknown molecules, probably proteins, in aphid saliva. Therefore, it is likely that aphids, like plant pathogens, deliver proteins (effectors) inside their hosts to modulate host cell processes, suppress plant defenses and promote infestation. We exploited publicly available aphid salivary gland expressed sequence tags (ESTs) to apply a functional genomics approach for identification of candidate effectors from *Myzus persicae* (green peach aphid), based on common features of plant pathogen effectors. A total of 48 effector candidates were identified, cloned, and subjected to transient overexpression in *Nicotiana benthamiana* to assay for elicitation of a phenotype, suppression of the Pathogen-Associated Molecular Pattern (PAMP)-mediated oxidative burst, and effects on aphid reproductive performance. We identified one candidate effector, Mp10, which specifically induced chlorosis and local cell death in *Nicotiana benthamiana* and conferred avirulence to recombinant *Potato virus X* (PVX) expressing Mp10, PVX-Mp10, in *N. tabacum* indicating that this protein may trigger plant defenses. The ubiquitin-ligase associated protein SGT1 was required for the Mp10-mediated chlorosis response in *N. benthamiana*. Mp10 also suppressed the oxidative burst induced by flg22, but not by chitin. Aphid fecundity assays revealed that *in planta* overexpression of Mp10 and Mp42 reduced aphid fecundity, whereas another effector candidate, MpC002, enhanced aphid fecundity. Thus, these results suggest that, although Mp10 suppresses flg22-triggered immunity, it triggers a defense response, resulting in an overall decrease in aphid performance in the fecundity assays. Overall, we identified aphid salivary proteins that share features with plant pathogen effectors and therefore may function as aphid effectors by perturbing host cellular processes.

Author summary

Aphids are insects that can induce feeding damage, achieve high population densities, and most importantly, transmit economically important plant diseases worldwide. To develop durable approaches to control aphids it is critical to understand how aphids interact with plants at the molecular level. Aphid feeding induces plant defenses, which can be suppressed by aphid saliva. Thus, aphids can alter plant cellular processes to promote infestation of plants. Suppression of plant defenses is common in plant pathogens and involves secretion of effector proteins that modulate host cell processes. Evidence suggests that aphids, like plant pathogens, deliver effectors inside their host cells to promote infestation. However, the identity of these effectors and their functions remain elusive. Here, we report a novel approach based on a combination of bioinformatics and functional assays to identify candidate effectors from the aphid species *Myzus persicae*. Using this approach we identified three candidate effectors that affect plant defense responses and/or aphid reproductive performance. Further characterization of these candidates promises to reveal new insights into the plant cellular processes targeted by aphids.

Introduction

Like most plant parasites, aphids require intimate associations with their host plants to gain access to nutrients. Aphids predominantly feed from the plant phloem sieve elements, and use their stylets to navigate between the cells of different layers of leaf tissue during which plant defenses may be triggered. Indeed, aphid feeding induces responses such as clogging of phloem sieve elements and callose formation, which are suppressed by the aphid in successful interactions with plant hosts [1]. In addition, some aphid species can alter host plant phenotypes, by for example inducing the formation of galls or causing leaf curling [2] indicating that there is an active interplay between host and aphid at the molecular level. During probing and feeding, aphids secrete two types of saliva: gelling saliva, which is thought to protect stylets during penetration, and watery saliva, which is secreted into various plant host cell types and the phloem [3]. The secretion of aphid saliva directly into the host-stylet interface [4], suggests that molecules present in the saliva may perturb plant

cellular processes while aphids progress through different feeding stages. Interestingly, the knock-down of the *C002* salivary gene in *Acyrthosiphon pisum* (pea aphid) negatively impacts survival rates of this aphid on plant hosts [5, 6]. Furthermore, proteomics studies based on artificial aphid diets showed the presence of secreted proteins, including C002, in aphid saliva indicating that these proteins are delivered inside the host plant during feeding [7, 8]. However, whether and how these aphid salivary proteins function in the plant host remains elusive.

Suppression of host defenses and altering host plant phenotypes is common in plant-pathogen interactions and involves secretion of molecules (effectors) that modulate host cell processes [9, 10]. Therefore it is likely that aphids, similar to plant pathogens, deliver effectors inside their hosts to manipulate host cell process enabling successful infestation of plants [9]. Effector-mediated suppression of plant defenses, such as Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity (PTI), generally involves the targeting of a plant virulence target, or operative target [11]. However, plant pathogen effectors that are deployed to suppress host defenses are recognized by plant disease resistance (R) proteins in particular host genotypes, resulting in effector-triggered immunity (ETI) [12]. Interestingly, the R proteins that recognize plant pathogens and those that confer resistance to aphids, such as *Mi-1.2* and *Vat*, share a similar structure, and contain a nucleotide binding site (NBS) domain and leucine rich repeat (LRR) regions [13-15]. The *Mi-1.2* resistance gene confers resistance in tomato to certain clones of *Macrosiphum euphorbiae* (potato aphid), two whitefly biotypes, a psyllid, and three nematode species [16-19], indicating that there is significant overlap in plant pathogen and aphid recognition in plants. In addition, aphid resistance conferred by several resistance genes was shown to be race-specific [16,20]. This suggests that depending on their genotype, certain aphid clones may be able to avoid and/or suppress plant defenses and fits with the gene-for-gene model in plant-pathogen interactions [21]. Therefore, it is likely that not only plant pathogens, but also aphids, secrete effectors that in addition to targeting host cell processes may trigger ETI depending on the host genotype.

Plant pathogen effectors generally share the common feature of modulating host cell processes [22]. Various assays have been developed to identify the functions of effectors from bacterial and eukaryotic filamentous plant pathogens [22-24]. One important and common function of plant pathogen effectors is the suppression of PTI. This activity is especially common among type III secretion system (T3SS) effectors. For example, the large majority of *Pseudomonas syringae* DC3000 effectors can suppress PTI responses, including the oxidative burst [25]. However, effectors from eukaryotic filamentous plant pathogens can also suppress PTI, as demonstrated for the AVR3a effector from *Phytophthora infestans*, which suppresses cell death induced by the PAMP-like elicitor INF1 [26, 27]. Another activity of plant pathogen effectors is the induction of phenotypes in plants. For example, several effectors, including CRN2 and INF1, from the oomycete plant pathogen *P. infestans* induce cell death upon overexpression *in planta* [28, 29], whereas other effectors, like AvrB from *P. syringae* DC3000 induce chlorosis [30]. Also, overexpression of effector proteins from plant pathogenic nematodes in host plants can affect plant phenotypes, as shown for the *Heterodera glycines* CLE protein Hg-SYV46 that alters host cell differentiation [31]. As effectors exhibit functions important for pathogenicity, their deletion can have detrimental effects on pathogen virulence. However, due to redundancy, the knock-down or deletion of single effectors does not always impact virulence. On the other hand, overexpression of plant pathogen effectors can enhance pathogen virulence, as shown for active AvrPtoB, which enhances virulence to *P. syringae* DC3000 in Arabidopsis [32], and for the *H. schachtii* effector 10A06 that, in addition to altering host plant morphology, increases nematode susceptibility in *Arabidopsis* [33].

We exploited publicly available aphid salivary gland sequences to develop a functional genomics approach for the identification of candidate aphid effector proteins from the aphid species *Myzus persicae* (green peach aphid) based on common features of plant pathogen effectors. Data mining of salivary gland expressed sequences tags (ESTs) identified 46 *M. persicae* predicted secreted

proteins. Functional analyses showed that one of these proteins, Mp10, induced chlorosis and weak cell death in *Nicotiana benthamiana*, and suppressed the oxidative burst induced by the bacterial PAMP flg22. In addition, we developed a medium-throughput assay, based on transient overexpression in *N. benthamiana*, that allows screening for effects of aphid candidate effectors on aphid performance. Using this screen, we identified two candidate effectors, Mp10 and Mp42, that reduced aphid performance and one effector candidate, MpC002, that enhanced aphid performance. In summary, we found aphid secreted salivary proteins that share features with plant pathogen effectors and therefore may function as aphid effectors by perturbing host cellular processes.

Results

Description of Functional Genomics Screen

We developed a functional genomics approach to identify candidate effectors from *M. persicae* using 3233 publicly available aphid salivary gland ESTs [34]. We hypothesized that aphid effectors are most likely secreted proteins that are delivered into the saliva through the classical eukaryotic endoplasmic reticulum (ER)-Golgi pathway of the salivary glands. A feature of proteins secreted through this pathway is the presence of an N-terminal signal peptide. Therefore, we used the SignalP v3.0 program [35] to predict the presence of signal peptides in the amino acid sequences encoded by the open reading frames (ORFs) found in salivary gland ESTs. Out of 5919 amino acid sequences corresponding to predicted ORFs, we identified 134 nonredundant sequences with signal peptide (Figure 1A). Out of these 134 proteins, 19 were predicted to contain a transmembrane domain in addition to the signal peptide, and are therefore likely to remain in the salivary gland membrane upon secretion. Hence, 115 predicted secreted proteins remained. In order to investigate the *M. persicae* candidate effector protein in functional assays, we started with the cloning of 46 candidates that corresponded to full-length sequences within the set of 115 candidates. Effectors are subject to diversifying selection because of the co-evolutionary arms race between host and pathogen proteins [36, 37]. Therefore, we used the presence of amino acid polymorphisms among alignments of deduced

protein sequences of *M. persicae* and *A. pisum* ESTs as an additional criterion. Three candidates did not fulfill this criterion and were removed from our candidate set bringing the total to 43 candidates.

