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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Functional Characterization of Aphid Salivary Proteins and Their Role in Modulating
Plant Defense

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Genetics, Genomics and Bioinformatics

by

Ritu Chaudhary

March 2014

Dissertation Committee:

Dr. Isgouhi Kaloshian, Chairperson
Dr. Linda Walling
Dr. Anupama Dahanukar

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This Dissertation of Ritu Chaudhary is approved:

Committee Chairperson

University of California, Riverside

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DEDICATIONS

To My Family

ABSTRACT OF THE DISSERTATION

Functional Characterization of Aphid Salivary Proteins and Their Role in Modulating
Plant Defense

by

Ritu Chaudhary

Doctor of Philosophy, Graduate Program in Genetics, Genomics and Bioinformatics
University of California, Riverside, March 2014
Dr. Isgouhi Kaloshian, Chairperson

Aphids (Hemiptera: Aphididae) are among the most destructive agricultural pests that cause extensive economic losses to cultivated crops worldwide. Aphids feed on the phloem sap by using their specialized mouthparts known as stylets. While feeding, aphids secrete large quantities of saliva into the host cells both intercellularly and intracellularly. Aphid saliva is of two types: soluble and gelling saliva. Aphid saliva is presumed to contain a large number of proteins and some of them have been shown to be involved in altering plant defenses. Potato aphid is one of the major agricultural pests for tomato and potato. In Chapter One, by using mass spectrometry we identified plethora of proteins present in the potato aphid saliva, by feeding them on two different diets using *in vitro* feeding chambers. Interestingly, several of these were of aphid endosymbiont *Buchnera aphidicola* origin. One of these proteins GroEL that is known to induce immune responses in mammals was chosen for further characterization. This GroEL

characterization is described in Chapter Two. Our analysis showed that delivering GroEL *in planta* by bacterial type-three secretion or expressing GroEL *in planta* by making stable transgenic *Arabidopsis thaliana* lines enhanced resistance against aphids and as well as bacteria. Additional experiments showed that GroEL is also recognized extracellularly and this extracellular recognition required the coreceptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1. This study identified a novel role of endosymbionts in inducing plant defense responses.

Another indirect approach to identify secreted proteins is based on prediction of secretion signal. Using this approach, potato aphid salivary gland transcriptome was sequenced as described in Chapter Three. Putative salivary gland proteins predicted to be secreted were identified by SignalP and TargetP programs. *In planta* functional characterization of eight of these putative aphid secreted proteins identified roles for two, Me10 and Me23, in altering plant responses to aphid's advantage. Me10 enhanced aphid performance on tomato and *Nicotiana benthamiana* plants and was chosen for further analysis described in Chapter Four. A yeast two-hybrid screen with tomato cDNA library revealed that Me10 interacts with several tomato proteins including 14-3-3, which is involved in plant-pathogen immune responses.

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General Introduction

Since the beginning of human civilization, agriculture has been the main source of food and livelihood. We rely on plants and their products to survive. Plants are the first link in the food chain as everything we eat comes directly or indirectly from plants. Unlike animals, plants are sessile; therefore, they can be continuously exposed to biotic and abiotic stresses. In addition, they lack the adaptive immunity of vertebrates and thus depend on active innate immune responses against pests and pathogens that are cell autonomous (Jones and Dangl 2006, Millet *et al.* 2010, Schwessinger and Ronald 2012, Spoel and Dong 2012). Plant defense responses also include various physical or chemical barriers (Bednarek and Osbourn 2009, Hematy *et al.* 2009), and preformed antimicrobial compounds (Van Poecke 2007).

Plants come in contact with various types of microbes and herbivores including insect pests. Insect pests cause great damage to crops both during larval and adult life stages by feeding on vegetative and reproductive parts as well as burrowing in stems and roots. Among these are insect pests belonging to the orders Orthoptera, Hemiptera, Coleoptera, Lepidoptera, and Diptera (Sorensen 1988). In addition to direct damage, insects can cause indirect damage by transmitting bacterial, viral and fungal pathogens (Hogenhout *et al.* 2008, Stavrinides *et al.* 2009, Steinkraus 2006). Among the most destructive insect pests of cultivated plants, are aphids. Aphids belong to the superfamily Aphidoidea in the order Hemiptera (McGavin 1993). Aphids are difficult to control because of their great reproductive capability and their ability to develop resistance to insecticides (Bass and Field 2011, Minks and Harrewijn 1987, Silva *et al.* 2012).

The potato aphid (*Macrosiphum euphorbiae*) is one of the most damaging pests of tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*) crops worldwide (McKinlay *et al.* 1992, Walgenbach 1997). Potato aphids have a wide host range. In addition to potato and tomato, this pest infests a large number of plant species including eggplant, sunflower, peppers, peas, beans, apple, turnip, corn, sweet potato, asparagus, clover, and roses. Besides direct damage caused by feeding, potato aphids also transmit several potato and tomato viruses and cause symptoms on hosts such as mosaics, leaf roll, and spindle tuber (Blancard 2012, Wootford 1992). Green peach aphid (GPA, *Myzus persicae*) is also one of the most agriculturally important aphid pests as it has one of the broadest host ranges encompassing more than 50 plant families, including vegetables, field crops, ornamental plants and fruit trees. Among the hosts of GPA is the model plant *Arabidopsis thaliana* (Arabidopsis). Arabidopsis has been successfully used to study plant-insect interactions, and contributed to unraveling mechanisms involved in plant resistance and insect susceptibility (Louis *et al.* 2012).

Aphid morphology

Aphids are small, soft-bodied, pear-shaped insects with long legs and antennae. They vary in color from green, yellow, red, purple, brown, pink, black to colorless. Coloration in aphids could be due to different factors including carotenoid biosynthetic genes of fungal origin and the presence of certain secondary endosymbionts (Moran and Jarvik 2010, Tsuchida *et al.* 2010).

Aphids have antennae, which bear many sensilla and are important in chemoperception, gustation and perception of leaf surface (McGavin 1993). Aphids are phloem feeders and have piercing-sucking mouthparts known as stylets. Stylets are formed by pair of the mandibles and maxillae, enclosed in a sheath known as rostrum (Stroyan 1997). Mandibular stylets tightly enclose the maxillary pair. The maxillary stylets are interlocked and appear as a single structure enclosing two canals - a food canal for the uptake of phloem sap and a salivary duct for injecting saliva. Aphids have three pairs of legs and two-jointed two-clawed tarsi. They also have a pair of cornicles or siphunculi at the posterior end of their body. The cornicles secrete a defensive fluid containing alarm pheromone (E- β -farnesene) that warns aphids of predators, other enemies and aphid density (Vandermoten *et al.* 2012). Aphids have a tail-like protrusion called a "cauda" at the end of their abdomen. They have two compound eyes, and an ocular tubercle behind and above each eye, made up of three lenses known as triommatidia (Dixon 1998).

The excretory system of aphids is not well defined, as they don't have Malpighian tubules, the excretory system present in most insect species (Bradley 1985). It is known that aphids secrete nitrogenous waste in form of ammonia in the honeydew from their anus (Sasaki *et al.* 1990). Additional information, suggests that aphid salivary glands function as an auxiliary excretory gland (described below).

Aphid salivary glands are composed of a pair of organs (Ponsen 1972). Each individual gland has a principal salivary gland and an accessory salivary gland both having secretory cells. The glands are connected to the salivary pump by a duct. The

principal gland of GPA has two lobes, anterior lobe possesses six *Deckzellen* cells or cover cells and the posterior lobe have fifteen *Hauptzellen* cells or main cells, each with a nucleus (Ponsen 1972, Weidemann 1968). The average size of nuclei and nucleoli differs in different cell types and is correlated with the composition of cytoplasm and cell size (Ponsen 1972). The accessory gland on the other hand is composed of fewer cells. In GPA it is made of 3-4 cells of uniform size and showing extensive microvilli externally (directed towards hemolymph) (Ponsen 1972). The presence of these external microvilli suggests absorption of material from the hemolymph and its transport into glandular cells. Both gland types also possess extensive microvilli directed towards the ducts, suggesting resorption from the duct fluid into the cells and back into hemolymph (Ponsen 1972).

Aphid reproduction, life cycle and wing dimorphism

Aphids exhibit a high reproductive rate due to peculiarities of their reproductive biology. They possess a cyclical parthenogenetic life cycle with alternating sexual and asexual reproductive phases (Blackman and Eastop 2000), producing eggs or live nymphs and switching between herbaceous and woody host plants. The overwintering eggs hatch in the spring producing females only, called fundatrices. During spring and summer, the females reproduce by parthenogenesis and vivipary (Hales *et al.* 2002). The embryos develop within the mother's ovarioles and produce live first-instar female nymphs that are morphologically identical to the mother. In autumn, aphids undergo sexual oviparous reproduction. A change in temperature, photoperiod, lower food quantity or quality,

causes females to parthenogenetically produce both sexual females and males. The males are genetically identical to their mothers except that they have one less sex chromosome (Hales et al. 2002). Sexual females and males mate, and females lay eggs that develop outside the mother. The eggs survive the winter and emerge as females the following spring. These females can be winged or wingless. Aphid wings are membranous and winged aphids are known as alatae while wingless aphids are called apterae. Different aphid species exhibit wing dimorphism at various stages of their life cycle. The wing dimorphism can be environmentally induced (polyphenism, primarily among parthenogenetic females) or genetically determined (polymorphism, only in males) (Braendle *et al.* 2005, Brisson 2010). If an aphid colony is crowded, aphids may develop wings to fly to a new host plant. The mechanism of this switch between these two morphs is unclear. However, it is believed that these switches are regulated by insect hormones, such as juvenile and ecdysone hormones (Ishikawa *et al.* 2013, Zera 2003).

Aphid feeding behavior and plant damage

Aphids have a diverse host range. Based on this characteristic, aphids can be divided into generalists and specialists. Among the specialists, are the mustard aphid (*Lipaphis erysimi*) and cabbage aphid (*Brevicoryne brassicae*), which feed only on cruciferous plants and pea aphid (*Acyrtosiphon pisum*) feeding on leguminous host plants, including peas, clover and alfalfa (Blackman and Eastop 2007). While potato aphid and GPA represent the generalist group that can feed on plants from distinct families as stated above. GPA, is considered as the most polyphagous of all aphids (Baker 1982, Cabreray

Poch *et al.* 1998). Aphids cause little tissue damage while feeding as they employ their specialized mouth parts the stylets to penetrate mostly intercellularly and consume phloem sap (Tjallingii and Hogen Esch 1993). Regardless of plant feeding specialization, heavy aphid infestation can weaken a plant by depleting nutrients while feeding, stunt plant growth, cause leaves to curl or wilt, delay fruit or flower development and produce galls on leaves, stems or even roots (Dedryver *et al.* 2010, Quisenberry and Ni 2007).

For chemoreception and perception of the leaf surface, aphids use olfactory receptor neurons that are housed inside the sensilla on the antennae (Bromley and Anderson 1982). They scan the host plant surface with the tip of their proboscis. The tactile receptors present on the tip of the proboscis respond to contact and surface texture and enable aphids to detect their preferred feeding site (Tjallingii 1978). They then probe into the plant with their stylets. When aphids puncture plant tissue, they sample the cell content before determining if a particular plant is a suitable host or not (Powell *et al.* 2006). As the stylets move through plant tissue, aphids continuously secrete sheath or gelling saliva from the principal salivary gland (Cherqui and Tjallingii 2000, Ponsen 1972). The gelling saliva solidifies soon after release forming a sheath around the stylets. The sheath material contains 10% phospholipids and the lipid layer is thought to provide protection from the host chemicals (Miles 1990, Tjallingii 1995, Tjallingii 2006, Will *et al.* 2012).

In addition to the gelling saliva, aphids also secrete watery saliva (Miles 1959). The watery saliva is also secreted into the plant tissues from the onset of stylet penetration (Moreno *et al.* 2011), and gets mixed with the phloem sap as it is taken up

from the sieve element (Cherqui and Tjallingii 2000, Prado and Tjallingii 1994). The majority of the watery saliva is secreted inside the phloem, however, during stylet probing, watery saliva is also secreted intercellularly as well as intracellularly while penetrating mesophyll cells (Tjallingii 2006).

Aphid feeding affects plant metabolism, sugar transport and enhances senescence (de Vos *et al.* 2007, Moran and Thompson 2001, Pegadaraju *et al.* 2005, Voelckel *et al.* 2004). It is thought that aphid salivary proteins also affect these processes (de Vos and Jander 2009). Besides affecting plant nutritional health, some aphid salivary secretions i.e, Russian wheat aphid (*Diuraphis noxia*) can be toxic to the host plant and can cause necrosis and hypersensitive response (HR) (Zemetra *et al.* 1993).

When the aphid stylets puncture the phloem vessel, the very high pressure in the phloem tubes forces the phloem sap into the insect. This phloem sap, which has high sucrose concentration, increases the sugar content inside the aphid stomach causing hyperosmosis, which can lead to transfer of water from hemolymph to stomach causing insect death. To avoid this, aphids regulate osmosis several ways. The first strategy is to metabolize sucrose to oligosaccharides which are then secreted as honeydew from the anus (Ashford *et al.* 2000). Secondly, by transferring water from the hindgut to the stomach through aquaporins that dilutes the ingested sap and thus prevents osmotic loss of water from hemolymph to the gut (Shakesby *et al.* 2009). Thirdly, by ingesting xylem sap which has low amounts of sugars and carbohydrates than the phloem sap and thus ingestion of xylem sap replenishes the water balance (Pompon *et al.* 2010, Spiller *et al.* 1990).

As honeydew is excreted, it drops onto different plant organs, supports growth of a black sooty mold fungus, which covers plant surfaces and reduces the rate of photosynthesis. In addition to sooty mold, aphid honeydew attracts and is a food source to other insects including flies, hornets, yellow jackets, and ants. Aphids have formed mutualistic relationships with at least one of these insect species. Some ant species that are attracted to honeydew, "farm" the aphids by protecting them from their predators and these ants can carry the aphids one plant to another (Linda 2008, Stadler and Dixon 2005, Stadler and Dixon 2008). Farming ants may also assist aphids to overwinter by gathering and storing aphid eggs in their nests over the winter season (Way 1963).

Aphid endosymbionts

Most aphid species represent the outcome of more than one organism living in harmony or mutual symbiosis. Aphids house two kinds of endosymbionts: the primary (P) obligate endosymbionts, *Buchnera aphidicola*, and the secondary (S) facultative endosymbionts belonging to a number of distinct species (Baumann *et al.* 1995, Buchner 1965, Fukatsu *et al.* 2000, Munson *et al.* 1991b, Rouhbachsh *et al.* 1994). *Buchnera* belongs to the γ subdivision of the division Proteobacteria (Unterman *et al.* 1989). The P-symbionts can not survive when they are removed from the aphid host cells; similarly, aphids die or do not reproduce when deprived of *Buchnera* (Houk and Griffiths 1980, Ohtaka and Ishikawa 1991). It has been shown that, in a rare case, the S-facultative endosymbionts can partially compensate for the essential role of *Buchnera* by positively affecting host survival and reproduction (Koga *et al.* 2003). Aphid diet, the phloem sap, is rich in

carbohydrates but is deficient in nitrogenous compounds including essential amino acids (Douglas 1993, Raven 1983, Sandstrom *et al.* 2000). Experimental and genome sequence information (explained below; (Shigenobu *et al.* 2000)), indicate that *Buchnera* provides essential amino acids to aphids, supplementing the limited supply of these nutrients in the phloem sap (Akman Gunduz and Douglas 2009, Douglas and Prosser 1992). S-endosymbionts are found in many but not all aphid species, they are not essential for aphid survival but confer tolerance to biotic and abiotic stresses (Oliver *et al.* 2010).

The symbiotic relationship between aphid and *Buchnera* began 200-250 million years ago (Baumann *et al.* 1995). As mentioned earlier, symbiotic relationship is obligate and mutualistic as aphids provide a niche and nutrients to *Buchnera* and in return obtain essential amino acids and other nutrients from it (Baumann *et al.* 1995, Dixon 1998, Douglas 1998). Sequencing of the *Buchnera* genome revealed that *Buchnera* has lost most of the genes required for biosynthesis of nutrients and non-essential amino acids (glutamate, aspartate, serine, glutamine, alanine, proline, and asparagine), however, the genes for biosynthesis of most of the essential amino acids have been retained (Shigenobu *et al.* 2000). *Buchnera*'s genome also includes genes for enzymes in pathways for the nonessential amino acids tyrosine, glycine, and cysteine but lacks the genes in five essential amino acid pathways (leucine, isoleucine, valine, methionine, and phenylalanine) (Shigenobu *et al.* 2000). Interestingly, aphid bacteriocyte gene expression analysis revealed aphid-*Buchnera* cooperate in the amino acid production pathways as *Buchnera* depends on the host to synthesize nonessential amino acids and the

bacteriocyte, of host origin, is able to recycle ammonia into amino acids by using enzymes encoded by both genomes (Hansen and Moran 2011).

Buchnera is transmitted from mother to offspring by transovarial transfer (Braendle *et al.* 2003, Miura *et al.* 2003). Many studies have shown that the *Buchnera* symbionts from distantly related aphid species have a single origin as indicated by the presence of conserved 16S rDNA sequences (Fukatsu and Ishikawa 1993, Moran and Baumann 1994, Morioka and Ishikawa 1993, Munson *et al.* 1991a). *Buchnera* is present in almost all aphid species (Baumann *et al.* 1995, Buchner 1965) except in a few aphids in which *Buchnera* has been replaced by yeast-like symbionts belonging to the subphylum Ascomycotina and class Pyrenomycetes (Fukatsu and Ishikawa 1992, Fukatsu and Ishikawa 1996).

Inside aphids, *Buchnera* is present within specialized polyploid cells called bacteriocytes (Buchner 1965, Griffiths and Beck 1973, Houk and Griffiths 1980). The bacteriocyte contains a large nucleus, nucleolus, ribosomes, golgi apparatus, endoplasmic reticulum, transparent vacuoles, granular bodies, multivesicular bodies, and microfilaments (Griffiths and Beck 1975, McLean and Houk 1973). Bacteriocytes are grouped to form a bilobed structure called bacteriome and are located in the abdominal cavity, dorsal to the gut (Griffiths and Beck 1973). In young aphids, it is surrounded by a sheath that consists of a thin layer of flattened and frequently syncytial cells (Buchner 1965, Douglas and Dixon 1987, Houk and Griffiths 1980). In adult aphids, the bacteriome breaks apart and bacteriocytes become dispersed throughout the abdomen into the hemocoel (Brough and Dixon 1990, Douglas and Dixon 1987, Hinde 1971).

Buchnera cells are 2-5 µm in diameter, can be spherical or oval in shape and have a cell wall resembling that of gram-negative eubacteria (Griffiths and Beck 1973, Houk and Griffiths 1980, McLean and Houk 1973). It also has two endogenous peripheral membranes and a third membrane of host origin (Houk and Griffiths 1980). The first endogenous membrane is M1 or cytoplasmic membrane. It surrounds the symbiont cytoplasm and sometimes it shows infoldings just like bacterial mesosomes (Griffiths and Beck 1975). The second membrane is M2 or the cell wall. It represents the lipopolysaccharide-lipoprotein layer characteristics of gram-negative bacteria. A thin layer of peptidoglycan is also detected in between the two unit membranes (Houk *et al.* 1977). The third layer or M3 is of insect host origin and is shown to be continuous with the endoplasmic reticulum (ER) and in unique cases with the plasma membrane (Griffiths and Beck 1975). M3 is lost during the transovarial infection process and a new M3 is formed after entry into the embryonic mycetocytes. This could result due to endocytosis of the plasma membrane or via *de novo* expansion of host endoplasmic reticulum (Hinde 1970, Korner and Feldhage 1970). *Buchnera* reproduces by binary fission without the formation of cross walls. Aphids control the number and location of *Buchnera* from one generation to another by three ways (Hinde 1970, Hinde 1971) - (a) regulating the passage of symbionts into developing embryo, (b) lysosomal breakdown in the bacteriocyte, and (c) hemocytic phagocytosis outside bacteriocyte.

Buchnera is a close relative of *Escherichia coli*. However it contains 120 genomic copies per cell, the cells are much larger in volume than *E. coli* cells and they don't divide as frequently (Komaki and Ishikawa 1999). The genome size of *Buchnera* is 1/7th

of that of *E. coli* (Charles *et al.* 1999). The genome of *Buchnera* sp. strain APS has a single chromosome of 640,681-base-pair and two small plasmids representing about 620 genes (Shigenobu *et al.* 2000). *Buchnera* lacks genes needed for recombination function (*recA* and *recF*) thus eliminating the events of gene acquisition (Tamas *et al.* 2002). A comparative analysis of the orthologous genes of *Buchnera*, *E. coli* and *Vibrio cholerae* has shown that the reduction in the genome size of *Buchnera* occurred from multiple events of gene disintegration and deletions, often including genes of unrelated functions, distributed over the whole genome (Moran and Mira 2001, Silva *et al.* 2001). The reduction of *Buchnera* genome could be explained by horizontal gene transfer of *Buchnera* genes to the aphid host. However, a recent study ruled out the possibility that the reduction in the genome size of *Buchnera* is due to lateral transfer of genes from *Buchnera* to the aphid (Nikoh *et al.* 2010).

The most abundant *Buchnera* protein is the chaperonin GroEL, which constitutes 10% of its total protein content (Clark *et al.* 1996, Hara *et al.* 1990). It has been shown that GroEL is present in the aphid hemolymph where it is able to adhere to virus particles and assist with virus transmission (Filichkin *et al.* 1997, Hogenhout *et al.* 2000). Compared to other genes in *Buchnera*, that accumulate non-synonymous changes due to effect of genetic drift, *groEL* is subjected to a strong purifying selection suggesting that this protein is functionally important (Fares *et al.* 2002). GroEL is a bacterial chaperone with the key role in protein folding (Henderson *et al.* 2013). Bacterial GroEL also induces several humoral and cell-mediated immune responses in mammals. For example, GroEL of *Mycobacterium tuberculosis* is antigenic in mammals and activates both B and

T lymphocytes (Lamb *et al.* 1989). Similarly, GroEL from *E. coli* induces mRNA levels of cytokines and interleukin-1 secretion in macrophage cultures (Retzlaff *et al.* 1994). Mice immunized with GroEL from *Salmonella typhi* show a significant increase in lymphocyte proliferation and cytokine levels (Bansal *et al.* 2010). A recent study has shown that recombinant GroEL from *Salmonella typhi* can be potentially used as a candidate to develop vaccine against several bacterial pathogens including *Shigella dysenteriae* type I, *S. flexneri*, *S. boydii*, enteropathogenic *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Chitradevi *et al.* 2013). GroEL does not possess a secretion signal sequence or any motif indicating its export and was therefore thought to be localized to the cytoplasm. However, GroEL from several human pathogenic bacteria has been detected on their surface presumably helping them attach to the surface of host cell (Bergonzelli *et al.* 2006, Hennequin *et al.* 2001, Tsugawa *et al.* 2007).

S-endosymbionts present in aphids can be one of the following: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Spiroplasma*, *Rickettsia*, and *Arsenophonus* species (Fukatsu *et al.* 2000, Fukatsu *et al.* 2001, Koga *et al.* 2003, Moran *et al.* 2005). S-endosymbionts differ markedly in morphology and localization in different aphid species (Fukatsu and Ishikawa 1998, Fukatsu *et al.* 2000). They are found in two different types of host cells of the *Buchnera* bacteriome, the sheath cells and special cells called S-bacteriocytes (Fukatsu *et al.* 2000). In nature, S-endosymbionts are mainly transmitted maternally although paternal transmissions have also been observed for *R. insecticola* and *H. defensa* (Moran and Dunbar 2006). They may not infect all the individuals in an aphid population and artificial transmission of S-symbionts, using

microinjection of insect hemolymph, between individual of the same species or different species is possible indicating that they can be transmitted horizontally (Chen *et al.* 1996, Oliver *et al.* 2008, Oliver *et al.* 2006). The ability to artificial transmit the S-endosymbionts has enabled research to define their contributions to aphid welfare.

Although S-endosymbionts are not essential for aphid survival, they provide protection against biotic and abiotic stresses. *R. insecticola* provides protection against aphid-specific fungal entomopathogen *Pandora neoaphidis* (Scarborough *et al.* 2005). In pea aphids, presence of *S. symbiotica* and *H. defensa*, reduces parasitism by the endoparasitoid wasp *Aphidius ervi* by killing the developing larvae (Oliver *et al.* 2006, Oliver *et al.* 2003). In a different study it was shown that levels of protection vary significantly, from small reductions in parasitism to nearly complete protection, depending on the strain of *H. defensa* (Oliver *et al.* 2005). Interestingly, superinfection with both *S. symbiotica* and *H. defensa* has a cumulative effect and enhances the aphid's resistance to parasitism than those harboring a single endosymbiont (Oliver *et al.* 2006). Also, parasitized aphids bearing *H. defensa*, but not *S. symbiotica*, produce significantly more offspring than parasitized aphids lacking *H. defensa*. However, in the absence of parasitism, this superinfection is very costly to aphids as it reduces aphid fecundity in infected compared to uninfected aphids. In addition to protection to biotic stresses, S-endosymbionts also provide protection against heat stress (Chen *et al.* 1996, Montllor *et al.* 2002). Moreover, presence of certain S- endosymbionts such as *R. insecticola* enhances aphid performance on certain host plants suggesting a role for these S-endosymbionts in plant host range (Ferrari *et al.* 2004, Leonardo and Muir 2003).

Aphids as virus vectors

Aphids are common vectors of plant viruses and over 200 aphid species are known to transmit pathogenic plant viruses (Ng and Perry 2004). Plant viruses show vector specificity and each is transmitted by a limited number of aphid species (Gray and Gildow 2003, Hogenhout et al. 2008). Insect-transmitted plant viruses, seem to modulate both host and aphid behavior to enhance their transmission. These modifications are based on the mode of transmission of the virus (Mauck *et al.* 2012). With regards to transmission, plant viruses can be categorized as persistent, semi-persistent and non-persistent (Ng and Falk 2006). In non-persistent transmission, aphids inoculate viruses into the plants a few minutes after acquisition and retain the virus only for a very short time. In persistent transmission, aphids can inoculate the acquired virus for days and even weeks after acquisition, while in semi-persistent transmission aphids retain the virus for a few hours to a few days.

Viruses can also be categorized as circulative and non-circulative based on their relationship with the vector (Hogenhout et al. 2008). Circulative viruses are persistent viruses. Taken up by the aphid during feeding, they have to cross organs and multiple membrane barriers before they are transported into the salivary glands and ultimately exit in the saliva. Circulative viruses are further categorized as propogative, which replicate both in the plant host and aphid vector, and non propogative, which replicate only in the plant host. On the other hand, non-circulative viruses can be trasnmitted both in semi-persistent and non-persistent manner as they have more of a superficial and transient relationship with the aphid and are only associated with the stylets and foregut. These

viruses are acquired by aphids with a single probe, within seconds, and can be transmitted to a healthy plant within seconds, too, and are therefore retained by the aphid for only a short period of only an hour or two (Ng and Falk 2006, Uzest *et al.* 2010).

GPA is capable of transmitting both persistent and non-persistent viruses. Both nymphs and adults are capable of transmitting viruses (Namba and Sylvester 1981). There are more than 200 viruses transmitted by GPA (Blackman and Eastop 2000), majority being Potyviruses and Luteoviruses (Ng and Perry 2004). In contrast to GPA, potato aphids are not efficient vectors of viruses and mainly transmit non-persistent viruses including *Potato leafroll virus (PLRV)*, *Beet mild yellowing virus*, *Lettuce mosaic virus*, *Cucumber mosaic virus* and *Potato virus Y* (Blackman and Eastop 2000).

The *Buchnera* chaperonin GroEL has been associated with virus transmission by aphids (Hogenhout *et al.* 2008, Kliot and Ghanim 2013, van den Heuvel *et al.* 1999). GroEL is implicated in the transmission of viruses belonging to the family Luteoviridae and it binds to the readthrough domain of the *PLRV* and *Barley yellow dwarf virus (BYDV)* *in vitro* (Filichkin *et al.* 1997, van den Heuvel *et al.* 1997). It has been shown that hemolymph extracts from GPA contains GroEL, and *Buchnera*-cured aphids are not able to transmit these viruses suggesting that GroEL is required for virus transmission and may protect the virus against proteolytic breakdown in the hemolymph (van den Heuvel *et al.* 1994).

Plant innate immunity and microbial effectors

Plant pathogenic microbes enter the host either by direct penetration of tissues or indirectly through wounding or natural openings like stomata or hydathodes. Plant immune responses can be activated by “general elicitors” of pathogen or plant origin, including oligosaccharides, lipids, polypeptides, and glycoproteins (Nurnberger *et al.* 2004). One of the early plant defense responses are triggered by microbe-associated molecular patterns (MAMPs), indispensable conserved motifs of a certain class of microbes, which are recognized by host cell surface receptors known as pattern recognition receptors (PRRs) (Boller and Felix 2009, Dodds and Rathjen 2010). The recognition of MAMPs by PRRs initiates pattern-triggered immunity (PTI). Responses associated with PTI includes rapid ion fluxes across the plasma membrane, activation of mitogen-activated protein kinase (MAPK), production of reactive-oxygen (ROS) species, activation of defense genes, cell wall reinforcement and production of ethylene (Boller and Felix 2009, Zipfel 2009).

Microbial pathogens have developed strategies to avoid this recognition whereas others secrete effector molecules to suppress PTI, making the plant a suitable host for their growth and reproduction. This plant-pathogen interaction is referred to as compatible interaction (Chisholm *et al.* 2006). During compatible interactions, the host plant still has a weak inducible defense known as basal resistance, which is not effective to eliminate the pathogen but it is able to limit infection severity (Abramovitch and Martin 2004).

A variety of phytopathogenic organisms including bacteria, fungi, oomycetes, nematodes, and insects are able to secrete effector proteins to modulate host processes to their advantage (Hogenhout *et al.* 2009, Muthamilarasan and Prasad 2013). In response to microbial effectors, plants have acquired resistance (*R*) genes to recognize effectors directly or indirectly (Dodds and Rathjen 2010, van der Hoorn and Kamoun 2008). Effectors that are recognized by *R* proteins are called avirulence (*Avr*) effectors and the *R*-*Avr* interaction is referred to as incompatible interaction (Flor 1955, Flor 1971). Incompatible interaction activates a very strong form of immune responses known as effector-triggered immunity (ETI) (Chisholm *et al.* 2006). ETI is often characterized by a localized cell death or hypersensitive (HR) response (Jones and Dangl 2006). The absence of either *R* or *Avr* proteins results in susceptibility. In turn, pathogens have evolved effectors that suppress ETI and the arms race between plants and pathogens continues. Therefore, PTI and ETI are evolving to counter the coevolving pathogens (Boller and Felix 2009, Nurnberger and Lipka 2005).

Effectors are also evolving in a fast pace. Genes that encode effector proteins evolve at highly accelerated rates relative to pathogen core genome and often exhibit higher rates of non-synonymous to synonymous nucleotide substitutions demonstrating high levels of positive selection (Dodds *et al.* 2006, McCann and Guttman 2008). Effectors secreted by microbes can either act in the apoplastic space at the plant-microbe interface or are delivered inside the plant cell (Kamoun 2006). For example, many plant pathogenic bacteria deliver effectors inside host cells via type three secretion system (T3SS) (McCann and Guttman 2008), whereas biotrophic fungi and oomycetes have

evolved haustoria for effector delivery (Whisson *et al.* 2007). Insects and nematodes use their specialized mouthparts or stylets for this purpose.

During infection, pathogenic bacteria can deliver 20-40 effectors into the host plant cell (Cui *et al.* 2009), which frequently promote pathogenicity and virulence (Alfano and Collmer 2004, Mudgett 2005). For example, in tomato, *AvrPto*, an effector from the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), induces ETI when recognized by the *Pto* R-gene (Pedley and Martin 2003). However, in the absence of *Pto*, *AvrPto* enhances bacterial growth and virulence (Shan *et al.* 2000). *AvrPto* can also block callose deposition induced by T3SS-deficient *P. syringae* strains (Hauck *et al.* 2003). In addition, *AvrPto* and *AvrPtoB* directly target the PRR FLAGELLIN-SENSING 2 (FLS2) and suppress PTI including early defense gene transcription and MAPK signaling in *Arabidopsis* (He *et al.* 2006, Xiang *et al.* 2011, Xiang *et al.* 2008). Additional effectors such as *AvrRpt2*, *AvrRpm1*, and *HopAII* suppress defense responses elicited either by T3SS-defective mutant bacteria or the flagellin-derived peptide flg22 (Kim *et al.* 2005, Li *et al.* 2005, Oh and Collmer 2005). Effectors also have the ability to inhibit and protect against plant hydrolytic enzymes, such as protease, glucanase, chitinase, tyrosine phosphatase, E3 ligase, ADP-ribosyltransferase, phosphothreonine lyase and acetyltransferase (Deslandes and Rivas 2012, Misas-Villamil and van der Hoorn 2008).

