Control of *Turnip yellows virus*: Assessing impact on oilseed rape quality traits and dissecting circulative transmission by aphids

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Abstract

Turnip yellows virus (TuYV) is one of the most significant viral diseases of oilseed rape and may be one of main reasons why commercial oilseed rape crops do not reach their genetic potential. TuYV is transmitted by aphids, sap-sucking hemipteroid insects, and the green peach aphid (GPA) is the predominant vector. TuYV can reduce oilseed rape yield by up to 26% in the UK and may also affect oil quality. Current control measures rely on insecticides; however, changing legislation and reduced effectiveness necessitate novel approaches to virus control. In this thesis, the impact of TuYV on the UK commercial oilseed rape crop was established and sources of partial resistance to TuYV and aphids were investigated. TuYV reduces yield and has a subtle impact on seed physiology including small changes to fatty acid profiles and glucosinolate content. Furthermore, these changes appear to be genotype-dependent and not as a result of virus accumulation in the plant. To learn more about TuYV transmission by aphids, a novel, functional-genomics tool was developed to silence aphid genes by plant-mediated RNA interference (PMRi). Highly specific protein interactions between virus particles and aphid proteins are critical determinants of circulative transmission, a process whereby virus particles can move between aphid cell layers. However, the aphid components underlying these processes are poorly understood. As the GPA Rack1 protein has been implicated in transcytosis of TuYV particles across the aphid gut barrier, PMRi was used to dissect its role in the circulative transmission process. This revealed that Rack1 may have a direct role in TuYV acquisition by GPA. This work further demonstrates the potential of PMRi as a post-genomics tool in aphids and similar insects, but also as a direct means of aphid and/or virus control. These contrasting research strategies have provided a two-pronged approach towards improving TuYV control.

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List of common abbreviations

AAP – Acquisition Access Period

ASG – Accessory Salivary Gland

C(t) – Threshold Cycle

CP – Coat Protein

dsRNA – Double-stranded RNA

EPG – Electrical Penetration Graph

FAMEs – Fatty Acid Methyl Esters

GFP – Green Fluorescent Protein

GLM – Generalized Linear Model

GPA – Green Peach Aphid (*Myzus persicae*)

IAP – Inoculation Access Period

MpC002 – *Myzus persicae* homolog of C002

MpPInt02 – *Myzus persicae* Progeny Increase to Over-expression 2

nt – Nucleotide

PMRi – Plant-Mediated RNA Interference

qRT-PCR – Quantitative Reverse Transcriptase - Polymerase Chain Reaction

Rack1 – Receptor for Activated C Kinase 1

RdRp – RNA-dependant RNA polymerase

RISC – RNA-Induced Silencing Complex

RNAi – RNA interference

RT – Room Temperature

RTD – Read-Through Domain

siRNA – Small interfering RNA

ssRNA – Single stranded RNA

TAS-ELISA – Triple Antibody Sandwich - Enzyme-Linked Immuno-Sorbancy Assay

TuYV – *Turnip yellows virus*

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Finally, I would like to dedicate this thesis to my parents. My mum has always encouraged me to follow my interests and has strived selflessly to give her children the opportunities she never had. My dad has taught me the value of hard work and to proverbially measure twice, cut once. Together they have supported me throughout my life in every way possible, for which I will be forever grateful.

"The pessimist complains about the wind; the optimist expects it to change; the realist adjusts the sails"

~ William Arthur Ward

"Research is to see what everybody else has seen and to think what nobody else has thought"

~ Albert Szent-Györgi

1. Introduction

1.1. TuYV is a major disease of oilseed rape

Oilseed rape (*Brassica napus* L.) is one of the most important crops in UK agriculture, yet despite improvements in breeding and agronomic practices, oilseed rape yields have remained relatively static in recent years (Diepenbrock, 2000). *Turnip yellows virus* (TuYV, polerovirus, *Luteoviridae*) is one of the most significant viral diseases of oilseed rape and may be one of main reasons why commercial oilseed rape crops do not reach their genetic potential (Stevens *et al*., 2008). TuYV is distributed worldwide and is also capable of infecting a wide variety of other crops such as lettuce, cauliflower, cabbage, spinach and pea as well as various weed species which can provide a reservoir for infection (Walkey & Pink, 1990; Graichen & Rabenstein, 1996; Stevens *et al.*, 2008). TuYV incidence in oilseed rape crops are extremely variable, ranging from less than 10% to up to 85% infection (Stevens *et al*., 2008).

Oilseed rape plants infected with TuYV exhibit a variety of symptoms, some of which are dwarfing, reddening of leaf margins, interveinal yellowing or reddening, leaf curling, leaf rolling and brittleness (**Figure 1.1**) (Stevens *et al.*, 2008; ICTV, 2010). Most of these symptoms resemble water stress and nutrient deficiency hence TuYV infections often go unnoticed (Stevens *et al.*, 2008). In some varieties, TuYV-induced symptoms are less conspicuous, requiring detection by serological techniques such as Enzyme-Linked Immunosorbent Assay (ELISA) to confirm the presence of the virus. For these reasons, the economic importance of TuYV is likely underestimated.

Figure 1.1. | TuYV symptoms on oilseed rape plants. TuYV infected oilseed rape plants produce a range of symptoms, shown here are interveinal yellowing and purple blotching (photo: Dr. Mark Stevens, Broom's Barn, UK).

Experiments conducted in the UK showed that TuYV can decrease oilseed rape yield by up to 26% and from this all yield parameters (including the number of primary branches, numbers of seeds per pod and percentage oil content per seed) were shown to be affected (Stevens *et al.*, 2008). A previous study comparing the yields of infected and lightly infected plots showed a yield decrease of 13% due to the effect on oil and seed yields (Jay *et al.*, 1999). Also, a three-year experiment in Germany showed that oilseed rape plots with 90% to 100% TuYV infections yielded 12% to 34% fewer seeds than nearly virus-free plots (Graichen & Schliephake, 1999). Moreover, yield losses can further increase when TuYV infected plants are infected with other viruses (Stevens *et al.*, 2008). TuYV may also affect the chemical composition of seed and therefore the quality of oil but this is not known.

TuYV is a persistent virus that is transmitted by small, sap-sucking insects called aphids (Chapter 1.3) and its epidemiology is intrinsically linked to aphid population dynamics. Climatic conditions have a major influence on the spread of TuYV. Aphids may develop earlier in the growing season due to milder winters or early spring conditions and warmer temperatures in autumn or winter encourage the migration and later development of aphid vectors which may increase virus spread (Stevens *et al.*, 2008). Oilseed rape losses can further increase when TuYV infected plants are infected with other viruses (Stevens *et al.*, 2008). Increased UK temperatures due to climate change (Semenov, 2007) could therefore extend the potential damage

caused by this virus. A study in the hotter, drier climate of Australia demonstrated that TuYV infection produced yield losses over 40% with up to 3% decreased oil content and significant increases in erucic acid (Jones *et al.*, 2007), which could have negative impacts on animal health (Kimber & McGregor, 1995).

Oilseed rape cultivation has more than doubled in the past decade and is now the third most grown crop in UK agriculture (DEFRA, 2012). Oilseed rape is the third most important source of edible oil in the world, following soybean and palm oil (El-Beltagi & Mohamed, 2010). Rapeseed oil has also become the primary source for biodiesel in Europe, and the processing by-products provide high-protein animal feed. According to statistics from the Department for Environment, Food and Rural Affairs (DEFRA), 705,000 hectares of oilseed rape were grown in 2011, producing a record harvest of 2.8 million tons of oilseed in the UK, an increase of 24% on 2010 (DEFRA, 2012). This trend is likely to continue as approximately 750,000 hectares of oilseed rape were grown in the UK for harvest in 2012, yielding 2.6 million tons of oilseed (DEFRA, 2012). With current prices at approximately £400 per ton, the economic loss from TuYV infection could equate to over £150m a year to UK oilseed rape growers alone.

1.2. Characteristics and history of *Turnip yellows virus*

TuYV belongs to the genus *Polerovirus*, one of three distinct genera in the family *Luteoviridae* (luteovirids)*.* TuYV was formerly referred to as *Beet western yellows virus* (BWYV); however, European non-sugar beet infecting strains have since been reclassified by the International Committee for the Taxonomy of Viruses (ICTV) as an independent species in the *Polerovirus* genus (Mayo, 2002). The separation of the beet-infecting and non-infecting isolates of BWYV as two distinct viruses has been supported with molecular evidence (Beuve *et al.*, 2008; Hauser *et al.*, 2000; Stevens *et al.*, 2005).

Poleroviruses are spherical, non-enveloped particles approximately 25-30 nm in diameter (**Figure 1.2A**). The protein shell is composed of 180 coat proteins, orientated into T=3 icosahedral symmetry (**Figure 1.2B**) (ViralZone, 2013). Virus particles contain a single-stranded positive-sense RNA molecule, typically of about 6 kilobasepair (kbp) (Hull, 2001). This RNA is infectious and serves as both the genome

and viral messenger RNA. The viral RNA contains several open reading frames (ORF) and gene expression involves a complex series of different mechanisms (**Figure 1.2C**).

The concentration of virus particles within the infected plant is low (less than 100 µg/L of sap) as the particles replicate almost exclusively in the phloem tissue within cytoplasmic viral factories (van den Heuvel *et al.*, 1994; ViralZone, 2013) and have not been observed outside of the phloem. After penetration into the host cell, un-coating of virus particles occurs, releasing the viral genomic RNA. The VPg protein (Viral protein genome-linked) is covalently attached to the 5′ end of the viral RNA and acts as a primer during RNA synthesis. ORF0 protein (P0), ORF1 polyprotein (P1) and the polymerase (ORF1-2) are translated directly from genomic RNA forming the RdRp (RNA-dependent RNA polymerase) fusion protein by a -1 ribosomal frameshift near the end of ORF1. Translation of the Rap1 protein initiates approximately 1500 nt downstream of the 5' end of the viral gRNA by an unusual internal ribosome entry site (IRES). A dsRNA genome is synthesized from the genomic RNA which is then transcribed/replicated to provide viral mRNAs/new RNA genomes. All other ORFs are translated from subgenomic RNAs (sgRNAs). Leaky scanning of ORF3 and ORF4 produces the coat protein (CP) and movement protein (MP). Suppression of termination of the CP stop codon produces CP-RTD, a fusion protein of 78 kDa that is composed of the 24 kDa CP at the N-terminus and the 54 kDa readthrough domain (RTD) at the C-terminus (**Figure 1.2C**). Virion particles are assembled and cell-to-cell transfer of virion particles is mediated by the viral movement protein (ViralZone, 2013).
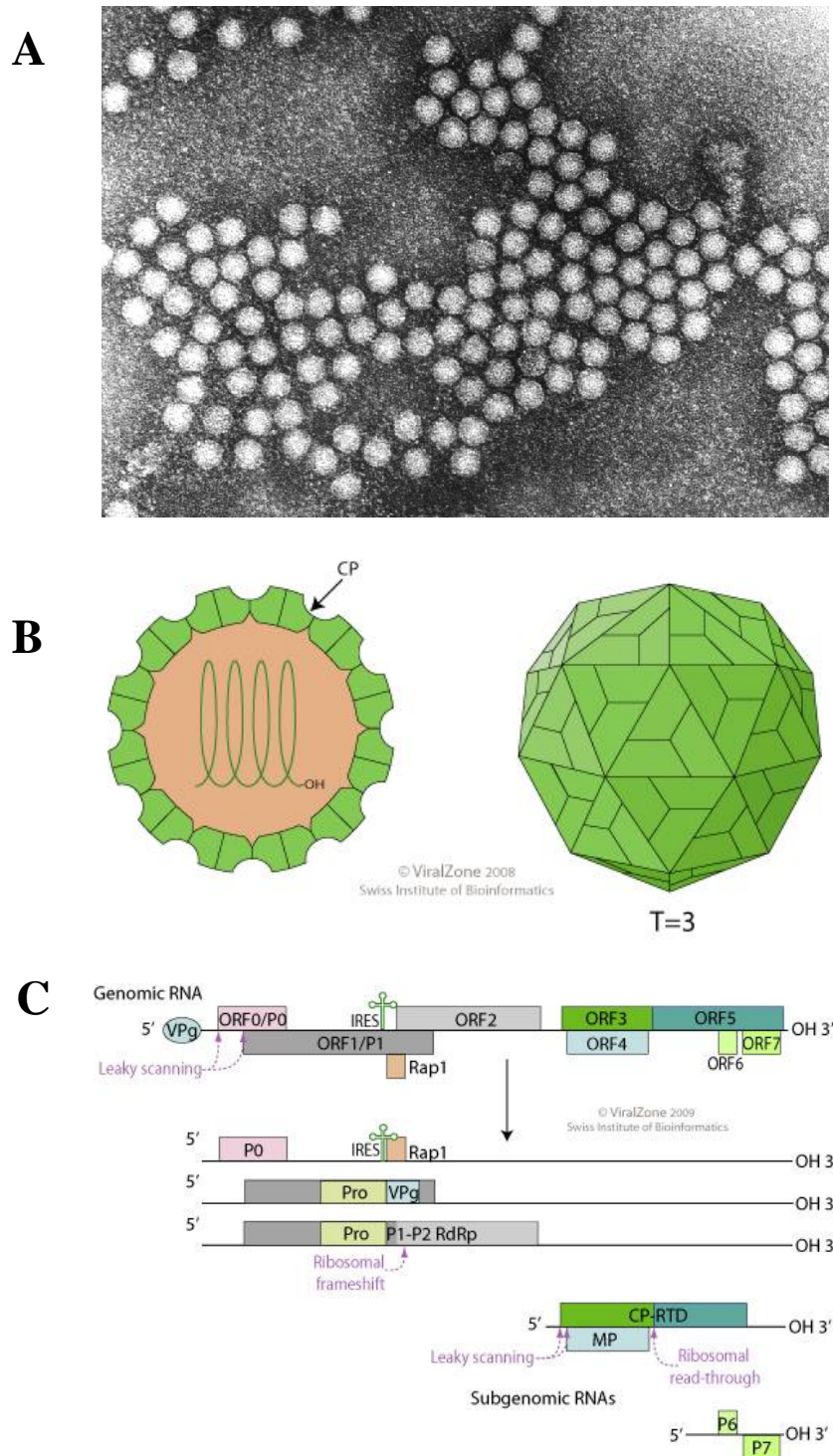
Figure 1.2. | Luteovirus particle structure, genome and replication. (**A**) TEM image of TuYV particles from a purified virus sample (photo: Mark Stevens, Broom's Barn, UK). (**B**) The virus genome is contained inside the protein shell which displays T=3 icosahedral symmetry (ViralZone, 2008). (**C**) The polerovirus genome consists of a single-stranded positive-sense RNA molecule. Gene expression mechanisms such as subgenomic RNA, ribosomal frameshifting, ribosome leaky scanning, suppression of termination, and polyprotein expression are employed. Replication occurs in cytoplasmic viral factories within host-plant phloem cells (ViralZone, 2008).