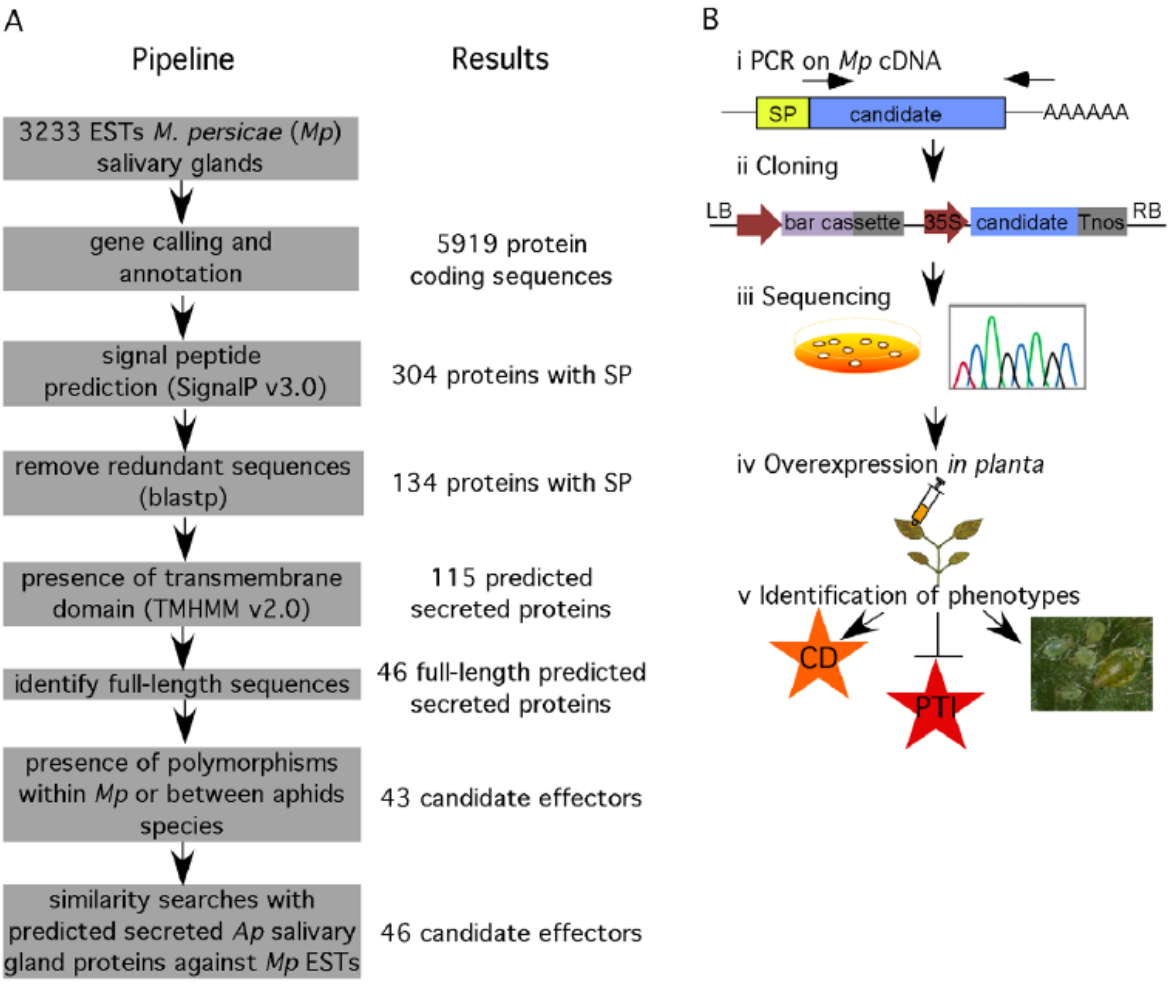

Figure 1. Overview of functional genomics pipeline to identify candidate effectors from *Myzus persicae*.

(A) Bioinformatics pipeline for data mining of *M. persicae* salivary gland expressed sequence tags (ESTs). (B) Cloning and functional analyses of candidates to identify effector activities. i) PCR amplification was performed on *M. persicae* cDNA. ii) Amplicons were cloned in the pCB302-3 vector under control of a 35S promoter and constructs were transformed into *Agrobacterium tumefaciens*. iii) Multiple clones were sequenced to identify polymorphic candidates. Clones were stored and cultured for subsequent functional assays. iv) Candidate effectors were overexpressed in *Nicotiana benthamiana* by agroinfiltration to determine whether they induce a phenotype *in planta*, such as cell death, suppress basal plant defences, PAMP-triggered immunity (PTI), and affect aphid performance.

We applied a similar data mining approach as described above to 4517 publicly available salivary gland ESTs from *A. pisum*, thereby identifying 24 candidates (Table S1). In the *A. pisum* salivary gland ESTs we predicted only 1751 ORFs, explaining the relatively low number of *A. pisum* candidates. A total of three candidates were found in both *M. persicae* and *A. pisum* datasets (combinations Mp1/Ap1, Mp5/Ap7 and Mp16/Ap4). The remaining 21 non-overlapping *A. pisum* candidates were subjected to BLAST searches (E value < 10^{-15}) against all available *M. persicae* ESTs to identify putative *M. persicae* homologs. This led to the identification of three *M. persicae* sequences (Mp3, Mp54 and MpC002) that were added to the *M. persicae* candidate effector dataset bringing the total to 46 (Figure 1A, Table S2).

Interestingly, for two candidates, Mp39 and Mp49, no similar sequences were present in the publicly available aphid sequence datasets, including the *A. pisum* genome sequence (Table S2). Also, no homologs of these proteins were identified by BLAST searches against GenBank nucleotide and protein databases (E value < 10^{-5}). This suggests these proteins may be specific to *M. persicae*. A total of 11 candidates were shared between the independent salivary gland EST datasets from *M. persicae* and *A. pisum* but were not present in gut ESTs from *M. persicae* (Table S2) providing support that the corresponding proteins may share a similar function in both these aphid species. For four candidates matches were found in gut ESTs from *M. persicae*, suggesting these proteins may be derived from salivary gland contaminants in dissected gut tissues and not function uniquely in the salivary gland or saliva. Indeed, gene expression analysis of *Mp51* in various aphid tissues dissected from aphids fed on *N. benthamiana* confirmed that this gene is specifically expressed in the aphid gut (Figure S1). In contrast, candidate effector genes *Mp1*, *Mp2*, *Mp10*, *Mp30*, *Mp42*, *Mp47*, *Mp50* and *MpCOO2*, were expressed in aphid heads and salivary glands but not in aphid guts (Figure S1), suggesting that their corresponding proteins are indeed produced in the salivary glands. Furthermore, Mp1 and MpCOO2 were previously identified in saliva of *M. persicae* using a

proteomics-based approach [7] confirming that these two proteins are secreted into aphid saliva.

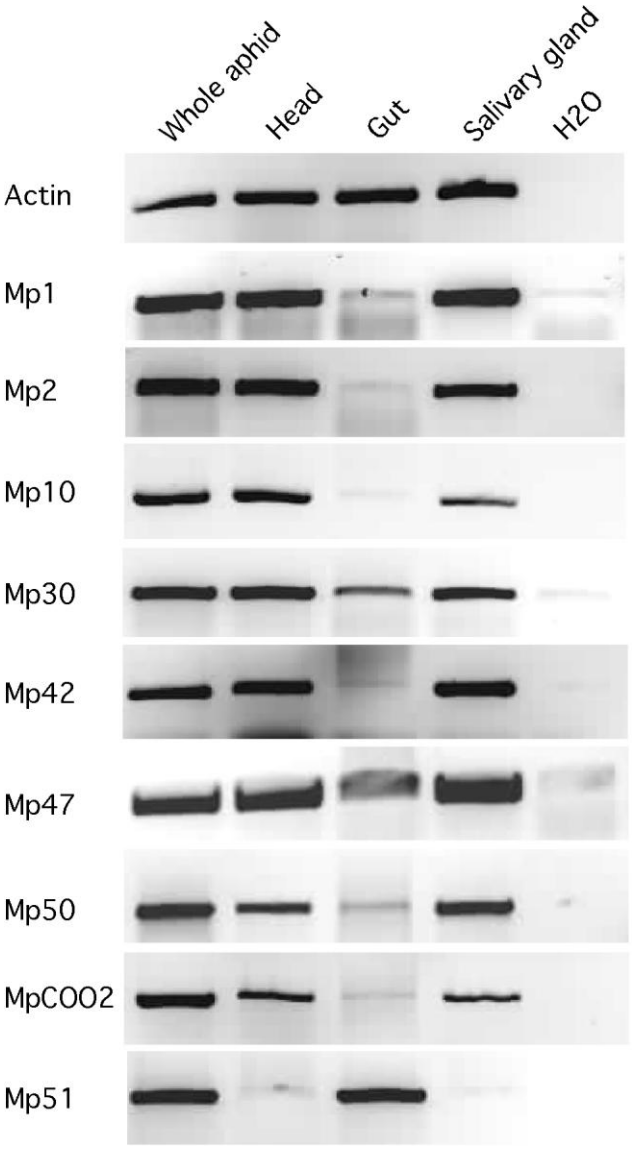

Figure S1

Gene expression analyses of candidate effectors in various aphid tissues. RT-PCR was performed on cDNA prepared from whole aphids fed on *N. benthamiana*, dissected heads, guts, salivary glands and on H_2O (control). Candidates were amplified using gene specific primers. Actin primers were used as a control for equal template amounts.

To investigate the functions of the 46 effector candidates, we amplified the corresponding sequences encoding the mature proteins, without the signal peptide

encoding sequences, from *M. persicae* cDNA for cloning (Figure 1B). To preserve the authentic sequence in the 3' end of the ORF, we designed reverse primers in the 3' untranslated regions (UTRs) based on EST sequences when possible. Amplicons were cloned in a 35S cassette and corresponding constructs were transformed directly into *Agrobacterium tumefaciens* followed by sequencing (Figure 1B). Two out of the 46 candidates, *Mp7* and *Mp38* could not be amplified from *M. persicae* cDNA. Of the remaining 44 candidates, four (*Mp6*, *Mp17*, *Mp33* and *Mp35*) were represented by two polymorphic forms, with polymorphisms within the mature protein portion. Except for one of the polymorphic *Mp6* sequences, all sequences were identical to those in the *M. persicae* EST databases. To rule out that the polymorphism in *Mp6* was due to PCR errors, we repeated the *Mp6* PCR and sequencing several times on individual aphids with similar results. Both forms of the four polymorphic candidates were cloned resulting in a total of 48 cloned *M. persicae* effector candidates. Functional assays were performed based on transient over-expression in *N. benthamiana* to assess whether the *M. persicae* candidate effectors 1) induce a phenotype *in planta*, 2) suppress PAMP-triggered immunity and 3) affect the ability of *M. persicae* aphids to reproduce (fecundity) (Figure 1B). We assessed fecundity of *M. persicae* lineage RRes (genotype O), which can utilize *N. benthamiana* as a host.