Plant components targeted by effectors have been identified using a number of approaches. The tomato targets of the XopN effector from the bacterial pathogen *Xanthomonas campestris* were identified using a yeast-two hybrid screen. Several plant targets were identified including the Tomato Atypical Receptor-Like Kinase1 (TARK1)

and TFT1 that encodes a tomato 14-3-3 protein (Kim *et al.* 2009, Taylor *et al.* 2012). Similarly, the targets for HopQ1 effector from *Pst* strain *DC3000* were identified by performing coimmunoprecipitations coupled with mass spectrometry (MS) (Li *et al.* 2013). These targets included different 14-3-3 isoforms, TFT1 and TFT5, suggesting that bacterial effectors target scaffold proteins, some of which have been implicated in PTI.

The specific effector examples given above are either powerful bacterial effectors or are recognized by an R protein and their functions have been identified through deletion mutants or inoculation on resistant hosts. However, frequently effectors have redundant functions and hence knocking out an individual effector does not result in a phenotype or gives a very subtle phenotype (Cunnac *et al.* 2011). Therefore, an alternate way to find the virulence function of an individual effector is to express it transgenically *in planta*. Using this approach, the functions of a number of bacterial effectors have been identified and shown to suppress plant defense and enhance bacterial growth (Guo *et al.* 2009, Jamir *et al.* 2004).

MAMPs and PRRs

Identification of MAMPs and their corresponding host PRR is a subject of intense research interest. A number of MAMPs and their corresponding PRR have been identified and characterized from bacteria, fungi and oomycetes (Newman *et al.* 2013, Schwessinger and Ronald 2012, Spoel and Dong 2012). Here, I describe only a few well-characterized examples.

The best-characterized MAMP is the bacterial flagellin. The flagellum is an important part of bacteria required for motility and survival. Plants recognize a very conserved 22-amino-acid epitope, flg22, at the N-terminus of flagella (Felix *et al.* 1999, Gomez-Gomez *et al.* 1999). In Arabidopsis, flg22 is perceived by a leucine-rich repeat receptor-like kinase (LRR-RLK) FLS2 (Chinchilla *et al.* 2006, Gomez-Gomez and Boller 2000). Functional orthologs of FLS2 have been identified in the Solanaceous plants, *Nicotiana benthamiana* and tomato (Hann and Rathjen 2007, Robatzek *et al.* 2007), and sequences homologous to *FLS2* are present in most sequenced plant species (Boller and Felix 2009). Flg22 perception by FLS2 induces ROS production, activates MAPK pathway, and upregulation of *WRKY-29* transcription factor, *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE*), and *PR* genes (Asai *et al.* 2002). Pretreatment of plants with flg22 peptide makes plants more resistant to subsequent infection with the pathogenic bacterium *Pst DC3000*, while plants mutated in FLS2 are more susceptible to spray inoculation of this bacterium (Zipfel *et al.* 2004). In addition, flg22 as well as flagellin, induce HR in Arabidopsis (Naito *et al.* 2008) invalidating the dogma that MAMPs generally do not induce HR.

Another well-characterized MAMP is the bacterial most abundant protein the elongation factor (EF-Tu). EF-Tu has a highly conserved N-acetylated 18-amino acid peptide, elf18 that is characterized as a MAMP in Arabidopsis and members of the family Brassicaceae only (Kunze *et al.* 2004). EF-Tu is an intracellular protein and is released by lysis of the dying bacteria during plant colonization, and is sufficient to trigger defense responses at sub-nanomolar concentrations. In Arabidopsis, EF-Tu is also perceived by

an LRR-RLK, EF-TU RECEPTOR (EFR) (Zipfel *et al.* 2006). Interestingly, the peptide elf18 from *Pst DC3000*, differing from nonpathogenic *E. coli* elf18 peptide at positions 1, 6, 8, 9 and 12, exhibits highly reduced elicitor activity suggesting that virulent bacteria are able to evade PTI by mutating the MAMP signature. However, elf18 from the plant pathogenic bacteria *Agrobacterium tumefaciens* is fully active as an elicitor in *Arabidopsis* indicating that not all virulent bacteria are able to evade recognition (Kunze *et al.* 2004). Interestingly, *efr* mutant plants show enhanced susceptibility to *Pst DC3000* only when spray inoculated with higher bacterial inoculum levels 1×10^9 colony forming units (CFU)/mL (Saijo *et al.* 2009) and not at lower inoculum 1×10^7 CFU/mL (Nekrasov *et al.* 2009).

Additional bacterial MAMPs include peptidoglycans (PGNs), present in both gram-positive and gram-negative bacteria and are recognized in *Arabidopsis* (Erbs *et al.* 2008, Gust *et al.* 2007) via three lysin-motif (LysM) domain proteins LYM1, LYM3, and CERK1 (Chitin Elicitor Receptor Kinase 1) (Willmann *et al.* 2011). Similarly, lipopolysaccharides (LPS) in the outer membrane of gram-negative bacteria act as a MAMP in dicots and monocots, however, no plant PRR have been characterized for LPS (Newman *et al.* 2013). Bacterial cold shock proteins (CSPs) have a highly conserved RNA-binding motif RNP-1 that acts as MAMP in the Solanaceae by the recognition of the 22-amino acid core of RNP-1 (CSP22) (Felix and Boller 2003). No PRR for CSP have been identified yet.

Similarly, several MAMPs have been identified in phytopathogenic fungi. Chitin, a major component of fungal cell walls, is a polymer of N-acetyl-D-glucosamine, is

recognized as a MAMP, which induces defense responses in many plant species (Wan *et al.* 2008). In rice (*Oryza sativa*) the two the essential components for chitin perception and signaling in plants are *OsCERK1* (Shimizu *et al.* 2010) and *OsCEBiP* (Chitin oligosaccharide Elicitor- Binding Protein) (Kaku *et al.* 2006), whereas in Arabidopsis *AtCERK1* is sufficient for chitin perception (Shinya *et al.* 2012). An ethylene-inducing xylanase (EIX), 22-kD fungal protein (β -1-4-endoxylanase) from the pathogenic fungus *Trichoderma viride*, is a potent elicitor of plant defense responses in specific cultivars of tobacco and tomato and is perceived by the receptor-like proteins (RLPs) *LeEix1* and *LeEix2* (Ron and Avni 2004).

Many PRRs require an additional well-explored LRR-RLK, BRASSINOSTEROID-INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1)/ SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), to activate plant immune responses (Dou and Zhou 2012). BAK1 is required for signaling by FLS2 and EFR (Chinchilla *et al.* 2007, Heese *et al.* 2007, Roux *et al.* 2011). BAK1 interacts with FLS2 in a ligand-dependent manner soon after flg22 elicitation (Chinchilla *et al.* 2007) and leads to rapid phosphorylation of FLS2 and BAK1 (Schulze *et al.* 2010). Beside interacting with FLS2, BAK1 also bind to the C-terminus of flg22 that is bound to the FLS2 ectodomain (Sun *et al.* 2013). Attenuating *BAK1* expression in Arabidopsis and *N. benthamiana* affects responses to diverse MAMPs including LPS, PGN, CSP22, chitin, EIX, and the oomycete INF1 and *BAK1*-silenced or mutant plants are more susceptible to certain pathogens (Bar *et al.* 2010, He *et al.* 2008, Heese *et al.* 2007, Roux *et al.* 2011, Shan *et al.* 2008).

Plant *R* genes against piercing sucking insects

The majority of *R* genes cloned to date encode proteins with LRRs, a central nucleotide binding site (NBS) and either an N-terminal coiled-coil (CC) or toll/interleukin like receptor (TIR) motifs (Gururania *et al.* 2012, Liu *et al.* 2007). These confer resistance against diverse class of pathogens and pests (Martin *et al.* 2003, Muthamilarasan and Prasad 2013).

Mi-1, *Bph14* and *Vat* genes are the only *R* genes cloned that confer resistance to insect pests (Dogimont *et al.* 2008, Du *et al.* 2009, Kaloshian *et al.* 1998, Milligan *et al.* 1998, Rossi *et al.* 1998). *Mi-1* confers resistance to three distinct insect pest including potato aphids (Kaloshian *et al.* 1995, Milligan *et al.* 1998, Rossi *et al.* 1998), whiteflies (*Bemisia tabaci*) (Nombela *et al.* 2003) and psyllids (*Bactericera cockerelli*) (Casteel *et al.* 2006). In addition to resistance to insects, *Mi-1* also confers resistance to three species of root-knot nematodes (*Meloidogyne arenaria*, *M. incognita*, and *M. javanica*) (Milligan *et al.* 1998, Roberts and Thomason 1989). In the early 1940's, the *Mi-1* gene was identified in wild relative of tomato *S. peruvianum* and was later introgressed into cultivated tomato (Smith 1944). *Mi-1* encodes a CC-NB-LRR protein and is one of the two *R*-genes that give resistance to two very distinct animals. The other *R*-gene with such diverse resistance is the *Cf-2* gene conferring resistance to the fungus *Cladosporium fulvum* and the cyst nematode *Globodera rostochiensis* (Lozano-Torres *et al.* 2012). *Mi-1*-mediated resistance to root-knot nematodes breaks down at temperatures above 28°C (Dropkin 1969). It is not known whether resistance to insects is also influenced by temperature. Some field isolates of potato aphids, as well as root- knot nematodes, have

overcome the resistance mediated by the *Mi-1* (Goggin *et al.* 2001, Kaloshian *et al.* 1996, Rossi *et al.* 1998).

Although the exact resistance mechanism(s) mediated by *Mi-1* remains unclear, there is evidence that the *Mi-1*-mediated resistance to aphids and nematodes is regulated differentially. Tomato plants carrying *Mi-1* are resistant to nematodes early in development, whereas the resistance against whiteflies and potato aphids is developmentally regulated where only four- to five- week-old plants are insect resistant (Kaloshian *et al.* 1995, Pascual *et al.* 2000). The fully expanded leaves of adult tomato plants are resistant to aphids and the resistance is independent of leaf position (Kaloshian *et al.* 1997). The resistance against root-knot nematodes is accompanied by HR in root tissue near the head of the feeding infective-stage juvenile, which causes either nematode death from starvation or exit the root (Dropkin 1969). In contrast, no HR is detected on *Mi-1* tomato plants infested with aphids (Martinez de Ilarduya *et al.* 2003). *Mi-1* affects the development, longevity, and fecundity of potato aphids (Kaloshian *et al.* 1997, Kaloshian *et al.* 2000). Adult aphids and nymphs die as early as 24 h after exposure to *Mi-1* tomato plants. This death is most likely due to starvation and desiccation, because aphids seem to fully recover when transferred from resistant to susceptible tomato plants (Kaloshian *et al.* 1997). Studies with electrical penetration graphs have shown that aphid stylets are able to penetrate *Mi-1* containing tomato leaves and can successfully reach the sieve elements. Nevertheless, they ingest smaller amounts of vascular fluids from *Mi-1* containing tomato plants compared to susceptible plants (Kaloshian *et al.* 2000).

An additional gene, *Rme1*, required for *Mi-1* function was identified in a suppressor screen of *Mi-1*-mediated root-knot nematode resistance. *Rme1*, is also required for resistance to aphids and whiteflies and functions early in the *Mi-1* signaling cascade (Martinez de Ilarduya *et al.* 2001, Martinez de Ilarduya *et al.* 2004). In addition, *Rme1* seem to be specific for *Mi-1* function as no additional functions have been attributed to this gene (Martinez de Ilarduya *et al.* 2001). In addition to *Rme1*, *Mi-1*-mediated resistance to aphids and nematodes require the heat shock protein *Hsp90-1*, the co-chaperone like gene *Sgt1* (Bhattarai *et al.* 2007), and transcription factors *WRKY70*, *WRKY72a* and *WRKY72b* (Atamian *et al.* 2012, Bhattarai *et al.* 2010). Interestingly, the tomato *SISERK1*, encoding an LRR-RLK, is required for only *Mi-1*-mediated aphid resistance but not for nematode resistance distinguishing the requirement for resistance to these two pests (Mantelin *et al.* 2011).

The *Vat* gene of melon confers resistance to cotton aphid (*Aphis gossypii*) and the plant viruses transmitted by this aphid (Dogimont *et al.* 2007). Similar to *Mi-1*, it encodes a protein with a CC-NB-LRR domains (Dogimont *et al.* 2008). The third aphid *R* gene is *AKR* from *Medicago truncatula* cv Jester also encodes a CC-NBS-LRR protein and confers resistance to *Aphis kondoi* (Klingler *et al.* 2005).

Aphid salivary components and their role in plant interaction

Aphid feeding alters expression of genes involved in plant defense including oxidative stress, pathogenesis-related proteins and defense signaling as well as those involved in metabolism (de Vos *et al.* 2005, Dubey *et al.* 2013, Moran *et al.* 2002). GPA feeding on

Arabidopsis plants induces transcripts levels of *PR-1* and *BETA-1-3 GLUCANASE 2* (*BGL-2*), known to be induced by the defense hormone salicylic acid (SA), as well as transcripts of *PDF1.2*, known to be induced by both defense hormones jasmonic acid (JA) and ethylene (ET) (Ellis *et al.* 2002, Martinez de Ilarduya *et al.* 2003, Moran and Thompson 2001). Similarly on tomato, potato aphid feeding induces both SA and JA/ET regulated *PR* gene expression (Martinez de Ilarduya *et al.* 2003). In Brassicaceae, accumulation of phytoalexins and other secondary metabolites such as camalexin and glucosinolate metabolites, is also associated with aphid-induced defense responses (Kettles *et al.* 2013, Kim *et al.* 2008). Moreover, it has been shown that infiltration of diet containing aphid saliva results in differential expression of plant genes related to signal transduction, secondary metabolite biosynthesis and genes encoding various stress-associated transcription factors indicating that components of the saliva are able to induce plant defenses (de Vos and Jander 2009).

Aphid stylet penetration is accompanied by the sieve tube elements occlusion triggered by accumulation of Ca^{+2} ions (Will and van Bel 2006). An aphid counteraction mechanism is thought to be elimination of this plugging. Forisomes are proteinaceous inclusions in the sieve element of legumes that undergo Ca^{+2} -dependent conformational changes. In an *in vitro* experiment, aphid saliva caused reversion of dispersed forisomes, that cause plugging, suggesting that aphids are able to reverse sieve element occlusion (Will *et al.* 2007). However, a recent study showed that aphid salivation does not induce any reversion of the dispersed forisomes *in vivo* (Medina-Ortega and Walker 2013).

Therefore, sieve element occlusion is not one of the aphid arsenals to counteract plant defense responses.

As described above, aphid salivary proteins play an important role in aphid-plant interactions. Both types of saliva, gelling and watery, can be collected by feeding aphids on artificial diets using feeding chambers (Miles 1965). The feeding chamber consists of a plastic cylinder, on one side containing the diet and the other side used as an opening to introduce the aphids. The diet is enclosed in between two layers of stretched parafilm. Aphid stylets probe through the parafilm membrane, feed on the diet and during this feeding they salivate into the diet. Aphid saliva can be collected from the parafilm pouch in the diet that is now mixed with saliva. The gelling saliva, also secreted during probing, can be seen as “a string of pearls” structure attached to the lower parafilm layer of the pouch (Miles 1965, Will et al. 2012). Saliva from different aphid species have been collected by feeding them on artificial diets, usually containing sucrose and a cocktail of amino acids, using similar feeding chambers. Different approaches are implemented to identify the proteins secreted in the aphid saliva. Earlier, aphid salivary proteins were characterized based on enzymatic activity assays or separating the proteins on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) (Miles 1999). More recently, aphid salivary proteins have been identified using MS or gel electrophoresis coupled with MS (GE-MS/MS).

Several enzyme activities have been detected in aphid saliva and a few are highlighted in this section (Madhusudhan *et al.* 1994, Madhusudhan and Miles 1998, Ni *et al.* 2000). Two cell wall degrading enzymes, pectinases and polygalacturonases, have

been detected in the saliva of green bug *Schizaphis graminum* by performing gel assays (Ma *et al.* 1990). Similarly, two oxidoreductases, polyphenol oxidase and peroxidase were detected in the grain aphid, *Sitobion avenae*, stylet sheath (gelling saliva) only polyphenol oxidase activity was detected in the watery saliva (Urbanska *et al.* 1998).

SDS-PAGE approach have been used to perform a comparative analysis of salivary secretions from five different aphid species namely pea aphid, green bug (*S. graminum*), Russian wheat aphid, western wheat aphid (*D. tritici*) and another wheat aphid *D. mexicana*, by feeding them on an artificial diet prepared with 15% sucrose (Cooper *et al.* 2011). On the basis of banding pattern, it was found that a protein(s) of molecular weight 132 kDa was commonly present in the saliva of all aphids. Additionally, saliva from all these aphid species showed alkaline phosphatase activity based on a fluorescence substrate assay. Moreover, nine bands were uniquely detected in the saliva of all the three *Diuraphis* aphid species, while ten unique protein bands were detected in the saliva of only pea aphid suggesting that aphid salivary protein composition varies among aphid genera.

Another study reported on SDS-PAGE analysis of both gelling and liquid saliva of the vetch aphid (*Megoura viciae*) fed on two different types of diets, one matching to sieve-elements sap (15% sucrose, L-serine, 100 mM L-methionine and 100 mM L-aspartic acid with a pH of 7.2) and the other similar to cell-wall milieu (20 mM KCl, 1 mM CaCl₂, 10 mM MES, adjusted to pH 5.5) (Will *et al.* 2012). Based on visual observations, the authors concluded that the diet composition influences the salivary composition of both watery and gelling saliva.

A direct identification of salivary proteins is done by subjecting aphid saliva to MS. Using GE-MS/MS approach 17 salivary proteins were identified in the saliva of GPA (Harmel *et al.* 2008), nine in pea aphid (Carolan *et al.* 2009), 34 in Russian wheat aphid (Nicholson *et al.* 2012) and 12 and seven in the saliva of the cereal aphids *S. avenae* and *Metopolophium dirhodum*, respectively (Rao *et al.* 2013). The composition of the salivary proteins from these aphids were very different with only a few common proteins detected in the saliva of two or more aphid species. These common proteins are two glucose dehydrogenases, a trehalase, a sheath-like protein (ACYPI009881) and an uncharacterized protein (ACYPI004904). However, similar proteins were identified in the saliva of both cereal aphids. Therefore, the differences in the salivary proteome among the aphid species could be due to differences in their host range or host adaptation. Another explanation of the diversity in saliva composition could be because of the differences in the composition of the artificial diets used for saliva collection and the variable number of aphids. Therefore, it may be premature to speculate on the role of host range and aphid host adaptation on the salivary protein composition.

An alternative approach to identify secreted salivary proteins is by sequencing the salivary gland transcriptome and using bioinformatics tools such as SignalP algorithm to predict the secretion signal peptides at the N-terminal of putative proteins corresponding to the transcripts (Atamian *et al.* 2013, Bos *et al.* 2010, Carolan *et al.* 2011, Ramsey *et al.* 2007). Using Signal P and the salivary gland transcriptome of pea aphid and GPA, 33% (273 of 835 contigs) and 24% (45 of 186 contigs) of the transcripts, respectively, were predicted to have signal peptides and therefore could be secreted (Carolan *et al.* 2011,

Ramsey et al. 2007). Using a similar approach, we identified putative potato aphid salivary proteins (Atamian et al. 2013) described in Chapter Three of my dissertation.

Functional characterization of aphid effectors

To date, no mutant populations of aphids exist and genetic transformation of aphids has not been achieved. However, the availability of a genome sequence for the pea aphid (Consortium 2010) has made this species a good candidate for aphid effector studies and also provided a valuable resource to investigate aphid biology at the genome level for other aphid species.

Several methods have been used to functionally characterize genes in aphids including aphid effectors. One of these approaches is using RNA interference (RNAi) to downregulate or silence target gene transcript levels (Mutti *et al.* 2006). Double-stranded RNA (dsRNA) can be delivered into aphids through artificial diets or microinjection. Injection of siRNA corresponding to the *ApC002* transcript, the most abundant transcript of the pea aphid salivary gland, into the pea aphid hemocoel led to knockdown of the *ApC002* transcript levels in the salivary glands and reduced the lifespan of the aphids (Mutti *et al.* 2008, Mutti *et al.* 2006). In another study with pea aphids, the expression levels of two additional genes calreticulin (ubiquitously expressed in aphid body) and cathepsin (gut specific) decreased by 40% after microinjection of dsRNA corresponding to their respective transcripts (Jaubert-Possamai *et al.* 2007). Although these microinjection-based RNA deliveries were successful, the aphid mortality rate due to injection in control aphids was quite high (30-45%). Because of this high mortality rate,

other methods for delivery of the dsRNA were employed. One of such approaches is to supplement artificial aphid diets with dsRNA. Feeding pea aphids on an artificial diet containing dsRNA corresponding to an aquaporin transcript *ApAQPI*, led to more than 2-fold decrease in transcript levels within 24 hours, and resulted in elevated osmotic pressure in the aphid hemolymph (Shakesby et al. 2009).

Another approach for silencing genes in insect pests is through plant-mediated RNAi delivery. This approach enables the study of pest-related aphid gene functions, while insects are feeding on the host plants, which is not possible to evaluate when feeding on artificial diet. Recently, this approach has been used to silence two GPA genes expressed in distinct tissues, *MpC002* (salivary gland) and a *Receptor of Activated Kinase C* (*Rack-1*) (gut). Aphids were fed on transgenic plants that either transiently (*N. benthamiana*) or stably (*Arabidopsis*) expressed dsRNA corresponding to portions of *MpC002* and *MpRack-1*. Silencing either gene resulted in reduced aphid fecundity on both plant hosts (Pitino et al. 2011).

For functional characterization of aphid effectors, an alternate approach to silencing is overexpression of aphid genes *in planta* and evaluating aphid performance on these plants. A high-throughput transient overexpression assay in *N. benthamiana* was developed and used to screen 46 candidate GPA effectors. These effectors were selected from an aphid salivary gland library based on the presence of N-terminal signal peptide in the absence of a transmembrane domain (Bos et al. 2010, Ramsey et al. 2007). Feeding aphids on *N. benthamiana* leaf discs overexpressing the candidate effectors *Mp10* and *Mp42*, both encoding unknown proteins, reduced aphid fecundity while overexpressing

MpC002 enhanced aphid fecundity (Bos et al. 2010). Interestingly, in spite of reducing aphid fecundity, *Mp10* overexpression was shown to suppress flg22-induced ROS response indicating *Mp10* is able to suppress PTI. In addition, *Mp10* induced chlorosis and cell death in *N. benthamiana* but not in *N. tabacum* suggesting the presence of a disease resistance protein in the former recognizing this effector (Bos et al. 2010). Moreover, expression of *Mp10* was shown to reduce the levels of *Agrobacterium*-mediated overexpression of unrelated proteins. *Mp10* seem to regulate both SA- and JA-signaling pathways as transient overexpression of *Mp10* activated JA- and SA-signaling pathways and resulted in decrease susceptibility to *Phytophthora capsici* (Rodriguez et al. 2013). The induction of the defense hormone signaling pathways may be responsible for the negative effect of *Mp10* overexpression on aphid fecundity. These studies also suggest that *Mp10* could possibly have dual activity in suppressing and activating host defense responses.

A similar overexpression assay in *N. benthamiana* and an alternate effector delivery approach were used to characterize potato aphid putative effectors identified by sequencing the salivary gland transcripts (Atamian et al. 2013). This work is described in Chapter Three of my thesis.

Objectives of dissertation research

A detailed understanding of the aphid biology and plant-aphid interactions is required to enhance plant resistance to aphids. A sustainable level of resistance is crucial to reduce the damage cause by aphids without using insecticides for improving crop production.

As mentioned above aphid salivary components perturb host cellular and metabolic processes. Therefore, it is essential to identify the components of the aphid saliva and functionally characterize these proteins to develop targeted defense strategies. Salivary proteins from several aphid species have been identified and a few have been characterized for their role in interaction with the plant host (Elzinga and Jander 2013, Hogenhout and Bos 2011). However, the salivary proteins of the potato aphid, one of the major pests of Solanaceous crops, have not been reported yet. Therefore, the first objective of my dissertation was to identify the potato aphid salivary proteins. To achieve this aim we used two different approaches. The first approach, described in Chapter One, was direct identification of proteins secreted in the potato aphid saliva using LC-MS/MS. Two different diets were used to collect the potato aphid saliva. A comparative analysis of the proteins secreted with two different diets and identification of unique proteins in the potato aphid saliva were reported. The second approach, described in Chapter Three, utilized sequencing the salivary gland transcriptome and bioinformatics analysis to identify putative secreted proteins.

The second objective of my research was to develop assays to functionally characterize the role of aphid salivary proteins *in planta*. We adopted approaches used for characterization of bacterial and fungal effectors and used these with three different hosts namely, *Arabidopsis*, tomato and *N. benthamiana* to assay with two different aphid pests potato aphid and GPA. This work is described in Chapter Two and Chapter Three.

My last objective was to identify the defense signaling- or metabolic- pathways targeted by one of these aphid effectors. This work is described in Chapter Four.

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CHAPTER ONE

Proteomic analysis of potato aphid saliva

Abstract

Aphids are phloem-feeding insects and while feeding they secrete two kinds of saliva: soluble saliva and gelling saliva. Aphid saliva contains proteins that have been shown to alter plant defenses. Proteins from the saliva of several aphid species have been identified and handful of them have been functionally characterized. Here, we report the identified proteins in the saliva of potato aphid by using mass spectrometry (MS). We collected soluble saliva after feeding of mixed developmental stages of potato aphids on water supplemented with the neurostimulant resorcinol. In addition, both soluble and gelling saliva were collected from aphids feeding on water only diet. We found resorcinol enhanced aphid salivary secretions and can be successfully used to collect aphid saliva. In both diets, we identified a plethora of proteins in the potato aphid saliva with the majority being uncharacterized, while others involved in various metabolic processes, stress responses and protein folding. In the water-only diet, we found several proteins of aphid endosymbiont *Buchnera aphidicola* origin. In soluble saliva in either diet and gelling saliva in water diet, approximately 46% of proteins were predicted to be secreted. The remaining proteins not predicted for secretion have housekeeping or cytoskeletal functions and possibly originate from other organs than the salivary glands and moved through the salivary glands for secretion. A comparative proteomic analysis was performed with aphid soluble salivary proteins identified in both diet types and those reported from six distinct aphid species. Only one protein was present in the saliva of all six aphid species. A few of the proteins, like metabolic enzymes, a sheath protein,

cytoskeleton components and oxidases were found to be shared between potato aphid and atleast one other aphid species.

Introduction

Aphids (Hemiptera: Aphididae) are sap-sucking insects that cause serious economic losses to cultivated crops directly by feeding on plant sap and indirectly by transmitting viruses (Ng and Perry 2004). Aphids have worldwide distribution and some have a broad host range, while others infest restricted a number of host plants. The potato aphid (*Macrosiphum euphorbiae*) is one of the most damaging pests of tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*) crops worldwide. Potato aphids have a wide host range that include plants from non-solanaceous species such as sunflower, peas, beans, apple, turnip, corn, asparagus, clover, and roses.

Aphids inject saliva into the plant host and withdraw nutrients from the plant phloem sieve elements using a specialized mouthpart or stylets. On susceptible hosts, aphids can feed continuously on a single sieve element for hours and even days (Tjallingii 1995). While feeding, aphids keep the sieve element alive and prevent coagulation of the sieve plates by the phloem proteins (p-proteins) and callose deposits (Prado and Tjallingii 1994, Tjallingii and Hogen Esch 1993). The phloem sap is rich in sugars but is deficient in essential amino acids providing an incomplete source of nutrient to aphids (Douglas 1993, Sandstrom and Moran 1999). These essential amino acids are supplied by its intracellular primary obligate symbiont *Buchnera aphidicola* (Douglas 1998, Hansen and Moran 2011, Shigenobu *et al.* 2000, Shigenobu and Wilson 2011), and in return, aphids provide a suitable host niche to *Buchnera* (Gil *et al.* 2006).

Inside aphids, *Buchnera* is present in specialized cells known as bacteriocytes, which are grouped together to form the bacteriome, located at the posterior region of the

abdomen adjacent to the ovarioles (Braendle *et al.* 2003, McLean and Houk 1973). The cytoplasm of the aphid bacteriocyte contains a large nucleus and nucleolus and large numbers of mitochondria, endoplasmic reticulum and ribosomes (McLean and Houk 1973). In addition to the primary endosymbiont, aphids also have secondary endosymbionts but their presence may vary from one aphid species to another and even within clonal populations of the same aphid species (Fukatsu and Ishikawa 1998, Fukatsu *et al.* 2000, Griffiths and Beck 1973). These secondary endosymbionts are not required for aphid survival. However, they are capable of enhancing the aphid's tolerance to suboptimal temperatures (Russell and Moran 2006), provide protection against parasitoid wasps (Oliver *et al.* 2005) and entomopathogenic fungi (Oliver *et al.* 2005, Scarborough *et al.* 2005).

During feeding, aphids secrete two types of saliva: gelling saliva and soluble or liquid saliva (Fig. 1.1). Gelling saliva, also known as sheath saliva, is secreted during stylet penetration of host tissue and solidifies soon after exiting the stylet tip. It forms a continuous protective sheath around the stylet, and remains behind in the host tissues after the stylet is retracted (Fig. 1.1B and 1.1C) (Tjallingii and Hogen Esch 1993). Gelling saliva plays a role in minimizing aphid-feeding damage as it seals the punctured sites in various cell types and reduces loss of phloem sap due to stylet puncture. Majority of soluble saliva is secreted into sieve element lumen, however, some soluble saliva is also secreted intercellularly in the stylet path and intracellularly when aphids penetrating the mesophyll cells (Tjallingii 2006).

Plant pathogenic nematodes utilize stylet to inject their effector proteins inside the plant vascular cells and plant pathogenic bacteria use type-three secretion system (T3SS) to deliver effector molecules into the host cell (Abramovitch *et al.* 2006, Davis *et al.* 2008). Analogous to these phytopathogens aphids also deliver through their stylets molecules present in their saliva, which have implications in aphid's ability to colonize the plant (Elzinga and Jander 2013, Rodriguez and Bos 2013). A number of enzymatic activities have been detected in aphid saliva including pectinases, cellulases, polyphenol oxidases, peroxidases, and lipases (Campbell and Dreyer 1990, Cherqui and Tjallingii 2000, Miles 1990, Miles 1999). Such enzymes could assist in insect feeding, maintaining redox conditions and detoxification of phenolic compounds (Miles 1999, Miles and Oertli 1993, Tjallingii 2006).

In addition, aphid salivary components play an important role in modulating plant defense responses. Salivary proteins can suppress or enhance plant defense responses (Bos *et al.* 2010, de Vos and Jander 2009) while others alter aphid performance on the host plants expressing these proteins (Atamian *et al.* 2013, Bos *et al.* 2010, Mutti *et al.* 2008, Mutti *et al.* 2006). Besides evading or altering host defense responses, aphid feeding also modifies host plant physiology to their advantage (Pompon *et al.* 2010). Therefore, identification and characterization of the aphid salivary components could assist in developing resistance in crops effective against agriculturally important aphids.

Recently, two main approaches have been used to identify aphid salivary proteins: transcriptome or/and proteome analyses of aphid salivary gland tissues and proteome analysis of the saliva. Using salivary gland transcriptome and deduced amino acid

sequences, proteins with secretion signals or predicted for secretions were identified from a number of aphid species. Twenty four percent (45/186 contigs) of the *Myzus persicae* (Green peach aphid; GPA) (Ramsey *et al.* 2007), 33% (273/ 835 contigs) *Acyrtosiphon pisum* (Pea aphid) (Carolan *et al.* 2011), and 35% of the potato aphid (159/460) (Atamian *et al.* 2013) salivary gland transcripts were predicted for secretion. Proteome analysis of the salivary glands of the pea aphid showed that 17% (156/925) of the salivary gland proteins have a secretion signal (Carolan *et al.* 2011). In contrast, mass spectrometry (MS) analyses of the pea aphid (Carolan *et al.* 2009) and GPA (Harmel *et al.* 2008) saliva have identified only 9 and 17 secreted proteins, respectively, likely due to difficulty collecting large amount of aphid saliva.

Using MS technique, salivary proteins from three additional aphid species have been identified. Thirty-four, twelve and seven proteins have been identified in the saliva of the Russian wheat aphid (*Diuraphis noxia*) and two cereal aphids (*Sitobion avenae* and *Metopolophium dirhodum*), respectively (Nicholson *et al.* 2012, Rao *et al.* 2013). The composition of the salivary proteins varied greatly among these different aphid species. Variation in the composition of salivary proteins have been detected between aphid biotypes of the same species and even in saliva of aphids fed on different diets (Cooper *et al.* 2010, Cooper *et al.* 2011, Miles and Harrewijn 1991, Nicholson *et al.* 2012).

In the present study, we profiled the salivary proteins of the potato aphid using MS. We collected aphid saliva in water-only diet and in water supplemented with resorcinol. Resorcinol is a neurostimulant that has been used successfully to induce esophageal gland secretions plant parasitic nematodes (Bellafiore *et al.* 2008, Davis *et al.*

1994). Potato aphid salivary proteins identified in water-only or water with the neurostimulant were compared.

Data presented in this Chapter is an equal contribution by myself and a former graduate student in the lab Hagop Atamian.

Materials and Methods

Plant material and aphid colonies

Tomato (*Solanum lycopersicum*) cultivar Moneymaker plants and UC82B were maintained in growth rooms at 24°C with 16-hr light and 8-hr dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light and fertilized weekly with MiracleGro (18-18-21; Stern's MiracleGro Products).

A colony of the parthenogenetic potato aphids was reared on tomato cv. UC82B plants. The colony was maintained in insect cages in a pesticide-free greenhouse at 22-26°C supplemented with 75 μE light for 16-hr light photoperiod.