1.3. Aphids are major agricultural pests and vectors of plant viruses

Plant viruses have evolved a large diversity of strategies to be transferred efficiently from one host to the next, including transfer of infected sap, transmission through seed/pollen or via an insect, nematode or plasmodiophorid vector (DPV, 2013). Of these, insect-vector transmission is by far the most common as over 75% of the ~700 plant viruses officially recognized by the International Committee on Taxonomy of Viruses are transmitted by insects, predominantly those of the hemipteroid assemblage (ICTV, 2013). Hemipteroids include aphids, whiteflies, leafhoppers, planthoppers, and thrips (Hogenhout *et al.*, 2008). TuYV and other members of the *Luteoviridae* family are transmitted by aphids which are capable of transmitting nearly 30% of plant virus species to date (Ng & Perry, 2004; Hogenhout *et al.*, 2008). Due to their role in virus transmission aphids can be thought of as the 'mosquitoes of the plant world' (quoted from Dr. Saskia Hogenhout).

Aphids are members of the super family *Aphidoidea* in the suborder *Sternorrhyncha* (order *Hemiptera*). Over 4,000 species exist, distributed into 10 families (Blackman, 2000). Of these, approximately 250 are serious pests in agriculture and forestry. Aphids are distributed worldwide but are most commonly found in temperate zones (Blackman, 2000). Aphids vector many economically important viral species in these regions culminating in huge losses to crop yield and quality. Typical virus symptoms may include leaf yellowing (either of the whole leaf or in a pattern of stripes or blotches), leaf distortion (e.g. curling, gall formation) and/or other growth distortions (e.g. stunting of the whole plant, abnormalities in flower or fruit formation) (DPV, 2013). The characteristic symptoms of some economically significant aphid-vectored viruses are presented in Figure 1.3.
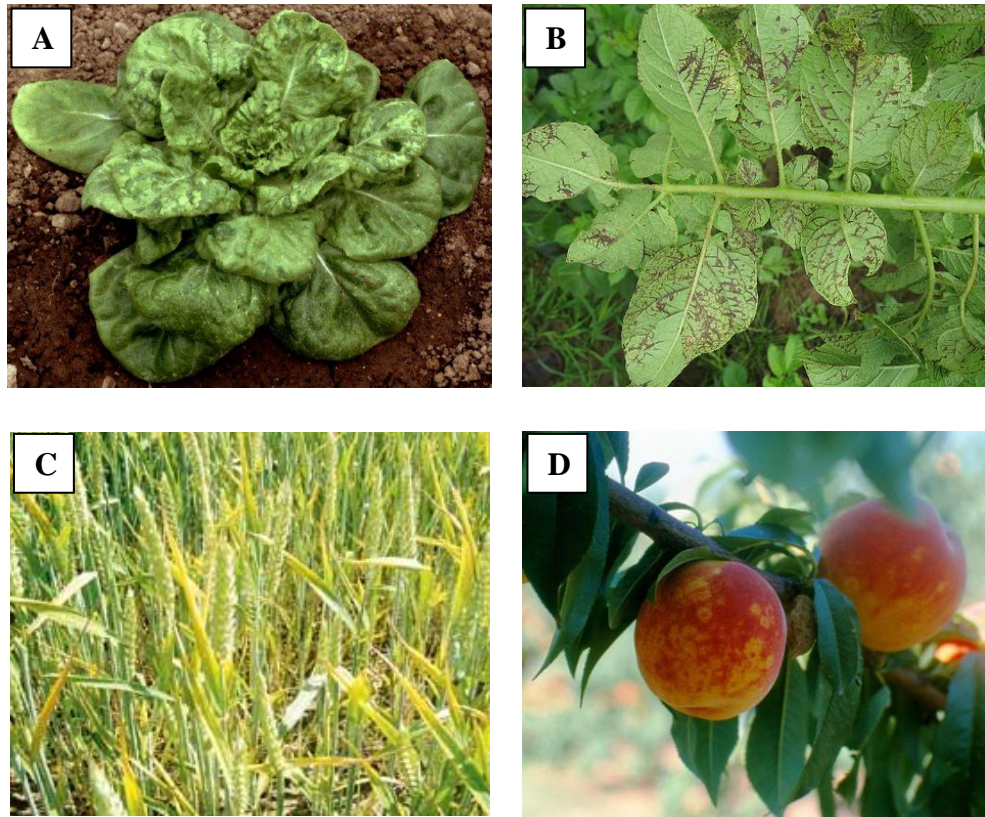
Figure 1.3. | Characteristic symptoms of some economically significant aphid-vectored viruses. (A) Yellow mosaic symptoms on lettuce leaves caused by the Potyvirus *Lettuce mosaic virus* (photo: National Institute of Agronomic Research, Avignon, France). **(B)** Necrotic lesions on potato leaves caused by the Potyvirus *Potato virus Y* (photo: Ollie Martin, WikiGardener). **(C)** Leaf yellowing in wheat caused by the luteovirid *Barley yellow dwarf virus* (photo: Farmer's Weekly, Sutton, UK). **(D)** Discolored rings and blotches on Peach fruit caused by the Potyvirus *Plum pox virus* (photo: Ontario Ministry of Agriculture, Food and Rural Affairs, Ontario, Canada).

Aphids are therefore of primary economic concern for their role in virus transmission but they also negatively impact plant productivity in other ways. For example, they can quickly build to high population densities on the plant, causing wilting or death of plants through removal of photoassimilates. Aphids also excrete large volumes of a sticky fluid called 'honeydew'. Honeydew can build up on colonized plants and promote fungal diseases which may further damage the plant and reduce photosynthetic efficiency. Additionally, salivary secretions of some aphids are phytotoxic, causing stunting, plant hormone imbalances, leaf deformation, and gall formation (Blackman, 2000).

Hemipteroids are characterized by their sap-sucking mouthparts which allow stealthy feeding from the phloem of host plants. Aphid mouthparts are highly

specialized and well adapted to their feeding habits. The piercing/sucking mouthparts known as 'stylets' are enclosed in a sheath called a rostrum, which is formed from modifications of the mandible and maxilla of the insect mouthparts (Chapman, 2000). The stylets enable them to remove plant fluids from the host; they are incredibly flexible and allow aphids to probe down extracellularly through multiple cell layers without damaging plant tissue (Pollard, 1973; Tjallingii & Esch, 1993). Aphids typically feed from phloem sieve elements (Tjallingii & Esch, 1993), they also feed from xylem tissue to balance osmotic potential (Pompon *et al.*, 2011). Prior to phloem feeding, an aphid will probe the plant by puncturing cells and sampling cell contents, once the aphid accepts the plant it can introduce its stylet further into the epidermis to the phloem sieve elements. The aphid stylet may take multiple routes in this process before a successful feeding site is established where upon it may feed for multiple hours (**Figure 1.4**).
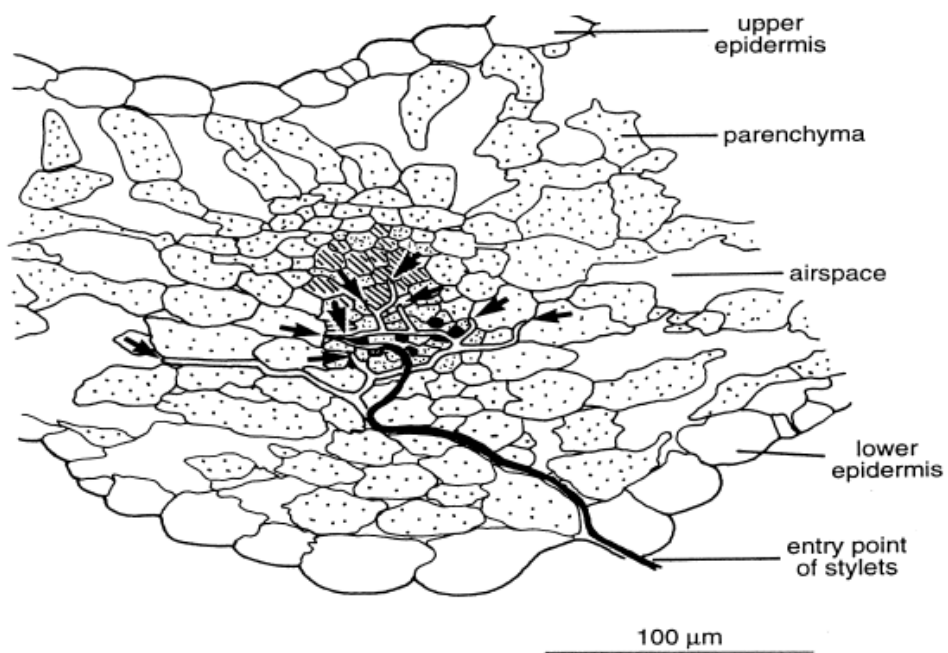
Figure 1.4. | Aphid stylet pathways in plants. Using the stylet, aphids probe extracellularly through multiple cell layers. Abortive pathways are shown white with the ends of the paths indicated by arrows. The final pathway, reaching the phloem, is shown in black. Phloem sieve tubes, black; xylem, crosshatched; parenchyma, stippled. Taken from: Chapman (2000).