M. persicae candidate effector Mp10 induces chlorosis upon overexpression in *Nicotiana benthamiana*

Several plant pathogen effectors induce a phenotype upon overexpression *in planta*, which may reflect their virulence activity [22]. Hence, we performed transient overexpression of the effector candidates in *N. benthamiana* by agroinfiltration to screen for the induction of phenotypes. Out of the 48, one candidate effector, Mp10, induced chlorosis starting from 2 days post inoculation (dpi) (Figure 2A). In addition, we observed local cell death in a low number of infiltration sites (Figure S2A-D). The phenotype was not affected by co-expression with the silencing suppressor p19 (Figure S2E). To independently confirm the phenotype, we expressed Mp10 in *N. benthamiana* using a *Potato virus X* (PVX)-based vector (PVX-Mp10). Systemic

PVX-based overexpression of Mp10 induced systemic chlorosis in *N. benthamiana* starting at 10 dpi (Figure 2B). This also suggests that the Mp10 response is not dependent on the presence of *Agrobacterium*. To determine whether the response to Mp10 was specific to *N. benthamiana*, we infected *N. tabacum*, *Solanum lycopersicum* (tomato) and *N. benthamiana* plants with PVX-Mp10 in parallel. Starting at around 10 dpi, systemic chlorosis was observed in *N. benthamiana* expressing PVX-Mp10, but not in control PVX-infected plants (Figure 2B). Whereas mosaic symptoms were observed in *S. lycopersicum*, indicative of PVX infection, no Mp10-induced chlorosis was observed (Figure 2C; Figure S3A,B). Mp10 expression was confirmed by semi-quantitative RT-PCR in systemically PVX-Mp10 infected leaves of *S. lycopersicum* suggesting that the lack of symptoms is not due to a loss of the Mp10 sequence from PVX-Mp10 (Figure 2E). In contrast, *N. tabacum* plants infected with PVX-Mp10 did not show mosaic symptoms indicative of virus infection, while *N. tabacum* inoculated with PVX alone did (Figure 2D; Figure S2B). No Mp10 expression could be detected in leaves of *N. tabacum* plants inoculated with PVX-Mp10, whereas expression of the viral coat protein was detected, indicating that PVX itself did systemically spread in *N. tabacum* (Figure 2E). In contrast, PVX-Mp42 did spread systemically in *N. benthamiana*, *N. tabacum* and *S. lycopersicum*, indicating that this aphid protein can be systemically expressed in these plant species using PVX (Figure S4). It is possible that PVX-Mp10 may evoke an avirulence response in *N. tabacum* causing the selection of PVX without the Mp10 insert. Loss of foreign gene fragments from the PVX genome has been reported previously and is most likely due to selection pressures forcing virus recombination [38]. The lack of mosaic symptoms in PVX-Mp10-inoculated *N. tabacum* plants is possibly due to the initially low abundance of recombined PVX-virus as compared to the vector control.

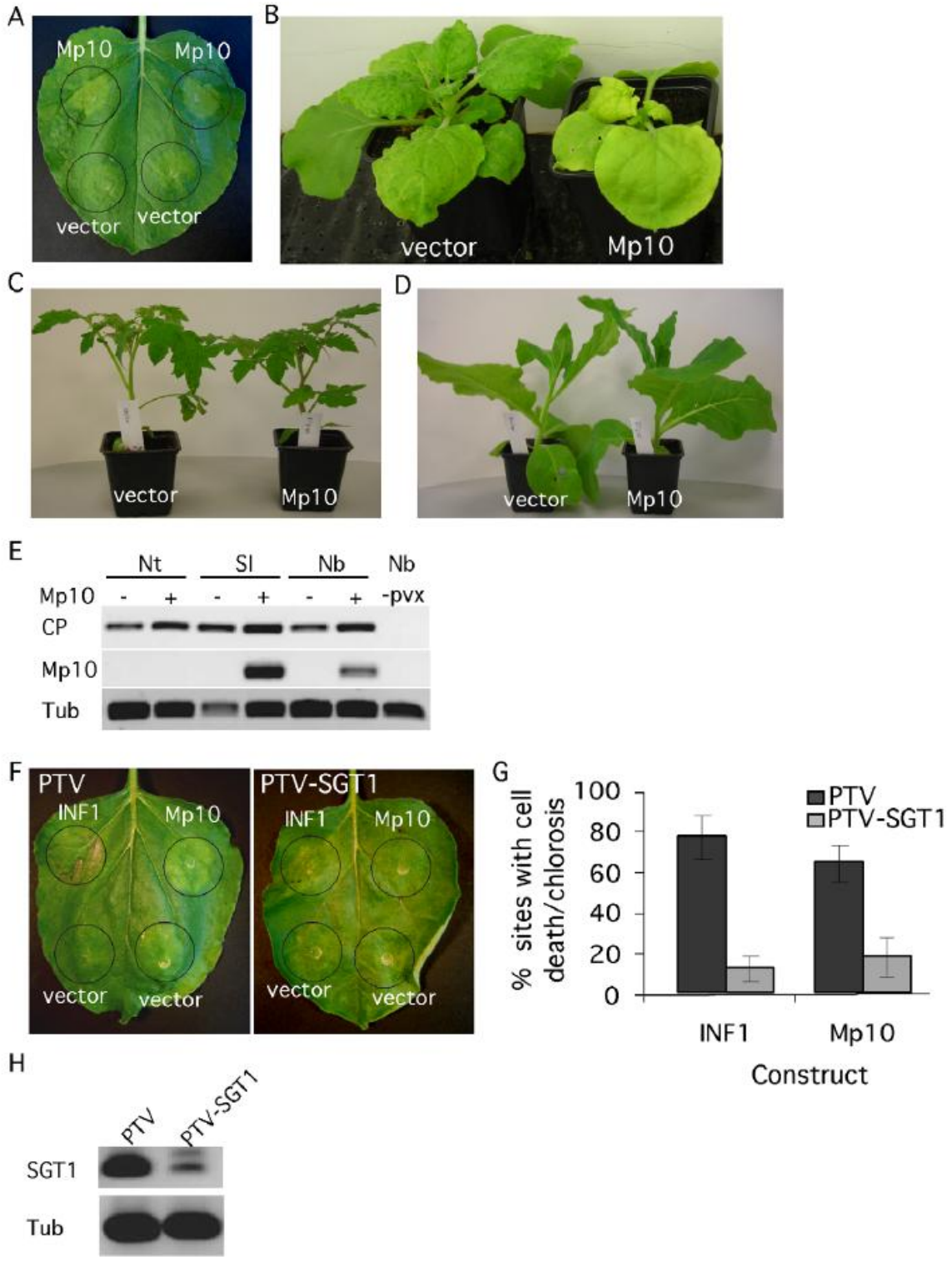

Figure 2. The candidate effector Mp10 induces chlorosis specifically in *Nicotiana benthamiana*. (A) Overexpression of 35S-Mp10 by agroinfiltration induces chlorosis in *N. benthamiana*. Symptoms of chlorosis started to appear from 2 days post infiltration (dpi). Photos were taken 4 dpi. (B) PVX-based

expression of Mp10 in *N. benthamiana*. Symptoms of chlorosis started to appear from 10 days post wound-inoculation (dpwi). Photos were taken 14 dpwi. (C) PVX-based expression of Mp10 in *Solanum lycopersicum* (tomato). Photos were taken 14 dpwi. D) PVX-based expression of Mp10 in *N. tabacum.* Photos were taken 14 dpwi. (E) Semi-quantitative RT-PCR on RNA from *N. benthamiana* (Nb), *N. tabacum* (Nt) and *S. lycopersicum* (Sl) plants infected with PVX-Δgfp (vector) or PVX-Mp10 as well as non-infected *N. benthamiana* plants (Nb, -pvx). Primers were used to amplify sequences corresponding to the PVX virus coat protein (CP) and Mp10. The plant tubulin gene (Tub) was used as a control for equal RNA levels. Plant tissues were harvested 14 dpwi (F) Over-expression of 35S-INF1 and 35S-Mp10 in *SGT1*-silenced (TRV-SGT1) and control (TRV) *N. benthamiana* plants. Photos were taken 4 dpi. (G) Percentage of infiltration sites showing either INF1 cell death or Mp10 chlorosis 4 dpi on *SGT1*-silenced and control *N. benthamiana* plants. The graphs show the averages calculated from 3 replicated experiments, with 8-10 infiltration sites per individual replicate. Error bars indicate the standard error. H) Semi-quantitative RT-PCR on *SGT1*-silenced and control *N. benthamiana* plants with *SGT1*-specific primers. The plant tubulin gene (Tub) was used as a control for equal RNA amounts.