Saliva collection

To collect saliva, about 100 aphids were introduced into a feeding chamber consisting of a plastic cylinder (Fig. 1.2). One end of the cylinder was covered with a double layer of parafilm forming a pouch, containing 150 μl of desired diet prepared in ultra pure autoclaved water. Aphids were introduced from the other end of the tube and given access to feed on the pouch for 16-hr under yellow light at 23°C. After aphid introduction, the open end of the cylinder was secured with cheesecloth. The liquid content of the pouches was collected with a fine pipette tip. To collect gelling saliva, the parafilm pouches were opened, rinsed well in sterile water and the internal surface of the lower parafilm was scraped using the dull side of a sterile surgical blade.

For the comparative analysis of aphid salivary proteins three different diets were used, 0.4% resorcinol in water, cocktail of amino acids with 15 % sucrose (Cherqui and Tjallingii 2000) and a water-only diet. Saliva was collected from 800 aphids in 1ml volume as described above and vacuum concentrated to 30µl. Samples were mixed with 2x Laemmli buffer and boiled for 10 minutes before loading on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Bands were visualized by staining the gel with SilverQuest Silver Staining Kit following manufacturer's protocol (Life Technologies, USA).

For MS analysis, saliva was collected from aphids fed on water and resorcinol. For the first experiment/replicate saliva was collected from 30,000 aphids in 30 ml volume by feeding them on 0.4% resorcinol and for the second experiment saliva was collected from 100,000 aphids in 98ml volume by feeding them on sterile autoclaved water. Collected saliva was stored at -80°C until ready for MS.

Saliva preparation and liquid chromatography (LC)-MS analysis

Collected saliva processing and liquid chromatography-electrospray ionization/multi-stage MS (LC ESI MS/MS) analysis was performed by Dr. Steve Briggs' group at UC San Diego as described in Bellafiore et al. (2008). The filtered MS/MS spectra were searched against three databases: 1) a NCBI non-redundant protein database limited to *Buchnera* taxonomy; and 2) potato aphid transcriptome database developed in our lab; and 3) the pea aphid proteome database downloaded from aphid base (ACYPI proteins v2.1). The potato aphid transcriptome database was constructed by performing 6-frame

translation of the potato aphid transcripts. ORFs with protein length less than 6 amino acids were discarded.

Staining gelling saliva

Gelling salivary sheaths were stained in tomato leaves or parafilm pouches from 100 feeding potato aphids. Parafilm pouches and tomato leaf disks (2-cm diameter) were stained overnight in a solution of 0.1% acid fuchsin in equal parts of 95% ethanol and glacial acetic acid. Parafilm was destained in a solution of equal parts of lactic acid, glycerol, and water, tissue samples were autoclaved for 10 min in the same de-staining solution. This experiment was repeated twice. Images were captured using a Nikon digital camera in the lab.

DAPI staining

Aphid saliva was collected in water as described above. Saliva from five feeding chambers with 1000 aphids each was pooled and vacuum concentrated. Adult aphids were dissected and ovaries were collected in phosphate buffered saline (PBS). The samples were fixed in 1% paraformaldehyde in PBS at room temperature for 30 min. After fixing, nuclei were stained in the dark for 20 min with 1 µg/ml of 4', 6'-diamino-2-phenylindole (DAPI, Sigma). After staining, samples were observed under fluorescent microscope (Nikon Eclipse Ti).

Annotation, gene ontology classification, signal peptide prediction and protein localization

Potato aphid transcripts matching the sequenced peptides were annotated by performing reciprocal TBLASTX analyses with pea aphid, a close relative of potato aphid with a genome sequence, predicted sequences (aphidbase_2.1_mRNA) and against NCBI nucleotide (nt/nr) database. The annotated sequences were assigned to different gene ontology (GO) categories based on available database containing GO assignments of all the publicly available pea aphid expressed sequence tags (EST) and also UniProt.

Amino-acid sequences of putative full-length pea aphid orthologs of the potato aphid secreted proteins were subjected to *de novo* signal peptide prediction analysis using SignalP 4.0 (Petersen *et al.* 2011) and TargetP 1.1 (Emanuelsson *et al.* 2000) programs. Hidden Markov model scores higher than 0.45 were considered for SignalP prediction, while for TargetP predictions were determined by predefined set of cutoffs that yielded specificity >0.95 on the TargetP test sets.

Amino-acid sequences of putative full-length pea aphid orthologs of the potato aphid secreted proteins were subjected to protein subcellular localization prediction by using the program WoLF PSORT (Horton *et al.* 2007)

Results

Protein profile of potato aphid gelling and soluble saliva obtained in different diets

Aphid gelling saliva was collected by feeding potato aphids on water diet and soluble saliva was collected and compared in three different diets. Soluble saliva was collected by feeding 800 potato aphids on 0.4% resorcinol, 15% sucrose plus amino acids (Cherqui and Tjallingii 2000) or water. Soluble saliva was concentrated by using vacuum and separated on one dimensional- SDS PAGE under denaturing conditions. Using silver staining, variable numbers of protein bands were detected from soluble saliva collected in the three diets. Relatively more intense and higher numbers of bands were visualized with the resorcinol diet compared to the other two diets (Fig. 1.3A). The soluble saliva in 0.4% resorcinol displayed about 42 protein bands, from sucrose plus amino acid diet about 33 proteins whereas from water-only diet about 22 bands were detected. Some of the lower molecular weight protein bands that were easily visible with the resorcinol diet were difficult to visualize in the water only diet.

Gelling saliva was collected by feeding 800 potato aphids on ultrapure water (Fig. 1.3B). The gelling saliva contained about 23 protein bands. Based on gel banding patterns, several similar size protein bands were present in the gelling and soluble saliva suggesting that there are common proteins present in both saliva types or there is cross contamination between the two saliva types during the collection process.

These results suggest that similar to nematodes, the neurostimulant resorcinol may have increased the stylet thrust in aphids thus forcing them to secrete more saliva

(Bradley 1985). Based on the results from SDS PAGE analysis of salivary proteins, we performed MS analysis of aphid secretome by feeding potato aphids on water-only and water-plus resorcinol diets.

Proteomics analysis of soluble and gelling saliva

For secretome analysis in the resorcinol diet, saliva was collected from about 25,000 aphids whereas more than 100,000 aphids were used to collect saliva in water-only diet. Soluble saliva was collected from resorcinol diet, whereas both soluble and gelling saliva samples were collected from water-diet treatment. Secreted proteins were identified by nanoLC ESI MS/MS. MS spectra with peptide FDR less than 0.1% were considered valid and the protein FDR was set at 0.4%. To identify salivary proteins of aphid origin, the MS spectra were searched against the predicted potato aphid proteome, which was derived from its transcriptome (Atamian Thesis), as the genome of the potato aphid has not been sequenced. In order to identify additional aphid salivary proteins that could be missed in the spectra searches against the potato aphid proteome, the MS spectra were also searched against the pea aphid proteome. Pea aphid is the only aphid species with a published and publically available genome resources (Consortium 2010). Peptides matching to orthologous pea aphid and potato aphid proteins were grouped together. Proteins with at least two valid MS/MS spectra are reported here and proteins with a single unique peptide but multiple spectra were reported after manual validation.

In searches against the potato aphid database, a total of 1,144 peptides representing 109 non-redundant proteins were identified in the potato aphid soluble saliva

in water-only diet (Table S1). Searches against the pea aphid database, identified an additional six proteins in the water-only diet (Table S1). Ninety-eight of the 109 proteins identified in the potato aphid searches were also present in pea aphid (Table S1). Therefore, 11 unique potato aphid salivary proteins were identified (Table S1). The majority of these proteins (86/115; 75%) were identified by detection of two or more peptides. The remaining 29 proteins were identified by several spectra but only one peptide, were manually validated and the spectra were shown in (Table S2). Of the 29 proteins identified by only one peptide, five have been previously predicted to be secreted in the pea aphid salivary gland proteome (Carolan et al. 2011) confirming the proper identification of a subset of these proteins.

For the proteins identified in the resorcinol diet after interrogation of the potato aphid database, a total of 550 peptides representing 75 non-redundant proteins were identified (Table S3). Searches against the pea aphid database identified an additional five proteins in the resorcinol diet (Table S3). Almost all proteins (72/75; 96%) identified in the potato aphid searches were also present in the pea aphid (Table S3). Therefore, only three unique potato aphid salivary proteins were identified (Table S3). Sixty-one proteins (61/80; 76%) were identified by the detection of two or more peptides while the remaining 19 proteins were identified by several spectra but only one peptide (Table S2). A subset of these 19 proteins was also identified in the water-only diet. Eight were represented by the same peptide and additional peptides, while three were represented by another unique peptide or peptides in the secretome of water-only diet. Taken together,

115 salivary proteins were identified in the water diet (Table S1) and 80 in the resorcinol diet (Table S3), with 68 proteins common in both diets.

In the potato aphid gelling saliva in the water only diet, a total of 211 peptides representing 92 non-redundant proteins were identified in searches against the potato aphid database (Table S1). Searches against the pea aphid database, identified an additional 19 proteins bringing the total number of proteins in the gelling saliva to 111 (Table S1). All 92 proteins identified in the potato aphid searches were also present in pea aphid. Only 43 proteins (43/111; 39%) were identified by the detection of two or more peptides (Table S1). The remaining 68 proteins were identified by only one peptide and were manually validated. The validated spectra were summarized in Table S2. Of the 68 proteins identified by only one peptide, nine have been previously predicted to be secreted in the pea aphid salivary gland proteome confirming the proper identification of a subset of these proteins (Carolan et al. 2011).

Comparing the proteins identified in both soluble and gelling saliva in the water-only diet, 53 proteins were present in both saliva types suggesting cross contamination between the two saliva types during collection (Table S1). Therefore 58 proteins were gelling-saliva specific.

To identify proteins of endosymbiont origin in the potato aphid saliva, the spectra obtained from both resorcinol diet and water-only diet, were searched against publically available *Buchnera*-predicted proteins. No endosymbiont-specific proteins were detected in the saliva collected from resorcinol diet. To our surprise, 11 *Buchnera* proteins were identified in water-only diet (Table S1). Remarkably, six of these proteins, including

GroEL, were detected in the gelling saliva, whereas seven were detected in the soluble saliva and two proteins were present in both the saliva types. All peptides matching to proteins from the endosymbiont *Buchnera* were manually validated (Table S2).

Protein annotation

Salivary protein-encoding transcripts were annotated by performing TBLASTX analysis against the pea aphid and NCBI databases. More than half of the salivary proteins from the resorcinol diet (45/80; 56%) were uncharacterized with no known function (Table S3). The remaining half represented proteins with a plethora of functions including glucose dehydrogenases, β -N-acetylglucosaminidase, trehalase, lipases, peroxidases, heat shock proteins and cytoskeletal proteins. Majority of the proteins (68/115, 59%) present in the soluble saliva collected from water-only diet were similar to the ones detected in the soluble saliva of resorcinol diet. Remaining represented proteins like alanyl aminopeptidase N, neuroendocrine convertase 1, isocitrate dehydrogenase, galactosylgalactosylxylosylprotein 3- β -glucuronosyltransferase, fatty acid synthase, gag protein, cyclic AMP-response element-binding protein A-like, glutathione S-transferase, heat shock proteins and some uncharacterized proteins.

Among the proteins present only in the gelling saliva, only a few were uncharacterized (40/111, 36%) and the remaining were majorly heat shock proteins, ribosomal proteins, elongation factors and components of cytoskeleton (Table S1).

Among the *Buchnera*-specific proteins detected with the water-only diet were the molecular chaperonin GroEL and co-chaperonin GroES, several types of

dehydrogenases, N-acetylglucosamine transferase, ATP synthase, ribosomal proteins, heat shock proteins and an unnamed protein (Table S1).

Signal Peptide Prediction

SignalP and TargetP programs were used to predict secreted proteins with presence of a cleavage site or predicted for secretions, respectively. Since not all of the potato aphid transcriptome sequences are full-length, we used the predicted proteins of their pea aphid orthologs to predict whether these proteins are secreted. Fifty percent (40/80) of the proteins found in the potato aphid saliva in the resorcinol diet were predicted to be secreted by SignalP 4.1 (Table S3). Moreover, TargetP identified eight additional secreted proteins that were not predicted by SignalP bringing the total number of predicted secreted proteins to 48 (48/80). Similarly, about 50% (61/115) of the proteins present in the soluble saliva in the water diet were predicted to be secreted by SignalP 4.1 (Table S1). TargetP identified 15 additional secreted proteins that were not predicted by SignalP bringing the total number of predicted secreted proteins to 76 (76/115). Forty-five proteins predicted for secretion were common in both resorcinol diet and water only diet, while the total number of unique proteins predicted for secretion in both diets were 79.

In the gelling saliva collected in the water diet, only 35% (39/111) of the proteins present were predicted to be secreted by SignalP 4.1 (Table S1). TargetP identified seven additional secreted proteins bringing the total number of predicted secreted protein to 46.

Among the *Buchnera*-specific proteins detected with the water only diet no protein was predicted to be secreted by SignalP 4.1 or TargetP 1.1 (Table S1).

Subcellular localization of secreted proteins

To predict the subcellular localization of the salivary proteins, the WoLF PSORT program was used (Horton *et al.* 2007). Among the proteins identified in the soluble saliva with resorcinol diet, 46% (37/80) were predicted to be extracellular, 12% (10/80) cytoplasmic, 12% (10/80) mitochondrial, 10% (8/80) endoplasmic reticulum (ER), 6% (5/80) nuclear and 4% (3/80) to both the cytosol and nucleus (Table S3).

In the water diet, about half of the proteins in the soluble saliva (55/115; 48%) were localized extracellularly, 15% (17/115) cytoplasmic, 10% (11/115) mitochondrial, 7% (8/115) ER, 5% (6/115) nuclear, 2% (2/115) to both cytosol and nucleus and 1% (1/115) to the peroxisome (Table S1). In the gelling saliva, 32% (35/111) proteins were localized extracellularly, 32% (36/111) cytoplasmic, 13% (14/111) mitochondrial, 9% (10/111) nuclear, 5% (5/111) to both cytosol and nucleus, 4% (4/111) ER, 2% (2/111) cytoskeleton and 1% (1/111) to the peroxisome (Table S1). Among the *Buchnera* specific proteins detected with the water only diet majority of the proteins were cytoplasmic (8/11, 73%), 18% (2/11) mitochondria and 9% (1/11) in the cytoskeleton (Table S1).

Gene ontology

A functional overview of the potato aphid salivary proteins was performed by exploring the publicly available pea aphid database and UniProt. The functions of the annotated

potato aphid secretome were determined by categorizing the gene ontology (GO) terms by biological process and molecular function.

Biological process was assigned to 28% (22/80) and 23% (27/115) of the aphid proteins in the soluble saliva from resorcinol and water-only diets, respectively (Table S1 and Table S3). In addition, in the water diet, biological processes were assigned to 50% of the aphid proteins (55/111) in the gelling saliva and 91% of the proteins (10/11) specific to endosymbiont *Buchnera* (Table S1). The most common assigned categories included metabolic processes such as alcohol, carbohydrate, lipid, trehalose and protein metabolism; biosynthesis of heme, proline and cellular macromolecules; proton and ion transport; stress responses (eg. oxidative stress); transcription regulation or translation and elongation; proteolysis; proteins and small molecules catabolism; protein folding and motility.

Molecular functions were assigned to 34% (27/80) and 28% (32/115) of the potato aphid proteins in the soluble saliva from resorcinol and water-only diets, respectively (Table S1 and Table S3). In addition, in the water diet, molecular functions were assigned to 57 (51%) of the potato aphid proteins present only in the gelling saliva and 82% (9/11) of the proteins specific to endosymbiont *Buchnera* (Table S1). The common molecular functions assigned to the annotated proteins included: oxidoreductase activity; peroxidase activity; peptidase activity; catalytic activity; structural constituents of ribosomes; binding to ATP; NAD; GTP; cation binding; nucleic acid binding and protein binding.

Salivary proteins lacking the predicted signal for secretion

Among the salivary proteins of aphid origin not predicted for secretion were proteins involved in cell maintenance such as chaperonins, heat shock proteins, ribosomal proteins, as well as proteins involved in energy metabolism, membrane trafficking, and components of the cytoskeleton. Saliva was collected within a period of 16-hr. The absence of dead aphids during this period eliminated the possibility that these proteins were products of histolysis. To identify a possible source of these unexpected proteins, saliva collections were stained for presence of nuclei using DAPI (4,6'-diamidino-2-phenylindole). No nuclei were detected in aphid saliva suggesting movement of these proteins from the insect hemocoel into the saliva (Fig. 1.4).

Comparative analysis of aphid salivary proteins identified by MS

A comparative analysis of potato aphid salivary proteins with previously published reports of proteins detected by MS analysis from five different aphid species was performed. These aphids were: pea aphid (Carolan et al. 2009), GPA (Harmel et al. 2008), two cereal aphids (Rao et al. 2013), and Russian wheat aphid (Cooper et al. 2010, Nicholson et al. 2012). Comparative analysis revealed only limited overlap among the secretomes of these aphids (Table 1.1). Present in the saliva of all six aphids were several different types of glucose dehydrogenases. The second most common proteins were trehalase and a sheath-like protein present in four aphid species including potato aphid. An uncharacterized protein ACYPI004904 was present in the saliva of potato aphid and the two cereal aphids. Two additional proteins, ACYPI008224 (*Me10*) and an

uncharacterized protein ACYPI006346 were detected in only pea aphid and potato aphid, respectively. Additionally, beta-N-acetylglucosaminidase, aminopeptidase N, cytochrome oxidase, carbonic anhydrase, actin and a hypothetical protein (ACYPI001606) were detected in the saliva of potato aphid and at least one other aphid species (Table 1.1).

Discussion

In the present study, the proteins in the saliva of potato aphids were identified and compared by feeding on different diet samples: water-only diet and water plus a neurostimulant resorcinol diet. Salivary proteins were resolved by SDS-PAGE. Higher numbers of protein bands were recovered in the soluble saliva with the resorcinol diet than water-only diet. To confirm these observations, MS was used for a detailed analysis of the salivary proteins in both diets. Based on the SDS PAGE analyses, fewer numbers of aphids were used with the resorcinol diet compared to water diet. We used 25,000 aphids to collect saliva from resorcinol diet whereas more than 100,000 aphids were used to collect saliva from the water-only diet. We identified 80 proteins in the soluble saliva from resorcinol diet and 115 proteins in the water only diet.

The MS analysis results further supported our SDS-PAGE data indicating that more salivary proteins per aphid can be collected when resorcinol is used. Our data suggested that by using almost one-fourth number of aphids with resorcinol diet we could detected over 70% of the proteins that were found with water only diet. Our analysis also suggested that 68 proteins were common in both the diets. The remaining proteins detected with the resorcinol diet were either uncharacterized proteins or were members of large gene families like heat shock proteins and components of cytoskeleton that were common with water diet. Taken together, resorcinol stimulates aphids to salivate more *in vitro* and can be successfully used to enhance aphid salivation to collect copious amounts of saliva by using relatively fewer numbers of aphids.

We also profiled the salivary proteins in the gelling saliva from the water-only diet and identified 111 proteins. Extreme care was taken to keep the soluble and gelling saliva separate during collection, by rinsing the internal surface of the parafilm forming the feeding pouch with ultra pure water before collecting the gelling saliva by scraping the membrane to recover. However, we found 53 common proteins in the soluble (53/115; 46%) and gelling (53/111; 48%) saliva in the water-only diet, suggesting either these proteins are part of the soluble saliva and have affinity to stick to the gelling matrix, or they are remnants of the soluble saliva on the parafilm pouches could have cross contaminated the gelling saliva. Alternatively, gelling saliva might have been collected inadvertently in the soluble fraction. Majority of the proteins detected in the gelling saliva were represented by a single peptide and most of these proteins were uncharacterized and the ones with annotation belonged to categories such as heat shock proteins, ribosomal proteins and cytoskeleton components. A number of these proteins represent members of gene families. We frequently found that a single peptide matched to more than one protein from these protein families and therefore it was difficult to identify the exact protein source of some peptides.

In the peptide searches against the pea aphid proteome, we identified additional proteins in both water-only and resorcinol diets that were missed in the searches against potato aphid proteome. This is most likely due to incomplete nature of the potato aphid transcriptome at the present time (Atamian Thesis). Interestingly, in the soluble saliva collected from both diet types water-only and resorcinol diets, we found only 11 and three proteins, respectively, specific to potato aphids. Considering that pea aphid and

potato aphid have a very different host range with pea aphids being more specialized in its host range and feeding mainly on leguminous plants, and the potato aphid has a wide host range that includes many crop plants, this overall similarity of the salivary proteins in these two aphid species was unexpected. However, this low number of potato-aphid specific salivary proteins could be an underestimate because of the incomplete nature of the potato aphid transcriptome (Atamian thesis). A better comparison of salivary proteins between these two aphids could be performed when potato aphid genome is sequenced.

Interestingly, in the peptide searches against endosymbiont *Buchnera*, we found several proteins of *Buchnera* origin. Among these was a molecular chaperonin protein GroEL. Although the presence of GroEL has previously been reported in aphid saliva using western blot analysis and antibody against *Escherichia coli* GroEL (Filichkin *et al.* 1997), it was not clear whether the GroEL was a contaminant from bacteria other than *Buchnera* and which saliva type contained the GroEL. In our analysis, GroEL was present in the gelling saliva only, as five of the 12 GroEL peptides were *Buchnera* specific. In addition, no GroEL peptides were identified in soluble saliva from either diets; these data strongly suggests that GroEL is present only in gelling saliva.

Taken together, 196 proteins were identified in soluble saliva and gelling saliva of the potato aphids. This number represented the total number of proteins detected with the two diets. To date our data is the first to report this large number of salivary proteins in aphids. This could be due to several reasons including larger number of aphids used for saliva collection, use of neurostimulant resorcinol or the use of highly sensitive MS equipment (Bellafigliore *et al.* 2008). For most of the aphid species whose saliva proteins

are reported to date the genomes are not sequenced, except for the pea aphid. Therefore, during their analysis the peptides were mapped against their existing ESTs, and/or pea aphid genome and NCBI and thereby some proteins could be missed. An additional reason, although unlikely, could be that potato aphids secrete larger quantities of salivary proteins than the other aphid species whose saliva have been investigated (Carolan et al. 2009, Cooper et al, 2011, Harmel et al. 2008, Nicholson et al. 2012, Rao et al. 2013). Another possibility is that in most of the published reports, aphid saliva was collected by feeding the aphids on sugar-containing diets, and to eliminate the sugars from saliva collection, filter with 3-5 kDa molecular weight cut off membranes were used (Carolan et al. 2009, Harmel et al. 2008). This process inevitably causes loss of proteins. In our analysis, the salivary protein samples were concentrated using vacuum centrifugation, which should minimize losses. In a previous study, with the pea aphid salivary-gland secretome, 324 proteins (324/925, 35%) were predicted to be secreted based on the presence of secretion signal (Carolan et al. 2011). Therefore, although in the current study with the potato aphid saliva, we identified a larger number of proteins predicted for secretion (125), it is likely that we are missing a number of salivary proteins that are secreted in low abundance and are beyond the MS detection limit.

We utilized the published information of proteins identified in the saliva of pea aphid, GPA, two cereal aphids, and Russian wheat aphids and performed a comparative analysis with the potato aphid salivary proteins identified in this Chapter. We found glucose dehydrogenases to be present in saliva from all six aphid species including potato aphids. Aphids feed on phloem sap which is very rich in sugars therefore the presence of

glucose dehydrogenases in aphid saliva is expected as they could be potentially be involved in sugar metabolism. Similarly, we also found trehalase and a sheath protein as common proteins in aphid saliva. Aphid trehalase may hydrolyze trehalose that has been associated with plant defense against aphids (Singh *et al.* 2011). Me10 was present in potato aphid saliva and in the saliva of pea aphid (Carolan et al. 2009). *Me10* encodes a protein with no known motifs, however, expression of Me10 *in planta* enhanced both GPA and potato aphid fecundity on *Nicotiana benthamiana* and tomato, respectively, Chapter Three (Atamian et al. 2013). Presence of Me10 in the saliva of pea aphid suggests that Me10 might also increase pea aphid performance on its host plant. Although pea aphid and potato aphid have a very different host ranges, as mentioned before, the presence of a common protein in their saliva and the effect of Me10 on GPA and potato aphid suggest that Me10 could be a virulence protein targeting a common component of plant defense signaling pathway.

Several other uncharacterized proteins were also common between different aphid species. Presence and absence of these proteins in the saliva of different aphid species may reflect the diverse host range they feed on. However, it is possible that more proteins are common in the saliva of distinct aphid species but have not been detected due to different experimental approaches and technical issues mentioned above related to their collection and identification.

Our results also indicate that almost half of the proteins present in both water-only diet and resorcinol diet are not predicted for secretion. These proteins not predicted for secretion belongs to the category of ribosomal proteins, heat shock proteins, enzymes

involved in metabolic processes and cytoskeleton components like actin, tubulin and myosin. Similar proteins lacking secretion signals have also been found in the saliva of the cereal aphid *S. avenae* (Rao et al. 2013). In Russian wheat aphid salivary protein study, where no secretion prediction analyses were performed, we re-analyzed the proteins reported by them and found the majority of the identified proteins (68%; 23/34) were not predicted for secretion. Therefore, a large number of proteins in the aphid saliva might be from sources other than the salivary glands. A possible source of origin of these proteins could be the bacteriocyte that contains eukaryotic organelles including nucleus, nucleolus, mitochondria, endoplasmic reticulum and ribosomes (McLean and Houk 1973). Since bacteriocytes disintegrate and disperse in the hemocoel in adult aphids and as they mature, it could be the source of these proteins (Douglas and Dixon 1987, Hinde 1971). Presence of these proteins in the saliva of different aphid species suggests that these proteins might be translocated from the hemocoel through the salivary glands and were delivered into the salivary secretory canal (Miles 1968). Aphid lacks Malpighian tubules, the excretory organ present in most of the insects species (Bradley 1985) and therefore it has been proposed that aphids utilize their salivary glands as an auxiliary excretory gland (Ponsen 1972). Both the principal and accessory salivary gland in aphids possess extensive microvilli directed towards the salivary ducts, suggesting passage of these proteins from the hemocoel into the salivary ducts (Ponsen 1972). Using artificial *in vitro* feeding assays, it has been shown that aphids have the ability to uptake dyes and radioactive isotopes while feeding on diets supplemented with these materials and subsequently deliver them into plants (Forrest and Noordink 1971, Madhusudhan and

Miles 1998). This suggests that molecules can also cross the gut, move through the hemocoel into the salivary gland and secreted through the saliva.

Among the proteins predicted for secretion, there were some with known functions involved in host defense or metabolic processes. The role of a few of these proteins is discussed here. We found proteins involved in chemical detoxification, namely, peroxidases and glutathione peroxidase. Studies have shown the presence of peroxidase activity in aphid saliva by performing enzymatic substrate-based assays but specific peroxidases secreted in the saliva have not been previously reported. Aphid feeding induces the production of reactive oxygen species (ROS), like hydrogen peroxide, in plants that is an early defense-signaling component. Presence of peroxidases in the aphid saliva could be a counter defense response to prevent damage due to oxidative stress and thus help aphids in successful host colonization.

Another enzyme identified was glutathione S-transferases (GST), with roles in detoxifications of insecticides, plant allelochemicals, and in counteracting oxidative stress responses (Fournier *et al.* 1992, Ranson *et al.* 2002, Vontas *et al.* 2001, Wang *et al.* 1991) which may ensure uninterrupted feeding. Other enzymes that were detected in aphid saliva and might be directly involved in aphid feeding were maltases that hydrolyze complex sugars like maltose to simple sugar glucose. Since aphids feed on plant phloem sap rich in sugars, the presence of maltase in the aphid saliva maybe necessary to utilize these sugary compounds as their dietary component. Similarly, lipases present in the aphid saliva might help in digesting plant lipids.

We also detected carbonic anhydrases (CAs) that play an important role in maintaining pH in the midgut (del Pilar Corena *et al.* 2005, Linser *et al.* 2009) which are also expressed in the saliva of ruminating animals to maintain the oral pH and sustain an essential environment required by the symbiotic bacteria for cellulose fermentation (Mau and Sudekum 2011). We speculate that CAs detected in aphid saliva might be performing a similar role in symbiotic bacterial maintenance.

Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) was also identified in the aphid saliva that is known to be involved in many cellular processes like endocytosis, phagocytosis, cell-cell adhesion, focal adhesions, stress responses, apoptosis and nuclear functions (van den Bout and Divecha 2009). The role of PIP5K in insect host interactions is unknown. In addition we found chitinases that are involved in regeneration of exoskeleton and gut lining in insects and may have a function in plant-aphid interactions (Nakabachi *et al.* 2010). Overexpressing aphid chitinases *in planta* enhances aphid fecundity and reduces pre-reproductive period (Saguez *et al.* 2005). The exact mode of action of aphid chitinases in plant-aphid interactions is not known.

Our analyses demonstrate the protein constituents in the saliva of potato aphid. We identified very diverse protein repertoire in the aphid saliva, which is more complex than what we were expected based on the saliva protein studies performed with other aphid species in the past. However, these novel and unique proteins needs to be further characterized to identify their role in altering host defense response to sustain long-term aphid feeding and successful aphid infestation.

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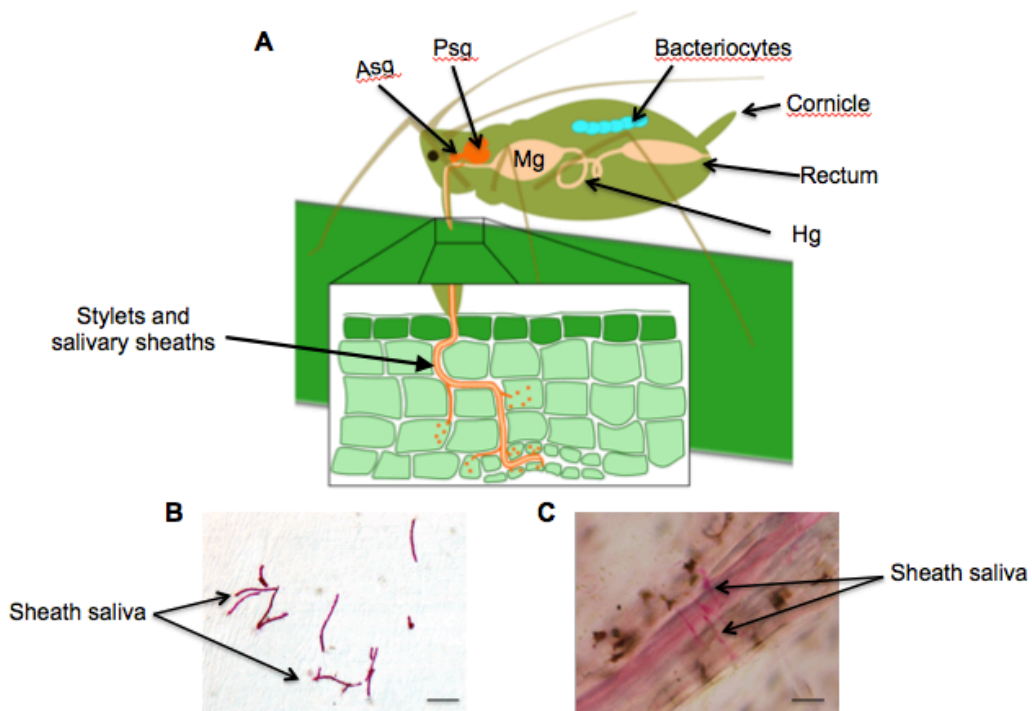


Fig. 1.1. Aphid morphology and feeding behavior. (A) Schematic diagram of an aphid feeding on a leaf. Aphid gelling/sheath saliva stained with acid fuchsin in leaf tissue (B) and a layer of a parafilm pouch (C) fed on by aphids. Arrows point to aphid gelling/sheath saliva stained pink. Scale bar = 50 μ M. Psg- Principal salivary gland, Asg- accessory salivary gland, Mg- midgut, Hg- hindgut.

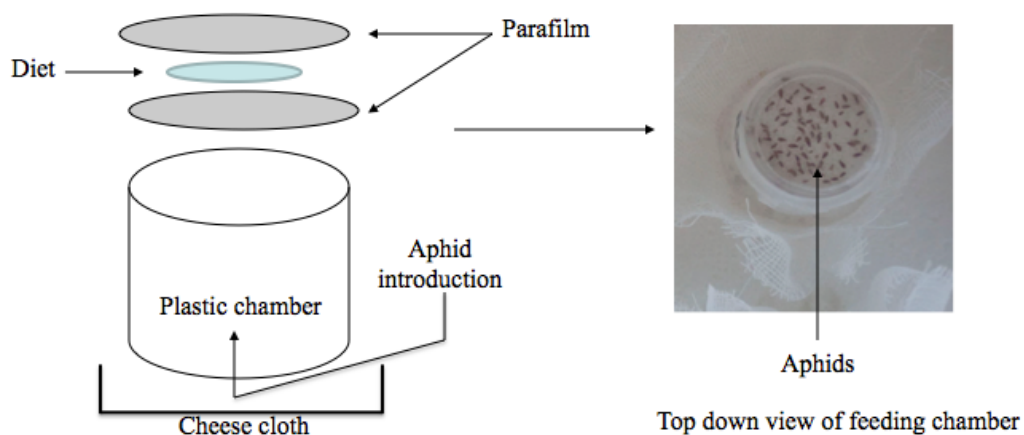


Fig. 1.2. System used for aphid saliva collection. Aphid feeding chamber consists of a plastic container covered with a double layer of parafilm forming a pouch on one side, containing 150 μ l of an artificial diet. Approximately 100 aphids were introduced from the other end of the cylinder and given access to feed on the pouch for 16-hr under yellow light at 23°C. After aphid introduction, the end was secured with cheesecloth.