Aphid feeding behavior is therefore highly conducive to virus transmission. As plant cells have a robust cell wall, viruses cannot penetrate them unaided, aphid feeding therefore provides a direct route for a virus into the plant. Furthermore, aphids are

mobile and capable of producing winged forms (alates) providing viruses with a route of dissemination across large distances. As luteovirids are phloem-limited, they are wholly reliant on their insect vectors for transmission. There is some evidence that luteovirids alter insect behavior to enhance their spread. Ingwell *et al* (2012) showed that the bird cherry-oat aphid (*Rhopalosiphum padi*), after acquiring *Barley yellow dwarf virus* (BYDV) during *in vitro* feeding, prefers healthy wheat plants, while non-viruliferous aphids prefer BYDV-infected plants (Ingwell *et al.*, 2012).

1.4. Aphid anatomy and reproductive biology

Aphids vary in length (1 to 10 millimeters) and color, have a soft cuticle and are pear-shaped (Blackman, 2000). They have fairly long antennae which can up to six segments, two compound eyes and a tail-like protrusion (cauda) above their rectal apertures (Dixon, 1998). Most aphids have a pair of cornicules (or siphunculi), which secrete a defensive fluid involved in the alarm response to predators and other enemies (Bowers *et al.*, 1972; Pickett *et al.*, 1992; Beale *et al.*, 2006). Aphids have a compartmentalized gut system and the salivary system consists of two pairs of glands, each with a small accessory salivary gland (ASG) and a larger principal salivary gland (Dixon, 1998). Hemolymph fills the interior (hemocoel) of the insect's body and surrounds all tissues (Dixon, 1998). Free-floating cells within the hemolymph (hemocytes) play a role in the arthropod immune system (Lavine & Strand, 2002). The key features of aphid anatomy are presented in **Figure 1.5**.
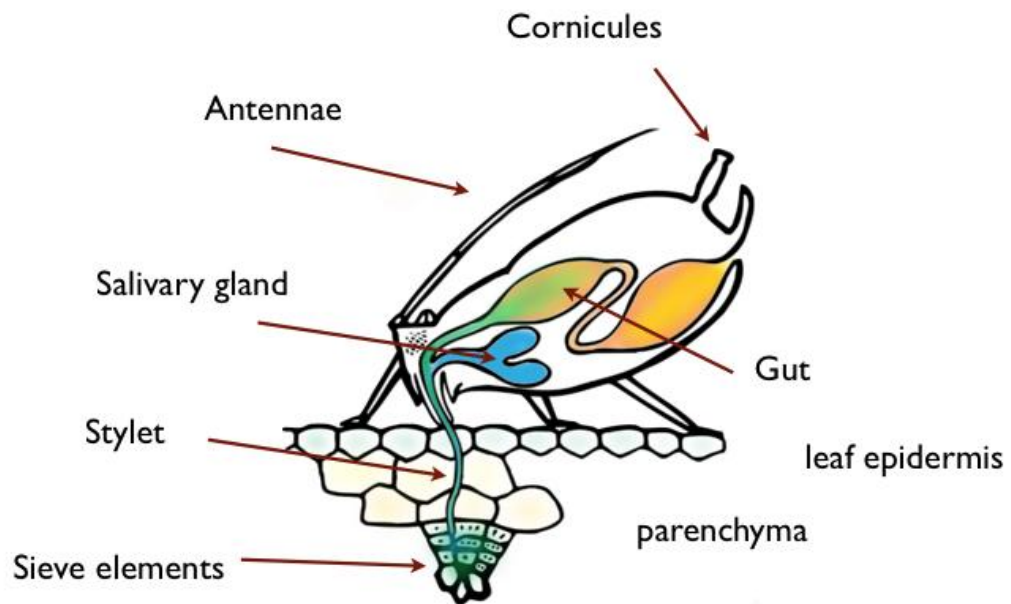
Figure 1.5. | Aphid anatomy. Summary of the key features of aphid anatomy. Taken from: Pitino (2012).

Aphids have a number of symbiotic relationships with bacterial communities. The best studied obligate endosymbiotic relationship for aphids is with *Buchnera aphidicola* (Shigenobu *et al.*, 2000). *Buchnera* are housed in specialized host cells called bacteriocytes located in the abdominal hemocoel (Douglas, 1998), and are essential for aphid metabolism, providing essential amino acids present only in low concentrations in phloem sap (Dale & Moran, 2006). *Buchnera* have also been shown to be important for aphid heat tolerance (Dunbar *et al.*, 2007). Additionally, other bacterial symbionts have an important role in resistance against aphid predators (Oliver *et al.*, 2003). These complex communities are vertically transmitted to aphid offspring (Douglas, 1998).

The lifecycle of aphids is complicated. Unlike the majority of insects, they can reproduce clonally and give birth to live young (viviparous reproduction) which facilitates a more rapid development to reproductive maturity (Goggin, 2007). Furthermore, this reproductive strategy is generationally telescopic as an aphid's embryonic development begins before its mother's birth (Goggin, 2007). Juvenile aphids (nymphs) molt about four times before becoming an adult (Blackman, 2000). Parthenogenetic females proliferate in the long-day summer months then short autumn day-length induces the production of sexual females and males (Shingleton, 2003). After mating, the females lay eggs which are able to withstand harsh winter

temperatures. Asexual females emerge from the eggs in spring to establish a new population. Asexuality enables aphid populations to quickly expand on a food source given the right environmental conditions and lack of predation. When host plant quality is compromised or conditions become crowded, some aphid species produce alates that can disperse to other food sources (Blackman, 2000). The lifecycle of the pea aphid is shown in **Figure 1.6**. Although slight variations exist in life cycle, it is similar across different aphid species (Shingleton, 2003).
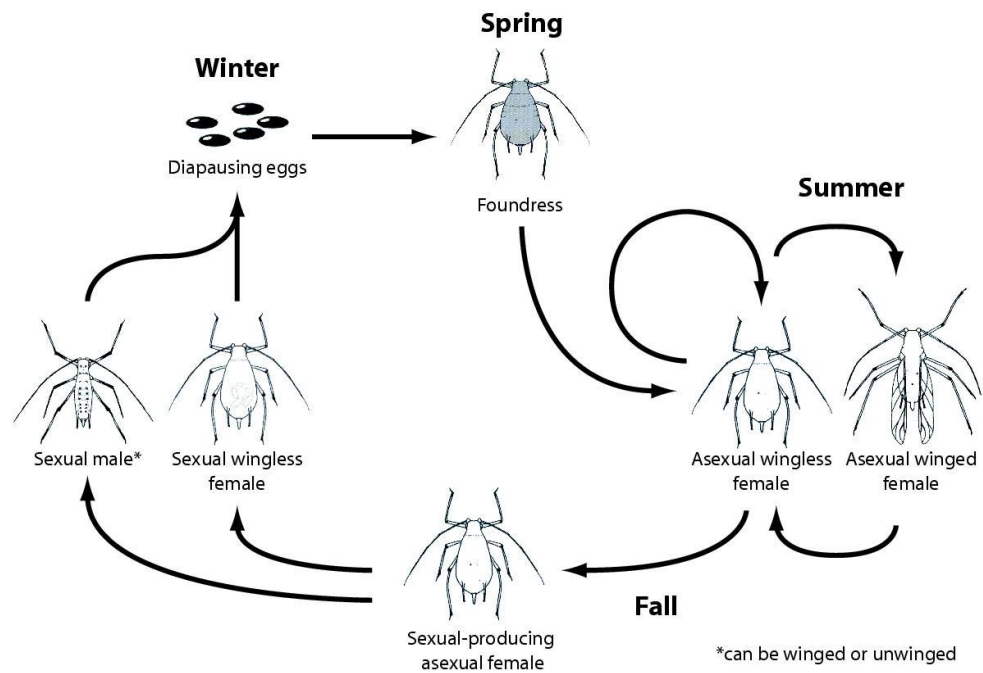
Figure 1.6. | Life cycle of the pea aphid. During the spring and summer months, reproduction is by parthenogenesis. Sexually-reproducing males and females and produced in autumn, these mate to produce eggs for overwintering. The eggs do not hatch until the following spring where upon a new population is established by an asexual 'foundress'. Taken from: Shingleton *et al* (2003).

1.5. Green peach aphid – the predominant vector of TuYV

TuYV can be transmitted by a number of aphid species such as the potato aphid (*Macrosiphum euphorbiae*) and the cabbage aphid (*Brevicoryne brassicae*). However, the green peach aphid (GPA) (*Myzus persicae*) (**Figure 1.7**), is generally regarded as the most important vector of TuYV due to a combination of factors. For example, GPA are highly efficient vectors of TuYV with transmission rates over 90% reported

experimentally (Schliephake *et al.*, 2000). GPA are also widespread across the UK and extremely polyphagous, feeding on over 40 different plant families including multiple arable crops (Vanendem *et al.*, 1969). As the majority of insect species feed on one or two different plant species (Schoonhoven, 2005), GPA therefore provides a large number of available hosts for TuYV dissemination.

GPA are a key species in TuYV epidemiology but are also one of the most significant insect pest species in agricultural crops, capable of efficiently transmitting over 100 different virus species including at least 7 of the 20 viruses listed in the *Luteoviridae* family (Schliephake *et al.*, 2000). The dominant GPA genotype in the UK is currently genotype O (Fenton *et al.*, 2010). TuYV epidemiology is tightly linked to yearly GPA host cycles and its biannual migration events. Populations develop in spring after over-wintering on winter host plants, causing a migration of viruliferous alates to summer hosts. Populations expand rapidly on summer hosts during favorable conditions. The subsequent migration of viruliferous alates to newly-planted winter crops (such as winter oilseed rape) in September & October is of particular concern to growers.

Figure 1.7. | GPA, the most important vector of TuYV. Different GPA asexual life stages (adults and nymphs) feeding on *A. thaliana* leaf midvein (photo: Andrew Davis, JIC, UK).

GPA reproduction is slightly different from other aphids such as the pea aphid (Figure 1.6). GPA also lay eggs, but they hatch and the nymphs overwinter, sometimes growing to adulthood. Under controlled environment conditions with little variation in

temperature, the asexual cycle can continue indefinitely with no appearance of the sexual morph. It is believed that GPA in the UK have lost the ability to form sexual morphs as the peach trees that this aphid uses as a host in its sexual reproduction stage are largely absent in the UK (Fenton *et al.*, 1998).

1.6. Plant defense and aphid colonization

Both constitutive and inducible mechanisms contribute to plant defense against aphids and these are generally classified as antixenosis or antibiosis (Painter, 1958; Kogan & Ortman, 1978). Antixenosis refers to a resistance mechanism employed by a plant to deter or prevent pest colonization (Kogan and Ortman, 1978), whereas antibiosis results from defenses that impact insect physiology leading to impairment of pest fitness (Smith & Clement, 2011). Constitutive defenses range from mechanical barriers to pre-formed toxins and compounds which reduce digestibility (Walling, 2008).

Inducible defense relies on successful perception of the insect by the plant. Plants have an intricate, multilayered immune system to detect and defend against potential threats (reviewed in Jones & Dangl, 2006; Chisholm *et al.*, 2006; Dodds & Rathjen, 2010). The first layer of this system is based on the perception of pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) at the plant cell surface. Examples of well studied PAMPs that elicit plant basal defense include bacterial flagellin or lipopolysaccharides, and fungal chitin (Zipfel, 2008). Plant PRRs identify these conserved, non-self molecules, inducing rapid initial responses followed by downstream defense activation. Plant responses to PAMPs are called PAMP-triggered immunity (PTI) and these early responses are similar when challenged by a variety of pathogens (e.g. fungal & bacterial pathogens). Plants are resistant to the majority of microbes through this innate immunity or non-host resistance. Successful pathogens produce 'effectors' to inhibit PTI, but plants, in turn, can perceive such effectors using resistance (R) proteins to mount a stronger, second layer of defense called effector-triggered immunity (ETI). R proteins are often characterized by their nucleotide binding site and leucine-rich repeat (NBS-LRR) structures (Belkhadir *et al.*, 2004).