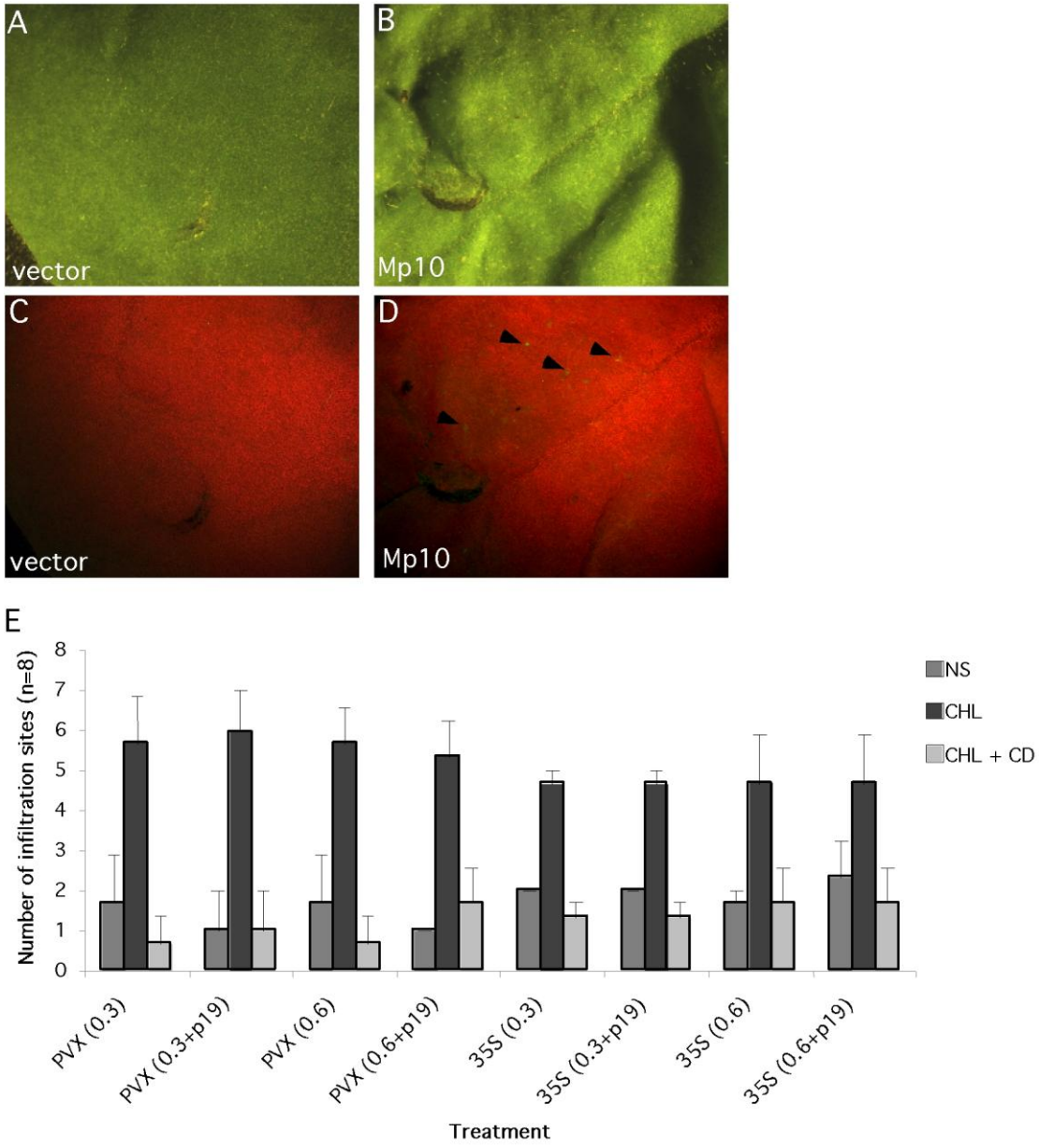

Figure S2

Mp10 induces weak local cell death in *N. benthamiana*. (A–D) Symptoms of *N. benthamiana*agroinfiltration sites expressing the 35S empty vector (control) or Mp10 under bright and ultraviolet (UV) light. Symptoms induced by the control (A) and Mp10 (B) were analyzed under a dissecting microscope. Accumulation of autofluorescent phenolic compounds associated with local cell death induced Mp10 (D), but not the control (C) were visualized under ultraviolet (UV) light (480/40 nm excitation filter; 510 barrier nm). Photographs were taken 5 days post infiltration. The black arrow heads indicate foci associated with autofluorescent phenolic compounds as a result of local cell death. (E) Percentages of infiltration sites showing induction of local macroscopic cell death upon expression of the Mp10 in *N. benthamiana* plants. Leaves were agroinfiltrated with *Agrobacterium* strains carrying

35S-Mp10 or PVX-Mp10 in the presence or absence of strains carrying p19 at an OD600 of 0.3 or 0.6. NS indicates no symptoms, CHL indicates chlorosis and CHL+CD indicates cell death. Symptoms were scored 4 days post infiltration. The average number of infiltration sites was based on 3 replicated experiments (n = 8 sites per experiment). Error bars indicate the standard error.

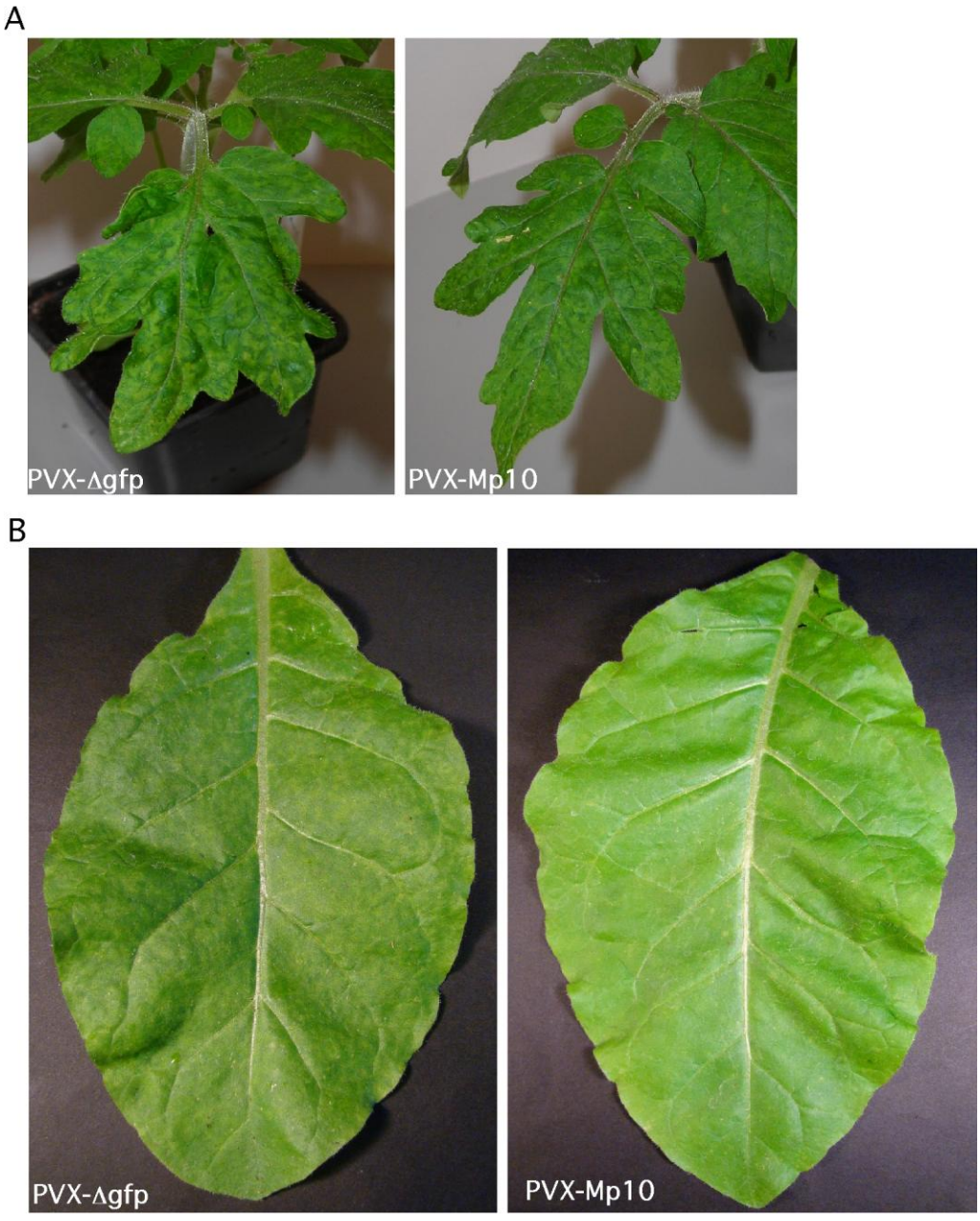

Figure S3

Symptoms of PVX-Mp10 infected *Solanum lycopersicum* (tomato) and *N. tabacum* plants. (A) Symptoms on a tomato plant infected with PVX-Δgfp (control) (left panel) and PVX-Mp10 (right panel).

(B) Symptoms on a *N. tabacum* plant infected with PVX-Δgfp (control) (left panel) and PVX-Mp10 (right panel). Pictures were taken 14 days after inoculation.

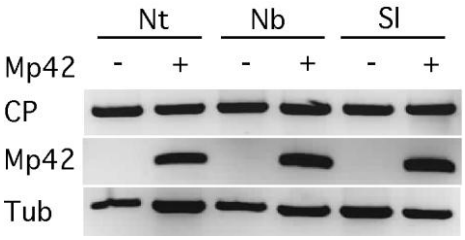

Figure S4

PVX-based expression of *Mp42* in various plant species. Leaf tissues from *N. benthamiana* (Nb), *N. tabacum* (Nt), and *Solanum lycopersicon* (Sl) were collected for RNA extractions 14 days post wound-inoculation (dpwi). For semi-quantitative RT-PCR primers were used to amplify sequences corresponding to the PVX virus coat protein (CP) and Mp42. The plant tubulin gene (Tub) was used as a control for equal RNA amounts.

The SGT1 protein, an ubiquitin-ligase associated protein, is required for plant cell death responses, including those involved in plant resistance [39]. To investigate whether SGT1 is required for the Mp10 chlorosis response, we generated *SGT1*-silenced *N. benthamiana* plants using *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS). Silenced plants (treated with TRV-SGT1) and control plants (treated with TRV) (Figure 2H) were infiltrated with *Agrobacterium* strains expressing Mp10 or the positive control INF1, an elicitin from *P. infestans* that induces cell death in control plants, but not in *SGT1*-silenced plants [40]. Both the Mp10-induced chlorosis and the INF1-induced cell death were pronouncedly reduced in the *SGT1*-silenced plants, but not in the TRV-treated control plants (Figure 2F and G), indicating SGT1 is required for these chlorosis and cell death responses.

Candidate effector Mp10 suppresses the flg22- but not the chitin-induced oxidative burst

Suppression of PTI induced by PAMPs like flg22 and chitin is a common feature of plant pathogen effectors. To determine whether aphid candidate effectors can suppress PTI, we assessed whether any of our 48 candidates suppressed the

oxidative burst response induced by the bacterial PAMP flg22. We decided to screen for suppression of the oxidative burst induced by flg22 only, as this PAMP gives a strong and consistent oxidative burst response in *N. benthamiana*, which is convenient for use in large screens. *N. benthamiana* leaf discs overexpressing the effector candidate genes under control of the 35S promoter were challenged with the flg22 elicitor and the production of reactive oxygen species (ROS) was measured using a luminol-based assay [41]. The bacterial effector AvrPtoB, a suppressor of the flg22-mediated oxidative burst response [42], was included as a positive control. We found that Mp10 suppresses the flg22-induced oxidative burst in leaf discs harvested 2 days post agroinfiltration (three replicated experiments) (Figure 3A), whereas other candidate effectors did not (data not shown). Although the level of suppression by Mp10 was significant compared to that of the empty vector control, it was not as effective as AvrPtoB. We tested whether Mp10 also suppressed the oxidative burst induced by a fungal PAMP, chitin, and found that while Mp10 suppressed the flg22 response, no suppression of the chitin-induced oxidative burst was observed (Figure 3B). Thus, Mp10 specifically suppresses the oxidative burst induced by the PAMP flg22.