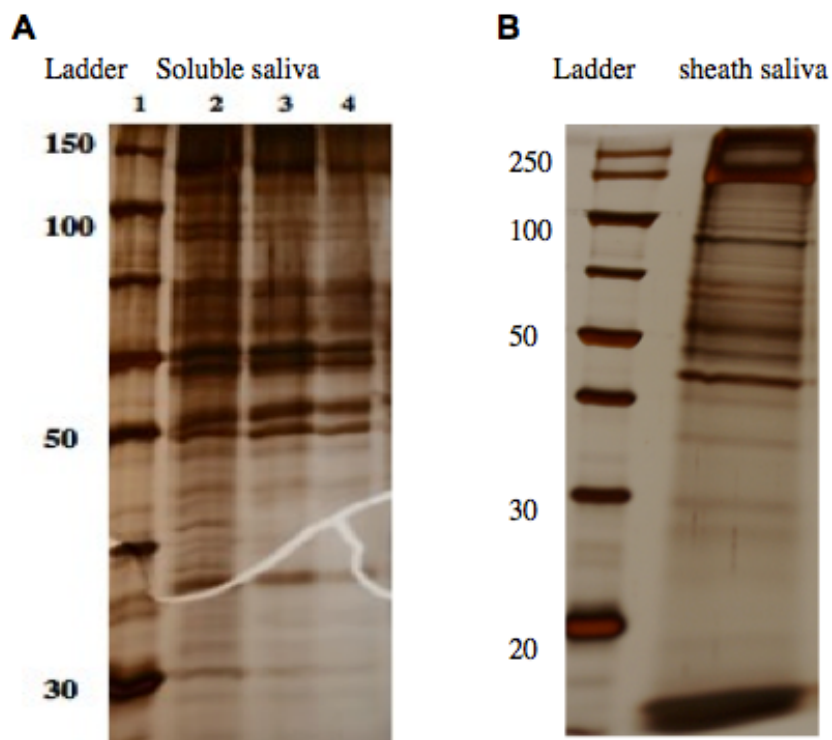


Fig. 1.3. Aphid salivary protein variation detected by SDS PAGE. (A) Soluble saliva collected after feeding 800 aphids on 0.4% resorcinol (lane 2), cocktail of amino acids in 15% sucrose (lane 3), and water-only (lane 4) diets. **(B)** Gelling/sheath saliva collected after feeding 800 aphids on water-only diets. All the saliva samples were concentrated by using vacuum and were separated by 12% SDS PAGE. To visualize the protein bands gels, were silver stained.

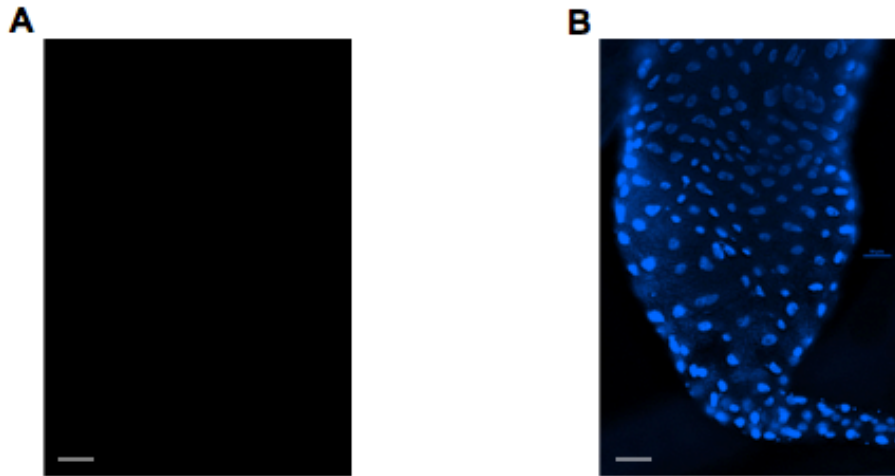


Fig. 1.4. No nuclei were detected in aphid saliva. DAPI (4,6'-diamidino-2-phenylindole) staining of aphid saliva (**A**) and ovaries (**B**; control). Saliva was collected from ~1000 aphids feeding for 24 h on parafilm pouches containing sterile water. Samples were observed under a fluorescent microscope. Scale bar = 10 μ M.

Table 1.1. Comparative analysis of potato aphid salivary proteins with five other aphid species, identified using MS. Proteins common in potato aphid and atleast one other species are reported.

Proteins	<i>Macrosiphum euphorbiae</i> (potato aphid)	<i>Acyrtosiphon pisum</i> (pea aphid)	<i>Myzus persicae</i> (GPA)	<i>Diuraphis noxia</i> (Russian wheat aphid)	<i>Sitobian avena</i> (cereal aphid)	<i>Metopolophium dirhodum</i> (cereal aphid)
Glucose dehydrogenase	Y	Y	Y	Y	Y	Y
Sheath like protein - ACYPI009881	Y	Y			Y	Y
Trehalase	Y			Y	Y	Y
Uncharacterized protein - ACYPI004904	Y				Y	Y
Actin	Y				Y	
Aminopeptidase - N	Y			Y		
Beta-N-acetylglucosaminidase	Y			Y		
ACYPI008224 (Me10)	Y	Y				
Uncharacterized protein - ACYPI006346	Y	Y				
Carbonic anhydrase	Y				Y	
Cytochrome oxidase	Y			Y		
Uncharacterized protein - ACYPI001606	Y					Y

CHAPTER TWO

The GroEL of the aphid endosymbiont *Buchnera* is delivered in the aphid saliva and is recognized by plant defense machinery

Abstract

Aphids are sap-feeding plant pests of great agricultural importance. Aphid saliva is known to modulate plant immune responses but limited information exists about the composition of aphid saliva. By means of mass spectrometry, we had identified the presence of the chaperonin GroEL from the *Buchnera aphidicola* in the saliva of the potato aphid *Macrosiphum euphorbiae*. *Buchnera* is a proteobacterium that lives endosymbiotically within bacteriocytes located in the hemocoel of the aphid. Intracellular delivery of GroEL by an effector delivery bacterial vector through *Pseudomonas fluorescence*, engineered with type three secretion system, enhanced resistance against aphids and bacteria in both tomato and *Arabidopsis thaliana* (*Arabidopsis*) plants. Similarly, stable transgenic *Arabidopsis* plants expressing GroEL induced pattern-triggered immunity (PTI) marker genes and were more resistant to aphids and bacteria. Applying purified recombinant GroEL also induced PTI responses. The induction of GroEL-mediated defense responses required the coreceptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1. Our findings indicate that plant-aphid interactions have a third element, the aphid endosymbiotic prokaryotic component, which induces plant immunity.

Introduction

Aphids are one of the most important pests of modern agriculture. They are phloem-feeding insects and use a specialized needle-like mouthpart known as the stylet to suck nutrients from the phloem sap. While feeding, they secrete copious amounts of saliva in plant tissues and compartments in the stylet path as well as into the phloem. Aphids secrete two types of saliva- gelling saliva and watery saliva. The gelling saliva gels rapidly after it is released from the stylet tip and forms a sheath around the stylets inside the plant tissue. This sheath remains behind after the stylet is retracted. The watery saliva is mainly secreted into the phloem cells but this type of saliva is also delivered intracellularly during cell puncture during stylet probing and penetration. Salivary gland transcriptome combined with bioinformatics analysis have identified a large number of putative secreted aphid proteins (Atamian *et al.* 2013, Carolan *et al.* 2011, Ramsey *et al.* 2007). Recently, proteomics analysis of aphid saliva from a number of aphid species has identified a number of secreted proteins (Carolan *et al.* 2009, Cooper *et al.* 2010, Harmel *et al.* 2008, Nicholson *et al.* 2012, Rao *et al.* 2013). In addition, recent work has shown that aphid saliva can modulate plant metabolism and defense responses (Bos *et al.* 2010, de Vos and Jander 2009, Will *et al.* 2007, Wilson *et al.* 2011).

Aphids harbor two distinct groups of endosymbionts classified as primary and secondary. The primary endosymbiont is *Buchnera aphidicola*, an obligate mutualist endosymbiotic γ -Proteobacterium that has co-evolved with the insect and is essential for its reproduction and survival (Douglas 1998, Moran *et al.* 1993). *Buchnera* is present in almost all aphid species (Baumann *et al.* 1995, Buchner 1965) except for few like

Astegopteryx styraci where it has been replaced by a yeast-like endosymbiotic fungus (Fukatsu and Ishikawa 1992, Fukatsu and Ishikawa 1996). *Buchnera* are housed in the hemocoel of the aphid within specialized cells of aphid origin known as bacteriocytes. *Buchnera* provide aphids with essential amino acids, which are lacking or are in low abundance in the plant phloem sap (Akman Gunduz and Douglas 2009, Sandstrom and Moran 1999, Wilson *et al.* 2010). In adults and older aphids, bacteriocytes disintegrate and disperse releasing their contents into the hemocoel (Brough and Dixon 1990, Douglas and Dixon 1987, Hinde 1971).

Contrary to the primary endosymbiont, secondary endosymbionts are not present in all aphid species and their nature and presence can differ even in clonal populations of the same aphid species (Fukatsu and Ishikawa 1998, Fukatsu *et al.* 2000). Secondary endosymbionts can be one of the following genera or species: *Spiroplasma*, *Rickettsia*, *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, and *Arsenophonus* species (Fukatsu *et al.* 2000, Fukatsu *et al.* 2001, Koga *et al.* 2003, Moran *et al.* 2005).

We profiled the potato aphid (*Macrosiphum euphorbiae*) salivary proteins and identified 11 proteins of *Buchnera* origin. Among these, was the chaperonin GroEL. Previously, using antibody against *Escherichia coli* GroEL, the presence of GroEL has been reported in aphid saliva (Filichkin *et al.* 1997). Interestingly, four of these proteins, including GroEL, were detected only in the gelling saliva. GroEL is the most abundant *Buchnera* protein constituting 10% of its total protein content (Baumann *et al.* 1996). GroEL is a highly conserved molecular chaperonin, present in large number of bacterial species and homologs of GroEL, Heat shock protein 60 (Hsp60) and Chaperonin 60

(Cpn60), have been identified in other organisms like bacteria, fungi, plants and humans (Zeilstra-Ryalls *et al.* 1991). GroEL is required for proper folding of unfolded or misfolded proteins and for this function, it requires a lid-like co-chaperonin protein complex GroES (Cheng *et al.* 1989, Horwich *et al.* 1993, Xu *et al.* 1997). GroEL contains 14 identical subunits of relative molecular mass 58kDa that are assembled as two heptameric rings stacked back to back, whereas GroES contains seven identical 10kDa subunits assembled as one heptameric ring (Sigler *et al.* 1998). There are duplicate or multiple copies of the *groEL* gene in certain bacteria like *Mycobacterium tuberculosis* and *Corynebacterium glutamicum* (Goyal *et al.* 2006). However, in aphids only a single copy of *groEL* has been reported (Hogenhout *et al.* 1998, Shigenobu *et al.* 2000).

GroEL has been implicated in aphid transmission of plant viruses and well as maintenance and persistence of virus particles inside the aphid hemocoel (Hogenhout *et al.* 1998, van den Heuvel *et al.* 1997). GroEL is involved in transmission of Luteoviruses and it binds to the readthrough domains of the *Potato leafroll virus (PLRV)* and *Barley yellow dwarf virus (BYDV) in vitro* (Filichkin *et al.* 1997, van den Heuvel *et al.* 1997). It is presumed that GroEL protects the virus against proteolytic breakdown in the hemolymph (van den Heuvel *et al.* 1994). Additional traits have also been attributed to GroEL. A homolog of GroEL produced by an endosymbiotic bacteria *Enterobacter aerogenes*, which lives in the saliva of the *Myrmeleon bore* (pit-making ant lion) larvae, acts as a neurotoxin (Yoshida *et al.* 2001). The predator mainly feeds on small arthropods like ants and paralyzes the prey before sucking their body content (Matsura

1986). In mammals, GroEL acts as an antigen and activates T-cells and B-cells- mediated defense responses. Lamb and coworkers showed that a 65kDa protein of *Mycobacteria*, with 60% homology with *E. coli* and human GroEL stress proteins, act as an immunodominant antigen for both T and B lymphocytes (Lamb *et al.* 1989). *E. coli* GroEL induces expression levels of cytokines and also induces secretion of interleukin-1 in macrophage cultures (Retzlaff *et al.* 1994). Purified GroEL protein from *Salmonella typhi* when immunized in mice induces lymphocyte proliferation and also induces cytokine levels (Bansal *et al.* 2010). Recombinant GroEL from *S. typhi* can be used as a candidate to develop vaccine against pathogenic bacteria (Chitradevi *et al.* 2013). Many other molecular chaperones related to GroEL, like Hsp60, Hsp70 and Hsp 90 acts as potent immunogens, active immunomodulators, and inducers of cross reactive immunity and autoimmunity (van Eden *et al.* 2005).

Arabidopsis thaliana (*Arabidopsis*) GroEL homologue, *LEN1* (Lesion Initiation 1), a chloroplast chaperonin 60 β , acts as a negative regulator of plant defense (Ishikawa *et al.* 2003). *Arabidopsis len1* T-DNA mutant plants showed cell death like lesions, induction of *pathogenesis-related* (*PR*) genes and enhanced resistance to virulent bacterial pathogen, *Pseudomonas syringae maculicola* ES4326 (Ishikawa *et al.* 2003).

Perception of microbial pathogens by the plant immune surveillance system is initiated by recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) (Boller and Felix 2009, Dodds and Rathjen 2010). This recognition activates a general form of defense response known as pattern-triggered immunity (PTI) that confers basal resistance (Boller and Felix 2009). MAMPs are

typically proteins or nucleic acids that are essential signature molecules of a class of microbes such as the bacterial flagellin (Felix *et al.* 1999, Gomez-Gomez *et al.* 1999) and elongation factor Tu (EF-Tu) (Kunze *et al.* 2004). The PTI response includes activation of mitogen-activated protein kinase (MAPK) cascade, production of reactive oxygen species (ROS), callose deposition, and induction of defense marker genes (Zhang and Zhou 2010, Zipfel 2009). Frequently, PTI responses require the well-characterized BAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1) coreceptor (Heese *et al.* 2007).

In the present study, we characterize the role of *Buchnera* GroEL in plant defense. To mimic aphid feeding behavior, we delivered GroEL intracellularly and in the apoplast. Our results show that GroEL is recognized by the plant innate immune surveillance system and induces PTI. We also demonstrate that the GroEL-induced defenses require BAK1 suggesting that GroEL is MAMP.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum*) cultivar Moneymaker plants were maintained in growth rooms at 24°C with 16-hr light and 8-hr dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and weekly fertilized with MiracleGro (18-18-21; Stern's MiracleGro Products). Arabidopsis wild-type Columbia (Col-0) and *bak1-4* (SALK_116202C) mutant plants were grown in a growth room at 23°C, 75 μE light with a 12-hr light photoperiod. Unless mentioned otherwise, five-week-old tomato and Arabidopsis plants were used for assays.

Aphid colonies and growth conditions

Colonies of the parthenogenetic potato aphid and green peach aphid (GPA, *Myzus persicae*) were reared on tomato cv. UC82B and mustard India (Burpee & Co., Warminster, PA) plants, respectively. The colonies were maintained in insect cages in a pesticide-free greenhouse at 22-26°C supplemented with light for a 16-hr light photoperiod. Age-synchronized one-day-old adult aphids were produced as described in (Bhattarai *et al.* 2007)

Sequence alignment

Full-length GroEL sequences were aligned using the GeneDoc 2.7.0. The GenBank accession numbers for the full-length GroEL protein used in the alignment are:

Acyrtosiphon pisum NP_239860, *M. euphorbiae* KF366417, *Myzus persicae* AF003957 and *E. coli* AAL55999.

Cloning in pVSP *PsSPdes* vector, aphid bioassays and bacterial growth curve assays

The open-reading frames corresponding to full-length *groEL* (accession number KF366417) and β -glucuronidase (*GUS*) genes were PCR amplified from potato aphid cDNA and pENTR-GUS (Invitrogen), respectively, using Phusion High fidelity DNA Polymerase (New England BioLabs). Sequences were cloned in pDONR221 entry vector (Invitrogen) and recombined into pVSP *PsSPdes* vector (Rentel *et al.* 2008) using GATEWAY® cloning technology (Invitrogen). A *Pseudomonas fluorescens* (*Pfo*) strain, engineered to deliver effectors into plant cells by T3SS (type-three secretion system) (Thomas *et al.* 2009), and wild type *Pfo* were transformed with the recombinant pVSP *PsSPdes* vectors and used for assays in tomato and Arabidopsis.

Transgenic and wild-type *Pfo* strains expressing GroEL or GUS were grown overnight at 30°C in King's B medium containing appropriate antibiotics. Leaves of five-week-old Arabidopsis plants were infiltrated with a suspension of *Pfo* at a density of 1×10^4 colony forming units (cfu)/ml in 10 mM MgCl₂ using a needleless syringe. Twenty-four hours post treatment (hpt) plants were infested with a single age-synchronized one-day-old adult GPA and 15 plants were used per treatment. Aphid fecundity was assessed by counting the number of nymphs daily for a period of five days. Experiments were repeated three times with similar results.

For tomato infections, plants were vacuum infiltrated at 1×10^4 cfu/ml in 10 mM MgCl₂ and 0.02% Silwet L-77. Tomato plants were infested with nine age-synchronized one-day-old adult potato aphids 24 hpt and six plants were used per treatment. Aphid fecundity was assessed by counting the number of nymphs daily for a period of five days. Experiments were repeated three times with similar results.

For *in planta* bacterial growth assays, Arabidopsis and tomato plants were infiltrated with *Pfo* strains as described above. At the indicated time points, leaf discs (total area 1 cm²) were harvested from the infiltrated tissues, ground in 10 mM MgCl₂ and plated in serial dilutions on appropriate media. Fifteen leaves were used per treatment. Experiments were repeated two times with similar results.

Immunoblot analysis of GroEL expression by pVSP *PsSPdes* vector

Five-week-old tomato plants were vacuum infiltrated with recombinant *Pfo* expressing GroEL-HA_pVSP at 1×10^9 cfu/ml in 10 mM MgCl₂ and 0.02% Silwet L-77. Leaf samples were harvested at 0 and 9 hpt. For protein extraction, the frozen leaf tissue was ground in cold protein extraction buffer and processed as described in (Sohn *et al.* 2007). Proteins were electrophoresed through a 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). Immunodetection was performed with a 1:2000 dilution of horseradish peroxidase–conjugated anti-HA antibody (Sigma-Aldrich) and visualized with an enhanced chemiluminescent substrate (Thermo Scientific) following manufacturer’s instructions and exposed to X-ray film.

Construction of transgenic plants expressing GroEL

Arabidopsis Col-0 plants were used to generate GroEL transgenic lines. The full-length *groEL* in pENTR221 vector was used to perform an LR reaction with the GATEWAY® compatible binary vector pMDC7 having a β -estradiol-inducible G1090::XVE promoter (Curtis and Grossniklaus 2003). The resulting clone was transformed into *Agrobacterium tumefaciens* strains GV3101 and stable transgenic *Arabidopsis* lines were generated using *A. tumefaciens*-mediated floral-dip transformation (Clough and Bent 1998). Independent transformed plant pools were kept separate for selection of independent transgenic lines. Transgenic plants selected on hygromycin (25 mg/L) and by PCR for the presence of *groEL* transgene (Table 2.1), were screened in the T2 generation for single locus insertions of *groEL* transgene and were propagated to successive generations to obtain homozygosity for the transgene. Experiments were repeated two times with similar results.

Aphid bioassays on transgenic Arabidopsis

Five-week-old transgenic plants grown in soil were sprayed with 20 μ M β -estradiol solution containing 0.02% silwet L-77 to induce the *groEL* transgene expression. Twenty-four hpt, each plant was infested with a single age-synchronized one-day-old adult GPA and allowed to feed on the induced plants for 24 hr. The adult aphids were moved to a fresh transgenic plant sprayed with β -estradiol for two more times. At each move and on the last day (days 2, 3, 4 and 5), the number of nymphs was counted and nymphs were removed. Fifteen plants were used per treatment.

For bacterial growth assays, transgenic lines and Col-0 plants were sprayed with estradiol and 12 hpt with *Pfo* strains as described above. At the indicated time points, leaf discs (total area 1 cm²) were harvested from the infiltrated tissues, ground in 10 mM MgCl₂ and plated in serial dilutions on appropriate media. Nine leaves were used per treatment. Experiments were repeated two times with similar results.

Expression and purification of proteins

groEL and *GUS* constructs in pDONR221 were recombined into the pDEST17 expression vector (Invitrogen) using GATEWAY® cloning technology to generate His-fusion proteins. The clones were transformed into *E. coli* BL21 cells and grown at 37°C to an OD₆₀₀ of 0.7. Protein expression was induced with 1mM IPTG at 16°C for 16 hr. For purification of the His-fusion protein, cells were lysed in Buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.2) with 20 mM imidazole, sonicated and insoluble protein removed by centrifugation. His-tagged protein was isolated from the supernatant by binding to Ni-NTA column (QIAGEN). The column was washed ten times in Buffer A with 40 mM imidazole. The bound protein was eluted in Buffer A with 250 mM imidazole. Pooled elutes were concentrated and equilibrated to phosphate-buffered saline (PBS) (pH 6.8) using an Amicon concentrator. Eluted GroEL protein was further fractionated on a 4.3 ml BioFox 17Q anion exchange column. The sample was loaded onto the resin and washed with 10 column volumes of 20 mM Tris-Cl (pH 8.0) at 0.5 ml/min. Bound protein was eluted with a linear gradient of 0 to 1 M NaCl in 20 mM Tris-Cl (pH 8.0). Protein

concentration was measured using Bradford assay. Anion exchange purification was performed by AthenaES (Athena Enzyme Systems Group, Baltimore, MD).

Aphid bioassays with purified GroEL protein

In preliminary experiments, defense gene induction after GroEL infiltration of *Arabidopsis* leaves indicated transient expression of *PR1* gene reaching maximum levels at 24 hr and returning to pre-induction levels at 48 hr. Based on this result, aphids were exposed to GroEL-infiltrated leaves for only 48 hr. *Arabidopsis* leaves were infiltrated with 1x PBS or 1.5 μ M of GroEL or GUS. Soon after infiltration, each leaf was infested with a single age-synchronized one-day-old adult GPA. After 48 hr, the adult aphids were moved to a freshly infiltrated leaf and were allowed to feed for an additional 48 hr. Nymphs were counted daily for a total of four days. Fifteen plants were used per treatment. Experiments were repeated two times with similar results.

Quantitative Real-time PCR analysis

Total RNA was extracted from leaves using Trizol reagent (Invitrogen) and isopropanol precipitation. RNA was treated with DNase I enzyme (New England BioLabs) and cDNA was synthesized using superscript III (Invitrogen). Quantitative RT-PCR was performed using iQTM SYBR[®] Green Supermix (Biorad) and gene-specific primers (Table 2.1) in iCycler Real-Time PCR System (BioRad). Relative expression of genes was calculated using actin (*ACT-2*) as a standard gene for *Arabidopsis* and ubiquitin (*Ubi3*) for tomato

(Table 2.1). Each set of experiment had three biological replicates with two technical replicates each and experiments were repeated at least twice.

For gene expression in transgenic *Arabidopsis* lines, five-week-old plants were sprayed with 20 μM β -estradiol or three-week-old seedlings, germinated on MS media, were transferred to MS supplemented with 5 μM β -estradiol. Leaf samples were harvested at the indicated hpt.

For gene induction by purified GroEL, five-week-old *Arabidopsis* Col-0 and *bak1-4* plants were infiltrated with 1.5 μM of GroEL, GUS or PBS. Leaf samples were harvested at the indicated hpt.

For gene expression after *Pfo* infection in tomato and *Arabidopsis*, plants were infected at a density of 1×10^9 cfu/ml as described earlier (Wei *et al.* 2013). Leaf samples were harvested at 6 hpt.

Oxidative burst measurement

ROS burst was determined by a luminol-based assay as described previously (Keppler and Baker 1989) with modifications. Three- to four-week-old *Arabidopsis* plants were excised into 2-mm slices and incubated overnight in 96-well plate with 200 μl H_2O to eliminate wounding effect. H_2O was replaced with 200 μl of 20 μM of luminol and 5 $\mu\text{g/ml}$ of horseradish peroxidase (Sigma) supplemented with 1.5 μM of GroEL or GUS and measurement was conducted with a luminometer (Mithras LB 940 Multimode Reader luminometer, Berthold Technologies). The mean values of relative light units

(RLU) for ROS production from 16 leaf slices per treatment were calculated.

Experiments were repeated three times with similar results.

Callose deposition

Callose deposition was performed as described previously (Felix et al. 1999) with modifications. Briefly, sixteen leaves of 5-week-old Col-0 and *bak1-4* mutant plants were infiltrated with 1.5 μ M GroEL. Leaves were excised 24 hpt, cleared in 95% ethanol and stained with 0.1% aniline blue in 0.15 M phosphate buffer (pH 9.5). Callose deposits were visualized under a UV filter using a fluorescence microscope and counted using ImageJ 1.43U software (Abramoff *et al.* 2004), <http://rsb.info.nih.gov/ij/>) as described previously (Shang *et al.* 2006). This experiment was repeated once with similar results.

Results

Delivery of GroEL into tomato and Arabidopsis negatively affects aphid fecundity

Buchnera groEL from potato aphids was cloned into the bacterial expression vector pVSP *PsSPdes* which is designed for delivery of effectors into plant cells through the bacterial T3SS (Rentel et al. 2008). We have successfully used this system to deliver aphid effectors into plant cells (Atamian et al. 2013). We introduced this construct into *Pfo* strain EtHAn (Effector-to-Host Analyzer), a non-pathogenic bacterium engineered with T3SS (*Pfo*+T3SS) (Thomas et al. 2009) (Fig. 2.1). To assay plants with *Pfo*+T3SS delivering GroEL with aphids, tomato plants were vacuum-infiltrated with recombinant *Pfo*+T3SS delivering GroEL or GUS, used as a control, and infested with age-synchronized one-day-old adult potato aphids. Nymph production was evaluated on daily basis for five days. Tomato plants infected with *Pfo*+T3SS delivering GroEL exhibited reduced aphid fecundity compared to the *Pfo*+T3SS delivering GUS control (Fig. 2.2A), suggesting that the plant immune surveillance system recognized GroEL and triggered defense responses.

GroEL could be present in the saliva of all aphids harboring *Buchnera* and be recognized by a multitude of plant host immune responses. Thus, we speculated that this induced host resistance might be seen in other plant-aphid combinations. Since *Buchnera* GroEL sequences from different aphid species are highly conserved (Fig. 2.3), we used the potato aphid *Buchnera groEL* to test this hypothesis. We syringe-inoculated Arabidopsis leaves with *Pfo*+T3SS delivering GroEL and assayed the plants with GPA.

Arabidopsis plants infested with age-synchronized one-day-old adult GPA were evaluated daily for aphid fecundity for a period of five days. As with tomato, GPA fecundity was reduced on Arabidopsis infected with *Pfo*+T3SS delivering GroEL compared to GUS control (Fig. 2.2B) indicating GroEL induced reduced susceptibility to aphids. To confirm that delivery of GroEL into the plant caused the reduced susceptibility phenotype in both hosts, we expressed GroEL in wild-type *Pfo* that lacks the T3SS, and infected both tomato and Arabidopsis and infested them with their respective aphid pests. In neither of these host-pest systems, an effect on aphid fecundity was observed (Fig. 2.2C and Fig. 2.2D), indicating that GroEL is the cause of the reduced susceptibility to both aphid pests.

To investigate whether the GroEL-induced reduced susceptibility to aphids is due to activation of plant defense responses, we performed PTI defense marker gene expression analysis using qRT-PCR (Navarro *et al.* 2004, Nguyen *et al.* 2010). Tomato leaves infected with *Pfo*+T3SS delivering GroEL exhibited increases in *SlPti5* and *SlWrrky28* transcript levels compared to the control *Pfo*+T3SS infected leaves indicating induction of PTI (Fig. 2.4).

Delivery of GroEL into tomato or Arabidopsis enhances resistance to *Pfo*

GroEL triggers immune responses against bacteria in animals and our data showed that it also triggers expression of PTI marker genes in plants. Therefore, it might also elicit immunity against bacteria in plants. To assess a role for GroEL in bacterial defense in plants, we evaluated *Pfo* titer in tomato and Arabidopsis plants infected with *Pfo*+T3SS

delivering GroEL or GUS. Both tomato (Fig. 2.5A) and Arabidopsis (Fig. 2.5B) infected with *Pfo*+T3SS with GroEL displayed reduced bacterial titers compared to *Pfo*+T3SS GUS control indicating enhanced resistance to *Pfo*. Secretion of GroEL by the T3SS was required for this enhanced bacterial resistance (Fig. 2.5C and Fig. 2.5D).

Arabidopsis Col-0 transgenic lines expressing GroEL exhibit enhanced resistance to aphids and bacteria

To substantiate the role of GroEL in immunity and aphid resistance, we developed stable transgenic Arabidopsis Col-0 lines that overexpressed GroEL from an estradiol-inducible promoter (Fig. 2.6A). To induce GroEL expression, plants were sprayed with estradiol and assayed for induction of defense marker genes. Wild-type Arabidopsis plants sprayed with estradiol were used as control. Plants treated with estradiol did not exhibit visible immune responses such as cell death. The late PTI marker gene (*PR1*) was upregulated transiently in Arabidopsis plants expressing GroEL where transcript accumulation was detected at 24-hr and levels were greatly reduced at 48-hr (Fig. 2.7C and Fig. 2.6A). Therefore, aphid screens were performed with plants sprayed 24-hr with estradiol and moved to freshly sprayed plants 24-hr later (Fig. 2.6B).

To test for aphid fecundity phenotype, plants sprayed with estradiol were infested with age-synchronized one-day-old adult GPA 24 hpt and aphid fecundity was recorded daily for four days. Nymph production was evaluated for four consecutive days. In this period, aphid fecundity was reduced on GroEL transgenic lines compared to control plants (Fig. 2.6B). This result was in agreement with our earlier finding confirming that

GroEL expression *in planta* enhances resistance to aphids. To examine if the GroEL Arabidopsis transgenic lines exhibit reduced susceptibility to bacteria as well, we evaluated *Pfo* titer in these transgenic plants. As suspected, GroEL transgenic lines infected with *Pfo* had reduced bacterial titers compared to Col-0 control plants indicating reduced susceptibility to *Pfo* (Fig. 2.7D).

We also tested expression of PTI early-induced marker genes, *WRKY29* and *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE*) and the late-induced marker gene *PR1* (Fig. 2.7A-C) (Asai *et al.* 2002) in transgenic GroEL plants. Transcript levels for both *FRK1* and *WRKY29* increased at 6-hr whereas those for *PR1* gene were induced at 24-hr after treatment with estradiol. Taken together, our data show that Arabidopsis Col-0 transgenic lines expressing GroEL display enhanced immunity to aphids and bacteria.

GroEL induced defense responses in Arabidopsis is *BAK1*-dependent

To further characterize GroEL-induced PTI, we expressed histidine (His) epitope-tagged GroEL in *E. coli* and purified the recombinant protein using Ni-NTA beads followed by anion exchange chromatography (Fig. 2.8). To mimic aphid feeding behavior and delivery of saliva into plant apoplast, we infiltrated Arabidopsis leaves with the purified GroEL and assayed for PTI responses. His-tagged and purified GUS was used as control. To test whether extracellular application of GroEL induces enhanced aphid resistance, GroEL-infiltrated plants were first assayed for aphid fecundity. Plants treated with GroEL did not exhibit a visible immune response such as cell death. However, they displayed reduced aphid fecundity (Fig. 2.9A) similar to *Pfo* delivery or transgenic expression of

GroEL. Similarly, infiltration of GroEL into leaves induced expression of both early- and late-induced PTI marker genes (Fig. 2.9B) (Asai et al. 2002). Furthermore, application of GroEL triggered ROS accumulation (Fig. 2.10B) and callose deposition in treated leaves (Fig. 2.10C). None of these defense responses were detected in the GUS-treated control leaves.

Since most bacterial PTI responses require the BAK1 coreceptor, we tested whether GroEL-induced defense responses are also *BAK1*-dependent. The ROS burst (Fig. 2.10B) and callose deposition (Fig. 2.10C) triggered by GroEL were both greatly reduced in the *bak1-4* null mutant indicating *BAK1* dependency. Similarly, expression of the PTI early-induced marker genes (*WRKY29* and *FRK1*), which are known to be *BAK1*-dependent, and not the late-induced marker gene (*PR1*), were impaired in *bak1-4* (Fig. 2.10A). Taken together, these results indicate that GroEL serves as a microbe/aphid-associated molecular signature that induces *BAK1*-dependent PTI.