These plant responses are fairly well characterized in the model systems *A. thaliana* and *N. benthamiana* with certain bacterial and fungal pathogens (Segonzac & Zipfel, 2011). However, responses to insect herbivory are less well characterized. There is growing evidence that the components underlying plant defense responses against bacteria and fungi have a similar role in defense against insects (Hogenhout, *et al.* 2009). There are examples of elicitors of plant defenses present in insect saliva which can be classified as Herbivory-Associated Molecular Patterns (HAMPs) (Wu & Baldwin, 2010; Hogenhout & Bos, 2011; Elzinger & Jander, 2013). Aphid saliva has also been shown to contain HAMPs (De Vos & Jander 2009; Bos *et al.*, 2010; Prince, 2012). During feeding, aphids produce different salivas with different compositions and functions (Miles, 1999; Will *et al.*, 2007, 2009; Fereres & Morano, 2009). Several studies have identified aphid salivary proteins, which suppress similar defense responses as those targeted by bacterial or fungal effectors (Will *et al.*, 2007; Mutti *et al.*, 2008; Bos *et al.*, 2010). Aphid saliva therefore plays an important role at the host interface through delivery of effector molecules which manipulate host physiology to facilitate colonization. A model of plant-aphid interactions underlying compatible and incompatible interactions is presented in **Figure 1.8**.
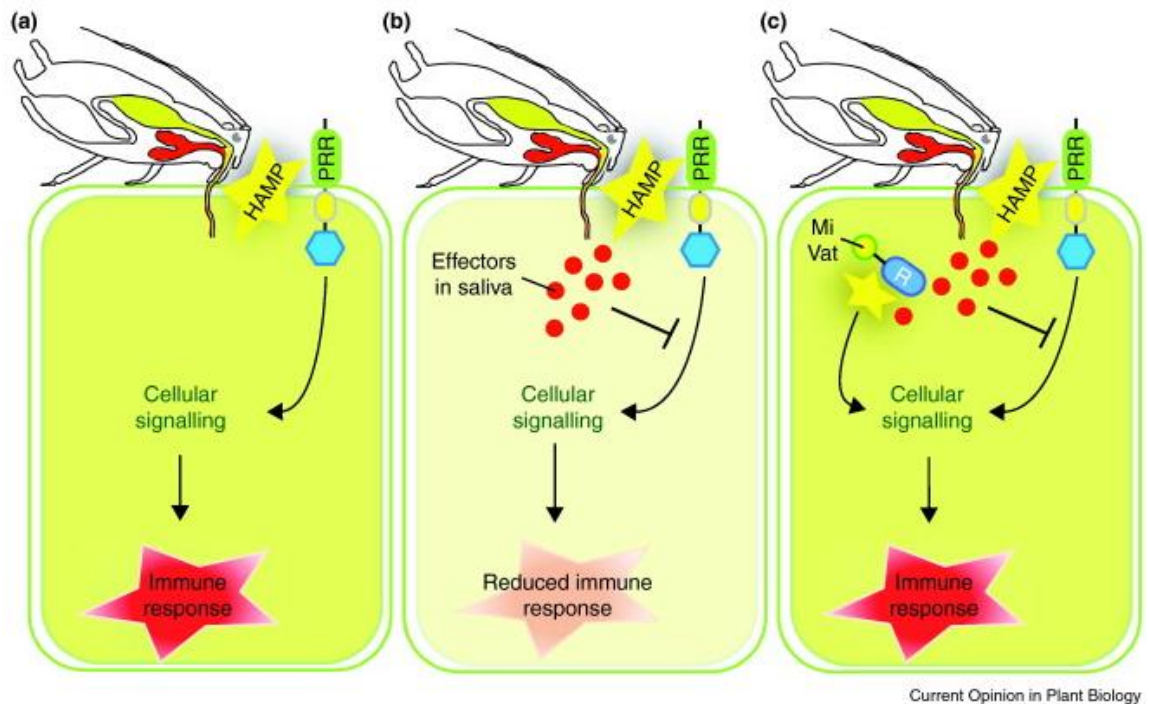
Figure 1.8. | Current model of the multi-layered plant defense response to aphid herbivory. (**A**) Incompatible interaction: Plant cells perceive aphid herbivore-associated molecular patterns (HAMPs) leading to HAMP-triggered immunity (HTI). (**B**) Compatible interaction: Although plants perceive the aphid HAMPs, the defense response is effectively suppressed by aphid effectors, resulting in aphid colonization. (**C**) Incompatible interaction: Aphid effectors effectively suppress HTI, but one or more effectors are recognized by R genes, leading to a reinstatement of the plant immune response. Taken from: Hogenhout & Bos (2011).

Plants have evolved to produce an array of secondary metabolites (or allelochemicals), many of which have defensive benefits against pathogens or pests. When subjected to pathogen attack, a plant needs to gauge the response appropriately to ensure that defense is successful and that resources otherwise used for growth and development are not wasted. Induction of defensive compounds is therefore usually highly localized and specific to the threat faced (Louis & Shah, 2013). Most of these metabolic pathways are regulated by the phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), which are involved to some degree in virtually all aspects of plant physiology (Bari & Jones, 2009). Interplay between these phytohormones activates distinct defense pathways, depending on the lifestyle of the invading pathogen (Glazebrook, 2005).

Secondary metabolites are typically unique to specific plant families. Brassicas (including oilseed rape) utilize glucosinolates (Björkman *et al.*, 2011) and their toxic

decomposition products (Halkier & Gershenzon, 2006; Broekgaarden *et al.*, 2008; Pratt *et al.*, 2008) as part of the defense response. Glucosinolates are always present in cells and function as a constitutive defense mechanism (Koroleva *et al.*, 2000). Glucosinolate synthesis is complexly regulated by SA, JA, and ET, and is typically induced after tissue damage (Mewis *et al.*, 2006). The glucosinolate content in plants is also affected by biotic and abiotic factors, while both the type and quantity of glucosinolate determines the susceptibility of plants to insect pests (Bonhinc *et al.*, 2012). Phloem feeders avoid cellular damage and have been shown to reduce total glucosinolate levels (Mewis *et al.*, 2006; Kempema *et al.*, 2007; Kim & Jander, 2007). It has been established that GPA avoids the insecticidal effects of glucosinolates by excretion in their honeydew (Kos *et al.*, 2011). However, this is not true for all glucosinolate groups as plants containing only indolic glucosinolates demonstrate higher resistance to GPA (Kim *et al.*, 2008).

Finally, microRNAs (miRNAs) have also been shown to play an important role in plant defense against aphids (Kettles *et al.*, 2013). MiRNAs are a class of endogenous RNAs which regulate the expression of genes involved in various biological and metabolic processes. These small RNAs also play important roles in resistance to plant viruses (Ding & Voinnet, 2007) and the complex responses against pathogens (Katiyar-Agarwal & Jin, 2010) and leaf-chewing insects (Pandey & Baldwin, 2007; Pandey *et al.*, 2008). *A. thaliana* plants deficient in miRNA processing show increased resistance to GPA via increased PAD3-mediated induction of camalexin (Kettles *et al.*, 2013). Camalexin also plays a role in plant defense against bacterial and fungal microbial pathogens (Kliebenstein *et al.*, 2005; Glawischnig, 2007).

1.7. Controlling TuYV infection

Chemical approaches have been effective, short term resolutions for aphid and therefore TuYV control. However, pesticides are becoming less effective due to increasing prominence of pesticide resistance in key pest species (Whalon *et al.*, 2008; Onstad, 2008). This is especially evident for GPA, which exhibit rapid adaptation to insecticides and have developed resistance to at least seventy different synthetic compounds (Silva *et al.*, 2012). Currently, six distinct insecticide resistance mechanisms in GPA have been reported worldwide, for example, modified

acetylcholinesterase (MACE) confers resistance to organophosphates and carbamate insecticides, plus *kdr* or *super kdr* (knockdown resistance) mutations in a voltage-gated sodium channel reduce the effectiveness of pyrethroids and organochlorines (Silva *et al.*, 2012). Reports of resistance against key pesticides in GPA lineages on several continents could have long-term impacts for aphid control in agriculture (Field *et al.*, 1988; Martinez-Torres *et al.*, 1999; Nauen & Denholm, 2005; Puinean *et al.*, 2010). Furthermore, as aphids are important primarily in virus transmission, insecticide application will have little benefit to plants that have already acquired a virus. As up to 72% of winged GPA carry TuYV (Stevens *et al.*, 1995; Stevens *et al.*, 2008), it is extremely difficult to prevent widespread primary infection of host crops even with regular pesticide use.

Critically, current European Union (EU) negotiations could lead to some chemical pesticides becoming restricted or withdrawn from use (Hillocks, 2012). There are significant financial costs as well as environmental and human health impacts associated with overuse or misuse of these chemicals (Hillocks, 2012). For example, pesticide usage and practices have recently been deemed as one of the main causative agents of colony collapse disorder (CCD), a phenomenon used to describe the devastatation of bee populations across the world (Oldroyd, 2007). Recently, several independent peer-reviewed studies were published showing that neonicotinoid pesticides pose a risk to bees and that increased usage has roughly correlated with rising bee deaths (Krupke *et al.*, 2012; Pettis *et al.*, 2012; Schneider *et al.*, 2012; Tapparo *et al.*, 2012). As a result of these concerns, in April 2013, the EU announced plans to restrict the use of several neonicotinoids for the following two years (European Commission, 2013). Neonicotinoid pesticides such as acetamiprid, clothianidin and imidacloprid are some of the most widely-used pesticides in the world (Gervais *et al.*, 2010). They are the predominant component of oilseed rape seed treatments and are the most important aphid control measure in oilseed rape crops (Gervais *et al.*, 2010). Without these insecticides, the incidence of TuYV will likely increase in future.

As pesticide usage is on the wane, alternative strategies for controlling TuYV are necessary. Control of TuYV may be achieved through genetic resistance to aphids. R genes (Chapter 1.6) are involved in crop resistance to aphids, however few of these have been reported and attempts at introducing aphid resistance into crops have had mixed success. In general, aphid resistance appears to be polygenic although there are

examples of single dominant R genes (Dedryver *et al.*, 2010; Dogimont *et al.*, 2010). For example, the nematode resistance gene '*Mi*' from tomato (*Solanum lycopersicum*) has been shown to confer resistance to certain aphid biotypes (Rossi *et al.*, 1998; Goggin *et al.*, 2001), and the '*Vat*' (virus aphid transmission) gene from melon (*Cucumis melo*) controls resistance to the cotton aphid (*Aphis gossypii*) (Klingler, 2005; Dogimont *et al.*, 2010). Both of these aphid R genes are members of the NBS-LRR family of resistance genes. Other putative R genes that are members of the NBS-LRR family and confer resistance to aphids have also been identified (Dogimont *et al.*, 2010). Although effective, R-gene-mediated resistance is often highly-specific to a particular genotype/biotype and can be broken down in as little as two years after commercial release in the field (McDonald & Linde, 2002). However, these R genes can be stacked to make it harder for pests to evolve counter-resistance and to provide multiple resistances to different attackers.

Other classical approaches towards aphid control include the use of biopesticides or biocontrol using predatory insects (e.g. ladybirds, parasitic wasps) or fungal/bacterial pathogens of aphids (Bhatia *et al.*, 2011). A wide range of natural predators of aphids exist which can be naturally encouraged using attractants, or artificially introduced to provide aphid biocontrol in crops. Ladybirds and their larvae are excellent aphid predators, as are some species of lacewing, hoverflies and even certain bird species (Blackman, 2000). The use of the parasitic wasps *Aphidius colemani* and *Aphidius ervi*, which oviposit inside aphid bodies, is an alternative aphid biocontrol strategy. However, there is evidence that aphid lineages emerge which have increased resistance to this control measure (Li *et al.*, 2002; Oliver *et al.*, 2003). Also, it is challenging to get sufficient numbers of natural enemies into open fields and for their population growth rates to keep pace with that of aphids. Other ways to prevent aphid colonization are the use of physical barriers to prevent access to the crop e.g. horticultural fleeces, nets, or insect traps. However, these methods are unsuitable for large-scale crop production and do not provide further protection once a single founding aphid reaches the crop.

As control of insect vectors has become increasingly problematic and use of transgenic crops is restricted, greater emphasis is being placed on searching for genetic resistance to TuYV. Novel germplasm for resistance breeding is limited and the only reported TuYV-resistant variety is the resynthesized oilseed rape line called 'R54'

(Graichen, 1994). By crossing this line with susceptible varieties, the resistance was shown to be heritable. From this, further work has revealed molecular markers from R54 that could assist in TuYV resistance breeding as well as a major quantitative trait locus (QTL) for TuYV resistance (Dreyer *et al.*, 2001). R54 resistance is not complete however (Juergens *et al.*, 2010), and is strongly influenced by environmental factors, particularly temperature (Dreyer *et al.*, 2001).