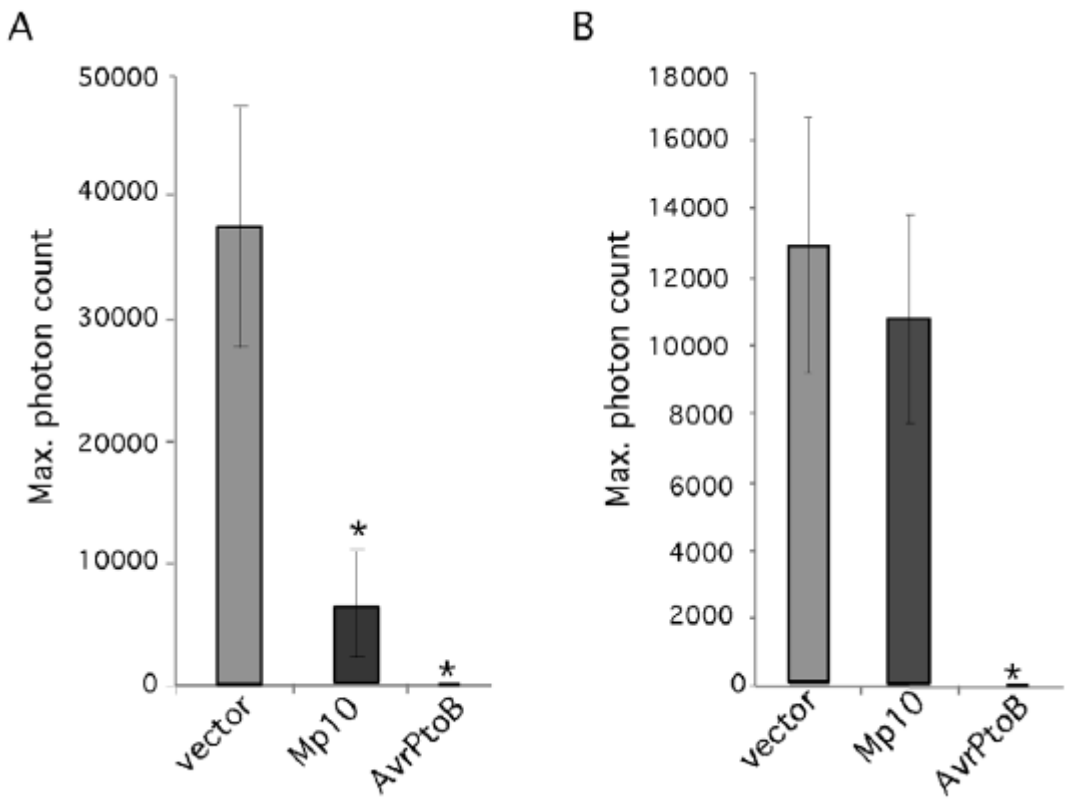

Figure 3. Mp10 suppresses the oxidative burst induced by flg22 but not chitin in *Nicotiana benthamiana*.

The induction of reactive oxygen species (ROS) induced by the flg22 and chitin was measured using a luminol-based assay. (A) The ROS response induced by flg22 in *N. benthamiana* leaf discs overexpressing Mp10, AvrPtoB (positive control) and the vector control upon agroinfiltration. The maximum photon count is based on the average of 8 leaf discs. The experiment was repeated 3 times with similar results. Error bars indicate standard error. Asterisks indicate statistical significance compared to the vector control ($p \leq 0.043$) (B) The ROS response induced by chitin in *N. benthamiana* leaf discs overexpressing Mp10, AvrPtoB (positive control) and the vector control upon agroinfiltration. The maximum photon count is based on the average of 8 leaf discs. The experiment was repeated 3 times with similar results. Error bars indicate standard error. Asterisks indicate statistical significance compared to the vector control ($p \leq 0.028$).

Candidate effectors Mp10, Mp42, and MpC002 alter aphid fecundity on *N. benthamiana*

We developed a medium-throughput 24-well plate assay to assess *M. persicae* fecundity on *N. benthamiana* leaves transiently overexpressing the 48 candidate effectors (Figure 4A). Leaf discs were harvested from infiltrated leaves one day after

agroinfiltration and placed upside down on water agar in 24-well plates. Four first-instar nymphs were placed on each leaf disc and the plate was incubated up-side-down under a light source. Aphids were moved every 6 days to plates with freshly infiltrated leaf discs, as expression levels of green fluorescent protein (GFP) in leaf discs were constant during 6 days and then tapered off (Figure S5). The aphids placed initially on the leaf discs generally started producing nymphs after about 10-11 days. Nymph production (fecundity) was assessed on day 12, 14 and 17 by counting and removing newly produced nymphs on each leaf disc. The total nymph production per adult was calculated and compared among the treatments and GFP and vector controls.

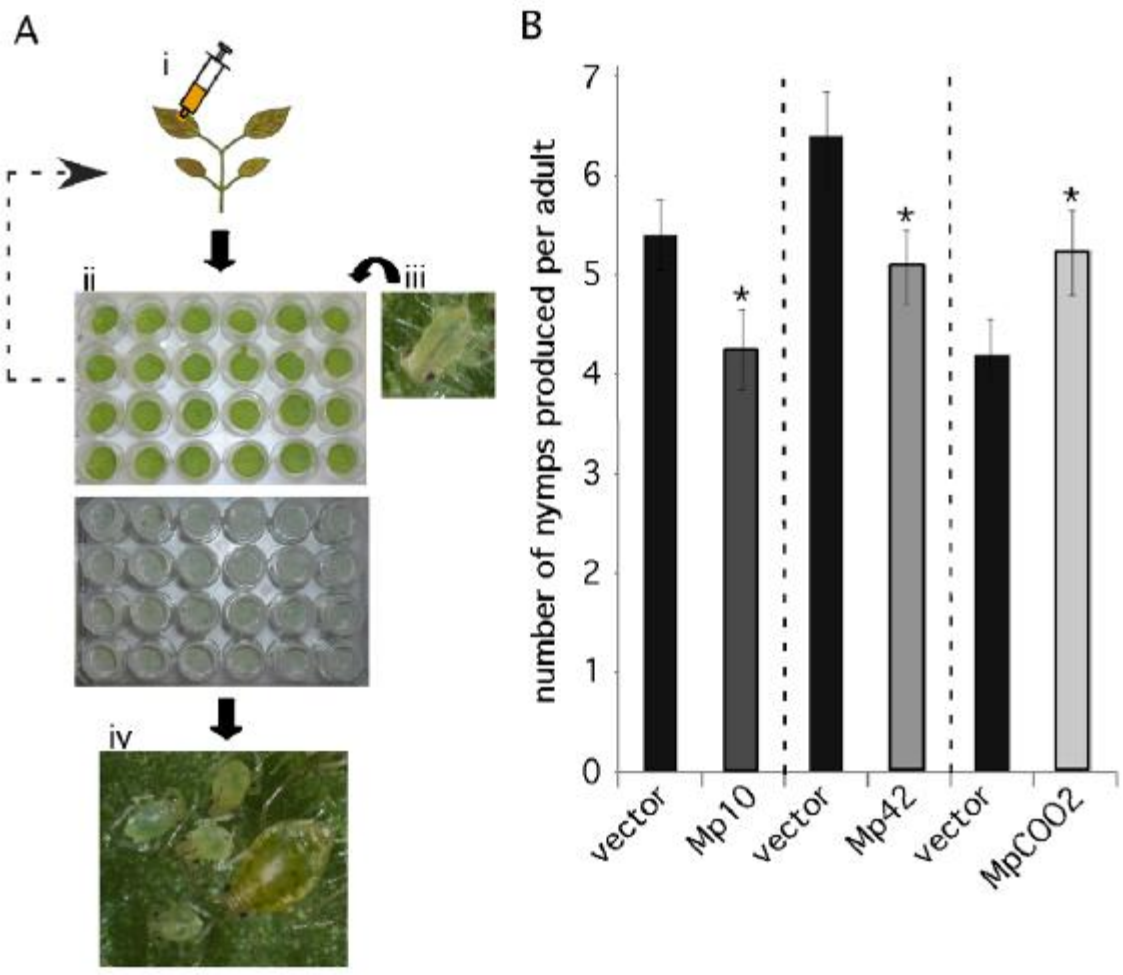

Figure 4. A medium-throughput leaf disc-based assay identifies *Myzus persicae* effector candidates that affect aphid performance.

(A) A novel medium-throughput assay to determine whether *in planta* overexpresssion of effector candidates affects aphid performance. i) Effector candidates are overexpressed in *Nicotiana benthamiana* by agroinfiltration. ii) One day after agroinfiltration leaf discs are harvested from infiltration sites using a cork borer. Leaf discs are placed upside-down on water agar in a 24-wells plate. iii) Four first-instar nymphs are placed on each leaf disc and wells are covered with individual mesh caps. Every six days these four aphids are moved to fresh leaf discs overexpressing the effector candidates. iv) Nymph production is assessed up to 17 days after placing first-instar nymphs on the leaf discs on day 1. (B) Overexpression of Mp10 and Mp42 reduces aphid nymph production (fecundity) and overexpression of MpC002 increases aphid nymph production. For each effector candidate, agroinfiltrations and aphid assays were performed side-by-side with the vector control (vector). Graphs show the average number of nymphs produced per adult based on 3 replicated

experiments, each consisting of 6 replicated leaf discs per candidate effector construct (n=18). Error bars indicate the standard error. Asterisks indicate statistical significance compared to the vector control based on a one-way ANOVA (Mp10: $p \leq 0.026$, Mp42: $p \leq 0.036$ and MpCOO2: $p \leq 0.038$).