Discussion

Aphids secrete a repertoire of proteins inside their hosts and many of these proteins may function as effectors to modulate plant cellular processes. However, only a handful of these putative effector molecules have been functionally characterized (Atamian et al. 2013, Bos et al. 2010, Mutti *et al.* 2008, Rodriguez *et al.* 2013). It has been shown that aphid saliva also contains an endosymbiont-derived proteins, chaperonin GroEL (Filichkin et al. 1997). Filichkin et al, showed the presence of GroEL in the saliva of the grain aphid *Sitobion avenae* fed on 30% sucrose in 10 mM phosphate buffer (pH 7.0), by performing a western blot analysis using anti-GroEL polyclonal antibody raised against *E. coli* GroEL. However, it is not clear if the authors detected the GroEL in the watery saliva or in the gelling saliva or in both. In our present analysis, we collected the two types of aphid saliva separately (as discussed in Chapter One) and identified 12 peptides corresponding to GroEL only in the gelling saliva and none in the watery saliva indicating that GroEL is present mainly in the gelling saliva.

It has been shown that infiltration of GPA saliva into Arabidopsis leaves induces defense marker gene expression and results in reduced susceptibility to aphids (de Vos and Jander 2009). These responses were induced by salivary proteins within 3-10 kDa molecular mass range. The molecular mass of GroEL is 58 kDa and therefore it is unlikely to be the salivary protein triggering these immune responses. Although the authors of this work did not specify what type of saliva were used for these assays, it is likely that they collected only watery saliva, which did not include *Buchnera* GroEL.

We were unable to evaluate the presence of *Buchnera* GroEL *in planta* after aphid feeding because available anti-GroEL polyclonal antibodies cross-reacted with plant proteins, most likely GroEL-related chaperones (data not shown). In addition, we were unable to generate *Buchnera*-specific monoclonal antibodies for a stretch of ~20 amino acid sequence that seemed to be specific to *Buchnera* GroEL. Nevertheless, our study demonstrates that *Buchnera* GroEL is present in the aphid gelling saliva, since 5 of the 12 GroEL peptides identified in our MS analysis were *Buchnera* specific (Table 2.2).

To evaluate the role of GroEL *in planta*, we delivered this protein intracellularly by using a soil bacterium *Pfo* engineered to inject effectors by utilizing T3SS. *Pfo* is the ideal bacterium for delivery pest or microbe effectors as it not pathogenic and does not have its own effectors, which could interfere with plant defenses and influence experimental results. Using pVSP *PsSPdes* vector and delivering GroEL through *Pfo*+ T3SS into the tomato or Arabidopsis plants, we were able to functionally characterize the role of GroEL in defense, as well as to evaluate its effect on potato aphid and GPA fecundity. In both host-pest systems, we found that GroEL induced defense responses and reduced aphid fecundity. GroEL also enhanced resistance against *Pfo* in both hosts. Considering that GroEL also induces immune responses in systems (Lamb et al. 1989), GroEL-induced PTI is likely an ancient immune response against bacterial infection that is broadly conserved in higher eukaryotic clades.

Delivery of GroEL extracellularly through syringe infiltration of leaves and intracellular by delivery through bacterial T3SS or transgenically, activated plant defense responses and resulted in reduced aphid fecundity. Therefore, GroEL was perceived both

extracellularly and intracellularly. Furthermore our data showed that the induction of GroEL-mediated defense responses also required the MAMP coreceptor BAK1. GroEL recognition both extracellularly and intracellularly was not surprising as it reflects the two cellular compartments where plants come in contact with aphid saliva. When aphids feed, gelling saliva forms a sheath around the stylet, which comes in contact with the host cell surface and intercellular spaces or the apoplast. However, gelling saliva is thought to seal intracellular stylet punctures and therefore may be exposed to the cellular milieu. Consistent with GroEL location as determined by proteomics analysis of gelling and watery saliva's (Chapter One). Therefore GroEL, GroEL could be perceived by the plant host both extracellularly and intracellularly.

An alternative explanation of this two types of host perceptions is that GroEL may be leaking outside the cell in the transgenic GroEL-overexpressing plants (Wei et al. 2013); in this scenario GroEL may be recognized extracellularly through a transmembrane receptor similar to well-characterized microbial MAMPs (Segonzac and Zipfel 2011). The receptor of GroEL and how it is recognized by the plant innate immunity is not yet known. Although BAK1 is a transmembrane receptor and is critical for GroEL perception, it is unlikely that BAK1 is the receptor for GroEL as BAK1 typically acts as a coreceptor for known MAMPs. Therefore, recognition of GroEL likely involves a yet unidentified receptor.

The presence of *Buchnera* proteins in the potato aphid saliva (Chapter One) indicates that plant-aphid interactions have a third element, the aphid endosymbiotic prokaryotic component, which induces plant immunity. Release of the endosymbiont

proteins in the insect hemocoel most likely is part of the *Buchnera* life cycle. Maintaining *Buchnera* by the aphid must have added advantage to the aphid that outweighs the penalty of GroEL recognition by the plant host immune surveillance system. This is consistent with the fact that *Buchnera* is essential for aphid survival and aphids cured from *Buchnera* by antibiotics treatments grow slowly and do not reproduce (Douglas 1998).

Although the ability of GroEL to elicit plant PTI was not surprising, it is interesting that this defense was effective against aphids. We hypothesized that GroEL is a microbial-associated molecular pattern, which also served as a signal for aphid attack. Since *Buchnera* related primary endosymbionts are present in a number of insects, our findings are likely to be broadly applicable to other arthropods. It is intriguing to speculate that plant defenses directly target the *Buchnera* endosymbiont to control insect pests. Since the aphid-*Buchnera* mutualism is obligate, where none of the partners can survive without the other, by targeting the endosymbiont the plant immune system is exploiting the strict mutual dependency of a host-insect with its symbiont to recognize the former as the intruder.

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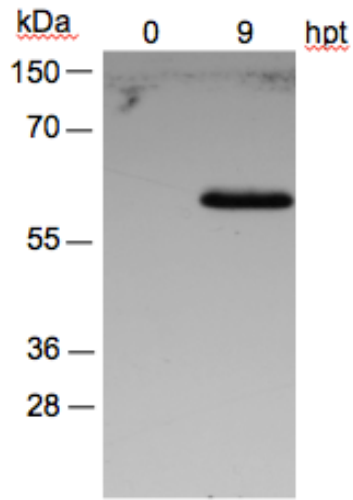


Fig. 2.1 GroEL is expressed by *Pseudomonas fluorescens* (Pfo). Five-week-old tomato plants were infiltrated with 1×10^9 cfu/ml of *Pfo*+T3SS expressing GroEL::3xHA. Leaf samples were harvested at 0 and 9 hpt for immunoblot analysis using anti-HA antibody. Samples were fractionated on 10% SDS-PAGE. Expected size of GroEL-3xHA is 60.6 kDa.

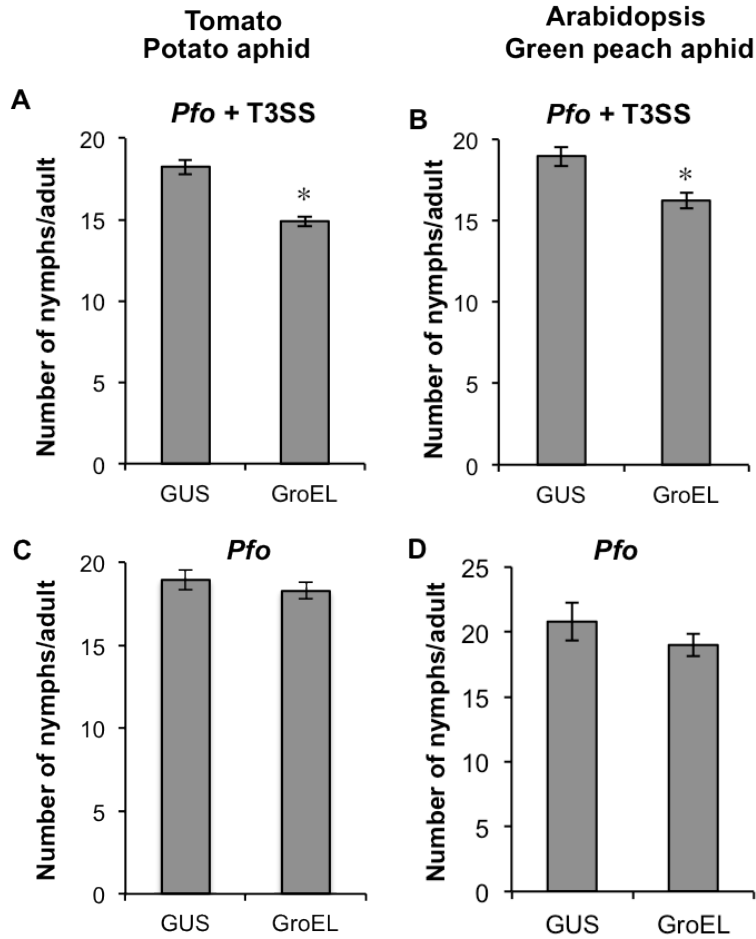


Fig. 2.2A-D. Delivery of GroEL into tomato and *Arabidopsis thaliana* negatively affects aphid fecundity. Tomato and Arabidopsis were infiltrated with *Pfo*+T3SS and or wild-type *Pfo*, expressing either β -glucuronidase (GUS; control) or GroEL, at a density of 1×10^4 colony forming units (cfu)/ml. Plants were assayed with parthenogenetic potato aphids (A), (C) or GPA (B), (D) and aphid fecundity was recorded daily over a 5-day period. Error bars represent $\pm$ SE of the mean (in A and C n=6; B and D n=15). Similar results were obtained in at least four independent experiments. Data from one experiment are presented. * indicates significant differences (Student's *t* test; $P < 0.05$).

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      *      20      *      40      *      60      *      80      *      100
MeB_GroEL : MAAKDVKEGNEARLKMRLGVNVLDADVKTLPKGRNVVLDKSFSGAPSTIKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTATLAAQIV : 100
ApB_GroEL : MAAKDVKEGNEARLKMRLGVNVLDADVKTLPKGRNVVLDKSFSGAPSTIKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTATLAAQIV : 100
MpB_GroEL : MAAKDVKEGNEARLKMRLGVNVLDADVKTLPKGRNVVLDKSFSGAPSTIKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTATLAAQIV : 100
E.coli_GroEL : MAAKDVKEGNEARLKMRLGVNVLDADVKTLPKGRNVVLDKSFSGAPSTIKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTATLAAQIV : 100

      *      120     *      140     *      160     *      180     *      200
MeB_GroEL : NEGLKAVAAGMNPMDLKRIGDKAVTAABEELKRLSVPCSDSKATIQVGTISANDEKVGSLTSEAMKVGNDGVITVEEETGLQDELIVVKGQDFDRGYL : 200
ApB_GroEL : NEGLKAVAAGMNPMDLKRIGDKAVTAABEELKRLSVPCSDSKATIQVGTISANDEKVGSLTSEAMKVGNDGVITVEEETGLQDELIVVKGQDFDRGYL : 200
MpB_GroEL : NEGLKAVAAGMNPMDLKRIGDKAVTAABEELKRLSVPCSDSKATIQVGTISANDEKVGSLTSEAMKVGNDGVITVEEETGLQDELIVVKGQDFDRGYL : 200
E.coli_GroEL : NEGLKAVAAGMNPMDLKRIGDKAVTAABEELKRLSVPCSDSKATIQVGTISANDEKVGSLTSEAMKVGNDGVITVEEETGLQDELIVVKGQDFDRGYL : 200

      *      220     *      240     *      260     *      280     *      300
MeB_GroEL : SPYFINKBETGVLELNPILLADKKISNREMLPILNVAKRGKPLLIISEDTEGEALATLVVNMRGIVKVAAVKAPGFGDRRKAMLODIATLTGGV : 300
ApB_GroEL : SPYFINKBETGVLELNPILLADKKISNREMLPILNVAKRGKPLLIISEDTEGEALATLVVNMRGIVKVAAVKAPGFGDRRKAMLODIATLTGGV : 300
MpB_GroEL : SPYFINKBETGVLELNPILLADKKISNREMLPILNVAKRGKPLLIISEDTEGEALATLVVNMRGIVKVAAVKAPGFGDRRKAMLODIATLTGGV : 300
E.coli_GroEL : SPYFINKBETGVLELNPILLADKKISNREMLPILNVAKRGKPLLIISEDTEGEALATLVVNMRGIVKVAAVKAPGFGDRRKAMLODIATLTGGV : 300

      *      320     *      340     *      360     *      380     *      400
MeB_GroEL : ISEELAMELEKRTLEDLGQAKRVVLEKDTTIIISGVGEKHSIQSRISQIRQEIQEATSDYDKEKLERLAKLGGGAVLRVGAATEVEMKEKKARVEDAL : 400
ApB_GroEL : ISEELAMELEKRTLEDLGQAKRVVLEKDTTIIISGVGEKHSIQSRISQIRQEIQEATSDYDKEKLERLAKLGGGAVLRVGAATEVEMKEKKARVEDAL : 400
MpB_GroEL : ISEELAMELEKRTLEDLGQAKRVVLEKDTTIIISGVGEKHSIQSRISQIRQEIQEATSDYDKEKLERLAKLGGGAVLRVGAATEVEMKEKKARVEDAL : 400
E.coli_GroEL : ISEELAMELEKRTLEDLGQAKRVVLEKDTTIIISGVGEKHSIQSRISQIRQEIQEATSDYDKEKLERLAKLGGGAVLRVGAATEVEMKEKKARVEDAL : 400

      *      420     *      440     *      460     *      480     *      500
MeB_GroEL : HATRAAVEGGVAGGGVALRVASKESNLRGQNEQDNVGRVALRAMEAPLRQIVNSGEEPSVVENNVKDGSGNYGYNAATDEYGMIDEGILDPTKVT : 500
ApB_GroEL : HATRAAVEGGVAGGGVALRVASKESNLRGQNEQDNVGRVALRAMEAPLRQIVNSGEEPSVVENNVKDGSGNYGYNAATDEYGMIDEGILDPTKVT : 500
MpB_GroEL : HATRAAVEGGVAGGGVALRVASKESNLRGQNEQDNVGRVALRAMEAPLRQIVNSGEEPSVVENNVKDGSGNYGYNAATDEYGMIDEGILDPTKVT : 500
E.coli_GroEL : HATRAAVEGGVAGGGVALRVASKESNLRGQNEQDNVGRVALRAMEAPLRQIVNSGEEPSVVENNVKDGSGNYGYNAATDEYGMIDEGILDPTKVT : 500

      *      520     *      540
MeB_GroEL : RSALQYAASVAGLMITTECMVTDLPKEDKSSDSNSSPAGGMGGMGMM : 548
ApB_GroEL : RSALQYAASVAGLMITTECMVTDLPKEDKSSDSNSSPAGGMGGMGMM : 548
MpB_GroEL : RSALQYAASVAGLMITTECMVTDLPKEDKSSDSNSSPAGGMGGMGMM : 548
E.coli_GroEL : RSALQYAASVAGLMITTECMVTDLPKEDKSSDSNSSPAGGMGGMGMM : 548

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Fig. 2.3. Sequence alignment of GroEL proteins. The deduced amino acid sequences of *Buchnera aphidicola* (B) GroEL amplified from aphids (Me, *Macrosiphum euphorbiae*; Ap, *Acyrtosiphon pisum*; Mp, *Myzus persicae*) and GroEL from *Escherichia coli* (*E. coli*). Black and grey shades indicate identical and highly conserved amino acids, respectively. *Buchnera* GroEL sequences from these aphids have 98-99% amino acid sequence identity.

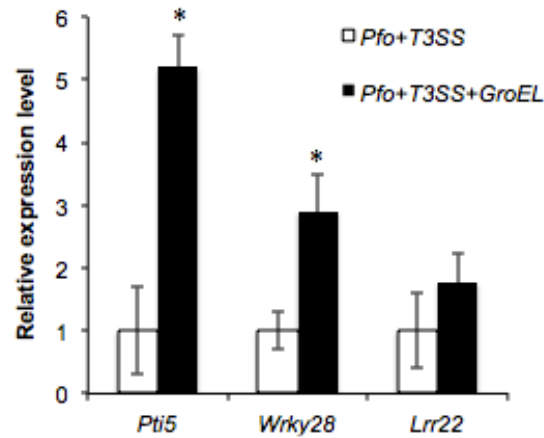


Fig. 2.4. *Pfo* expressing GroEL induces early-induced defense markers genes in tomato. Tomato plants were vacuum infiltrated with 1×10^9 cfu/ml of *Pfo* + T3SS or *Pfo* + T3SS delivering GroEL. Leaf samples were harvested 6 hpt. Relative expression levels of defense marker genes were evaluated by qRT-PCR. Expression levels in samples infiltrated with *Pfo* + T3SS were designated as 1. Error bars represent $\pm$ SE of the mean of two technical replicates and three biological replicates. This experiment was performed twice with similar results. Data from one experiment is presented. * indicates significant differences (Student's *t* test; $P <$

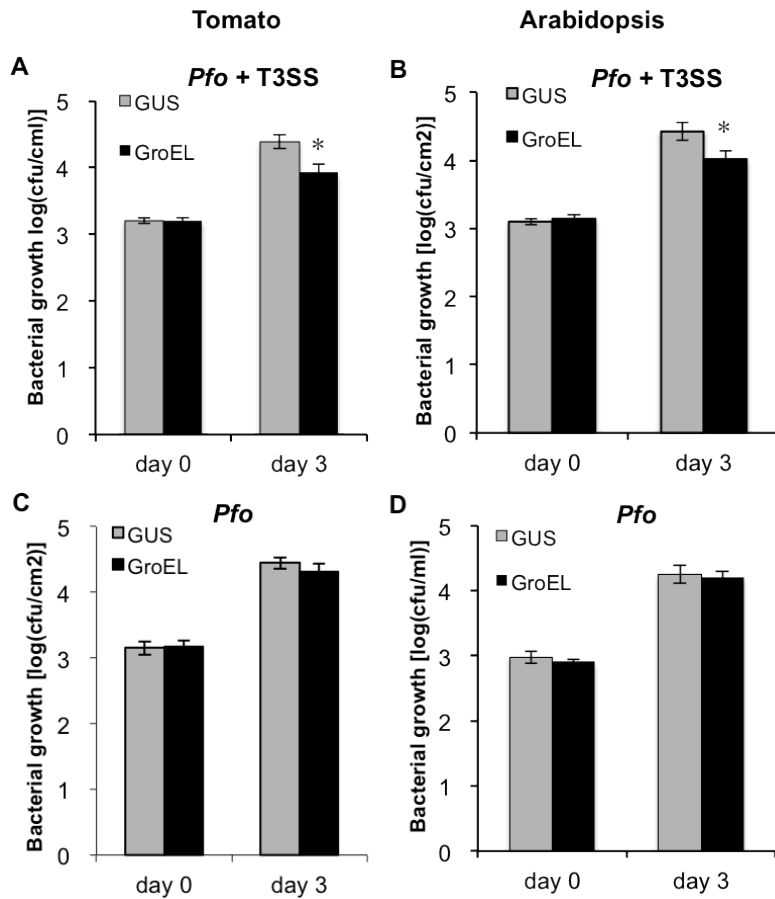


Fig. 2.5A-D. Delivery of GroEL into tomato or Arabidopsis enhances resistance to *Pfo*. Bacterial growth in tomato and Arabidopsis leaves infiltrated with *Pfo* + T3SS or wild-type *Pfo*, expressing either GUS (control) or GroEL, at a bacterial density of 1×10^4 cfu/ml. Error bars represent $\pm$ SE of the mean ($n=15$). Similar results were obtained in four independent experiments. Data from one experiment are presented. * indicates significant differences (Student's *t* test; $P < 0.05$).

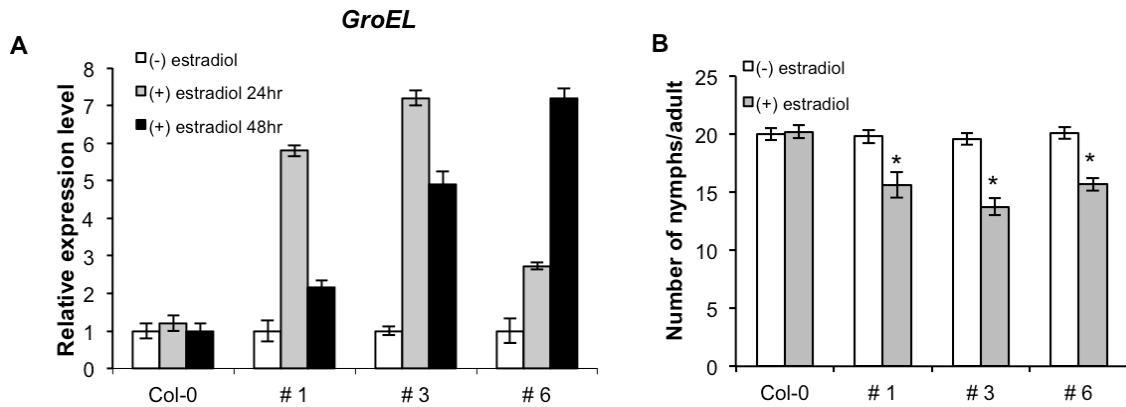


Fig. 2.6. Arabidopsis Col-0 transgenic lines expressing GroEL exhibit enhanced resistance to aphids. (A) *groEL* gene expression in the three Arabidopsis transgenic lines (# 1, # 3 and # 6), expressing *groEL* under control of an estradiol-inducible *G1090::XVE* promoter, were evaluated by qRT-PCR. Stable transgenic and control plants were sprayed with 20 μ M estradiol containing 0.02% silwet L-77 and harvested at the indicated time for expression analysis. Error bars represent $\pm$ SE of the mean of two technical replicates and three biological replicates. (B) Fecundity of GPA on Arabidopsis transgenic lines (# 1, # 3 and # 6) expressing β -estradiol inducible GroEL. Error bars represent $\pm$ SE of the mean (n=15). Similar results were obtained in three independent experiments. Data from one experiment are presented. * indicates significant differences (Student's *t*-test; $P < 0.05$).

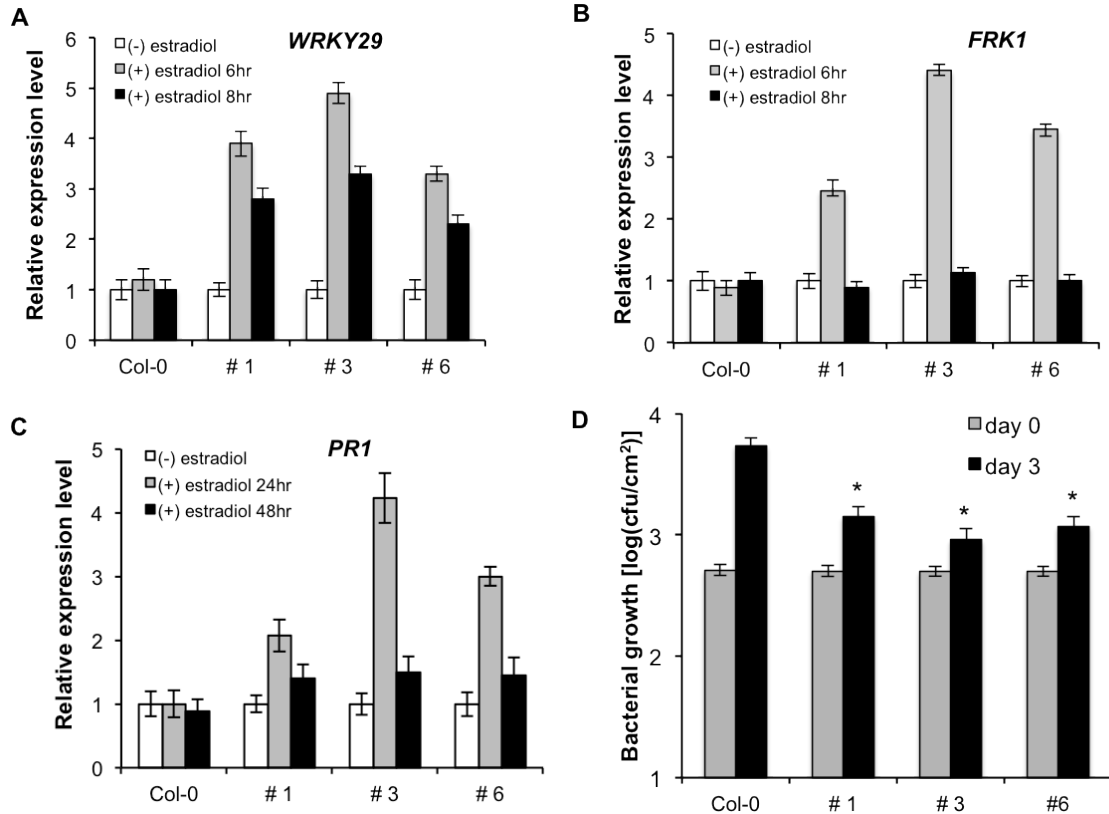


Fig. 2.7. Arabidopsis Col-0 transgenic lines expressing GroEL exhibit induced defense responses and enhanced resistance to bacteria. *WRKY29* (A) *FRK1* (B) and *PR1* (C) expression were evaluated in the three Arabidopsis transgenic lines. In (A-C) error bars represent $\pm$ SE of the mean of 3 biological replicates and 2 technical replicates each. Similar results were obtained in two independent experiments. **D**) Bacterial titre in Arabidopsis transgenic lines, expressing GroEL. Plants were treated with 20 μ M estradiol and 12 hpt later were syringe infiltrated with *Pfo* at a bacterial density of 1×10^4 cfu/ml. Error bars represent $\pm$ SE of the mean (n=9). Similar results were obtained in two independent experiments. Data from one experiment are presented. * indicates significant differences (Student's *t* test; $P < 0.05$).

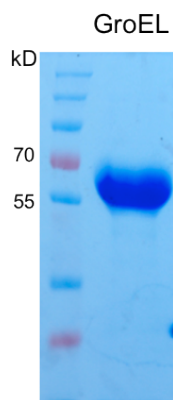


Fig. 2.8. SDS-PAGE of recombinant GroEL protein expressed in *E. coli*.

Twenty five μg of purified full-length 6xHis-GroEL protein was loaded onto 10% acrylamide gel and the gel was stained with Coomassie blue. Expected size of 6xHis-GroEL is 60.5 kD.

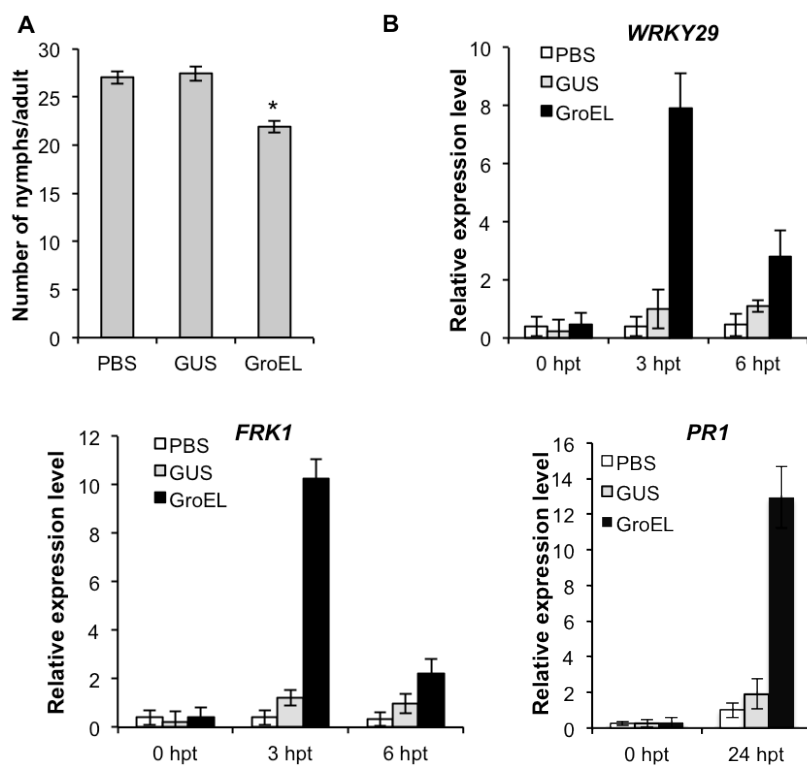


Fig. 2.9. GroEL induced defense responses in Arabidopsis. (A) GPA fecundity on WT Col-0 leaflet infiltrated with 1.5 μ M GroEL, PBS (buffer) or GUS. Soon after infiltration, ten plants (leaflets) per treatment were infested with a single one-day-old adult aphid each. After two days, aphids were moved to a freshly infiltrated leaflet. Aphid fecundity was recorded daily for a period of 4 days. * indicates significant differences ($P < 0.05$) compared with GUS control (Student's t -test). (B) *WRKY29*, *FRK1* and *PR1* expression in Col-0 infiltrated with 1.5 μ M GroEL, PBS or GUS at the indicated hpt and Error bars represents $\pm$ SE of the mean. For all assays similar results were obtained in at least two independent experiments. Data from one experiment is presented.

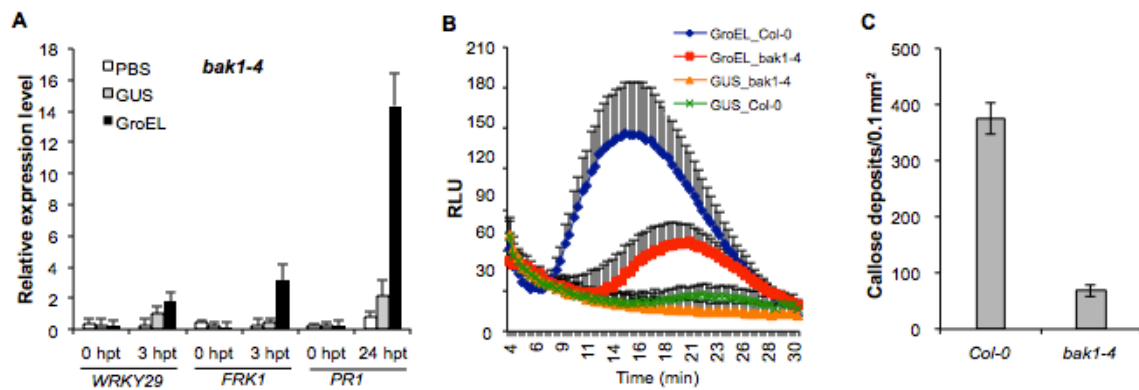


Fig. 2.10. GroEL induced defense responses in Arabidopsis in *bak1-4* dependent manner. (A) *WRKY29*, *FRK1* and *PR1* expression in *bak1-4* mutant infiltrated with 1.5 μ M GroEL, GUS or PBS at the indicated hpt, evaluated by qRT-PCR. Error bars represent $\pm$ SE of the mean of three biological replicate PCR results. (B) Oxidative burst triggered by 1.5 μ M GroEL or GUS in Col-0 and *bak1-4* mutant leaves measured in relative luminescence units (RLU). (C) Callose deposition in Col-0 and *bak1-4* at 24 hpt with 1.5 μ M GroEL stained with aniline blue and evaluated using ImageJ. Data are means of 16 leaf slices. Error bars represents $\pm$ SE of the mean. For all assays similar results were obtained in at least two independent experiments. Data from one experiment is presented.

Table 2.1. Sequences of the primers used in this study.

Primers	Sequences (5'-3')*	Purpose	Reference
GroEL-F	GGGGACAAGTTTGTACAAAAAAGCAGGC TccATGGCCGCTAAAGATGTGAAAT	Gateway cloning of <i>GroEL</i>	This study
GroEL-R	GGGCACCACTTTGTACAAGAAAGCTGGG TTACATCATTCGCCCCATGCC	Gateway cloning of <i>GroEL</i>	This study
GroEL-HA-R	GGGCACCACTTTGTACAAGAAAGCTGGG TcCATCATTCGCCCCATGCC	Gateway cloning of <i>GroEL</i>	This study
GUS-F	GGGGACAAGTTTGTACAAAAAAGCAGGC TccATGGTCCGTCCTGTAGAAAC	Gateway cloning of <i>GUS</i>	This study
GUS-R	GGGCACCACTTTGTACAAGAAAGCTGGG TTTATTGTTTGCCTCCCTGCT	Gateway cloning of <i>GUS</i>	This study
GroEL-qPCR-F	AATATCTAATTTACGTGGTCAAAATGA	qPCR	This study
GroEL-qPCR-R	CATCATTCGCCCCATGCC	qPCR	This study
pMDC32-F	TCCTTCGCAAGACCCTTCCTC	Genotyping	This study
pMDC7-F	GGTGGTAATGCCATGTAATATGC	Genotyping	This study
AtAct2-F	GATGAGGCAGGTCCAGGAATC	qPCR	(Kettles <i>et al.</i> 2013)
AtAct2-R	GTTTGTACACACAAGTGCATC	qPCR	(Kettles <i>et al.</i> 2013)
AtFrk1-F	AAGAGAGGTTGAAGATATCA	qPCR	This study
AtFrk1-R	CATCTTCGCTTGGAGCTTCT	qPCR	This study
AtWrky29-F	GCGTAAATACGGGCAGAAAC	qPCR	(Trujillo <i>et al.</i> 2008)
AtWrky29-R	GGTTTGGGTGGGAAGTTTT	qPCR	(Trujillo <i>et al.</i> 2008)
AtPR1-F	GTTGCAGCCTATGCTCGGAG	qPCR	(Chang and Pikaard 2005)
AtPR1-R	CCGCTACCCAGGCTAAGTT	qPCR	(Chang and Pikaard 2005)
SIUbi3-F	GTGTGGGCTCACCTACGTTT	qPCR	(Bhattarai <i>et al.</i> 2010)
SIUbi3-R	CCGTTTCATTCGACAAAAAGAA	qPCR	(Bhattarai <i>et al.</i> 2010)
SIWrky28-F	ACAGATGCAGCTACCTCATCCTCA	qPCR	(Kim <i>et al.</i> 2009)
SIWrky28-F	GTGCTCAAAGCCTCATGGTTCTTG	qPCR	(Kim <i>et al.</i> 2009)

Primers	Sequences (5'-3')*	Purpose	Reference
SILrr22-F	AAGATTGGAGGTTGCCATTGGAGC	qPCR	(Nguyen et al. 2010)
SILrr22-R	ATCGCGATGAATGATCGGTGGAGT	qPCR	(Nguyen et al. 2010)
SIPti5-F	ATTCGCGATTCGGCTAGACATGGT	qPCR	(Nguyen et al. 2010)
SIPti5-R	AGTAGTGCCTTAGCACCTCGCATT	qPCR	(Nguyen et al. 2010)

* Letters in red are part of Gateway recombination

* Letters in blue are additional nucleotides for inframe cloning in the vector

Table 2.2. GroEL peptides specific to *Buchnera*.