The search for natural sources of resistance to TuYV in Brassica germplasm is evidently an important goal for oilseed rape breeders, yet the current status of resistance to TuYV in UK oilseed rape varieties is unknown. By seeking natural resistance to aphids and TuYV, there is not only a possibility to protect an important agricultural crop, there is also a great financial incentive to be gained; even a minor improvement in yield could save a great deal of capital for UK oilseed rape growers (Stevens *et al.*, 2008).

1.8. Circular transmission of TuYV by aphids

Luteovirids are transmitted by aphids in a persistent, circulative and non-propagative manner (Gray & Gildow, 2003; Hogenhout *et al.*, 2008). This means that the vector can continue to transmit the virus throughout its life span (persistent transmission), the virus can move across cell layers in the insect vector (circulative transmission), and that viral replication takes place in the plant and not the insect (non-propagative transmission). The stylets of plant-feeding hemipteroids provide a route for uptake and inoculation of numerous plant viruses, including phloem-limited viruses such as TuYV (Brault *et al.*, 2010). TuYV particles are acquired in as little as 15 minutes (Stevens *et al.*, 2008) by ingesting infected sap. Upon this uptake, the virus begins part of its lifecycle in the aphid (Figure 1.9). The TuYV particles then move from the gut lumen into the hemolymph or other tissues, eventually reaching the ASG (Brault *et al.*, 2007). The virus is disseminated to a new host during insect feeding when the aphid injects virus particles along with saliva (Brault *et al.*, 2007).
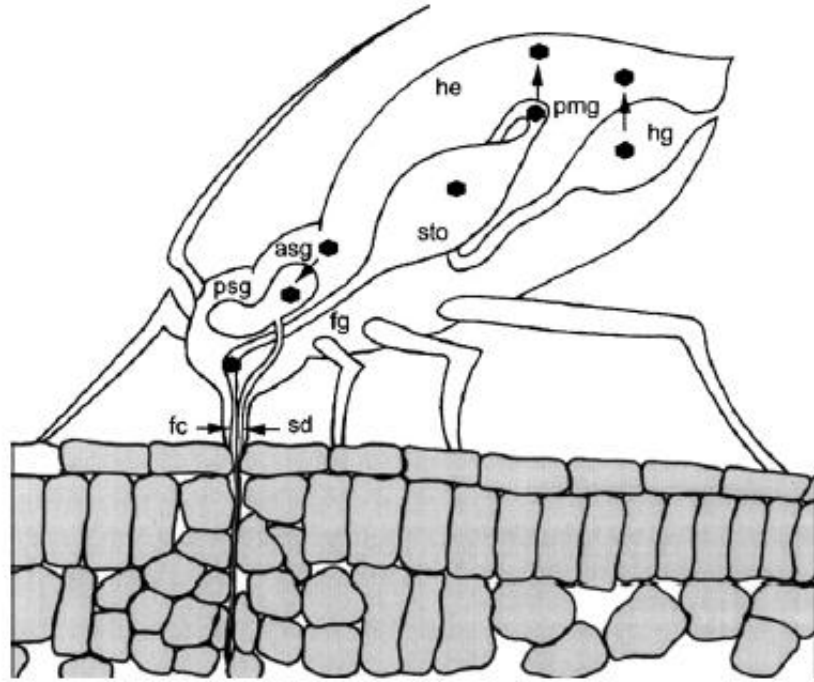
Figure 1.9. | Virus transmission in the aphid's body. TuYV virions are acquired in the food canal (**fc**), move across the posterior midgut (**pmg**) and/or hindgut (**hg**) to the hemolymph (**he**). TuYV virions cross into the accessory salivary gland (**asg**) for delivery into the plant through the salivary duct (**sd**). Also shown are the **fg**: foregut; **psg**: principal salivary gland; **sto**: stomach. Taken from: Brault *et al* (2007).

Transmission electron microscopy (TEM) observations have been extensively applied to follow the route of luteovirids in their vectors (Gildow & Rochow, 1980; Gildow & Gray, 1993; Brault *et al.*, 2007). TEM studies have sometimes been complemented with detection of viral RNA in different compartments of the aphid using molecular amplification techniques (Reinbold *et al.*, 2001; Chay *et al.,* 1996). At the gut level, the endocytosis mechanism seems to rely on a clathrin-mediated entry process and this is supported by luteovirids consistently observed in association with various vesicles in gut cells (Brault *et al.*, 2007). In the case of BWYV and *Cucurbit aphid-borne yellows virus* (CABYV), luteovirids have also been observed in the gut lumen in close proximity to the apical plasmalemma (Reinbold *et al.,* 2001; Reinbold *et al.*, 2003). The mechanism by which the virions traverse the ASG appears similar to the endocytosis–exocytosis process at the gut level but operates in the reverse direction (Gildow, 1993). Collectively, these studies indicate that all luteovirids follow a similar pathway through their aphid vectors.

Circulative virus particles therefore need to cross a number of physical barriers and endure in several diverse environments within the vector before reaching a new host (Gray & Gildow, 2003). For efficient virus transmission, successful adaptation to the vector is required to overcome each of these obstacles. As each species of luteovirid can only be efficiently transmitted by only one or two aphid species (Brault *et al.*, 2005), this implies a great deal of specificity and intimacy between virus and vector. The gut is one of the key sites which defines the high specificity of vector capability as many viruses not normally transmitted by aphids may be ingested into the gut and exit the aphid in the honeydew (Gildow & Gray, 1993). Highly specific protein interactions between virus particles and aphid proteins are therefore critical determinants of insect-transmission.

Two polerovirus structural proteins, the CP and RTD (Chapter 1.2), contain multiple functional domains that have been implicated in aphid transmission (Brault *et al.*, 1995; Bruyere *et al.*, 1997; Gildow *et al.*, 2000; Brault *et al.*, 2000; Lee *et al.*, 2005; Brault *et al.*, 2005; Seddas & Boissinot, 2006), and for efficient virus transport in the plant (Brault *et al.*, 1995; Chay *et al.*, 1996; Brault *et al.*, 2003; Peter *et al.*, 2009). Recently, structural information has been produced for the luteovirid *Potato leaf roll virus* (PLRV), revealing protein interaction topologies required for virion stability, aphid transmission, and virus interaction with plants (Chavez *et al.*, 2012).

Plant proteins may be potentially involved in circulative transmission. During ingestion, aphids sample virions along with sap. Therefore, any sap protein bound to virions will be acquired by the insects. Various sap proteins which bind to purified luteovirids have been described which facilitate increased transmission rates (Bencharki *et al.*, 2010). Interestingly, any soluble protein at sufficiently high concentration in the diet and acquired together with virions could stimulate virus transmission (Bencharki *et al.*, 2010).

1.9. Aphid genes involved in luteovirid transmission

Virus structural proteins are important for circulative transmission by aphids (Chapter 1.8), however, it is not fully understood which components of the aphid are involved in this process. Several aphid proteins with the ability to bind purified

luteovirid particles *in vitro* have been reported as well as some potential luteovirid-specific receptors implicated in the shuttling of virus particles between cell layers by transcytosis. These are summarized below.

Five proteins from GPA capable of binding PLRV have been identified (van den Heuvel *et al.*, 1994). One of these proteins is symbionin, an *Escherichia coli* GroEL homologue produced within the aphid by its endosymbiont *Buchnera* (Chapter 1.4). This demonstrates that endosymbiotic bacteria play a decisive role in determining the persistent nature of PLRV particles in the GPA hemolymph and that symbionin is a key protein in the interaction with PLRV, and perhaps other luteovirids. It has been suggested that symbionin protects the virus from recognition by the aphid immune system (Filichkin *et al.*, 1997).

A transcriptomic analysis of intestinal genes of the pea aphid was conducted following uptake of *Pea enation mosaic virus-1*, a virus complex made of two components; *PEMV-1* (family *Luteoviridae,* genus *Enamovirus*) and *PEMV-2* (genus *Umbravirus*) (Brault *et al.,* 2009). The study compared the transcriptome of viruliferous and non-viruliferous aphids using a cDNA chip microarray (Le Trionnaire *et al.,* 2009). Of the 6776 transcripts analyzed, 128 were significantly differentially regulated (105 genes down-regulated and 23 up-regulated) (Brault *et al.*, 2009). Five % of these were involved in processes related to the internalization and transport of virions. The major conclusion from this study was that PEMV hijacks a constitutive endocytosis-exocytosis mechanism without heavily altering cell metabolism *(Brault et al.*, 2009).

Using a co-immunoprecipitation technique to pull down virus binding proteins, Yang *et al* (2008) identified a luciferase-like protein and a cyclophilin-like protein potentially involved in receptor binding or targeting, and transport of luteovirids through cell cytoplasm (Yang *et al.,* 2008). These proteins were linked to the transmission phenotype of the aphid vector (Yang *et al.,* 2008). A similar approach also combined genetics and proteomics to link heritable aphid and endosymbiont protein expression to circulative polerovirus transmission yielding several putative luteovirid-interacting proteins (Cilia *et al.,* 2011). Lastly, several polypeptides from GPA bind *in vitro* to purified wild type or mutant particles of *Beet Mild Yellows Virus* (BMYV) (Seddas *et al.*, 2004). Three of these polypeptides were identified by mass spectrometry as Rack1, GAPDH3, and actin and are potentially involved in the epithelial transcytosis

of virus particles in the aphid vector. Rack1 was further found to interact with the RTD of other luteovirids (Gray *et al.*, 2013).

1.10. Rack1

Because poleroviruses are serologically inter-related (ICTV 2010), it is likely that TuYV interacts with GPA Rack1 in a similar way to other luteovirids (Seddas *et al.*, 2004; Gray *et al.*, 2013). Previous research has shown that Rack1 is important in regulating several cell surface receptors and intracellular protein kinases (Choi, *et al.*, 2003). Rack1 binds to integrins, which can interact with viruses (Albinsson and Kidd 1999), and which are components of the extracellular matrix basal lamella of invertebrates such as aphids (Pedersen, 1991).

Rack1 is a multifunctional, tryptophan/aspartate (WD) motif-containing protein that is conserved among plants, animals, and invertebrates and it is essential for cellular functions (Adams, 2011). Rack1 is an intracellular receptor that binds activated protein kinase C (PKC), an enzyme primarily involved in signal transduction cascades (hence named Receptor for Activated C Kinase 1) (Seddas *et al.*, 2004). Rack1 also produces signals required for the organization of actin in the cytoskeleton (Liliental & Chang, 1998) and is an integral component of the mammalian circadian clock (Robles *et al.*, 2010). It is a scaffold protein (crucial regulators of signaling pathways) that physically connects various signal transduction components into stable complexes (Chen *et al.* 2002). Due to its localization at the inner membrane leaflet, Rack1 is clearly not an extracellular receptor for luteovirids but evidence strongly suggests that this protein is a key component of the transcytosis mechanism (Seddas *et al.*, 2004).

Knockdown of Rack1 resulted in developmentally defective phenotypes in *Caenorhabditis elegans* including slow growth, embryonic lethality, egg laying defectiveness and sluggishness (Simmer *et al.*, 2003; Kamath *et al.*, 2003) as well as sterility and abnormal gonad development (Ciche & Sternberg, 2007). Rack1 in *Drosophila melanogaster* functions during oogenesis (Kadrmas *et al.*, 2007) and is required in early oocyte polarity (Kucherenko *et al.*, 2008).

1.11. RNAi process

{**Disclaimer:** Nomenclature on what to refer to target gene down-regulation is ambiguous. Generally, 90% or more down-regulation is referred to as gene 'silencing' or 'knockdown'. The term 'RNAi aphids', with the gene target as a pre-fix, will be used throughout this text to describe insects with target gene down-regulation up to, but not exceeding, 90%.}

RNAi interference (RNAi) is a natural, cellular process used by animals, plants and fungi, as a means of post-transcriptional gene regulation to maintain normal growth and development, as well as a method for defense against viruses or transposable elements (Hannon, 2002). This process was originally described as 'post-transcriptional gene silencing' (PTGS) in plant systems nearly 15 years ago but the mechanistic aspects of it at the time were not fully understood (Hamilton & Baulcombe, 1999). Since then, RNAi has been successfully used as a reverse genetics tool to study gene function in various organisms and as a practical tool in biotechnology and medicine. Inhibition of gene expression produced by RNAi resembles a loss-of-function or gene knockout mutation but is often quicker and easier to achieve allowing for rapid analysis (Ketting, 2011). This approach was initially documented for animal systems in the nematode species *Caenorhabditis elegans* (Fire *et al.*, 1998) and is now well-established in numerous eukaryotic systems e.g. *Arabidopsis thaliana* (Xie *et al.,* 2004) and *Drosophila melanogaster* (Elbashir *et al.*, 2001).