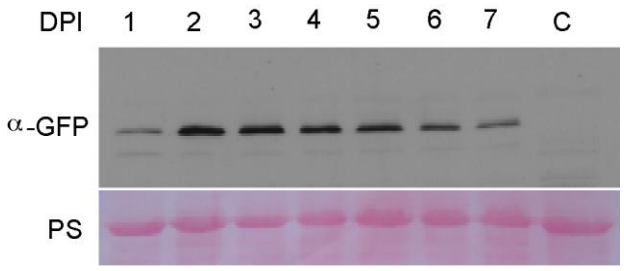

Figure S5

Expression of GFP in *N. benthamiana* leaf discs placed on water agar in a 24-well plate. Leaves were collected 24 after agroinfiltration with *Agrobacterium* strains expressing GFP and placed on top of water agar in a 24 wells plate. Leaf discs were collected every 24 hours from 1 to 7 days post infiltration (DPI) and ground in SDS-PAGE sample loading buffer to analyze the accumulation of GFP by western blotting with a GFP antibody. As a negative control (C) a 1-day old non-infiltrated *N. benthamiana* leaf disc was used. Ponceau S staining (PS) showed equal loading.

In our initial screens, in which candidate effector constructs were infiltrated on different leaves and not always side-by-side with the vector control, we identified 14 candidates that either enhanced or reduced aphid fecundity by one time the standard error compared to the empty vector (EV) control (Figure S6). To confirm the effect on aphid fecundity of these 14 candidates, we conducted additional assays in which the candidates were infiltrated side-by-side with the vector control (EV) on the same leaves. Two candidates, Mp10 and Mp42, reduced aphid fecundity in three repeated confirmation assays compared to the vector control (Figure 4B). In addition, one candidate, MpC002, enhanced aphid fecundity in three repeated confirmation assays compared to the vector control (Figure 4B). Transient overexpression of Mp10 did not induce chlorosis in leaf discs (Figure S7) or leaves that were detached from the plant 24hrs after infiltration (data not shown). Thus, leaves need to be attached to the plant for chlorosis to occur and the chlorosis itself was therefore not likely responsible for the observed reduction in aphid performance. In summary, we have developed a novel assay to screen for effects of *in planta* expressed aphid salivary proteins on

aphid performance and thereby identified three candidates that potentially function as effectors by eliciting plant defenses or promoting aphid infestation of host plants.

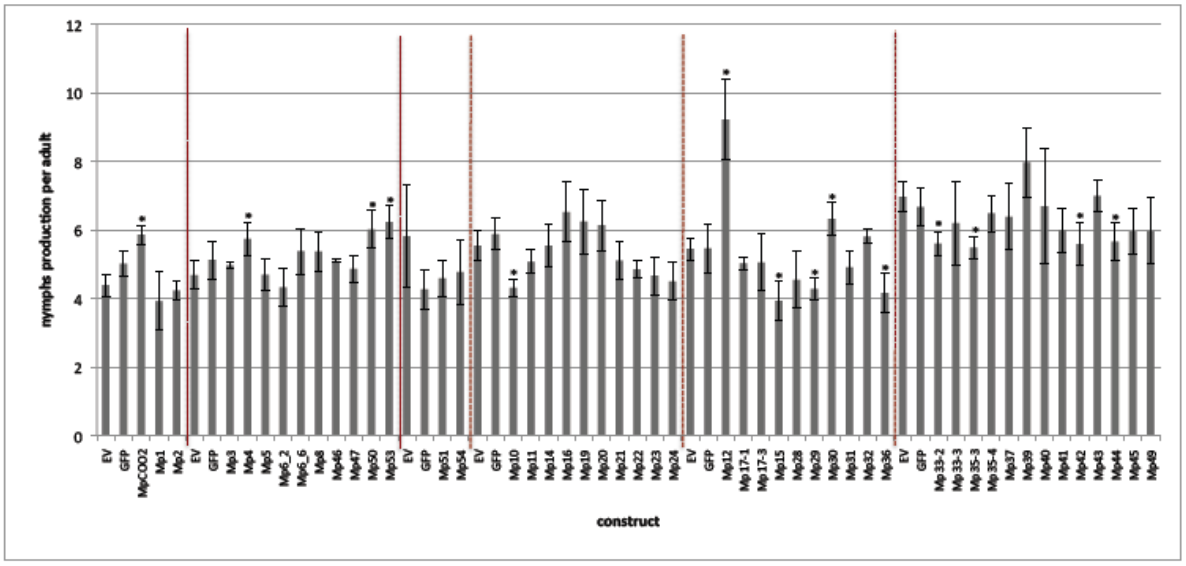

Figure S6

Overexpression of *M. persicae* candidate effector in *N. benthamiana* alters aphid reproductive performance (fecundity). Using the leaf disc-based assay, a set of 48 candidate effectors was expressed in *N. benthamiana* by agroinfiltration to screen for effects on aphid fecundity. Red dotted lines mark sets of candidates that were screened in parallel experiments. EV indicates the vector control and GFP indicates the GFP control. Nymph production was counted over a 17-day period. The average number of nymphs produced per adult was based on 3 replicated experiments. Error bars indicate the standard error. Asterisks indicate Mp candidates that were further tested in confirmation assays.

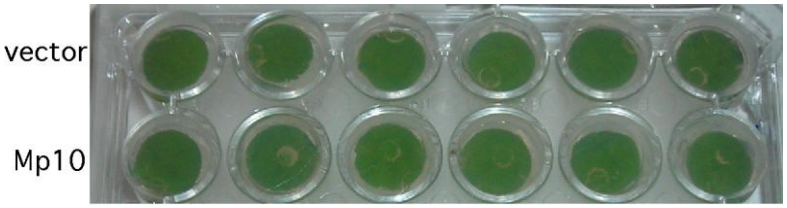

Figure S7

Symptoms of *N. benthamiana* infiltration sites expressing Mp10 during the leaf disc 24-well plate assay. Photo was taken 5 days after infiltration.

Homology searches of Mp10, Mp42, and MpC002

To determine whether the candidates that alter aphid fecundity, (i.e. Mp10, Mp42, and MpC002) share similarity to proteins of known or predicted function, we performed BLAST searches against the GenBank non-redundant (nr) protein database (E value < 10^{-5}). One of the three candidates, Mp10 showed homology to an insect protein of predicted function, the olfactory segment D2-like protein (OS-D2-like protein). The OS-D2-like protein is a member of a family of chemosensory proteins in aphids that contain the conserved cysteine pattern $CX_6CX_{18}CX_2C$ [43]. Mp10 also shows similarity to chemosensory proteins (CSPs) from other insects (E value < 10^{-5}), including the CSP5 protein from the mosquito *Anopheles gambiae* (Figure 5A). The four cysteines in Mp10 are conserved among different members of the CSP family [44, 45] (Figure 5A). Among the aphid sequences similar to Mp10, polymorphisms are predominantly present after the predicted signal peptide sequence, in the mature protein region. For Mp42 and MpC002, similar sequences were identified in the genome sequence of the aphid species *A. pisum* only, but these proteins have no similarities to proteins with known functions. Alignment of Mp42 to a putative *A. pisum* homolog shows strong sequence divergence especially in the mature protein regions (Figure 5B). Finally, alignment of MpC002 to *A. pisum* C002 shows sequence divergence consisting of both amino acid polymorphisms and a 45 amino acid gap in *A. pisum* C002 after the predicted signal peptide sequence (Figure 5C). The presence of polymorphisms mainly in the mature protein regions may reflect that the functional domains of these proteins have diversified due to distinct selective pressures.

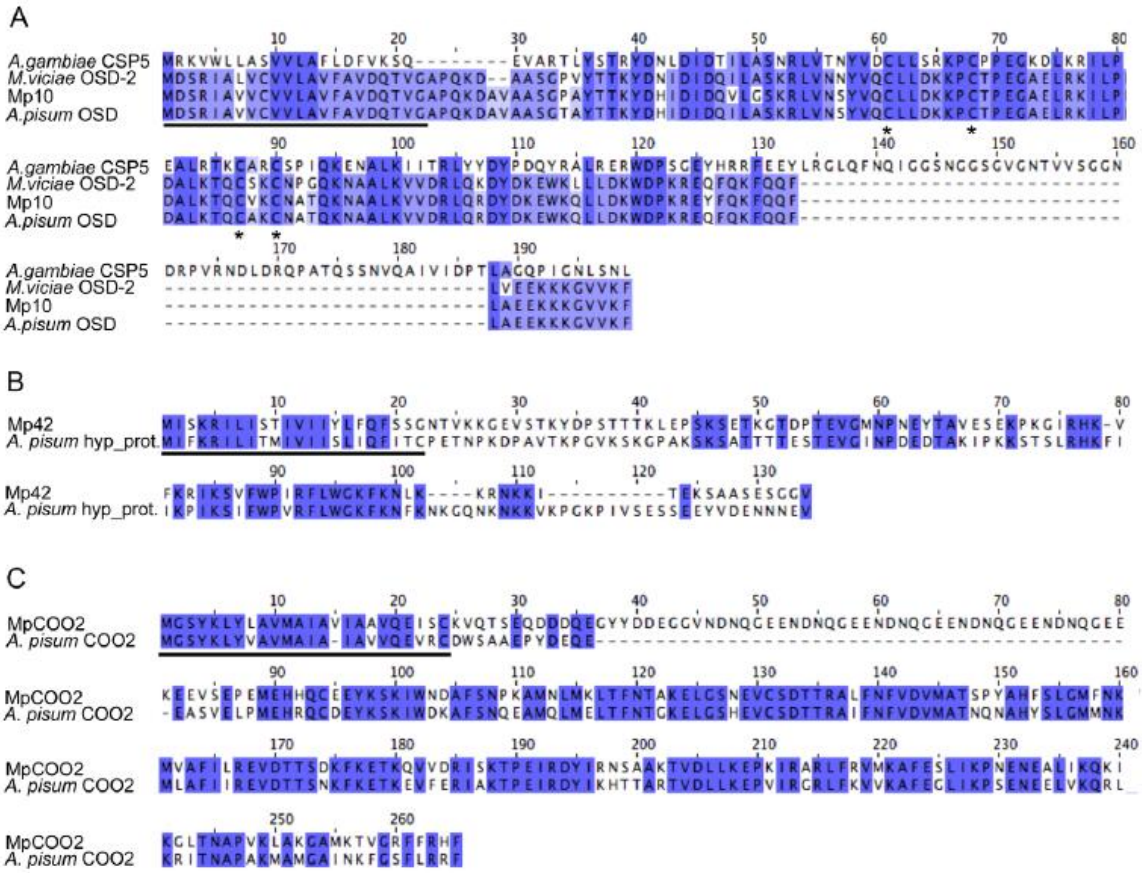

Figure 5. Amino acid alignments of *Myzus persicae* effector candidates that alter aphid fecundity.