Peptide	<i>Buchnera</i> specific
AITQVGTISANADEK	Yes
ANDAAGDGTATLLAQSIENEGLK	No
AVAAGmNPMDLK	No
AVISAVEELK	No
DTTTHGGVGEK	Yes
GVNVLADAVK	No
VEDALHATR	No
VGAATEVEMK	No
GQNEDQNVGIR	Yes
qEVQEATSDYDKEK	Yes
QIVSNSGEEPSVVTNNVK	Yes
STLEDLGQAK	No

CHAPTER THREE

In planta expression or delivery of potato aphid *Macrosiphum euphorbiae* effectors

Me10 and *Me23* enhances aphid fecundity

Abstract

The interactions between aphids and their host plants seem to be analogous to those of plant-microbial pathogens. Unlike microbial pathogen effectors, little is known about aphid effectors and their ability to interfere with host immunity. To date, only three functional aphid effectors have been reported. To identify potato aphid (*Macrosiphum euphorbiae*) effectors, we developed a salivary gland transcriptome using Illumina technology. We generated 85 million Illumina reads from salivary glands and assembled them into 646 contigs. *Ab initio* sequence analysis predicted secretion signal peptides in 24% of these sequences suggesting that they might be secreted into the plant during aphid feeding. Eight of these candidate effectors with secretion signal peptides were functionally characterized using *Agrobacterium tumefaciens*-mediated transient overexpression in *Nicotiana benthamiana*. Two candidate effectors, *Me10* and *Me23*, increased aphid fecundity suggesting their ability to suppress *N. benthamiana* defenses. Five of these candidate effectors, including *Me10* and *Me23*, were also analyzed in tomato by delivering them through the *Pseudomonas syringae* type three secretion system. In tomato, only *Me10* increased aphid fecundity. This work identified two additional aphid effectors with ability to manipulate the host for their advantage.

Introduction

Aphids (Hemiptera: Aphididae) are soft-bodied insects with piercing-sucking mouthparts that cause serious economic losses to cultivated crops. They damage plants directly by depleting nutrients and altering plant development, and indirectly by vectoring plant viruses and support the growth of the sooty mold fungus (Blackman and Eastop 2000). Some aphid species are globally distributed due to their polyphagous nature and ability to adapt to different environmental conditions (Margaritopoulos *et al.* 2009). The life cycle of aphids is somewhat complex, comprising of both sexual and asexual (parthenogenetic) modes of reproduction, the latter giving rise to live progeny (Blackman and Eastop 2000). Sexual reproduction occurs only in the fall season where eggs are laid on perennial plants for overwintering. Being hemimetabolous insects, aphids have no morphologically distinct larval or pupal stages. During asexual reproduction, females lay nymphs which, after three successive molts become adults (Moran 1992, Van Emden and Harrington 2007).

The potato aphid (*Macrosiphum euphorbiae*) has a broad host range including plants in Solanaceae, transmits a number of plant viruses and represents an aphid species of worldwide significance (Chan *et al.* 1991, Moeller 1973, Radcliffe and Ragsdale 2002). Its abundance and propensity to develop alatae makes this aphid species very important in viral epidemiology (Cerato *et al.* 1994, Singh and Boiteau 1984). In tomato (*Solanum lycopersicum*), resistance to potato aphids is mediated by the *Mi-1* gene that encodes a coiled-coil nucleotide-binding leucine-rich protein and requires the Somatic Embryogenesis Receptor Kinase 1 (*SISERK1*) (Mantelin *et al.* 2011, Rossi *et al.* 1998).

The aphid effector recognized directly or indirectly by Mi-1 remains elusive and further experimental evidences are required.

Aphids are phloem feeders that use a pair of slender stylets to mostly move between cells until they reach the sieve element, where they feed for a prolonged period of time. Although the stylets path is mainly intercellular, stylets do also puncture cells and cause cell wall disturbance and damage to the plasma membranes of mesophyll and parenchyma cells (Moran *et al.* 2002, Pollard 1973, Tjallingii and Hogen Esch 1993). Unlike chewing insects that cause extensive tissue damage, this specialized aphid feeding behavior and interaction with its host, avoiding extensive mechanical tissue damage, is analogous to plant-biotrophic pathogen interactions where the pathogen is sustained in a localized area and is dependent on living host plant cells. Consistent with these observations, induction of plant genes associated with pathogen-induced response pathways have been reported as a result of aphid feeding (de Vos *et al.* 2005, Kaloshian and Walling 2005, Martinez de Ilarduya *et al.* 2003, Thompson and Goggin 2006).

Aphids release two types of saliva during feeding, soluble saliva and gelling saliva. The soluble saliva is in liquid form and is delivered along the penetration path and in the sieve element whereas the gelling saliva forms a proteinaceous sheath around the stylets as soon as it exits the stylet tip (Miles 1999, Tjallingii 2006). It has been hypothesized that constituents of aphid salivary secretions play crucial roles in modulating plant responses. Aphid saliva consists of a suite of bio-reactive compounds, some of which may serve as cues to elicit plant defenses while others are expected to

function in suppressing or circumventing plant defenses (Harris *et al.* 2003, Hogenhout and Bos 2011, Miles 1999).

It is speculated that aphid-host interactions shadow the commonly accepted zig-zag model of plant-microbial pathogen defense evolution described by (Jones and Dangl 2006). In pathosystems, conserved sets of molecular signatures called pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) or general elicitors are recognized by pattern recognition receptors (PRRs) present at the host cell surface. PRR-mediated non-self recognition activates pattern-triggered immunity (PTI) in the host plant. PTI is associated with activation of downstream signaling pathways including mitogen activated protein kinases (MAPK) and WRKY transcription factors, induction of defense responses including production of reactive oxygen species, accumulation of pathogen-related (PR) proteins and callose deposition which collectively restrict microbial growth (Segonzac and Zipfel 2011). Pathogens can secrete small molecules or proteins known as effectors (Abramovitch *et al.* 2006, Birch *et al.* 2006, Davis *et al.* 2008, Kamoun 2006, van der Hoorn and Kamoun 2008) to counteract PTI and effectively parasitize and colonize the host plant. Extensive research using various approaches has led to the identification of hundreds of effectors secreted by bacterial, fungal and oomycete plant pathogens that were essential for understanding pathogenesis.

Unlike PAMPs and pathogen effectors, which have been extensively studied during the past decade, not much is known about herbivore associated molecular patterns (HAMPs) or herbivore effectors (Hogenhout and Bos 2011, Miles 1999, Tjallingii 2006). Proteinaceous elicitor(s) with a size between 3 and 10 kD in the saliva of green peach

aphid, *Myzus persicae*, have been shown to induce defense responses in *Arabidopsis* (de Vos and Jander 2009). Similarly, expressing the *M. persicae* proteins *Mp10* and *Mp42* in *Nicotiana benthamiana* resulted in a decrease in aphid fecundity (Bos *et al.* 2010). Moreover, induction of plant defense genes by feeding of piercing–sucking herbivores including aphids has been extensively demonstrated (Kempema *et al.* 2007, Martinez de Ilarduya *et al.* 2003, Rodriguez-Saona *et al.* 2010, Thompson and Goggin 2006). In contrast, little is known about how the salivary proteins of piercing-sucking herbivores may interfere with the plant immune system or manipulate host metabolites for their advantage. Aphids alter host primary metabolism and improve nutrient composition of the phloem sap to enhance their growth (Giordanengo *et al.* 2010, Wilson *et al.* 2011). However, it is not clear how aphids are able to cause these changes or the salivary secretion(s) responsible for these changes. A few aphid salivary proteins have been implicated in enhancing aphid performance. The salivary protein *MpC002* has been shown to enhance *M. persicae* fecundity or nymph production on *N. benthamiana* (Bos *et al.* 2010). *In vitro* analysis of the effect of the vetch aphid, *Megoura viciae*, saliva on forisomes provided direct evidence that aphid saliva has the ability to counteract plant defenses and prevent sieve tube plugging providing aphids with access to a continuous flow of phloem sap (Will *et al.* 2007). Moreover, some piercing-sucking insects have been shown to suppress the expression of plant defense genes and manipulate defense signaling pathways to their advantage (Zarate *et al.* 2007, Zhang *et al.* 2011). Taken together, the ultimate outcome of interactions of piercing-sucking herbivores with their host plant is likely to depend on the salivary secretion and effectors produced by the

herbivore and the ability of the plant to perceive these effectors and respond appropriately, and the ability of the salivary secretions to alter host metabolism.

A large number of pathogen effectors have been identified using homology-based searches while others were likely missed, as their sequences are unique. In aphids, candidate effector molecules have been identified using three approaches involving (1) sequencing the aphid salivary gland transcriptome or (2) the salivary gland proteome followed by prediction of the protein secretion signal, or (3) by direct sequencing the aphid salivary proteome (Bos et al. 2010, Carolan *et al.* 2011, Carolan *et al.* 2009, Harmel *et al.* 2008). In this study, we sequenced the salivary gland transcriptome of *M. euphorbiae* using the Illumina sequencing platform and assembled the reads into 646 contigs. Data mining of the assembled contigs identified 159 predicted *M. euphorbiae*-secreted proteins. Eight candidate effectors, with secretion signal peptides identified in the salivary transcriptome, were functionally characterized using *Agrobacterium tumefaciens*-mediated transient overexpression in *N. benthamiana* or delivered by *Pseudomonas syringae* type three secretion system (TTSS) into tomato plants. Using these assays, we identified two effectors, *Me10* and *Me23*, which enhanced aphid fecundity.

Data presented in this Chapter is in equal contribution work by a former graduate student in lab Hagop Atamian and myself.

Materials and Methods

Plant material and aphid colonies

Tomato (*Solanum lycopersicum*) cultivars UC82B and Moneymaker, *Nicotiana benthamiana* and tobacco (*Nicotiana tabacum*) NC-95 were used. Seedlings were transplanted into California mix II or sand. The plants were maintained in growth rooms at 24°C with 16-h-light and 8-h-dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and weekly fertilized with MiracleGro (18-18-21; Stern's MiracleGro Products).

Colonies of the parthenogenetic potato aphid (*Macrosiphum euphorbiae*) and green peach aphid (*Myzus persicae*) were reared on tomato cv. UC82B and tobacco NC-95 plants, respectively. The colonies were maintained in insect cages in a pesticide-free greenhouse at 22-26°C supplemented with light for 16-h daylength. Age-synchronized, one-day-old, adult *M. euphorbiae* aphids were produced as described in (Bhattarai *et al.* 2007).

RNA extraction and cDNA synthesis

RNA was isolated from 200 dissected *M. euphorbiae* salivary glands and used for RNA-Seq library preparation as described previously (Atamian and Kaloshian 2012).

Similarly, RNA was isolated from whole aphids and 20 dissected heads and salivary glands or guts. cDNA was synthesized from 100 ng DNase-treated whole body, head and salivary glands, or gut RNA using Superscript III (Invitrogen) reverse transcriptase enzyme and oligo-dT primers according to the manufacturer's recommendations.

Library construction, sequencing and *de novo* assembly

A detailed procedure of RNA-Seq library preparation from salivary gland tissues has been described previously (Atamian and Kaloshian 2012). One lane of single-end 75-nucleotide long sequencing was performed with an Illumina Genome Analyzer II instrument at the Institute for Integrative Genome Biology, University of California Riverside. Data from the Illumina sequencing run was processed using the Illumina standard pipeline version 1.4. The sequence data generated in this study have been deposited in NCBI's Short Read Archive, accession number SRR547988. Redundancies in the data set were removed with the SEED NGS clustering tool (Bao *et al.* 2011). The remaining sequences were assembled with the Velvet/Oases assembler as described in (Bao *et al.* 2011, Schulz *et al.* 2012) and deposited at DDBJ/EMBL/GenBank under the accession GAAF000000000 in NCBI or presented in Table S4.

Annotation and secretion signal prediction

Reciprocal TBLASTX analyses were performed between *M. euphorbiae* and *Acyrtosiphon pisum* sequences to identify the putative orthologs of the *M. euphorbiae* sequences generated in this study. These putative orthologs were annotated based on the latest *A. pisum* annotation (aphidbase_2.1_peptides). The *M. euphorbiae* sequences with no putative orthologs in *A. pisum* were annotated by performing reciprocal TBLASTX analysis against NCBI nucleotide (nt/nr) database. The annotated sequences were assigned to different GO categories based on available GO analysis. The full-length

putative *A. pisum* orthologs of the *M. euphorbiae* sequences were subjected to *de novo* signal peptide prediction analysis using SignalP 4.0 and TargetP 1.1 programs.

Cloning *M. euphorbiae* salivary gland EST sequences

Eight of *M. euphorbiae* salivary gland EST sequences with predicted secretion signal in their putative *A. pisum* orthologs were cloned. Gene-specific primers were designed excluding the secretion signal peptide at the 5'-end, based on the *A. pisum* full-length sequences, and a start codon added (Table 3.2). The PCR amplified products, obtained using Phusion High-Fidelity Polymerase (New England BioLabs) were cloned into the pDONR207 (Invitrogen) and recombined into the binary vector pEarleyGate100 (Earley *et al.* 2006) and sequenced using Sanger sequencing. The clones were transformed into *Agrobacterium tumefaciens* strain GV3101 for transient overexpression in *N. benthamiana*. These genes were cloned in pEarleyGate100 vector by a former postdoc Valeriano Dal Cin. A subset of these sequences were also recombined into the pVSP_PsSPdes vector (Rentel *et al.* 2008), sequenced and transformed into *Pseudomonas syringae* pv. *tomato* strain DC3000/ Δ avrPto/ Δ avrPtoB (*Pst*) for assay in tomato plants (Nguyen *et al.* 2010).

Aphid bioassay on *N. benthamiana*

Recombinant *A. tumefaciens* containing candidate effector or GFP were grown overnight in LB media supplemented with rifampicin (25 µg/ml), gentamycin (14 µg/ml) and kanamycin (50 µg/ml) at 28°C. The cultures were diluted to a final OD₆₀₀ of 0.3 in an

induction buffer (10 mM MES, 10 mM MgCl₂, 150 µM acetosyringone, pH = 5.6).

Leaves of 4–5 week-old *N. benthamiana* plants were agroinfiltrated using a needle less syringe. Two leaves of four plants were infiltrated per construct. On day two after infiltration, four *M. persicae* adults were caged on each infiltrated leaf. The following day, the adults were removed leaving four first-instar nymphs. On day 6, the four-day-old nymphs were moved to a leaf of a plant infiltrated 2 days earlier. On day 9, the adult aphids were moved again to a leaf of a plant infiltrated 2 days earlier. On day 12, the same adults were moved to a plant infiltrated 2 days earlier. Aphid survival was counted at 6, 9, 12 and 15 days after the start of the experiment and the number of newly produced nymphs was counted on day 9, 12 and 15. The average number of nymphs produced per leaf sample was calculated by dividing the number of nymphs produced by the number of live adult aphids on days 9, 12 and 15.

Aphid bioassay on tomato

The *Pst* culture was grown on King's B Medium (KBM) plates containing rifampicin (25 µg/ml), carbenicillin (100 µg/ml) and kanamycin (50 µg/ml) at 30°C for two days. A single colony was inoculated into 200 µl of liquid KBM, plated onto KBM and incubated for another day at 30°C. A good healthy lawn on the plate was resuspended in 10 mM MgCl₂. Whole plants were vacuum infiltrated with *Pst* (1 x 10³ CFU/ml) containing a candidate effector or GUS in 1 mM MgCl₂ and 0.02% Silwet L-77. Plants were infested with nine one-day-old adult *M. euphorbiae* 24 h after infiltration. Five plants were used

per construct. Aphid fecundity was assessed by counting the number of nymphs and removing them daily for five days.

Gene expression analysis

Semi-quantitative RT-PCR analysis was performed using cDNA prepared from whole aphids or dissected aphid heads and salivary glands or guts using gene-specific primer pairs (Table 3.3). *C002* (Mutti *et al.* 2008) and *Sucrase (Suc)* (Price *et al.* 2007) were used as tissue-specific markers (Table 3.3) and the ribosomal gene L27 (NM_001126221) was used as internal control. PCR was performed in 25 µl with 40 ng of cDNA template, 1 X PCR buffer, 2.5 mM MgCl₂, 0.4 mM dNTPs, 2 units of *Taq* DNA polymerase, and 10 µM of each forward and reverse primers. The PCR program was initialized at 94°C for 5 min, followed by 23 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Statistical analyses

For the aphid fecundity assays, data were normalized to GFP or GUS. Statistical analysis with aphid data from *N. benthamiana* plants was performed using one-tailed Flinger-Policello test followed by Bonferroni adjustment (BenMamoun 2006). Student's *t*-test was used for aphid data from tomato plants.

Results and discussion

Transcriptome sequencing, assembly and annotation

To analyze the *M. euphorbiae* salivary gland transcriptome, an RNA-Seq library was prepared from 200 dissected salivary glands of adult aphids. To determine the quality of the library, an aliquot was cloned and 10 clones were subjected to Sanger sequencing. TBLASTX analysis of the clones against the nonredundant database at National Center for Biotechnology Information (NCBI) identified unique sequences in each clone suggesting that the library was not biased for certain transcripts.

A total of 85 million reads were generated from this RNA-Seq library of which 4 million were unique. Initially, the generated reads were assembled using the Velvet/Oases assembler (Schulz et al. 2012). Further improvement of the assembly was achieved by reducing the redundancy in the data set with the SEED program, a novel clustering algorithm for next-generation sequencing data, which resulted in longer contigs (Bao et al. 2011). In total, 646 contigs were generated with 62% of the assembled contigs longer than 500 bp.

To determine how many of the *M. euphorbiae* salivary gland contigs have putative orthologs in the closely related species *Acyrtosiphon pisum*, whose genome has been sequenced, reciprocal TBLASTX analysis was performed. A total of 551 *M. euphorbiae* contigs were identified with sequences orthologous to 460 *A. pisum* transcripts (Table S5). Some of the *M. euphorbiae* contigs matched to non-overlapping regions of the same *A. pisum* transcript explaining the larger number of *M. euphorbiae*

orthologous contigs. Consequently, these contigs were annotated according to version 2 of the *A. pisum* annotation (Consortium 2010). Out of the 460 *A. pisum* transcripts identified in this study, 155 were also identified in the *A. pisum* salivary glands by (Carolan et al. 2011). Putative orthologs for an additional 41 contigs were identified by performing reciprocal BLASTX analysis against the Uniprot database and annotated accordingly (Table S5). Although these *M. euphorbiae* contigs did not have orthologs in *A. pisum*, we cannot exclude the possibility that they may be present in other aphid species. Orthologs could not be identified for 54 *M. euphorbiae* contigs (Table S5). They could be the result of inaccurate assembly of the Illumina reads, although it is possible that some of these sequences are correctly assembled and consequently unique to *M. euphorbiae*.

To determine the putative functions of the *M. euphorbiae* contigs, we used the database [Comment: would expect name of database here.] containing gene ontology (GO) assignments of all the publicly available *A. pisum* ESTs. The functional classification of the contigs based on GO terms showed enrichment for the classes “translation”, “metabolic process”, and “transport” in the GO category “biological process” (Fig. 3.1A). Aphids have a pair of salivary glands each consisting of a principal gland and two accessory glands (Ponsen 1972, Weidemann 1968). Besides the expected cell maintenance processes, the cells of the salivary glands undergo cycles of secretory activities (Miles 1999). Thus, transcripts grouped under the “translation” category are likely to serve both functions. To fulfill their respective roles, those proteins destined to be delivered in the saliva and potentially having roles in the interactions with the plant

host, are expected to have secretion signals allowing them to cross cell membranes into the salivary canal. Consequently, it is not surprising that transcripts predicted to have a transport function were enriched in this organ. On the other hand, the GO terms in the “molecular function” category showed more distributed and diverse enrichments of various molecular activity categories (Fig. 3.1B).

Identification of sequences with secretion signal peptides

The amino acid sequences of the putative full-length *A. pisum* orthologs of the identified *M. euphorbiae* contigs were analyzed with the SignalP 4.0 (Petersen *et al.* 2011) and TargetP (Emanuelsson *et al.* 2000) prediction softwares trained to identify signal peptides. Of the 460 examined sequences, 125 and 159 were predicted to have putative signal peptides predicted by SignalP 4.0 (hidden Markov model scores of higher than 0.45) and TargetP (predefined set of cutoffs that yields specificity >0.95 on the TargetP test sets), respectively (Table S5). TargetP predicted a signal peptide in 121 sequences predicted by SignalP. Moreover, it predicted an additional 38 sequences that were not predicted by SignalP. In the *A. pisum* salivary gland transcriptome, 30% (1074/324) of the transcripts were predicted to have signal peptides using SignalP 3.0 (Carolan *et al.* 2011). Using the version 4.0 of the same program we predicted 27% (460/125) of the salivary gland transcripts to have signal peptide. Around 42% of the sequences with predicted signal peptides in this study were also identified by (Carolan *et al.* 2011). This low overlap of sequences with signal peptides between the two studies suggests that the salivary gland sequences from both species are incomplete.

Selection of clones for functional analysis and their tissue-specific expression

An efficient way to investigate the roles of aphid candidate effectors *in planta*, is to transiently overexpress them in *N. benthamiana* using *Agrobacterium tumefaciens* and assay with a population of aphids that are adapted to plants with nicotine such as *M. persicae*. A similar approach, using *N. benthamiana* leaf discs, was successfully used to evaluate *M. persicae* effectors (Bos et al. 2010). In order to use this assay system, we choose *M. euphorbiae* effectors with secretion signals and putative orthologs in *M. persicae*. To identify the *M. persicae* orthologs of our set of salivary gland expressed *M. euphorbiae* contigs, we reassembled the publically available *M. persicae* ESTs and used them in reciprocal TBLASTX analysis with the *M. euphorbiae* salivary gland transcripts. We chose eight *M. euphorbiae* salivary gland contigs with putative orthologs in *M. persicae*, four with annotations and four encoding yet uncharacterized proteins (Table 3.1). We cloned the *M. euphorbiae* ORFs encoding the mature proteins corresponding to these sequences, excluding the signal peptide. These eight ORFs (*Me5*, *Me10*, *Me13*, *Me14*, *Me17*, *Me20*, *Me23*, and *Me25*) have 72-85% amino acid sequence identity to the corresponding *M. persicae* EST contig (Table 3.1). The relationship among the *M. persicae* and *A. pisum* orthologs of these eight *M. euphorbiae* clones was also demonstrated by reciprocal TBLASTX comparisons.

Four proteins were chosen for further study based on possible effector functions suggested by their annotation. Based on *A. pisum* annotation, *Me5* encodes a trehalase. Trehalose accumulation is associated with Arabidopsis defense against aphids (Singh *et al.* 2011). Therefore, trehalase secreted by the aphid may hydrolyze trehalose and

counteract host defenses. *Me14* encodes a lipase; members of this superfamily of proteins have diverse roles including defense against oxidative stress (Horne *et al.* 2009). *Me23* encodes a glutathione peroxidase (GPX) with potentially protective role against oxidative burst (Lamb and Dixon 1997); while *Me25* encodes a carbonic anhydrase 2, which may function in catalysis of aldehydes induced during aphid feeding (Gosset *et al.* 2009). Of the four uncharacterized proteins, conserved domain search identified known domains for two, *Me13* and *Me17* (Table 3.1).

To confirm that the eight genes were expressed in the salivary glands, we evaluated the accumulation of their transcripts in dissected head and glands or gut tissues of *M. euphorbiae* (Fig. 3.2). Seven genes were expressed in the head and glands and not in the gut suggesting that their corresponding proteins are produced in the salivary glands, while *Me5* transcripts were detected in all the tissues tested suggesting either tissue unspecific expression of this gene or expression of its paralogs in different tissues. As seven of the genes tested exhibited tissue-specific expression, it was unlikely that our tissue-specific cDNAs were contaminated. Orthologous transcripts of five of these genes, *Me10*, *Me13*, *Me17*, *Me20* and *Me23*, were also identified in *A. pisum* salivary gland transcriptome while peptides corresponding to *Me5*, *Me10* and *Me23* orthologs were identified in the *A. pisum* salivary gland proteome (Carolan *et al.* 2011). The presence of transcripts to most of our selected salivary gland genes in both *A. pisum* and *M. persicae*, aphid species with narrow and broad host ranges, respectively, suggests a general rather than specialized roles for these genes.

Evaluating the role of candidate *M. euphorbiae* effectors in aphid defense in *N. benthamiana*

To investigate the roles of *M. euphorbiae* candidate effectors *in planta*, we transiently expressed the selected *M. euphorbiae* proteins in *N. benthamiana* and assayed with a population of *M. persicae* adapted to feeding on tobacco (Kim and Jander 2007). For the transient expression assays, a large area of a *N. benthamiana* leaf was agroinfiltrated with the recombinant binary pEarleyGate100 vector expressing a candidate *M. euphorbiae* protein. Expression of green fluorescent protein (GFP) was used as control to monitor expression and aphid fecundity. Twenty-four hour after infiltration, four *M. persicae* adults were caged on each infiltrated leaf exposing the infiltrated area to aphids. The following day, the adults and newly born nymphs were removed leaving four first-instar nymphs. These remaining aphids were moved to a fresh plant with recently agroinfiltrated leaves on day 6, day 9 and day 12. This schedule was based on efficient GFP expression. These nymphs became adults on day 8 and their fecundity was evaluated on days 9, 12 and 15. The average nymph production per aphid was calculated and normalized to those on the GFP control. Two candidate effectors *Me10* ($P = 0.004$) and *Me23* ($P = 0.01$) increased significantly *M. persicae* fecundity compared to the GFP control (Fig. 3.3). There was no significant difference in aphid fecundity on the plants expressing the remaining six effectors (Fig. 3.3). Although the increase in aphid fecundity on plants expressing *Me10* or *Me23* was modest, this increase in aphid performance was consistent in three independent experiments. This suggests that *Me10* and *Me23* altered *N. benthamiana* responses for aphid's advantage. Contributions of a

single effector in manipulating host responses could be minor and this effect could be amplified when combined with additional components of the cocktail of effectors secreted by the aphid. Therefore, simultaneous expression of a combination of effectors, with no effect individually, may also result in enhancement of aphid performance.

To date, only a single aphid effector *MpC002*, has been identified to increase aphid fecundity (Bos et al. 2010). Although it remains unknown how *MpC002* alters *N. benthamiana* responses to *M. persicae*, the *A. pisum* ortholog *ApC002* has been implicated in aphid orientation and feeding (Mutti et al. 2008). *ApC002* is also secreted inside the plant host (Mutti et al. 2008). To date, nine and 17 salivary proteins have been identified from *A. pisum* and *M. persicae*, respectively (Carolan et al. 2009, Harmel et al. 2008). Interestingly, one of the *A. pisum* salivary proteins is the ortholog of *Me10* the effector with an unknown function. The second *M. euphorbiae* effector, *Me23*, we identified to increase aphid fecundity is predicted to encode a GPX and could be involved in reducing H₂O₂ and function as an antioxidant to enhance aphid virulence and reduce the effect of the oxidative burst triggered by aphid feeding (Martinez de Ilarduya et al. 2003).

Evaluating the role of candidate *M. euphorbiae* effectors in aphid defense in tomato

To feed, aphids probe the plant host tissue with a pair of stylets and secret both watery and gelling saliva in this process. During probing and stylet penetration cells are punctured and saliva is delivered inside the host cytoplasm. Therefore, aphid feeding can be compared with gram-negative plant pathogenic bacteria that possess a TTSS to invade

and colonize the host cell by injecting virulence effectors (Buttner and He 2009, Cornelis and Van Gijsegem 2000). Therefore, we took the advantage of an existing bacterial system to deliver candidate *M. euphorbiae* effectors into the tomato cells. The TTSS of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) have been successfully utilized to deliver oomycete *Hyaloperonospora parasitica* effectors into Arabidopsis cells (Rentel et al. 2008, Sohn *et al.* 2007).

We characterized the function of five of the eight candidate effectors tested in *N. benthamiana* by delivering them through the *Pst* TTSS into the tomato cell cytoplasm and assaying aphid performance on the plants. To deliver aphid effectors through the TTSS, we used the pVSP_*PsSPdes* expression vector that has the promoter and secretion-translocation signal of the *Pst* effector AvrRpm1 (Guttman and Greenberg 2001, Rentel et al. 2008). We introduced this vector in a less virulent strain of *Pst* DC3000, *Pst* DC3000 Δ *avrPto*/ Δ *avrPtoB* (Nguyen et al. 2010), lacking two strong virulent effectors *avrPto* and *avrPtoB*, to minimize the interference of bacterial effectors to better evaluate subtle differences in aphid performance. The roles of the five aphid effectors were evaluated in tomato by assessing their effect on the *M. euphorbiae* fecundity. To perform the fecundity assays, 4-5-week-old tomato plants were vacuum infiltrated with the recombinant *Pst* expressing the aphid constructs or β -glucuronidase (GUS) as control. Each plant was infested with nine one-day-old, age-synchronized, adult aphids 24 h post infiltration. Nymph production was evaluated for the next five days. The average nymph production per aphid was calculated and compared with the GUS control. Of the five candidate effectors tested, only *Me10* significantly increased ($P = 0.021$) aphid fecundity

on tomato (Fig. 3.4). Thus, indicating that *Me10* is able to manipulate tomato plants for aphid's advantage as it did in *N. benthamiana*. None of the remaining effectors affected aphid fecundity (Fig. 3.4). We did not find statistically significant differences in aphid performance in tomato plants infected with *Pst* expressing *Me23* compared to the GUS control suggesting that *Me23* is not able to alter tomato responses to detectable levels. It is possible that the different mode of effector delivery, the altered aphid assay used for tomato compared to *N. benthamiana* or the shorter time exposure of aphids to plant expressing the effector, did not allow detection of subtle differences in plant responses. Alternatively, *N. benthamiana* may be less tolerant than tomato to aphid infestation and, therefore, more suitable to detect the weak effects of *Me23* (Goodin *et al.* 2008).

Since *Me10* is uncharacterized and has no known functionally conserved domains, it is difficult to speculate how it manipulates plant responses. Future experiments should elucidate this role. Nevertheless, this experiment showed that *Pst* TTSS can be used for delivery of aphid effectors *in planta* to evaluate aphid performance. This approach will allow the evaluation of aphid effectors, without the need to express them by developing stable transgenic plants, in hosts in which *Agrobacterium*-mediated transient expression does not work consistently or is not feasible to perform.

***Me10* and *Me23* and their putative *A. pisum* and *M. persicae* orthologs**

Two genes of the *A. pisum* genome (ACYPI002439 and ACYPI38240) are annotated as glutathione peroxidases. The amino acid sequence identity between *Me23* and the *A. pisum* proteins ACYPI002439 and ACYPI38240 is 87% and 42%, respectively, while the

amino acid sequence identity between *Me23* and a putative *M. persicae* ortholog is 70% (Table 3.1). It is not clear how many *Me23* orthologs are present in the *M. persicae* genome, as the genome of this aphid has not been sequenced. The alignment of the amino acid sequences from the three aphid species shows blocks of conserved regions (Fig. 3.5). Near the N-terminus, within one of these conserved regions, a deletion of eight amino acids is present in the *M. persicae* protein. The presence of such insertion/deletions might alter the protein's function.

Me10 encodes an uncharacterized protein. BLAST analyses indicated that *Me10* is a single-copy gene in *A. pisum* (Table 3.1). The amino acid sequence of *Me10* has 76% and 88% sequence identity to *M. persicae* and *A. pisum* orthologs, respectively. Although these three aphids belong to the same tribe, Macrosiphini, within the aphid subfamily aphidinae, it is not surprising that *M. euphorbiae* sequences are more similar to *A. pisum*, since *M. euphorbiae* is phylogenetically more closely related to *A. pisum* than *M. persicae* (von Dohlen *et al.* 2006).