A simplified overview of the RNAi process is provided in **Figure 1.10**. Double-stranded RNA (dsRNA) can specifically lower the transcript abundance of a target gene when injected into an organism or introduced into cultured cells (Fire *et al.*, 1998). RNAi involves the cleavage of the dsRNA precursors into small-interfering RNA (siRNA) of approximately 21 to 23 nucleotides by the enzyme Dicer (Meister & Tuschl, 2004). These siRNAs are then incorporated into a RNA-induced silencing complex (RISC). Argonaute proteins (Ago), the catalytic components of RISC, use the siRNA as a template to recognize and degrade the complementary messenger RNA (mRNA) (Meister & Tuschl, 2004). RNAi can therefore be exploited to suppress gene expression through highly specific depletion of target transcripts.
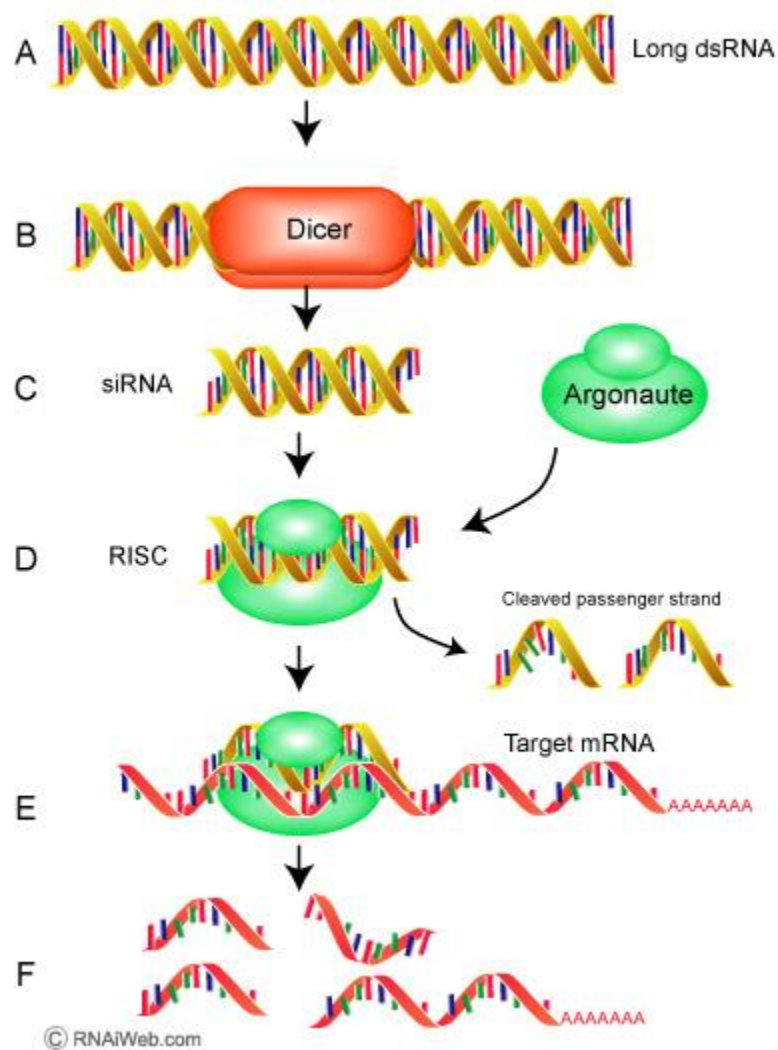
Figure 1.10. | RNAi process. (A) RNAi in the cell is triggered by dsRNA precursor molecules. **(B)** DsRNA is processed by the RNase III enzyme Dicer in an ATP-dependent reaction. **(C)** Long dsRNA is processed into 21-23nt siRNA with 2nt 3' overhangs. **(D)** The RISC complex consists of siRNA incorporated into an Ago protein. Ago cleaves and discards the passenger (sense) strand of the siRNA duplex. **(E)** The remaining (antisense) strand of the siRNA duplex serves as the guide strand and guides the activated RISC to its homologous mRNA. **(F)** Endonucleolytic cleavage of the target mRNA (RNAiWeb, 2013).

The most advanced and useful animal system for RNAi has been the nematode 'worm' *C. elegans* (Fire *et al.*, 1998). RNAi-induced knockdown in *C. elegans* is relatively easy as worms can either be soaked in or injected with a solution of dsRNA, or can be fed genetically transformed bacteria that express dsRNA. *C. elegans* was the first multicellular organism to have its genome completely sequenced (CeSC, 1998), and the abundance of sequence data makes reverse genetics approaches extremely viable. Moreover, the developmental processes of this organism are now well

understood so phenotypes generated by RNAi of specific target genes are comparatively easy to document. As a result, the majority of *C. elegans* genes have been knocked down, establishing a functional role for over 9% of the genome (Kamath *et al.*, 2003). Other nematode species such as *Caenorhabditis briggsae* (Stein *et al.*, 2003) and *Heterohabditis bacteriophora* (Ciche & Sternberg, 2007) have also been used as model organisms for RNAi based post-genomic studies or as a platform for comparative genomics.

1.12. RNAi in insect systems

Some of the earliest RNAi studies in insects include work on the fruit fly, *D. melanogaster* (Elbashir, 2001). Since then, RNAi has been successfully utilized in multiple insect systems using a variety of means, including direct injection of dsRNA/siRNA into larvae or adults, exogenous application of dsRNA/siRNA, transfection using bacterial or viral expression systems, and feeding of dsRNA/siRNA on artificial diets or via transgenic plant expression (Mao *et al.*, 2007; Yu *et al.*, 2013).

At least two pathways for uptake of dsRNA in insects have been described, the transmembrane channel-mediated uptake mechanism based on *C. elegans'* SID-1 protein (Winston 2007) and an 'alternative' endocytosis-mediated uptake mechanism (reviewed by: Huvenne & Smagghe, 2010; Gu & Knipple, 2013). Insects lack genes encoding an RNA-dependent RNA-polymerase (RdRP), the enzyme necessary for the siRNA amplification step that leads to persistent and systemic RNAi effects (Sijen *et al.*, 2001). The absence of dsRNA amplification implies that gene-knockdown effects produced by RNAi would be limited in insects, possibly only to cells directly exposed to dsRNA. However, numerous publications have shown that successful, systemic silencing can be achieved for insects, suggesting that the spread of dsRNA in insects is based on another mechanism(s) than that in nematodes. However these processes are not fully understood and there are differences between insects, for example, some insect species can be completely refractory to systemic RNAi whereas close to 100% knockdown can be achieved in others (Gu & Knipple, 2013).

Factors influencing efficacy of RNAi in insects include dsRNA concentration, nucleotide sequence, length of dsRNA fragment, and the life stage of the target

organism (Huvenne & Smagghe, 2010). For example, concentration and length of dsRNA have profound effects on the efficacy of the RNAi response in regard to both the initial efficiency and duration of the effect in red flour beetles (*Tribolium castaneum*) (Miller *et al.*, 2012). Furthermore, competitive inhibition of dsRNA can occur when multiple dsRNAs are injected together, influencing the effectiveness of RNAi (Miller *et al.*, 2012). Younger insects appear to be more susceptible to RNAi (Huvenne & Smagghe, 2010), for example, a stronger silencing effect was observed in 5th instar fall armyworm (*Spodoptera frugiperda*) larvae compared to adult moths (Griebler *et al.*, 2008).

RNAi has also been applied to Hemipteroid insects. Using either injection or ingestion, silencing of the salivary protein, salivary nitrophorin 2 (NP2) was achieved in the triatomine bug (*Rhodnius prolixus)*, allowing the role of this salivary protein to be assessed (Aruajo *et al.*, 2006). The *trehalose phosphate synthase* (TPS) gene in the brown planthopper (*Nilaparvata lugens*) was efficiently silenced after feeding insects on an artificial diet (Chen *et al.* 2010). Significant reductions in TPS enzymatic activity were observed, resulting in disturbed insect development and often lethality (Chen *et al.* 2010).

RNAi-mediated gene knockdown can be achieved in aphids through direct injection of dsRNA or small-interfering RNAs (siRNA) into aphid hemolymph. This approach was used to silence *C002*, a gene strongly expressed in the salivary glands of pea aphids (Mutti *et al.*, 2006). Silencing this gene resulted in lethality of the aphids on plants, but not on an artificial diet, indicating that C002 functions in aphid interaction with the plant host (Mutti *et al.*, 2006; Mutti *et al.*, 2008). Microinjection of long dsRNA into pea aphids also leads to silencing of genes encoding calreticulin and cathepsin by 30-40% (Jaubert-Possamai *et al.*, 2007). Calreticulin is a calcium-binding protein that is produced in most aphid tissues, while cathepsin is specifically produced in the pea aphid gut. Thus, gene silencing appears to occur in different aphid tissues (Jaubert-Possamai *et al.*, 2007).

Feeding of dsRNA from an artificial diet can also suppress expression of the corresponding aphid gene. Pea aphids fed on an artificial diet containing dsRNA corresponding to the aquaporin transcript lead to down-regulation by more than 2-fold within 24 hours (Shakesby *et al.*, 2009). Since aquaporin is involved in osmoregulation,

this resulted in elevated osmotic pressure in the hemolymph (Shakesby *et al.*, 2009). In a similar study, feeding of dsRNA targeting *vATPase* transcripts from an artificial diet achieved a 30% decrease in transcript levels in pea aphids and a significant increase in aphid mortality (Whyard *et al.*, 2009).

As well as use as a reverse-genetics tool, there is also potential to use RNAi as a means of pest control. A breakthrough study by Baum *et al* (2007) demonstrated the potential of RNAi to control coleopteran insect pests. Transgenic corn plants that were engineered to produce dsRNAs corresponding to the western corn rootworm resulted in significantly reduced feeding damage as a result of rootworm attack (Baum *et al.*, 2007). Silenced insects displayed larval stunting and increased mortality (Baum *et al.*, 2007). In another study, the model plants *N. tabacum* and *A. thaliana* were modified to produce dsRNA corresponding to cytochrome P450 gene of the cotton bollworm (Mao *et al.*, 2007). When larvae were fed transgenic leaves, levels of cytochrome P450 mRNA were reduced and larval growth retarded (Mao *et al.*, 2007).

1.13. Focus and aims described in this thesis

The aim of this study was to better understand TuYV transmission by aphids and to evaluate virus impact on commercial oilseed rape so that management practices can be improved.

Impact of TuYV on the UK commercial oilseed rape crop was established and sources of partial resistance to TuYV and aphids were investigated (Chapter 3). The objective of this was to evaluate the current resistance status in UK commercial varieties, to investigate TuYV impact on oil quality and yield, and to determine whether virus-induced changes correlate with virus accumulation in the plant.

To learn more about aphid genes involved in TuYV transmission, a novel, functional-genomics tool was developed to silence GPA genes by plant-mediated RNA interference (PMRi) (Chapter 4). The objective of this was to determine initially whether PMRi is feasible for aphids and whether genes expressed in different aphid tissues are equally susceptible to RNAi.

The PMRi tool was utilized to determine the role of GPA *Rack1* in TuYV transmission (Chapter 5). The objective of this was to determine whether RNAi of *Rack1* reduces the acquisition and transmission of TuYV, and to develop PMRi both as a post-genomics tool for plant-feeding hemipteroids but also as a direct means of controlling these insects and the viruses they transmit in agriculture.

2. Materials & Methods

Part of this work was published in: Coleman *et al.*, 2014. See Appendix A – II.