Black lines indicate the predicted signal peptide sequences. (A) Alignment of Mp10 with similar sequences from the aphid species *Acyrthosiphon pisum* (GenBank accession NP_001119652.1), *Megoura viciae* (GenBank accession CAG25435.1), and the mosquito species *Anopheles gambiae* (GenBank accession XP_317401.4). Asterisks indicate conserved cysteine residues. (B) Alignment of Mp42 with a similar sequence from *A. pisum* (GenBank accession XP_001948510). (C) Alignment of MpC002 with a similar sequence from *A. pisum* (GenBank accession XP_001948358.1).

Discussion

Aphids, like other plant parasites, deliver repertoires of proteins inside their hosts that function as effectors to modulate host cell processes. These insects most likely secrete effectors into their saliva while progressing through the different plant cell layers during probing and feeding. The identification and characterization of these proteins will reveal new insights into the molecular basis of plant-insect interactions. Here, we have described a functional genomics pipeline to identify *M. persicae* effector candidates as well as various assays to determine whether the candidates share features with plant pathogen effectors. Using this approach, we identified three candidate effectors, Mp10 and Mp42, MpC002 that modulate host cell processes and affect aphid performance.

The induction of chlorosis and local cell death by Mp10 can reflect a genuine effector activity of this aphid salivary protein. Ectopic expression of bacterial TTSS as well as filamentous plant pathogen effectors can affect host immunity and induce a variety of phenotypes in plants, ranging from chlorosis to necrosis [22, 28]. Both the *P. syringae* type III effectors AvrB [30] and HOPQ-1 [46] induce chlorosis and for AvrB this activity is plant genotype specific [47]. No Mp10 induced chlorosis was observed in tomato despite expression levels of PVX-Mp10 that were comparable to *N. benthamiana*. This suggests that the Mp10 response was specific for *N. benthamiana*. Interestingly, PVX-Mp10 was unable to infect *N. tabacum*, suggesting this protein may induce an unknown defense mechanism that is effective against PVX-Mp10.

There are several possibilities that may explain the Mp10 phenotype in a biologically relevant context. The first possibility is that the artificially high expression of Mp10 could lead to the induction of the chlorosis/local cell death phenotype and therefore this response could be an artifact of the *Agrobacterium*-mediated overexpression assay. However, in this case we would expect that the induction of chlorosis and local cell death by Mp10 would be more widespread in various plant species, and would also be observed in *N. benthamiana* leaf discs or detached leaves. Another

possibility is that the high expression of Mp10 could lead to excessive targeting of the operative target as well as other host proteins leading to an exaggeration of the true virulence activity [22]. Finally, the induction of chlorosis and local cell death could reflect avirulence activity of Mp10. Feeding of *M. persicae* is known to induce chlorosis and premature leaf senescence in plants, and this response is related to PAD4-mediated defense responses [48]. Therefore, Mp10 may exhibit an avirulence activity specifically in *Nicotiana* spp resulting in chlorosis and local cell death. The induction of chlorosis in *N. benthamiana* by *P. syringae* effector AvrB is thought to be due to weak activation of TAO1, an NBS-LRR protein, and requires the plant-signaling component Rar1 [49]. We found that chlorosis induction by Mp10 requires the co-chaperone SGT1, which is required for activation of NBS-LRR proteins and plant resistance responses [39]. Therefore, Mp10 may activate an NBS-LRR resistance protein resulting in ETI (further discussed below).

We also found that Mp10 suppressed the ROS response induced by flg22, suggesting that suppression of PTI may be a feature shared by plant pathogens and insects. Possibly, the flg22-induced signaling pathway may not be specific to bacteria as other (non-bacterial) PAMPs can induce this pathway. Also, plants may have a PTI pathway(s) that is induced by an unknown insect PAMP(s) and partially overlaps with the signaling pathway induced by flg22. To date the role of perception of PAMP-like molecules in plant-insect interactions remains elusive. However, chitin is a major structural component of the insect cuticle. Degradation of chitin by plant chitinases generates fragments that induce PTI [50]. Whether the chitin in the insect cuticle is degraded to induce plant defenses during interaction with host plants remains to be investigated. It has been hypothesized that sheath saliva protects the insect stylets, which mainly consist of chitin, from triggering plant defenses [51-53], potentially including PTI. Recent studies showed that insect saliva of both chewing insects [54] and aphids [55] contains elicitors that induce defense responses in host plants. The nature of these elicitors and their role in triggering PTI are unknown. Despite the lack of an understanding of the role in perception of PAMP-like molecules in plant-insect interactions, our data suggest that an aphid salivary protein, Mp10, can interfere with

a specific PAMP response in a *M. persicae* host plant. It is therefore possible that Mp10 is a genuine suppressor of PTI. Alternatively, the overexpression of Mp10 may perturb a signaling component in the PTI pathway that is required for recognition of flg22. As Mp10 induces weak chlorosis starting from 2 dpi, it is possible that this response itself is responsible for loss of the oxidative burst response to PAMPs. However, the Mp10 chlorosis response does not interfere with the oxidative burst triggered by chitin. This suggests that the induction of chlorosis itself may not be sufficient to block the oxidative burst induced by flg22, but that Mp10 specifically interferes with the flg22-triggered signaling cascade.

Despite the suppression of the flg22-mediated oxidative burst by Mp10, its overexpression in *N. benthamiana* reduced aphid fecundity. A plausible explanation for this contradictory observation is that Mp10 may activate an NBS-LRR resistance protein resulting in ETI, thereby reducing aphid performance. Thus, the recognition of Mp10, potentially through ETI, in *Nicotiana* spp may mask the true virulence activity of this protein. If true, this recognition may be suppressed by other effectors during plant-aphid interactions so that Mp10 can exhibit its virulence function.

The leaf disc assay allowed us to generate vast amounts of functional data and directly implicated three effector candidates in plant-aphid interactions. The differences in aphid fecundity observed in our screens were quite variable, requiring replication of experiments. Despite the variation, Mp10, Mp42, and MpC002 showed consistent effects on aphid fecundity throughout the individual replicates (data not show). The fecundity was affected by Mp10, Mp42, and MpC002 by around 1-1.5 nymph produced per adult over a nymph production period of about 6 days. Although these differences may seem small, they are expected to have a large impact on the population size of aphids. Furthermore, *M. persicae* does not perform as well on *N. benthamiana* as it does on other hosts, such as *Arabidopsis thaliana*. Despite the low reproduction level on *N. benthamiana*, the fecundity differences found in our screens are similar to those observed over a 2-day period on *A. thaliana* in a study by Pegadaraju et al. [56] which shows that overexpression of PAD4 reduced aphid

fecundity by about 1.5 nymphs per adult. The number of candidate effectors with an effect on aphid fecundity identified in this study may have been limited by our approach. For example, when the amount of an effector secreted by the aphid is sufficient to modulate host cell processes to promote feeding, *in planta* overexpression may not necessarily further enhance this effect. Also, there could be differences in plant responses to aphids in leaf discs versus whole plants as certain plant responses to aphids may require an intact plant transport system. Despite these limitations, the development of a novel leaf disc-based assay allowed us to identify three effector candidates from the aphid species *M. persicae*.

Out of the three candidates that affect aphid fecundity in the leaf-disc assays, only Mp10 shows homology to a protein of predicted function, namely OS-D2, a member of a family of predicted chemosensory proteins. Insect chemosensory proteins (CSP) are thought to be involved in olfaction and gustation. Indeed, several CSPs have been specifically found in chemosensory organs and are predicted to function in chemoperception [43, 57, 58]. However, for some members of this large protein family functions have been identified in insect development [59] and leg regeneration [60], suggesting that CSPs may have divergent functions. This is further supported by gene expression studies, which show that some CSPs are specifically expressed in antenna [61] or mouthparts [62], whereas others are expressed throughout the insect [63]. CSPs are thought to bind small molecules, such as fatty acids, and for some members of this protein family there is evidence that they bind to pheromones [64, 65]. In the aphid species *Megoura viciae* a Mp10 homolog was found to be expressed in aphid heads without antenna, indicating that it is not an antenna specific CSP [43]. Interestingly, in mosquitos, members of a family of odorant binding-related proteins, also with predicted functions in olfaction and gustation, are secreted into host cells to manipulate host physiology by for example scavenging host amines [66]. Counteracting host amines has evolved in various blood-feeding insects independently through adaptation of members of the lipocalin or odorant-binding protein families [66]. It is possible that also in plant feeding insects, proteins

predicted to be involved in chemosensing are actually involved in early plant host recognition and plant host cell manipulation.

For Mp42 and MpC002 no homology was found to proteins of known or predicted function. This is not surprising as most plant pathogen effectors described to date do not show similarity to proteins of known function based on amino acid alignments. The reduction in aphid performance upon overexpression of Mp42 could reflect that Mp42 induces defense responses against aphids in the plant. In contrast, the enhancement of aphid fecundity by MpC002 suggests that this protein may exhibit an effector activity to promote aphid infestation. Indeed, the *A. pisum* homolog of MpC002, ApC002, has been implicated in aphid feeding [5]. Interestingly, ApC002 is secreted into plant tissues during aphid feeding and silencing of *ApC002* gene expression reduces aphid survival on plants, but does not affect when aphids feed from diet [67]. However, whether *A. pisum* performs better upon overexpression of *C002 in planta* is not known. Our data suggest that the MpC002 homolog may exhibit a similar role in *M. persicae*, and that this protein is important during plant-aphid interactions. Future studies will be aimed at further characterizing these candidates to identify their plant targets and the molecular processes they perturb.

Methods

Sequence databases

We downloaded the following datasets in November 2008 from GenBank for bioinformatics analyses. A total of 3233 *M. persicae* salivary gland ESTs, 27868 *M. persicae* ESTs (all available ESTs), and 2558 *M. persicae* gut ESTs [34], as well as 4517 *A. pisum* salivary gland ESTs (GenBank accessions DV747494-DV752010). For similarity searches against the *A. pisum* genome sequence, we obtained the whole shotgun genome sequence scaffolds from GenBank (accessions EQ110773-EQ133570) in May 2010.