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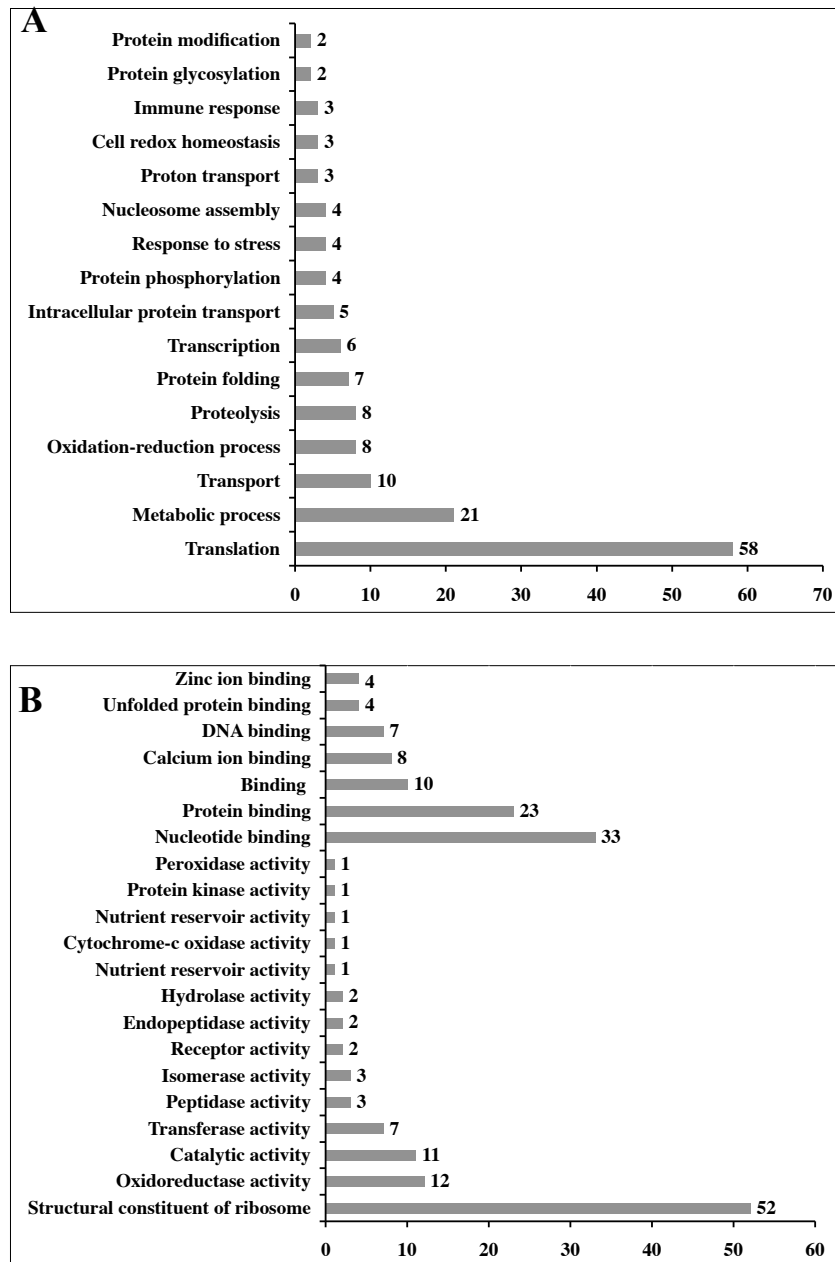


Fig. 3.1A-B Classification of the *Macrosiphum euphorbiae* contigs using the *Acyrtosiphon pisum* Gene Ontology (GO) terms. The contigs were annotated according to **A**, the biological process that they are predicted or known to be part of or **B**, the known or predicted molecular function.

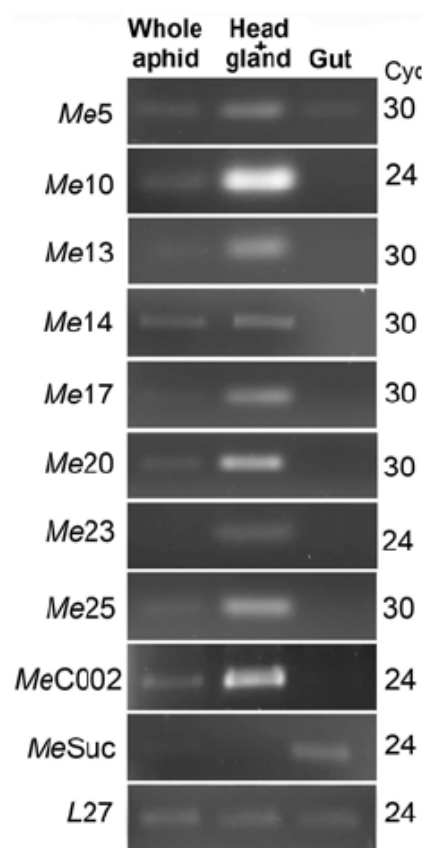


Fig. 3.2 Tissue-specific expression analysis of the eight *Macrosiphum euphorbiae* candidate effectors. RNA isolated from whole aphids or dissected aphid heads and salivary glands or guts was used in reverse transcription and semi-quantitative PCR with gene-specific primers. PCR cycles are indicated to the right of the panel. Expression of *MeC002* and *MeSuc* were used as controls for salivary glands and gut, respectively. Ribosomal gene *L27* was used as an internal control for cDNA.

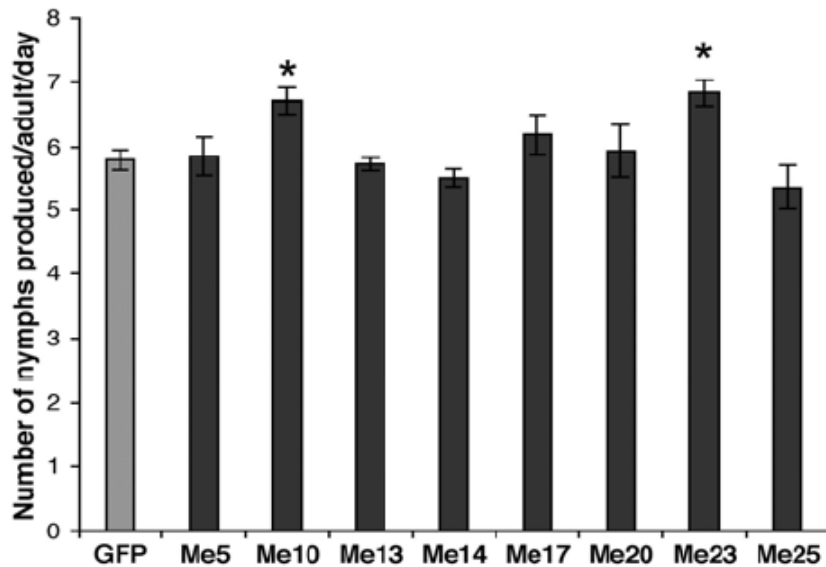


Fig. 3.3 *Myzus persicae* performance on *Nicotiana benthamiana* plants expressing *Macrosiphum euphorbiae* candidate effectors. *M. euphorbiae* candidate effectors were transiently overexpressed in *N. benthamiana* using *Agrobacterium tumefaciens*. One day after agroinfiltration, each leaf sample was caged with four adult *M. persicae*. The following day, adults were removed leaving four first-instar nymphs. The four nymphs were moved to a freshly agroinfiltrated leaf expressing the candidate effector on days 6, 9 and 12. Nymph production was evaluated up to day 15. GFP was used as expression and aphid assay control. Graphs show the average number of nymphs produced per adult from one experiment. Two leaves per plant and 4 plants per construct were used (n=8). Data from one experiment is presented with error bars indicating the standard error. Asterisks indicate statistical significance compared to the GFP control (*Me10* $P = 0.004$; *Me23* $P = 0.01$). This experiment was performed three times with essentially identical results.

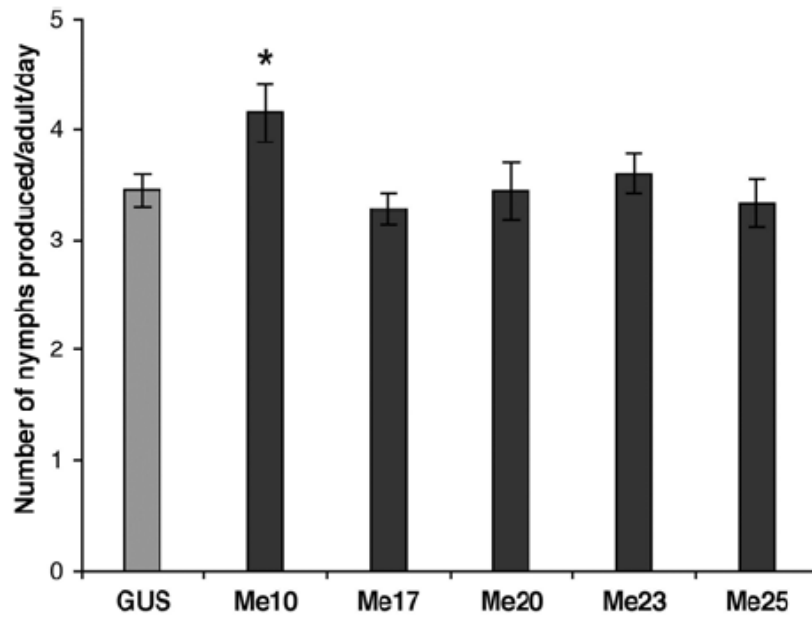


Fig. 3.4 *Macrosiphum euphorbiae* performance on tomato plants expressing *M. euphorbiae* candidate effectors. Five-week-old tomato plants were vacuum infiltrated with recombinant *Pseudomonas syringae* pv. *tomato* DC3000 Δ avrPto/ Δ avrPtoB (*Pst*) delivering individual aphid effector or GUS used as control. Five plants per treatment were vacuum infiltrated with recombinant *Pst* 1×10^3 CFU/ml and 24 h post infiltration each plant was infested with 9 age-synchronized one-day-old adult potato aphids. Aphid progeny was evaluated for 5 days. Graphs show the average number of nymphs produced per adult aphid ($n=5$). Data from one experiment is presented with error bars indicating the standard error. Asterisk indicates statistical significance compared to the GUS ($P = 0.021$). This experiment was performed three times with essentially identical results.

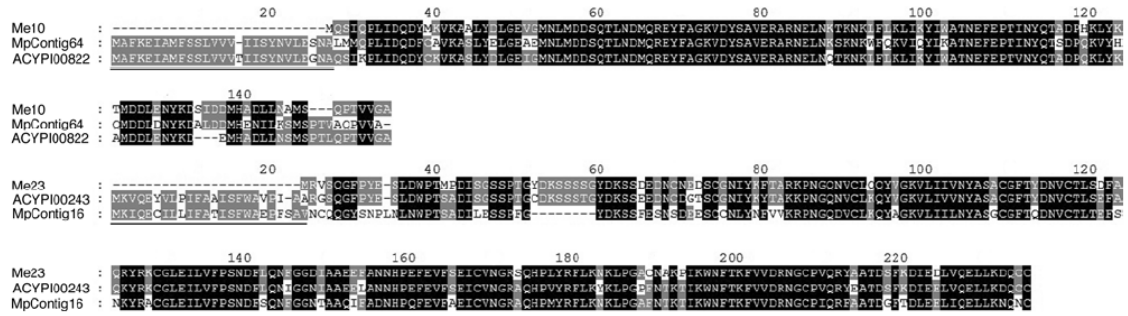


Fig. 3.5A-B Alignment of deduced amino acid sequences of *Macrosiphum euphorbiae* (Me) effectors Me10 (A) and Me23 (B), with putative orthologs from *Acyrthosiphon pisum* (ACYP) and *Myzus persicae* (Mp). Black and grey shades indicate identical and highly conserved amino acids, respectively. Putative secretion signal peptide sequences are underlined.

Table 3.1. *Macrosiphum euphorbiae* selected genes with predicted secretion peptide and their *Acyrtosiphum pisum* and *Myzus persicae* putative orthologs

Gene	ID ^a	Size ^b	Ortholog and E-value		Conserved domain	Annotation	Phenotype ^c	
			<i>A. pisum</i>	<i>Myzus persicae</i>			<i>N. b.</i>	Tomato
<i>Me5</i>	<i>Me_SG098</i>	1,713	ACYPI002298 (10 ⁻¹⁹³)	<i>Mp</i> Contig8632 (10 ⁻⁴⁵)	Bacterial alpha-L-rhamnosidase	Trehalase	No	NA
<i>Me10</i>	<i>Me_SG525</i>	387	ACYPI008224 (10 ⁻⁶⁹)	<i>Mp</i> Contig6453 (10 ⁻⁴⁹)	No conserved domain	Uncharacterized	Yes	Yes
<i>Me13</i>	<i>Me_SG442</i>	324	ACYPI56566 (10 ⁻⁶³)	<i>Mp</i> Contig11439 (10 ⁻⁴⁷)	Cytochrome P450, Spumavirus gag protein	Uncharacterized	No	NA
<i>Me14</i>	<i>Me_SG355</i>	954	ACYPI21663 (10 ⁻¹⁷⁸)	<i>Mp</i> Contig3168 (10 ⁻¹²⁹)	Lipase superfamily	Lipase	No	No
<i>Me17</i>	<i>Me_SG427</i>	1,014	ACYPI53825 (10 ⁻¹⁵²)	<i>Mp</i> Contig11230 (10 ⁻¹⁰³)	Tat binding protein 1-interacting protein	Hypothetical	No	No
<i>Me20</i>	<i>Me_SG497</i>	1,131	ACYPI21412 (10 ⁻¹⁶⁶)	<i>Mp</i> Contig1043 (10 ⁻⁵⁷)	No conserved domain	Hypothetical	No	NA
<i>Me23</i>	<i>Me_SG130</i>	636	ACYPI002439 (10 ⁻¹²²)	<i>Mp</i> Contig1685 (10 ⁻⁹³)	Glutathione peroxidase	Glutathione peroxidase	Yes	No
<i>Me25</i>	<i>Me_SG229</i>	918	ACYPI006300 (10 ⁻¹⁷⁷)	<i>Mp</i> Contig2442 (10 ⁻⁸¹)	Carbonic anhydrase alpha (vertebrate-like)	Carbonic anhydrase 2-like	No	No

^a *M. euphorbiae* expressed sequence tag identification number.

^b Given in bp, excluding the signal peptide.

^c *N. b.* = *Nicotiana benthamiana*; NA = not tested.

Table 3.2. Gateway primers for cloning in the expression vectors pEarleyGate100 and pVSP_PsSPdes

Contig	Forward primer (5'-3')	Reverse primer (5'-3')
Me5	GGGGACAAGTTTGTACAAAA AAGCAGGCTAACAATGAATA ATCAGGAATTTGTTCAATTT	GGGGACCACTTTGTACAAGAAA GCTGGGTTCAGTTTCCGGTGCA TAGGCGAATAAGACG
Me10	GGGGACAAGTTTGTACAAAA AAGCAGGCTAGTGTACTAGA AGGAACAATGCAATCAATA	GGGGACCACTTTGTACAAGAAA GCTGGGTTTATGCTCCAACGAC TGTTGGTTGGGAC
Me13	GGGGACAAGTTTGTACAAAA AAGCAGGCTCGGACAAC TAG TAACAATGAGTTTGTTTCC	GGGGACCACTTTGTACAAGAAA GCTGGGTTTATCCGTTGTTGGT CGAAGATACATTTTT
Me14	GGGGACAAGTTTGTACAAAA AAGCAGGCTTGTTCCAAACA ATGCAGTTTCTAATAT	GGGGACCACTTTGTACAAGAAA GCTGGGTATATTACATAGGTAG TAATATATAAGCTTA
Me17	GGGGACAAGTTTGTACAAAA AAGCAGGCTACCAGCAGGTG TGCAACAATGGAACCGAC	GGGGACCACTTTGTACAAGAAA GCTGGGTGAGTTGGTGTATAAC GTCAACG
Me20	GGGGACAAGTTTGTACAAAA AAGCAGGCTATAACAATGTG GCCGGGATTCACCGTGGTC	GGGGACCACTTTGTACAAGAAA GCTGGGTATCGGTTCGTACTTC CTGGGATTTTGAGTG
Me23	GGGGACAAGTTTGTACAAAA AAGCAGGCTAACAATGAGGG TTTCCCAAGGATTTCCCTA	GGGGACCACTTTGTACAAGAAA GCTGGGTGTTTACGTAACATAA TTAGCAACATTGGTCC
Me25	GGGGACAAGTTTGTACAAAA AAGCAGGCTGCAACAATGTC ACCAAGTCACGGAGAAAACG	GGGGACCACTTTGTACAAGAAA GCTGGGTTCAGAATAGAGCTAG AGTAGCGAGTTTG
GUS	GGGGACAAGTTTGTACAAAA AAGCAGGCTCCATGGTCCGT CCTGTAGAAAC	CACCACTTTGTACAAGAAAGCT GGGTCTTATTGTTTGCCTCCCTG CT

Table 3.3. Primers used for gene expression analysis.

Contig	Forward primer (5'-3')	Reverse primer (5'-3')
Me5	CATTCAAGGCCTAGACAG GAC	TCTTCTCAAGTCGTTTGTCCC
Me10	CAAGACCAAAAATAAGT TGTTCT	GACATTGCGTTAAGAAGATCG
Me13	GAAGTCAATTGGCCATTA AACAA	CCATTTCTGCATTATTTCTGG
Me14	GTCATTTCGAGAGCGTACC AT	CAAAAGGACTCGATCGGTTG
Me17	GTTTGACGAGATGGACTT GCTTA	GTATCTCAACCTTCTTTTCAGCC
Me20	GAACCGAAGACGACCTC ATTCT	CAAGTCTCCATGGGCATCG
Me23	GGAAATTTGTGTAAATGG CAGA	CATTGGTCCTTTAACAGTTCTTG
Me25	CATGGACAATAACACA CAATTC	GAGCTAGAGTAGCGAGTTTGCA AC
L27	CCGAAAAGCTGTCATAAT GAAGAC	GGTGAAACCTTGTCTACTGTTAC ATCTT
MeC002	GAGCAGGAAGAAGCGTC TGT	CTTGGTGGGAGCATTGGTTA
MeSuc	GAGATCGATCCTATTTAT GGC	CATTCCATTCCCACGGAGATC

CHAPTER FOUR

Potato aphid *Me10* effector interacts with the tomato defense component 14-3-3

Abstract

Aphid saliva contains large number of proteins, which are delivered into plant apoplast and cells to modulate plant responses to aphid's advantage. The salivary gland transcriptome of the potato aphid (*Macrosiphum euphorbiae*) was sequenced and several proteins were predicted to be secreted based on the presence of an N-terminal signal peptide. Among these, was a putative protein encoded by *Me10*. *Me10* is an uncharacterized protein and has no known function. Expressing *Me10* in tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* enhanced aphid reproduction. To determine *Me10* tomato target(s), a yeast two-hybrid screen was performed and seven proteins were identified as potential interactors. Among these were the tomato scaffold proteins Fourteen-Three-Three isoform 7 (TFT7), which is known to be involved in plant immune signaling. Cellular localization and immunoprecipitation analyses confirmed *in planta* interaction between *Me10* and TFT7. *TFT7* is up regulated transiently after aphid feeding. Our results indicate that aphid salivary protein *Me10* directly targets a plant defense component and this interaction likely plays a role in suppressing plant immune responses against aphids.

Introduction

Plants employ a number of defense strategies to protect themselves against pests and microbial pathogens. In addition to various physical and chemical barriers, plants utilize immune responses that are activated in response to these intruders. Plants use surface localized pattern recognition receptors (PRRs) to recognize conserved microbe-associated molecular patterns (MAMPs) and this recognition activates pattern-triggered immunity (PTI) (Boller and He 2009, Jones and Dangl 2006). PTI is characterized by activation of basal defense including downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) cascade, production of reactive oxygen species (ROS), callose deposition, and defense gene activation (Boller and Felix 2009, Zipfel 2009). To suppress PTI, pathogen and pests deliver effector molecules into the host cell (Hogenhout *et al.* 2009, Muthamilarasan and Prasad 2013). In response, plants have evolved another layer of defense signaling known as effector triggered immunity (ETI) (Jones and Dangl 2006). ETI involves recognition of pathogen/pest avirulence effectors by host disease resistance (R) proteins and is typically characterized by hypersensitive response (HR) or programmed cell death limiting the growth and invasion of the pathogen (Jones and Dangl 2006).

Aphids are important agricultural pests that predominantly feed on the plant phloem sieve element. To reach the phloem, aphids use their stylets, slender syringe-like mouthparts, to navigate between different layers of cells in the leaf tissues. Aphid feeding induces defense responses such as clogging of phloem sieve elements, callose formation, and transcriptional reprogramming of various defense response genes (de Vos *et al.* 2005,

Moran *et al.* 2002, Moran and Thompson 2001). Similar types of defense responses are also induced by infiltration of aphid saliva into leaf tissues. Infiltration of green peach aphid (GPA, *Myzus persicae*) saliva into Arabidopsis leaves induced aphid resistance and defense response genes (de Vos and Jander 2009). Infiltration of leaves with different molecular weight aphid salivary fractions revealed that the defense elicitor is a proteinaceous molecule within a size range of 3-10 kDa (de Vos and Jander 2009).

Aphids secrete two types of saliva while feeding: gelling and watery (Miles 1999). As soon as gelling saliva gels is released from the stylet tip it forms a proteinaceous sheath around the stylets inside the plant tissue. Watery saliva on the other hand is mainly secreted in the plant phloem cells but is also secreted in the apoplast and in the cells along the path due to cell puncture during stylet probing and penetration (Tjallingii 2006). To identify aphid salivary components, aphid saliva is collected by feeding the insects on artificial diets contained in a parafilm sachet in feeding chambers (Miles 1965). Profiling gelling and liquid salivary proteins from aphids, collected from different diets, on SDS- polyacrylamide gel electrophoresis (PAGE), Will and coauthors indicated that aphids are able to adapt salivary secretion based on the stylet tip environment (Will *et al.* 2012).

To identify salivary secretions, the transcriptome of salivary glands from several aphid species have been sequenced and subjected to bioinformatics analysis to predict the presence/absence of secretion signal peptides. Based on this approach, the putative salivary proteins of the pea aphid (*Acyrtosiphon pisum*) (Carolan *et al.* 2011), potato aphid (*Macrosiphum euphorbiae*) (Atamian *et al.* 2013) and GPA (Bos *et al.* 2010) have

been reported. Potato aphid salivary gland transcriptome was developed using Illumina technology and the contigs were assembled using Velvet/Oases assembler (Atamian et al, 2013). Out of 460 gland transcriptome contigs, 125 were predicted to have putative signal peptides predicted by SignalP 4.0. Eight of these candidate effector proteins were functionally characterized in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*–mediated transient overexpression. Five of the eight proteins were also characterized in tomato by delivering them into plant cells through the *Pseudomonas syringae* type-three secretion system (T3SS) (Atamian et al. 2013). One of these proteins Me10 enhanced both GPA performance on *N. benthamiana* and potato aphid performance on tomato (Atamian et al, 2013). Me10 was also identified in the saliva of potato aphid collected in feeding chambers using mass spectrometry as described in Chapter One. *Me10* encodes a previously uncharacterized protein and homologs exist in many aphid species. The wide prevalence of Me10 among aphid species and the ability to enhance aphid performance on two different hosts suggest a role for this protein in aphid virulence.

One of the best-characterized aphid salivary proteins to date is C002. *C002* was identified as the most abundant transcript in the salivary gland cDNA library of pea aphid (Mutti 2006). It has been shown that *A. tumefaciens*–mediated transient overexpression of MpC002 (*M. persicae* C002) enhances GPA performance in *N. benthamiana* (Bos et al. 2010). In contrast, GPA fecundity decreased after feeding them on *N. benthamiana* leaf discs transiently producing *MpC002* dsRNA and on transgenic Arabidopsis stably producing *MpC002* dsRNA (Pitino *et al.* 2011). Similarly, RNAi-mediated knock-down

of *ApC002* (*A. pisum* *C002*) by injecting *C002* siRNA reduced pea aphid reproduction, survival and feeding ability on a host plant (Mutti *et al.* 2008, Mutti *et al.* 2006).

A few other aphid salivary proteins have also been characterized. *A. tumefaciens*–mediated transient overexpression of Mp10 (no relationship to Me10) and Mp42 reduced GPA performance in *N. benthamiana* (Bos *et al.* 2010). Mp10 overexpression also suppressed the flagellin-derived peptide flg22 mediated ROS burst, triggered SGT1 dependent chlorosis in *N. benthamiana* and conferred avirulence on *N. tabacum* (Bos *et al.* 2010). SGT1 is a co-chaperone required for most *R*-gene mediated HR. Recently it has been shown that transient over-expression of Mp10 activates jasmonic acid- and salicylic acid- defense signaling pathways and reduces susceptibility to the hemibiotrophic oomycete pathogen *Phytophthora capsici* (Rodriguez *et al.* 2013). These results suggest that Mp10 could be playing a dual role in suppressing and activating plant defense responses by some uncharacterized pathways.

Aphid effectors act in a species-specific as well as host-specific manner. For example, GPA reproduction increases on transgenic Arabidopsis plants expressing the GPA MpC002, and the putative secreted proteins Mp1 (PIntO1) and Mp2 (PIntO2) (Pitino and Hogenhout 2013). In contrast, GPA reproduction did not increase on Arabidopsis expressing the pea aphid orthologs of these proteins (Pitino and Hogenhout 2013). *PIntO1* and *PIntO2* were identified in the salivary gland transcriptome of the GPA and encode unknown proteins with secretion signal peptides (Bos *et al.* 2010). In addition, transient over-expression of the GPA *PIntO1* and *PIntO2* in *N. benthamiana* also did not affect GPA reproduction. Similarly, in our previous study we showed that

transient overexpression of potato aphid effectors Me10 and Me23 enhanced GPA performance in *N. benthamiana* but only Me10 enhanced potato aphid performance in tomato (Atamian et al. 2013). Taken together, these data indicate that effectors from different aphid species do not trigger similar responses on the same host plant and different plant hosts have distinct abilities for recognition the same aphid effector.

In the past few years, our understanding about aphid effectors and their role in manipulating plant defense responses has greatly increased, but the molecular mechanism by which these effectors alter plant immune responses remains largely unknown. There are cues that aphid effectors behave in a similar manner as microbial effectors by targeting plant defense signaling components (Bos et al. 2010, Rodriguez et al. 2013). However, aphid effector direct host targets has not been identified. In this study, we functionally characterize the role of potato aphid effector *Me10*. Using a yeast two-hybrid screen, we identified several Me10 interactors, among these the Tomato Fourteen-Three-Three isoform 7 (TFT7) has known functional roles in plant defense. The interaction between TFT7 and *Me10* was confirmed *in planta* by immunoprecipitation. *TFT7* expression is up regulated early in response to aphid feeding suggesting it could be an aphid virulence target.

Material and Methods

Plants used and growth conditions

Tomato (*S. lycopersicum*) cultivars UC82B (*mi/mi*), Motelle (*Mi/Mi*) and Moneymaker (*mi/mi*), and *N. benthamiana* were used. Seedlings were transplanted into California mix II. Plants were maintained in growth rooms at 24°C with a 16-hr-light and 8-hr-dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and were fertilized weekly with MiracleGro (18-18-21; Stern's MiracleGro Products, Port Washington, NY, U.S.A.).

Aphid colony and time course aphid infestations

A colony of the parthenogenetic potato aphid was reared on tomato cultivar UC82B. The colony was maintained in insect cages in a pesticide-free greenhouse at 22 to 26°C supplemented with light for a 16-hr day length. Time course aphid infestation experiments were performed as described in (Bhattarai *et al.* 2010).

Yeast constructs and two-hybrid screen

Gene-specific primers were designed to amplify *Me10* (JX134488) sequences corresponding to the ORF encoding the mature protein excluding the signal peptide sequences (Table 4.1). The amplified product, obtained using Phusion high-fidelity polymerase (New England BioLabs), was cloned into the pDONR207 (Invitrogen, Carlsbad, CA), recombined into the destination bait vector pGBKT7 Gal4-BD (Clontech, Madison, WI), and sequenced using Sanger sequencing. This plasmid was transformed

into *Saccharomyces cerevisiae* Y2H Gold yeast strain (Clontech). Yeast transformation was performed by LiAc/PEG method as described in the Yeast Protocols Handbook (Clontech). Yeast transformants were screened on Synthetic Defined (SD) media lacking tryptophan (SD-T). Gal4-BD-Me10 bait construct was screened for toxicity and autoactivation using manufacturer's protocol (Clontech).

Prey cDNA library was prepared from tomato leaves infested with potato aphids. Tomato plants of cultivar *Mi/Mi* and *mi/mi*, potato aphid resistant and susceptible cultivars, respectively, were infested with mixed developmental stages of aphids. Leaf samples were harvested at 0, 12, 24 and 48-hr post infestation (hpi) and total RNA was extracted using Trizol. RNAs from each time point were pooled together and cDNA library was prepared using SMART cDNA Library Construction Kit (Clontech). The library was transformed into *S. cerevisiae* Y187 yeast strain and primary transformants were screened on SD media lacking leucine (SD-L). Colonies were scraped from plates, washed, suspended in 15% glycerol, and stored in 1-ml aliquots at -80°C. The tomato cDNA library was prepared by Dr. Tokuji Tsuchiya.

The prey cDNA library in pGADT7 vector was mated overnight with bait *Me10* in pGBKT7 vector following manufacturer's protocol (Clontech) by slowly shaking the cells at 30°C for 20-hr. Overnight mated cells were plated on high stringency selection media containing SD media lacking leucine, tryptophan, histidine, arginine (SD-LTHA) supplemented with antibiotics aureobasidin A (AbA) and kanamycin. The colonies that grew were rescreened individually on high stringency selection media.

To confirm the presence of prey cDNA, colony PCR was performed on the rescreened colonies. To identify duplicate clones, PCR products were subjected to restriction digest using the 4-bp cutter *RsaI* restriction enzyme.

To confirm interaction, individual pGADT7-prey plasmids were isolated and retransformed into *S. cerevisiae* Y2H Gold yeast strain containing Gal4-BD-Me10. Transformants were plated on SD-LTHA + AbA selection media and yeast growth was observed for the next three days. To eliminate false positives, all the positive prey cDNAs that interacted with Gal4-BD-Me10 were also mated with Gal4-BD-p53 bait construct provided with the Clontech kit. Additionally, T-antigen prey vector, provided with the kit, was also mated with Gal4-BD-Me10 to confirm the specificity of Me10 interactions with the prey plasmids.

Me10 bait construct expression analysis in yeast

Gold yeast strain expressing Gal4-BD-Me10 bait construct were streaked out on SD plates lacking tryptophan. Two days later, 2-3 medium sized colonies were re-suspended into a lysis buffer (0.5 M NaOH, 2-mercaptoethanol) and crude protein extracts were electrophoresed through a 12% SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad). Membrane was incubated with a 1:2000 dilution of anti-Myc antibody (Sigma-Aldrich) followed by anti-Mouse IgG-HRP conjugate (Bio-Rad) at a concentration of 1:3000. Signals were visualized with an enhanced chemiluminescent substrate (Thermo Scientific) following manufacturer's instructions and exposed to the

X-ray film. The expected molecular weight for Gal4-BD fused to Me10-Myc is approximately 36.5 kDa.

Constructs for *in planta* cellular localization and confocal microscopy

To create Me10-Yellow Fluorescent Protein (YFP)-tagged construct, full-length *Me10* gene lacking the signal peptide and C-terminal stop codon, but with added start codon, was PCR amplified using gene-specific primers (Table 4.1) and Phusion high-fidelity polymerase (New England BioLabs). The PCR product was cloned into pDONR207 vector followed by recombination into the binary vector pEarleyGate 101 (Earley *et al.* 2006). Full-length CDS of *TFT7* (X95905) was amplified from tomato cDNA using gene-specific primers and cloned into pEarleyGate 102 binary vectors with a C-terminal Cyan Fluorescent Protein (CFP) tag (Table 4.1).

Both binary plasmids were transformed into *A. tumefaciens* strain GV3101. Strains were grown overnight at 28°C in Luria Broth medium containing the appropriate antibiotics (gentamycin 14 µg/ml, rifampicin 25 µg/ml and kanamycin 50 µg/ml). Bacteria were collected, resuspended in infiltration media (10 mM MES, pH 5.6, 10 mM MgCl₂, and 200 mM acetosyringone), adjust to an OD₆₀₀ = 0.5 and incubated 4-hr in dark at room temperature before inoculation. Equal volumes of Me10 and TFT7 *A. tumefaciens* strains were mixed before infiltration. Leaves were hand-inoculated using a needle-less syringe and plants were incubated at room temperature under continuous low light for 2 to 3 d.

Leaf samples expressing the tagged constructs were visualized using 40x water objective lens on a Leica SP5 inverted confocal microscope. Microscopy was performed using 436- and 517-nm filters to excite the CFP or YFP, respectively, and images were collected through band emission filters at 488- and 527-nm, respectively.