2.1. Plant and insect growth/maintenance conditions

The GPA lineage used in this work is *Myzus persicae* RRes (genotype O) (Bos *et al.*, 2010). GPA were reared on *Nicotiana tabacum* plants for *Nicotiana benthamiana* leaf disc assays and on Chinese cabbage (*Brassica rapa*) for all other purposes (excluding the maintenance of TuYV – see Chapter 2.2). Plants and insects were maintained in custom-built acrylic cages (**Figure 2.1A**) located in controlled environment conditions at 18°C under 16 hours of light.
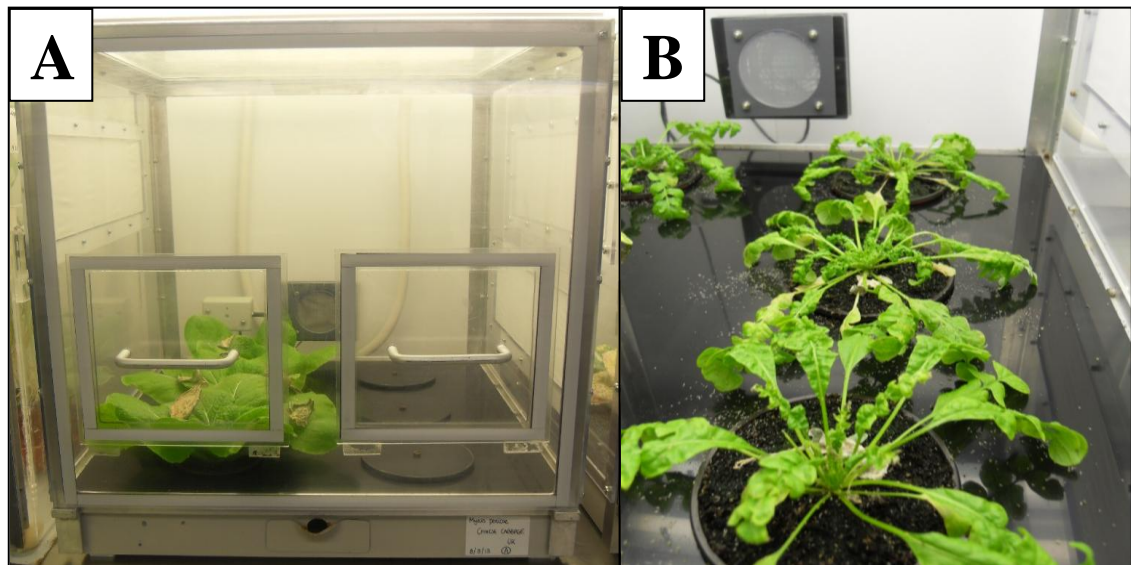
Figure 2.1. | Insectary stock cages for plants/GPA. GPA were maintained on Chinese cabbage (*Brassica rapa*) **(A)** or TuYV viruliferous insects maintained on Shepherd's Purse (*Capsella bursa-pastoris*) **(B)**.

2.2. Maintaining stock cages of TuYV infected *Capsella*

Shepherd's purse (*Capsella bursa-pastoris*) plants infected with TuYV isolate 'BW1' (Stevens *et al.*, 2005) were obtained from Broom's Barn Research Centre, Suffolk, UK. GPA were introduced to infected plants and allowed to feed. Un-infected *Capsella* plants were placed in an adjacent cabinet and viruliferous aphids moved across from infected plants. Two weeks after aphid inoculation, TAS-ELISA (Chapter 2.4) was used to determine whether plants had become infected with TuYV. Fresh *Capsella* seedlings were inoculated approximately every two weeks by introduction of viruliferous aphids. Plants and insects were maintained in custom-built acrylic cages

(**Figure 2.1B**) located in controlled environment conditions at 18°C under 16 hours of light.

2.3. Oilseed rape variety field trials

Field trials were designed and carried out at Broom's Barn Research Centre, Suffolk, UK, under the direction of Dr. Mark Stevens. In the 2009-2010 growing season, 49 *B. napus* varieties were grown in field trial micro-plots measuring 6 m long x 1.5 m wide. The plots were drilled at an equivalent of 5 kg seed/hectare at the start of September 2009 and seedlings at the four-five true-leaf stage were inoculated with viruliferous aphids by scattering leaves cut from TuYV infected *Capsella* plants (with aphids) onto plots to achieve approximately 10 aphids per plant. Plots were sprayed with a pirimicarb-based insecticide according to the manufacturer's instructions (Pirimor 50®, Syngenta, Jealott's Hill, UK) to remove aphids after 7 days. Plots corresponding to each variety were replicated four times overall in a randomized block design with two blocks inoculated with TuYV infected aphids and two blocks remaining un-inoculated. A discard strip of 12 m between the inoculated and un-inoculated blocks was sown with seed treated with the insecticide Modesto (Bayer Crop Science, Cambridge, UK) in order to prevent movement of viruliferous aphids from inoculated plots. In the 2010-2011 growing season, the ten oilseed rape varieties were grown in larger plots 4 m wide x 12 m long to allow seed to be harvested at the end of the growing season. Varieties were drilled and later inoculated with viruliferous aphids according to the same timescale and procedure as the previous trial. Plots for each variety were replicated eight times overall in a randomized block design with four inoculated and four un-inoculated blocks separated by a discard strip similar to the previous trial.

2.4. Triple Antibody Sandwich – Enzyme-linked Immunosorbent Assay (TAS-ELISA) protocol for testing leaves for TuYV (using BMYV monoclonal antibody)

TAS-ELISA was performed as in Stevens *et al.* (1994). Immuno MaxiSorp® 96 well plates (Fisher Scientific, Loughborough, UK) were coated (100 µL per well) with

BMYV polyclonal IgG (Neogen Europe, Auchincruive, UK) at a dilution of 1:1000 BMYV polyclonal:coating buffer (1.59 g Sodium Carbonate and 2.93 g Sodium Bicarbonate in 1 L distilled water). Plates were covered using Clingfilm and incubated at 37°C for one hour then washed with Phosphate Buffered Saline (PBS) + 1% Tween-20 (PBS-T). Two hundred μL of blocking buffer (1x PBS, 1% w/v milk powder) was added to each well and incubated at RT for one hour, plates were then emptied and stored at 4°C until needed. Leaf samples were crushed in a 1:9 weight ratio of leaf:extract buffer (1x PBS, 1% w/v milk powder, 10% Tween) and 100 μL of the resulting sap solution pipetted into a plate well. Each plate also contained healthy and virus infected leaf samples as controls. Plates were covered and left at 4°C overnight, then the sap was emptied and the plates rinsed with distilled water. Plates were washed with PBS-T and dried. A 1:1000 dilution (100 μL) of BMYV monoclonal antibody (Neogen Europe) in extract buffer solution was pipetted into each well of the plates. Plates were covered and incubated at 37°C for two hours then washed with PBS-T and dried. A 1:1000 dilution (100 μL) of Anti-Mouse (A1902) (Sigma-Aldrich, Gillingham, UK) in extract buffer solution was pipetted into each well of the plates. Plates were covered and incubated at 37°C for two hours then emptied and washed with PBS-T. One 5 mg phosphatase substrate tablet (Sigma-Aldrich) per plate was crushed in 10 mL substrate buffer (10% diethanolamine, pH 9.8). The solution (100 μL) was pipetted into each well then plates incubated at RT for one hour. Yellow color developed to show presence of TuYV and this was quantified using a Spectra Max 340PC plate reader (Bucher Biotec AG, Basel Switzerland) set at a wavelength of 405 nm.

2.5. Selection of oilseed rape varieties by TuYV titer

During the 2009-2010 growing season, 49 oilseed rape varieties from the HGCA recommended list (HGCA, 2012) were compared for TuYV accumulation within the plant. In March, field trial plots were visually scored for virus symptoms and ten plants per plot were randomly selected by walking a 'V'-shape in each plot and a leaf (4th or 5th leaf) sampled from a plant every meter. These were tested for TuYV infection by TAS-ELISA using TuYV-specific antibodies as previously described (Chapter 2.4). Ten varieties representing a range of TuYV titers were then selected for further investigation of yield impact, oil quality and aphid fecundity during the 2010-2011 growing season.

2.6. Oilseed rape seed processing

Plots from the 2010-11 trial were harvested by combining and weight of seed per plot was recorded by the combine. Seed moistures corresponding to each plot were determined by AP6060 moisture meter (Sinar Technology, Camberley, UK). Harvested seed was dried in an oven to standardize moisture content. Yield as tons per hectare at 9% seed moisture was calculated using the average seed weight for each variety from inoculated plots vs. un-inoculated plots adjusted to the 9% moisture standard. Seed mass in grams was calculated by weighing 20 seeds per plot in triplicate at 9% seed moisture. Approximately 2.5-5 Kg of seed was obtained for each plot; this seed was sampled for the various seed assessments described. The mean seed mass obtained from inoculated plots vs. un-inoculated plots was calculated for each variety. Ten seeds were also weighed and run whole using Nuclear Magnetic Resonance (NMR) Oxford instrument MQA 7005 to quantify oil percentage of seeds w/w, using the protocol described by O'Neill *et al* (2011). Five separate batches of 10 seeds were weighed per biological sample.

2.7. FAMES analysis

Fatty acid methyl esters (FAMEs) were prepared as follows. Twenty seeds per plot were ground using a pestle and mortar and the contents were transferred to a glass vial. A total of 2 mL of fatty acid (FA) extraction mixture (methanol:toluene:2,2-dimethoxypropane:sulphuric acid – ratio 33:14:2:1) was added together with 1 mL of n-hexane. The mixture was incubated at 80°C for one hour. FAMEs were analyzed by Gas chromatography–mass spectrometry (GC-MS). A concentration of 2.4 mg/mL of heptadecanoic acid (17:0) was used as an internal standard to quantify the relative amounts. The percentage of each component in the sample was calculated and expressed as a mass fraction in percent, using the following formula:

Corrected Total Area (CTA) = Total Area (TA) – Internal Standard Area (ISA)

% each FA = (FA peak area/ CTA)*100

The ester content (C) was calculated and expressed as a mass fraction in percent, using the following formula:

$$C = \frac{TA - ISA}{ISA} \times \frac{IS\left(\frac{mg}{mL}\right) \times IS\,(mL)}{Sample\,(g)} \times 100$$

2.8. Glucosinolates analysis

Glucosinolates were extracted from 10 seeds per sample from the 10 varieties using the protocol described by Mugford $et\,al$ (2010). Briefly, samples were extracted in 70°C methanol containing internal standard sinigrin. After centrifugation at 3000 g, the supernatant was loaded onto columns containing A25 Sephadex and washed through with water and acetic acid. Glucosinolates were then desulphated overnight with sulphatase, and eluted in water and frozen until HPLC analysis. Glucosinolates were separated by reverse-phase HPLC and measured by UV absorption at 229 nm relative to the internal standard using response factors. Each analysis was performed twice for each for the four biological repeats in the trial.