Bioinformatics analyses

The pipeline for the identification of *M. persicae* candidate effectors was developed as follows. The 3233 salivary gland ESTs from *M. persicae* were subjected to ORF calling. More specifically, we performed translations of all possible ORFs of 70+ amino acids, defined by an ATG to stop or an ATG to the end of a sequence, from both strands of the cDNA. We then applied the SignalP v 3.0 program [35] to predict the presence of signal peptides in the amino acid sequences with an HMM score cut-off value of >0.9 and a predicted cleavage site within the amino acid region 1-30. As some predicted secreted proteins were represented multiple times within the *M. persicae* salivary gland EST dataset, we used BLASTP searches to remove redundant sequences. Alignments were inspected manually and sequences that showed >95% identity throughout most of the alignment with an E value < 10^{-10} were classified as being redundant. To remove sequences that in addition the signal peptide also contained a transmembrane domain we used TMHMM v.2.0. The remaining sequences were searched using TBLASTN (E value < 10^{-5}) against all *M. persicae* and *A. pisum* ESTs in our datasets as well as the *A. pisum* genome sequence to assess whether they encoded full-length proteins. Criteria for selecting full-length sequences were: 1) the presence of a conserved start and stop site in ESTs within the alignments; 2) the absence of a methionine within the alignments upstream of the methionine predicted to be the start of the ORF; 3) similarity to a predicted full-length *A. pisum* protein, when available. The remaining predicted secreted protein sequences were then assessed for the presence of polymorphisms within the alignments described above. Sequences not showing any sequence variation in alignments with *M. persicae* sequences and that contained up to one amino acid difference in alignments of the mature protein regions with *A. pisum* sequences were removed from the candidate list.

The 4517 salivary gland ESTs from *A. pisum* were analyzed with the same procedures except that no analyses was performed for the presence of polymorphisms. The amino acid sequences of the predicted secreted proteins (Table S1) were searched using BLASTP (E value of < 10^{-5}) against the amino acid

sequences of the *M. persicae* candidates to identify overlap in the datasets. *A. pisum* candidates without a hit were then searched using TBLASTN against all available *M. persicae* ESTs (E value of < 10^{-5}) to identify *M. persicae* predicted secreted proteins with sequence similarity. The *M. persicae* candidates identified using our pipeline and subjected to cloning were designated MpC002, Mp1-12, Mp14-17, Mp19-24, Mp28-33, Mp35-37, Mp39-47, Mp49-51, Mp53-54, wherein Mp stands for *M. persicae* (Table S2).

Aphids

The *M. persicae* colony of lineage RRes (genotype O) was maintained in cages on *N. tabacum* plants. Cages were located in a contained growth room at 18°C under 16 hours of light.

Microbial strains and growth conditions

A. tumefaciens strain GV3101 was used in molecular cloning and agroinfiltration experiments and were routinely cultured at 28°C in Luria-Bertani (LB) media using appropriate antibiotics [68]. All bacterial DNA transformations were conducted by electroporation using standard protocols [68].

Cloning of Mp candidates

Primers were designed for amplification of sequences corresponding to the ORFs encoding the mature proteins (after the signal peptide encoding sequences) (Table S3). To confirm the 3' end of the ORFs, we designed, where possible, the 3'-primer in the 3'UTR sequence. Sequences were amplified from *M. persicae* cDNA using Phusion polymerase (Finnzymes) and ligated into *Spe*I/*Bam*HI, *Spe*I/*Bgl*II or *Bgl*II/*Bam*HI digested pCB302-3 vector [69] to generate 35S-constructs. To assess whether sequences were polymorphic within the *M. persicae* clonal lineage used in our studies, we performed sequence analyses of 4 clones per construct. To generate constructs for PVX-based expression, we amplified sequences encoding mature ORFs and ligated these into *Cla*I/*Not*I digested pGR106 vector. The PTV vectors used in this study have been described previously [40].

Gene expression analyses by semi-quantitative RT-PCR

Aphids were dissected in PBS and tissues stored in RNA later. We collected 25 salivary glands, 10 guts, 5 heads and 5 whole aphids. RNA extractions were performed with the NucleoSpin RNA XS kit (Macherey-Nagel, Germany). cDNA was synthesized from 80 ng total RNA per sample using expand reverse transcriptase (Roche Diagnostics Ltd). RT-PCR was performed with gene specific primers for each effector candidate indicated in table S3. MpActin primers were used as a control for equal cDNA template amounts.

For RT-PCR on plant tissues, 50 mg leaf tissue was ground in liquid nitrogen and RNA was extracted with the RNeasy Plant minikit (Qiagen). cDNA was synthesized from 500ng DNase treated RNA and subjected to PCR reactions with primer pairs Mp10-pvx-F/R and Mp42-pvx-F/R (Table S3) for amplification of Mp10 and Mp42 expressed in PVX, respectively. For amplification of the PVX coat protein we used primer pair PVX-CP-F/R and for amplification of plant tubulin we used the primer pair Tub-F/R (Table S3). Primers used for RT-PCR on RNA extracted from SGT- and HSP90-silenced plants were described elsewhere [26].

PVX agroinfection and agroinfiltration assays

Recombinant *A. tumefaciens* strains were grown as described elsewhere [70] except that the culturing steps were performed in LB media supplemented with 50 μg/mL of kanamycin. Agroinfiltration experiments were performed on 4-6 week-old *N. benthamiana* plants. Plants were grown and maintained throughout the experiments in a growth chamber with an ambient temperature of 22°-25°C and high light intensity.

For transient overexpression of candidate effectors by agroinfiltration, leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* strain GV3101 carrying the respective constructs at a final OD_{600} of 0.3 in induction buffer (10mM MES, 10mM $MgCl_2$, 150 μM acetosyringone, pH=5.6).

For agroinfection assays, cotelydons of *N. benthamiana*, *N. tabacum* (cv Petite Gerard) or *S. lycopersicum* (MoneyMaker) were wound-inoculated with candidate effector clones using P200 pipette tips. Each strain was assayed on 2 replicated plants. As a control, plants were wound-inoculated with *A. tumefaciens* strains carrying pGR106-Δgfp [26]. Systemic PVX symptoms were scored 14 days post inoculation.

TRV-induced gene silencing

We performed gene silencing as described elsewhere [40]. *A. tumefaciens* suspensions expressing the binary TRV-RNA 1 construct, pBINTRA6, and the TRV-RNA2 vector, PTV00 or PTV-SGT1 were mixed in 1:1 ratio (RNA1- RNA2) in induction buffer (final OD600 is 0.6). Leaves were challenged with *Agrobacterium* strains carrying 35S-Mp10 and 35S-INF1 or the 35S vector.

Aphid fecundity assays in 24-well plates

We developed a medium-throughput 24-well assay to test whether overexpression *in planta* of effector candidates affects aphid nymph production rates. For this purpose, we overexpressed the candidates (35S-constructs) by agroinfiltration in *N. benthamiana* at a final OD600 of 0.3. One day after infiltration, leaf discs were collected using a cork borer (No. 7) from the infiltration sites and placed upside-down on top of 1ml water agar in 24-well plates. A total of 6 infiltration sites, from 6 different leaves, were used per construct and a total of 4 different constructs per 24-well plate. In initial screens, we infiltrated multiple sets of 4 candidate effectors at the same time, with one set including the vector and GFP controls (two candidate effectors plus the two controls). The 4 candidates within a set were infiltrated side-by-side on the same 6 leaves. Leaf discs from each set of candidates were placed in one 24-well plate (6 discs times 4 candidates). For the confirmation assays, we performed infiltrations of each candidate effector with the vector control side-by-side on the same 6 leaves, and leaf discs were placed in one 24-wells plate. On each leaf disc, we placed 4 *M. persicae* first-instar nymphs. The wells in the plate were individually

sealed off using a cap of a 5ml BD Falcon™ round bottomed test tub with the top of the cap removed and covered with mesh. After 6 days, the nymphs were moved to a new 24-wells plate with fresh leaf discs infiltrated with the candidate effector constructs. Another 6 days later, the now adult aphids were again moved to a new 24-well plate with freshly infiltrated leaf discs. The numbers of adults (initially first-instar nymphs) were counted 6, 12, 14 and 17 days after setting up the first 24-wells plate and the number of newly produced nymphs were counted on day 12, 14 and 17. The newly produced nymphs were removed from the wells during counting. Wells wherein all 4 aphids that were initially placed on the discs died were taken out of the analyses. To calculate the production of nymphs per adult aphid, we calculated the average number of nymphs produced per adult by combining the average production rates throughout the experiment. These average production rates were obtained by dividing the number of nymphs on day 12 by the number of adults on day 6 (calculated per well), dividing the number of nymphs on day 14 by the number of adults on day 12, and dividing the number of nymphs on day 17 by the number of adults on day 14. To obtain the total average production rate, we calculated the sum of the average production rates for days 12, 14 and 17.

Measurements of reactive oxygen species

N. benthamiana leaf discs transiently overexpressing the effector candidates were subjected to a luminescence-based assay [41]. Leaf discs were floated overnight in 200ul water in a 96-well plate. The production of ROS was measured after replacing the water with a solution of luminol (20uM) and horseradish peroxidase (1ug) supplemented with either flg22 peptide (100nM) or chitin (100 µg/ml). Luminescence was measured using a Varioskan Flash plate reader. A total of 8 discs per construct, obtained from 4 different infiltration sites, were used per replicate. Assays with flg22 to screen the 48 candidates for suppression activity were repeated two times. The assays with chitin and flg22 were repeated three times.

Statistical analyses

All statistical analyses were conducted using Genstat 11. ROS assay was analysed using a two-sample t-test. Leaf discs fecundity assays were analysed using one-way ANOVA with “construct” as the treatment and “repeat” as the block. Data was checked for approximate normal distribution by visualising the residuals.

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Author contributions

Conceived and designed the experiments: JIBB MP SAH. Performed the experiments: JIBB DP MP JW. Analyzed the data: JIBB DP MP JW SAH. Contributed reagents/materials/analysis tools: JIBB DP MP MEM JW SAH. Wrote the paper: JIBB DP SAH.

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