Constructs and assays for immunoprecipitation in *N. benthamiana*

The full-length Me10-YFP construct described above also express a C-terminal hemagglutinin (HA) tag and was therefore used for immunoprecipitation. *TFT7* was cloned into binary vector p35S:GATA-HF (Mustroph *et al.* 2010) possessing a C-terminal FLAG-Histidine (His) tandem tags. For the co-immunoprecipitation assay, proteins were coexpressed in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression assay as described above. Forty-eight hpi; leaf samples were harvested and frozen in liquid N₂. For protein extraction, samples were ground in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM NaF, 1 mM Na₂MnO₄, 10% glycerol, 1% PVPP, 1% Nonidet P40, 10 mM DTT and plant protein protease inhibitor cocktail (33 µl/ 1gm tissue) [Roche]). Samples were solubilized in extraction buffer and centrifuged for 15 min at 12,000 g at 4°C. This step was repeated twice. For individual protein expression, 5 µg total protein was loaded in two separate lanes on 12% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad). Immunodetection was performed with a 1:2,000 dilution of horseradish peroxidase–conjugated anti-HA or anti-FLAG antibodies (Sigma-Aldrich), visualized with an

enhanced chemiluminescent substrate (Thermo Scientific) following manufacturer's instructions, and exposed to an X-ray film.

For co-immunoprecipitation analysis, protein concentration was adjusted to 1 mg/ml in a 1-ml volume. The protein extract was incubated overnight at 4°C with 20 µl of FLAG M2 Magnetic Beads (Qiagen) with gentle rocking. Immunoprecipitated proteins were washed three times with extraction buffer. Proteins were eluted with 50 µl sample buffer (100 mM glycine, pH 3.0) and 5 µg of protein was loaded on 12% SDS-PAGE, electrotransferred to a nitrocellulose membrane (Bio-Rad) and gel blot was processed using anti-HA antisera as described above.

RNA extraction and tissue specific expression

Leaf, stem and root samples were harvested from five-week-old tomato plants, whereas flowers were harvested from 10-week-old plants and stored in liquid N₂ until ready for processing. RNA was extracted using TRIzol (Invitrogen) and treated with DNase I (New England Biolabs). For each sample, 2 µg RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo-dT primer. For quantitative PCR, transcripts were amplified from 1 µl of 5× diluted cDNA in a 15µl reaction using gene-specific primers (Table 4.1) and iQ SYBR Green Supermix (Bio-Rad). PCRs were performed with two biological replicates each with two technical replicates. The PCR amplification consisted of 3 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C, 15 min at 72°C, followed by the generation of a dissociation curve. The

generated threshold cycle (C_T) was used to calculate the transcript abundance relative to tomato *Ubiquitin (Ubi3)* gene (Bhattarai et al. 2010).

Results

Me10 interacts with tomato scaffold proteins

The potato aphid salivary protein Me10 enhances aphid fecundity when expressed in tomato and *N. benthamiana*. Me10 is uncharacterized protein with no known function and homologs seem to be present in a large number of aphid species as well as in corn borer (*Ostrinia nubilalis*) and the bacterium *Clostridium sordellii*. To elucidate how Me10 enhanced aphid fecundity, plant targets of Me10 were identified using a yeast two-hybrid screen. A Matchmaker Gold yeast two-hybrid library from resistant and susceptible tomato leaflets, healthy and infested with potato aphids, was developed for this purpose. This library was used as the prey and the Gal4-BD-Me10 served as the bait. Expression of Gal4-BD-Me10 protein in the Gold yeast strain was confirmed by western blot using Myc antisera and empty vector Matchmaker Gold yeast strain was used as a negative control (Fig. 4.1).

We screened six million primary yeast transformants and identified 58 positive interactors. Using restriction digests, 33 distinct clones were identified. From these clones, plasmids were isolated and re-mated with Gal4-BD-Me10 bait construct. Seventeen clones were found to be true interactors. Sequencing these clones indicated that cDNAs represented seven different proteins (Table 4.2). Six tomato interactors (SGN-U580341, SGN-U591656, SGN-U298236, SGN-U572916, SGN-U582594 and SGN-U577153) shared homology with proteins containing a Rieske domain, a chlorophyll A/B binding protein, a BODYGUARD 1 domain, a Ribokinase, a WD-40

and a Copper-Zinc superoxide dismutase, respectively (Table 4.2). These interactors were represented by partial cDNAs and were not further characterized in this study.

Interestingly, the other cDNA clones encoded Tomato 14-3-3 isoform 7 (TFT7) known to be involved in immune signaling and therefore was selected for further analysis. Nine different cDNAs encoding TFT7 were detected in the screen, most of these cDNAs represented almost full-length TFT7 ORF. Me10 binding with TFT7 was strong as yeast growth was observed within three days on SD- LTHA media (Fig. 4.2).

Localization of Me10 and TFT7 *in planta*

We next examined the localization patterns of Me10 and TFT7 *in planta*. Co-localization of the two proteins in the same cellular compartments would indicate that there is a high possibility that they could interact within the plant cell. Both Me10 and TFT7 were tagged with fluorescent-tagged fusion proteins (Me10-YFP and TFT7-CFP) and were overexpressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Protein localization was then determined using confocal microscopy. Both Me10-YFP and TFT7-CFP were localized to the cytoplasm as well as nucleus (Fig. 4.3). The size of Me10-YFP fusion protein is about 46.5 kDa and that of TFT7-CYP fusion protein is about 60.5 kDa. The size limit for passive diffusion of molecules through the nuclear pores is about 40 kDa (Merkle 2003), therefore, eliminating the possibility of random diffusion of Me10 and TFT7 proteins into the nucleus. Our results demonstrate that the localization pattern of Me10 overlaps with its putative binding partner TFT7, and suggests that these proteins could physically interact with each other within the plant cell.

Me10 interacts with TFT7 *in planta*

To confirm Me10 and TFT7 interaction *in planta*, *N. benthamiana* extracts were used in a FLAG M2 affinity pull-down assay. *N. benthamiana* leaves were hand-infiltrated with 2×10^8 cfu/mL suspension of *A. tumefaciens* coexpressing Me10-YFP-HA and TFT7-FLAG. Total plant protein was isolated from leaves 48 hpi, and Me10-TFT7 complex was pull-down using FLAG M2 magnetic beads and the interaction was confirmed using anti-HA antibodies. Protein gel blot analysis showed that Me10-YFP-HA co-purified with TFT7-FLAG (Fig. 4.4). Taken together these studies demonstrate that Me10-YFP-HA physically associates with TFT7-FLAG in plant extracts.

TFT7 is ubiquitously expressed in tomato tissues

TFT7 tissue-specific expression pattern has not been evaluated previously. To examine *TFT7* expression pattern, transcript levels were measured in different tissues of tomato plant using quantitative real-time (qRT-PCR). *TFT7* transcripts were detected in all tissues tested although with variable levels of expression (Fig. 4.5). *TFT7* is preferentially expressed in shoots, with the highest expression levels in flower, followed by stem and leaf.

TFT7 is induced after aphid feeding

Earlier it was shown that *TFT7* is a positive regulator of defense (Oh *et al.* 2010). To explore the possibility that *TFT7* is also regulated by aphid feeding, we examined the temporal expression pattern of *TFT7* in leaves of susceptible tomato plants at multiple

time points after potato aphid infestation using qRT-PCR. The transcript levels of *TFT7* were significantly up-regulated at 6-hr and 12-hr after aphid infestation (Fig. 4.6).

Discussion

In this study we isolated putative Me10-interacting proteins and confirmed the interaction *in planta* with one of them. We performed yeast two-hybrid screen of a tomato cDNA library prepared from healthy and aphid-infested tomato leaves and identified seven different proteins to physically interact with Me10 (Table. 4.2). Interestingly, a subset of these proteins is known to be involved in plant defense and metabolic processes. TFT7 was selected for further analysis because of its known role in plant immunity (Oh et al. 2010). The remaining Me10 plant interactors will be characterized in the future.

In planta localization of Me10 and TFT7 proteins revealed that both proteins are localized to the cytoplasm and nucleus (Fig. 4.3). As the localization pattern of these two proteins overlapped, this suggests that these proteins have the potential to physically interact with each other *in planta*. The physical interaction between Me10 and TFT7 proteins was confirmed by co-immunoprecipitation where both proteins were found in the same complex (Fig. 4.4).

14-3-3 proteins are present in eukaryotes as diverse as fungi, human and plants (Darling *et al.* 2005, Roberts 2003). Within an organism, 14-3-3s constitute a set of diverse members of a protein family. These proteins can form homo- and heterodimers and the dimerized structure is capable of binding to two different proteins serving as adapters or scaffolds between the proteins (Bridges and Moorhead 2005, Roberts 2003). 14-3-3 proteins are involved in various biological processes including signal transduction, regulation of carbon and nitrogen metabolism, transcription, apoptosis, protein trafficking, plant growth and development and stress responses (Darling et al.

2005, Roberts 2003, Sehnke *et al.* 2002). In plants, 14-3-3 proteins were first identified in maize and barley (Brandt *et al.* 1992, de Vetten *et al.* 1992). There are 15 14-3-3 isoforms in Arabidopsis (Rosenquist *et al.* 2001), while only 12 members exist in tomato including TFT7 (Xu and Shi 2006).

14-3-3 binds to various proteins at one of three known discrete binding motifs, which are phosphorylation dependent. Mode I consensus motif is RXXpS/pTX, mode II is RXXXpS/pTXP and mode III consensus motif is RXXpS/pTX-COOH, where X can be any amino acid and p indicates the site of phosphorylation (Bridges and Moorhead 2005, Smith *et al.* 2011). None of these three binding motifs are present in Me10. Although uncommon, studies have shown that in addition to binding with proteins containing these phosphorylated motifs; 14-3-3s also bind to nonphosphorylated substrates (Bridges and Moorhead 2005). These substrates may contain negatively charged amino acids such as glutamic and aspartic acid residues which can mimic the phosphorylated serine and explain how 14-3-3 bind to these proteins (Hallberg 2002). In a small number of these non-phosphorylated substrates, interaction has been narrowed down to small divergent region with no consensus (Campbell *et al.* 1997, Henriksson *et al.* 2002, Higashida *et al.* 2001, Petosa *et al.* 1998). In our analysis, we used full-length Me10. No Me10 deletion mutants have yet been tested for TFT7 interaction. Therefore, Me10 deletion analysis should be a priority to narrow down the TFT7 interaction region.

Interestingly, members of 14-3-3 proteins are also involved in plant-pathogen interactions. Increase in 14-3-3 transcript levels have been reported at early time points after a number of bacterial and fungal pathogens. An increase in transcript levels of 14-3-

3 at 6-hr and 8-hr after infection has been reported in barley leaves by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Brandt et al. 1992, Gregersen *et al.* 1997). Similarly, an increase in 14-3-3 transcript levels have been described in soybean leaves infected with *P. syringae* pv. *glycinea* (Seehaus and Tenhaken 1998), as well as in tomato by the fungal pathogen *Cladosporium fulvum* toxin fusicoccin (Roberts and Bowles 1999). An early increase in *TFT1* transcript levels was reported in response to a strain of the bacterial pathogen *Xanthomonas campestris* lacking the T3SS but not in strains with T3SS suggesting a positive regulator for *TFT1* in PTI (Taylor *et al.* 2012). Our results showed that aphid feeding enhances transcript levels of *TFT7* at 6-hr and 12-hr after aphid infestation, possibly suggesting a role for *TFT7* as positive regulator in aphid PTI. A role for *TFT7* or 14-3-3 in plant-herbivore interactions in aphid resistance has not been reported previously.

14-3-3s can also act as negative regulators of plant defense responses. In rice, silencing GF14e (14-3-3 isoform), led to accumulation of ROS and enhanced resistance to the bacterial pathogen *X. oryzae* and the fungal pathogen *Rhizoctonia solani* (Manosalva *et al.* 2011).

Additional studies indicate that plant 14-3-3s are also involved in ETI. In Arabidopsis, the 14-3-3 λ protein, GF14 λ , was reported to interact with the R protein RPW8.2 that confers broad-spectrum resistance to the fungal powdery mildew pathogen (Yang *et al.* 2009). The T-DNA GF14 λ mutant plants were compromised in basal and RPW8.2-mediated resistance to powdery mildew. Over-expression of GF14 λ resulted in constitutive HR and enhanced resistance to powdery mildew indicating GF14 λ is a

positive regulator of ETI (Yang et al. 2009). In tomato, TFT7 interacts with MAPK Kinase Kinase α (MAPKKK α) and is required for HR caused by the interaction between the R protein Pto and its cognate avirulence effector AvrPto (Oh et al. 2010). Interestingly, TFT7 also interacts with the MAPK Kinase which acts downstream of MAPKKK α (Oh and Martin 2011). In this study they showed that TFT7 forms homodimerizes *in vivo*, and may possibly act as a scaffold between MAPK Kinase and MAPKKK α proteins for signal transfer. The interaction between TFT7 and both of these client proteins is through mode II motif (Oh and Martin 2011). Since the MAPK pathway is downstream of a number for *R*-mediated resistance signaling, *TFT7* is required for and is an important component for *R*-gene mediated resistances (Oh et al. 2010).

TFTs are known virulence target of phytopathogenic bacterial. *P. syringae* effector HopQ1. HopQ1 interacts with multiple tomato TFTs while HopM1 interacts with the Arabidopsis GF14 κ (an isoform of 14-3-3 in Arabidopsis) (Li et al. 2013, Nomura et al. 2006). Both HopQ1 and HopM1 encode proteins with no known function, however, HopM1 have been shown to degrade a host protein via the proteasome (Nomura et al. 2006). In addition to the *P. syringae* effectors, the bacterial pathogen *X. campestris* effector XopN targets tomato TFT1 to suppress PTI (Kim et al. 2009, Taylor et al. 2012). Both HopQ1 and XopN interact with the TFTs in phosphorylation-dependent manner, HopQ1 uses mode I while XopN uses mode II motifs (Li et al. 2013, Taylor et al. 2012).

It has been hypothesized that TFTs can act as scaffold proteins that facilitate effector virulence activity within the plant cell. Alternatively, TFTs may act as positive regulators of plant defense targeted by effectors to promote virulence, and lastly, act as

negative regulator of defense whose activity is positively regulated by effectors.

Additional experiments such as silencing *TFT7* in tomato and assaying for aphid performance will shed light on which one of these three possible roles does TFT7 play in aphid defense.

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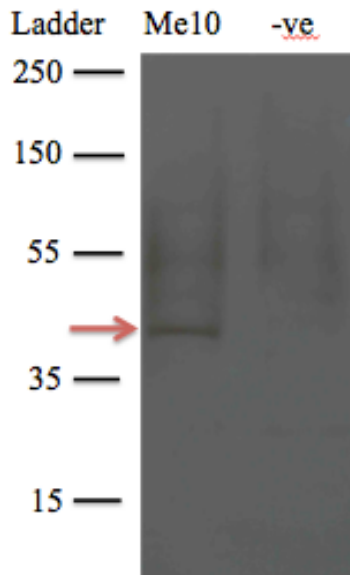


Fig. 4.1. Me10 is expressed in yeast. Gel blot analysis of proteins isolated from the Gold yeast strain expressing Me10. Gel blot analysis was performed using Myc antisera. The expected molecular weights for Gal4-BD fused to Me10 is approximately 36.5 kDa. An arrowhead indicates the corresponding protein. Empty vector Matchmaker gold yeast strain was used as negative control,

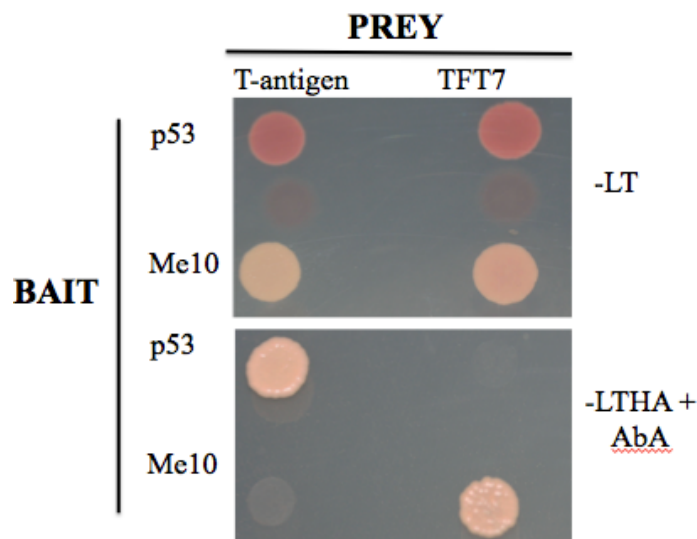


Fig. 4.2. Me10 interacts with TFT7 in yeast. Matchmaker gold yeast strain carrying pGBKT7 vector containing Me10 or p53 (negative bait construct) were transformed with the prey vector pGADT7 carrying TFT7 or T-antigen (negative prey construct). P53 interacts with T-antigen. Strains were spotted on nonselective (SD-LT) and selective (SD-LTHA) media supplemented with 0.1ug/ml AbA and incubated at 30°C for 3 days.

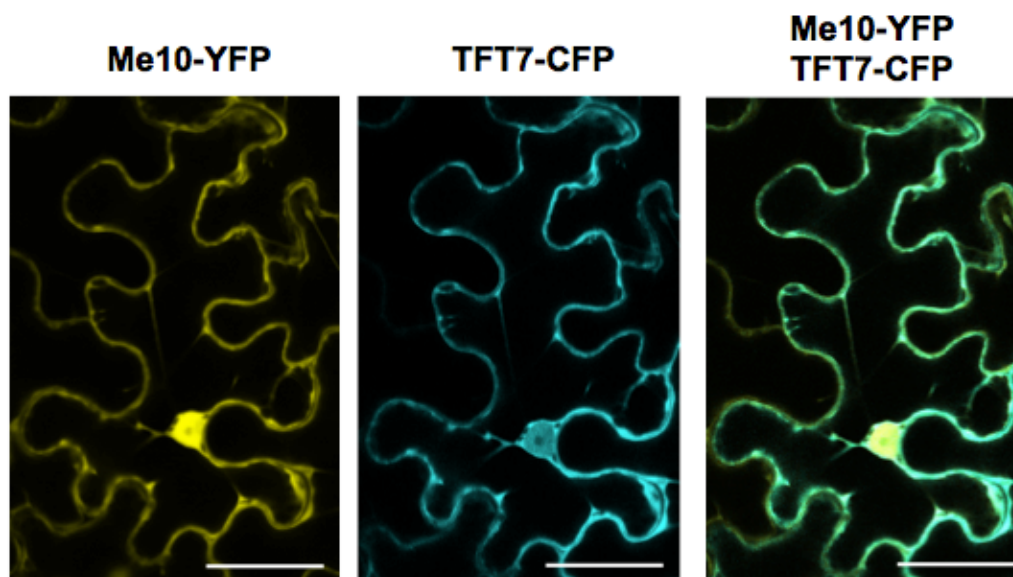


Fig. 4.3. Me10 and Tft7 subcellular localization *in planta*. *Agrobacterium*-mediated transient expression of Me10-YFP and Tft7-CFP in *N. benthamiana* leaves. Leaves were hand infiltrated with a suspension of two *A. tumefaciens* strains expressing Me10-YFP or Tft7-CFP. Leaf epidermal cells were imaged after 48-hr by confocal microscopy at 40x. Bars = 50 μ m. This experiment have been repeated twice.

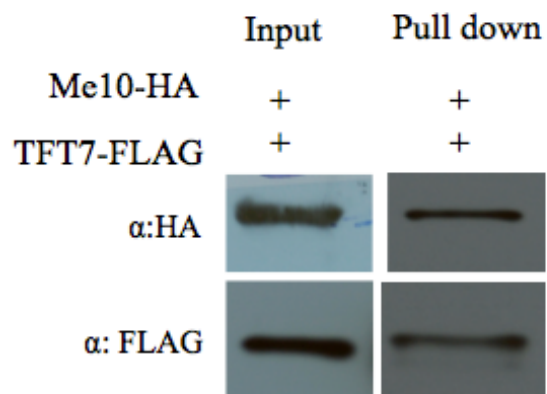


Fig. 4.4. Me10 interacts with TFT7 *in planta*. Pull-down analysis of Me10-HA and TFT7-FLAG in *N. benthamiana* extracts. *N. benthamiana* leaves were inoculated with a suspension of two *A. tumefaciens* strains expressing Me10-HA or TFT7-FLAG. After 48-hr, total protein was extracted, purified by FLAG M2 magnetic bead, and subjected to protein gel blot analysis using anti-HA or anti-FLAG sera.

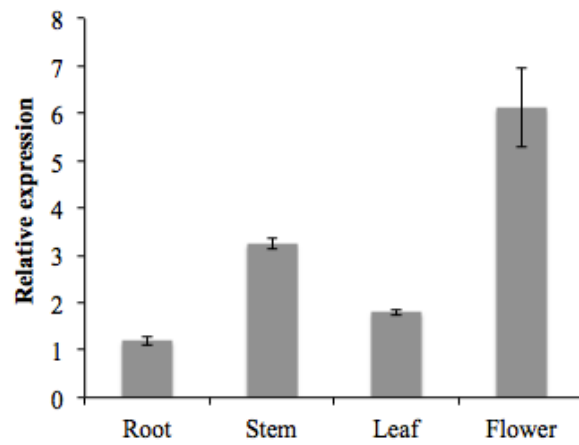


Fig. 4.5. Tissue-specific expression of *TFT7*. Transcript levels for *TFT7* were evaluated in four different tomato tissues/organ. Gene expression was quantified by qRT-PCR and normalized to *Ubi3*. Error bars represent $\pm$ SE of the mean of 2 technical replicates each.

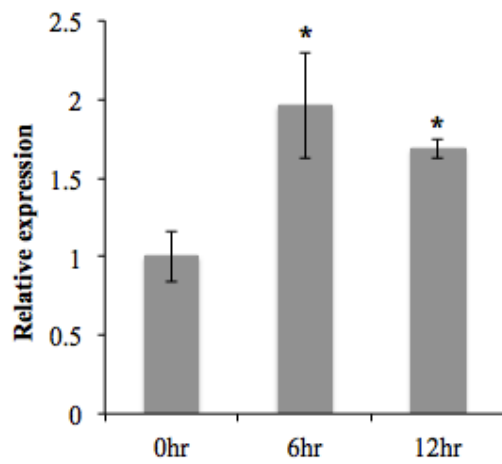


Fig. 4.6. *TFT7* expression is upregulated after aphid feeding. *TFT7* transcript levels were evaluated in tomato cultivar Moneymaker leaflets after aphid infestation. Gene expression was quantified by qRT-PCR, and levels at 0-hr time point were set to ‘1’ after normalization to *Ubi3*. Two independent experiments were performed with similar results and data from one are presented. Error bars represent $\pm$ SE of the mean of 2 technical replicates each. * indicates significant differences (Student’s *t*-test; $P < 0.05$).

Table 4.1 – Primers used in this study.

Primers	Sequences (5'-3')*	Purpose	Reference
Me10bait-F	GGGGACAAGTTTGTACAAAAAAGCA GGCT ^{cg} ATGCAATCAATACAACCATT AATAGAC	Gateway cloning of <i>Me10</i> bait construct	This study
M10bait-R	CACCACTTTGTACAAGAAAGCTGGGT TTATGCTCCAACGACTGTTGGTT	Gateway cloning of <i>Me10</i> bait construct	This study
Me10-Co-IP-F	GGGGACAAGTTTGTACAAAAAAGCA GGCTATGCAATCAATACAACCATTAA TAGACC	Gateway cloning of <i>Me10</i> Co-IP construct	This study
Me10-Co-IP-R	CACCACTTTGTACAAGAAAGCTGGGT ^g TGCTCCAACGACTGTTGGTT	Gateway cloning of <i>Me10</i> Co-IP construct	This study
TFT7-CoIP-F	GGGGACAAGTTTGTACAAAAAAGCA GGCTATGGAGAAGGAAAGAGAAAAA CAGG	Gateway cloning of <i>TFT7</i> Co-IP construct	This study
TFT7-CoIP-R	CACCACTTTGTACAAGAAAGCTGGGT ^g GTTCTCTCCCTGGCGCTCAT	Gateway cloning of <i>TFT7</i> Co-IP construct	This study
TFT7-qPCR-F	CACTTTGTGGACATCAGATCTTG	qPCR	This study
TFT7-qPCR-R	CAATAGGCATGAATCCCTGAAC	qPCR	This study
SIUbi3-F	GTGTGGGCTCACCTACGTTT	qPCR	(Bhattarai, et al. 2010)
SIUbi3-R	CCGTTTCATTGACAAAAAGAA	qPCR	(Bhattarai, et al. 2010)

* Letters in red are part of Gateway recombination

* Letters in blue are additional nucleotides for inframe cloning in the vector

Table 4.2. Me10-interacting proteins identified in a yeast two-hybrid screen using a tomato cDNA library.

Tomato gene ID	Annotation	Conserved domains NCBI	E value conserved domain	CDS length isolated	# of clones
SGN-U580341	Uncharacterized protein	Rieske domain	5.30E-16	198/828	1
SGN-U591656	Chlorophyll a-b binding protein CP26	Chlorophyll A-B binding protein	6.23E-74	357/858	1
SGN-U298236	Uncharacterized protein	BODYGUARD 1 domain containing hydrolase	3.94E-05	279/1347	2
SGN-U572916	Ribokinase-like	Ribokinase	4.48e-109	399/1263	1
SGN-U577153	Copper/zinc superoxide dismutase	Zinc superoxide dismutase	3.88E-148	276/897	1
SGN-U584943	TFT7	14-3-3 domain	5.78E-129	747/759	9
SGN-U582594	RACK1A	WD 40 domain	3.27E-83	175/981	2

General Conclusions

Many insects including aphids depend on plants for their food. Aphid feeding damages crop plants, trees and ornamentals and thereby causing huge losses throughout the world. Aphids also transmit viruses and make the plants diseased and non-edible for humans. For this reason it is important to protect the crop plants and trees from aphid infestation. The most common control methods for aphids includes use of insecticides, however, aphids have great ability to develop-resistance to these insecticides (Silva *et al.* 2012a, Silva *et al.* 2012b). Another approach is the use of disease resistant crop varieties but the number of aphid-resistant plant varieties is limited (Will and Vilcinskas 2013). Therefore, it is important to understand the plant-aphid interactions at the molecular level to develop an efficient pest control strategy.

Aphids are phloem-feeding insects and belong to order Hemiptera. They feed on the phloem sap, which is rich in sugars but deficient in nitrogenous amino acid supplements, and this shortfall is partly compensated for by the primary endosymbiotic bacterium *Buchnera aphidicola*. Aphids suck up plant nutrients by using their specialized piercing sucking mouthpart known as stylets. While feeding on the host plant, aphids secrete copious amount of saliva. Aphid saliva is of two kinds – soluble saliva and gelling saliva (Miles 1959). There is evidence that aphids can manipulate plants to their advantage by altering plant metabolism, reallocation of plant nutrients and possibly by suppressing plant immunity (de Vos *et al.* 2005, Giordanengo *et al.* 2010, Hogenhout and Bos 2011). Several studies have shown that aphid feeding induces various kinds of defense responses in plants (de Vos *et al.* 2007, Martinez de Ilarduya *et al.* 2003, Moran

and Thompson 2001). Additional studies have shown that aphid salivary components also play a similar role in modulating plant defense responses (Atamian *et al.* 2013, Bos *et al.* 2010, de Vos and Jander 2009, Mutti *et al.* 2008, Rodriguez *et al.* 2013). Therefore, identifying the proteins present in aphid saliva could potentially lead to an alternative control approach of aphid pest in crops.

Two different approaches have commonly been used to identify aphid-secreted proteins. The first is collecting the aphid saliva *in vitro* and identifying the protein content using proteomics approaches like mass spectrometry. The other is an indirect approach by analyzing the salivary gland transcriptome to identify the predicted secretory proteins using bioinformatics tools like SignalP. Both approaches have been used previously to identify the salivary proteins of several aphid species (Elzinga and Jander 2013, Hogenhout and Bos 2011). The potato aphid is one of the most damaging pests of tomato and potato crops worldwide. In this study, we identified the potato aphid salivary proteins using both these approaches as discussed in Chapter One and Three. We also performed functional characterization of a few of these aphid salivary proteins and this work is discussed in Chapter Two, Three and Four.

In previous investigations, researchers have used an *in vitro* system (Miles 1965) to feed aphids on artificial diets containing sugars and amino acids (Cherqui and Tjallingii 2000) to match the phloem sap composition. However, aphids secrete very little saliva in this *in vitro* feeding system. In addition, to analyze salivary proteins, elimination of sugars in the collected saliva is required; this usually is accomplished by filters that frequently cause loss of proteins. Therefore, to enhance potato aphid salivary secretions

we used the neurostimulant resorcinol in water diet and collected potato aphid soluble saliva. We also collected the watery and gelling saliva after aphids fed on water only diet. This work is summarized in Chapter One that is an equal collaboration work by myself and a former graduate student Hagop Atamian. We found a plethora of proteins present in the potato aphid saliva and some of these proteins were derived from endosymbiont *Buchnera*. Our analysis showed that presence of neurostimulant resorcinol in the aphid diet enhances their salivary secretions and therefore it can be successfully used to collect aphid saliva. Many of the proteins identified in the saliva were uncharacterized proteins and the ones with known annotations were proteins involved in sugar-, alcohol-, and lipid metabolisms, oxidative stress, heat shock proteins (HSP), chaperonins, ribosomal proteins and components of cytoskeleton. These unexpected proteins like HSPs, ribosomal proteins and cytoskeleton components were not predicted to be secreted and it is likely that could originate from organs other than the salivary glands such as the gut, the aphid component of the bacteriocyte or hemocoel and moved through the salivary glands and were delivered into the salivary secretory canal.

Among the several *Buchnera* origin proteins was the molecular chaperonin protein GroEL. In aphids, role of GroEL has been implicated in the transmission of viruses (Hogenhout *et al.* 2000). And interestingly, in mammalian systems several studies have demonstrated a role for GroEL in activating immune responses (Lamb *et al.* 1989). Therefore, we examined the role of GroEL in plant defense responses and the results are summarized in Chapter Two. We delivered GroEL intracellularly in plants, by bacterial type three-secretion system and evaluated the aphid performance

and bacterial titre on these plants. Our data suggested that GroEL induces resistance both against aphids and bacteria in both tomato and Arabidopsis plants. Additional experiments demonstrated that transgenic Arabidopsis plants expressing GroEL were also resistant to aphids and bacteria and these transgenic Arabidopsis plants showed increased expression of early and late defense-marker genes. Our data also suggested that GroEL protein, expressed and purified from *E. coli*, when applied extracellularly to Arabidopsis plants, enhanced resistance to aphids, induced early and late defense marker genes, induced production of reactive oxygen species and callose deposition. I also showed that this extracellular recognition of GroEL is dependent on the co-receptor BRASSINOSTEROID-INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1) as these defense responses were attenuated in the Arabidopsis *bak1* mutant plants. Our data suggested that GroEL is recognized both extracellularly and intracellularly *in planta* and this may reflect aphid salivation behavior during probing and feeding. Our findings indicated that plant-aphid interactions have a third element, the aphid endosymbiotic prokaryotic component, which induced plant immunity.

We also used a second approach, described above, to identify potato aphid putative secreted proteins. For this analysis, the potato aphid salivary gland transcriptome was sequenced and results are reported in Chapter Three that is an equal contribution work by a former graduate student Hagop Atamian and myself. RNA-Seq library was prepared from about 200 aphid salivary glands and sequencing was performed using Illumina technology. By utilizing the pea aphid genome (Consortium 2010), the putative

full-length pea aphid orthologs of the potato aphid salivary gland transcripts were identified. A subset of these genes was predicted to be secreted by SignalP and TargetP programs. We selected eight putative secreted proteins and identified their orthologs in pea aphid and green peach aphid (GPA). The full-length cDNAs of these eight genes were cloned from potato aphid and sequenced. *In planta* functional characterization of these eight putative potato aphid secreted salivary gland proteins was performed using transient expression in *N. benthamiana* and delivery in tomato using bacterial type-three secretion system.

Our data suggested that two of these eight proteins, Me10 and Me23, enhanced aphid performance on *N. benthamiana* plants expressing these proteins compared to the control plants. Interestingly, only Me10 enhanced aphid performance in tomato plants when delivered via bacterial type three-secretion system. In this study we identified two aphid effector proteins enhancing aphid performance by manipulating the host plant to their advantage. Me23, encodes glutathione peroxidase, may suppress the plant oxidative burst induced by aphid feeding. Me10 encodes for an uncharacterized protein and has homologues in many aphid species. Therefore, we characterized the role of Me10 by identifying the plant components targeted by Me10, as described in Chapter Four.

To identifying plant interactors of Me10 a yeast two-hybrid screen of a tomato cDNA library was performed by using Gal-BD-Me10 as bait. The cDNA library was prepared by Dr. Tokuji Tsuchiya. Six million primary yeast transformants were screened. We identified seven different proteins as potentially interacting with Me10. Many of these like scaffold proteins tomato 14-3-3 and Rack1 have previously been reported to be

involved in plant immune signaling or plant metabolic pathways. We confirmed the *in planta* interaction between Me10 and TFT7 by immunoprecipitation. In addition, transcript levels of *TFT7* were induced by aphid feeding. These results indicate that the aphid effector Me10 is binding to the plant proteins TFT7 and is targeting the components of plant immune signaling pathways likely involved in pattern-triggered immunity (PTI). Sequence analysis of Me10 revealed that it doesn't contain any of the consensus-binding motifs that are known to facilitate association with 14-3-3 proteins. Therefore, further experiments are needed to find the exact motif of Me10 that is involved in interacting with these plant proteins.

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