2.9. Leaf disc GPA fecundity/survival on oilseed rape

Oilseed rape varieties were grown in medium grade compost (Scotts Levington F2) under greenhouse conditions of approximately, 12 h day/night cycle. Leaf discs were cut from four-week old oilseed rape plants using an 11mm diameter cork borer. Six discs per variety were placed in separate wells within 24-well plates on top of 1mL distilled water agar (1% agarose) with the abaxial (underside) leaf surface facing up (**Figure 2.2A**). Five 1st instar GPA nymphs reared on Chinese cabbage were transferred to each leaf disc then wells were individually sealed with custom made lids containing thin mesh for ventilation (**Figure 2.2B**). Plates were then laid with the lids facing down in controlled environment conditions at 18°C under 16 hours of light (**Figure 2.2C**). Leaf discs were changed every five days to prevent disc desiccation. Total counts of adults and nymphs were made at day 5, 10, 12 and 14 post start of experiment with nymphs removed at each time point. This was repeated to give six biological replicates.
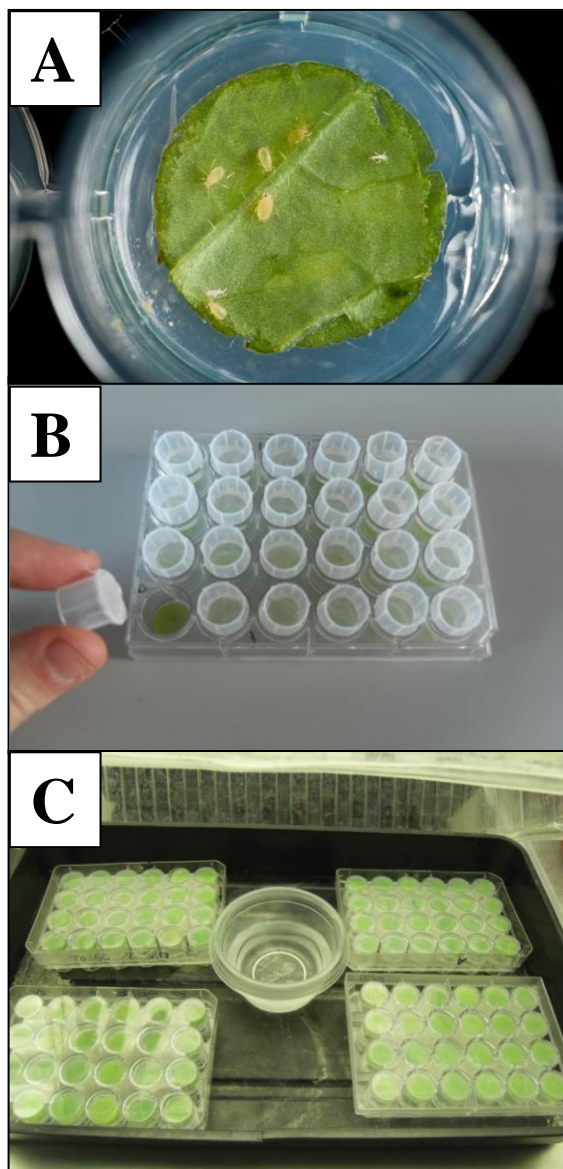
Figure 2.2. | Leaf disc assays. Leaf discs were laid on top of agar then 1st instar nymphs were added **(A)**. The 24 wells of the plate were individually sealed with mesh-covered lids **(B)**. Plates were laid upside down in controlled environment conditions **(C)**.

2.10. Total RNA extraction from plants/aphids and quality control

Working area and implements coming into contact with samples were wiped with RnaseZap® (Life Technologies, Paisley, UK). Plant/insect samples were ground in liquid nitrogen to a fine powder. Samples were kept at room temperature for two minutes then 1 mL of TRIzol® Reagent (Life Technologies) per 1 mg of tissue was added. The homogenate was incubated at RT for five minutes to allow the complete dissociation of nucleoprotein complexes. 0.2 mL of chloroform per 1 mL of TRIzol was

added and the homogenate mixed thoroughly for 15 seconds then incubated at room temperature for 10 minutes. Samples were centrifuged at 13,000 rpm for 15 min at 4°C to separate the homogenate into 3 phases: a lower red phenol-chloroform phase, an interphase and colorless upper aqueous phases. The aqueous upper phase (containing exclusively RNA) was transferred to a fresh tube then 0.5 mL isopropanol per 1mL of TRIzol added. The homogenate was mixed and incubated at room temperature for 10 minutes then centrifuged at 13,000 rpm for 10 minutes at 4°C to produce a gelatinous, white RNA precipitate on the side of the tube. The supernatant was removed and re-suspended in 1 mL of cold 75% ethanol before centrifugation at 13,000rpm for 5 minutes at 4°C. Ethanol was removed and samples were air dried for five minutes. The RNA was dissolved in 30-50 µL Rnase-free water (Qiagen) and the concentration/quality of RNA determined using a Nanodrop Spectrophotometer ND2000 (Thermo Scientific, Loughborough, UK). An A260/A280 ratio of 2.0 ± 0.1 was verified for each sample, corresponding to pure RNA. One µg total RNA was also visualized on an 1% agarose gel with 1x BPTE buffer [10x buffer consists of 100 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 300 mM Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane (Bis-Tris), 10 mM EDTA, pH 6.5] containing 0.5 µg/µL Ethidium Bromide. An equal volume of glyoxal loading dye (Life Technologies) to RNA was mixed and heated to 50°C for 30 min prior to loading. Gels were run at 120 V until dye reached the bottom of the gel. Gels were then visualized under UV light. Discrete, thick 28S and 18S ribosomal RNA (rRNA) gel bands at an approximate mass ratio of 2:1 were used as indications of high integrity. RNA samples were then stored at -20°C for short term or -80°C for long term.

GPA mRNA was also obtained using Dynabeads mRNA DIRECT kit (Life Technologies) according to the manufacturer's instructions. This allowed for high-throughput processing of GPA samples (in x5 batches) and maximized the RNA yield from single aphid samples.

2.11. CDNA preparation

CDNA was synthesized from aphid/plant total RNA samples for use in downstream reactions. For downstream qRT-PCR reactions, total RNA was subject to a genomic DNA removal treatment prior to cDNA synthesis. RQ1 Rnase-Free Dnase

(Promega) was used to treat total RNA samples according to the manufacturer's instructions. Subsequently, first-strand cDNA was made from 0.5-5 µg total RNA using the Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase Kit (Life Technologies) according to the manufacturer's instructions. The first-strand reaction was primed using 1 µL oligo (dT) (500 µg/mL) or random hexamers (250 ng/µL) using 1 µL of 10 mM dNTP mix (Life Technologies) per µg RNA. Completed cDNA was diluted to 100 µL with distilled water and mixed well before immediate use, or stored at -20°C.

GPA mRNA extractions obtained using the Dynabeads mRNA DIRECT kit (Life Technologies) were treated similarly using the inherent oligo (dT) attached to the beads to prime cDNA synthesis. These samples were diluted to 100 µL with distilled water and stored at 4°C. Both RNA extraction procedures gave identical downstream qRT-PCR results.

2.12. Cloning of constructs into pJawohl8-RNAi

Total RNA was extracted from whole GPA adults using the total RNA extraction protocol (Chapter 2.10) then cDNA was synthesized (Chapter 2.11). A 309bp region of the GPA *Rack1* transcript sequence starting at nucleotide position +49 (<u>G</u>GGTTAC) and ending at nucleotide position +358 (CGTCAA<u>A</u>) was amplified from GPA cDNA by PCR with specific primers containing additional attb1 and attb2 linkers for cloning with the Gateway® system (Life Technologies). The PCR product was introduced into the pDONR™207 plasmid (Life Technologies) to create an entry clone using Gateway® BP reaction according to the manufacturer's instructions (Life Technologies). Plasmid DNA was transformed into electrocompetent DH5α (Life Technologies) by electroporation and cultured at 37°C overnight on Luria Broth (LB) agar plates containing 7 µg/mL Gentamicin. Subsequent clones were sequenced to verify correct size and sequence of inserts using overlapping forward and reverse Sanger sequence reactions. Verified inserts were introduced into the pJawohl8-RNAi binary destination vector (I.E. Somssich, Max Planck Institute for Plant Breeding Research, Germany) using the Gateway® LR reaction according to the manufacturer's instructions (Life Technologies) in order to generate plasmids containing target gene fragments as inverted repeats. PJ*Rack1* constructs were transformed into *A. tumefaciens*

strain GV3101 containing pMP90RK plasmid (Hellens *et al.*, 2001) by electroporation. Bacterial cells were grown at 28°C for two days on Luria Broth (LB) agar containing 25 mg/L Kanamycin, 25 mg/L Gentamicin, 50 mg/L Rifampicin and 25 mg/L Carbenicillin until colony formation. Colonies containing pJ*Rack1* were verified by PCR using one gene specific and one vector specific primer. PJ*GFP*, pJMpC002 & pJ*MpPInt01* were previously cloned according to the same procedure by Marco Pitino in the Hogenhout lab (Pitino, 2012). All primers used for cloning are displayed below (Table 2.1).

Primer name	Sequence 5'-3'
GFP ATTB1 *	AAAAAGCAGGCTGGGAGTGGTCCCAGTTCTTGT
GFP ATTB2 *	AGAAAGCTGGGTGCTGCTAATTGAACGCTTCC
MpC002 ATTB1 *	AAAAAGCAGGCTCCATGAAGGTTCAGACTTCCG
MpC002 ATTB2 *	AGAAAGCTGGGTCTTAAAAATGTCTAAAGAAACGTCC
Rack1 ATTB1 *	AAAAAGCAGGCTCCGGGTTACGCAGATCGCCACC
Rack1 ATTB1 *	AGAAAGCTGGGTCTGTTTTGACGGTTGTCAGCAGAG
ATTB1 ADAPTER *	GGGGACAAGTTTGTACAAAAAAGCAGGCT
ATTB2 ADAPTER *	GGGGACCACTTTGTACAAGAAAGCTGGGT

Table 2.1. | Primer sequences used for cloning and verification of constructs. * Primer designed by Marco Pitino.

2.13. Agro-infiltration of *N. benthamiana*

Single *Agrobacterium* colonies harboring pJ*MpC002*, pJ*Rack1* or pJ*GFP* were inoculated into Luria Broth (LB) containing 25 mg/L Kanamicin, 25 mg/L Gentamicin, 50 mg/L Rifampicin and 25 mg/L Carbenicillin and grown (28°C at 225 rpm) until an Optical Density (OD600 nm) of 0.3 was reached (Eppendorf® BioPhotometer™, Eppendorf, Cambridge, UK). Cultures were re-suspended in infiltration medium (10 mM MgCl$_2$, 10 mM MES 2-(N-morpholino)ethanesulfonic acid, pH 5.6) with 150 µM Acetosyringone to initiate expression. Each construct was infiltrated into the youngest fully expanded leaves of 4–6-week old *N. benthamiana* plants. The plants were grown in a growth chamber under a short day regime at 22–25°C.

2.14. Transient leaf disc assay on *N. benthamiana* leaf discs

One day post *A. tumefaciens* infiltration of constructs (Chapter 2.13), six leaf discs per construct were cut from infiltrated areas using an 11mm diameter cork borer then placed on one mL solidified distilled water agar (DWA) in a 24 well plate with the abaxial leaf surface facing up (**Figure 2.2A**). Four 1st instar nymphs (1–2 days old) reared on *N. tabacum* were transferred to each leaf disc then wells were individually sealed with custom made lids containing thin mesh for ventilation (**Figure 2.2B**). Plates were then laid with the lids facing down in controlled environment conditions at 18°C under 16 hours of light (**Figure 2.2C**). Fresh leaves were infiltrated every six days to provide new leaf discs for the leaf disc assays. The new leaf discs were placed in a new 24 well plate as previously. Adult aphids were moved to new discs and a total count made (number of adults and nymphs). Six replicated experiments were used with total counts made at 6, 12, 14 & 17 days post start of the experiment.

2.15. Transformation and selection of T3 homozygous 35S::dsRNA lines

The pJawohl8:RNAi constructs were transformed into *A. thaliana* ecotype Col-0 using the floral dip method (Bechtold *et al.,* 1993) and seed harvested from the dipped plants. Seeds were sown out in compost (Scotts Levington F2) and grown under 18°C, long-day conditions to encourage quick flowering. Seedlings were sprayed with BASTA solution (120 mg/L phosphinothricin) to select for transformants, then seed from surviving plants was harvested. T2 seeds were germinated on 0.8% Murashige and Skoog (MS) medium supplemented with 20 mg/mL BASTA for selection. Plants displaying 3:1 dead/alive (evidence of single insertion) segregation ratio were taken forward to T3. T3 seeds were sown on MS+BASTA and lines with 100% survival ratio (homozygous) selected. The presence of each construct insert was determined by PCR and sequencing. Three independent lines were generated for each construct.

2.16. Preparation of *A. thaliana* and aged GPA for whole-plant bioassays

All transgenic and wild-type *A. thaliana* used in insectary bioassays were prepared similarly to allow generation of aged test insects. *A. thaliana* were grown in medium-grade compost (Scotts Levington F2) and initially maintained under controlled environment conditions of 18°C, 10 hours light, 60% humidity. At approximately 10-14 days after sowing, plants were transferred individually to single wells (approximately 5cm^3) of p24 cell trays. Four-week old plants were transferred to the insectary and placed individually inside sealed experimental cages consisting of a transparent, plastic cover containing the entire plant, with mesh on the top to allow plant transpiration and prevent insect escape (Figure 2.3A). Subsequently, GPA adults from the stock colony on Chinese cabbage (Chapter 2.1) were introduced to the plants. After two days, all adults were removed leaving 'aged GPA nymphs' between 0-2 days old on the plants to be used as the experimental insects. All *A. thaliana* whole-plant bioassays with GPA were performed under controlled environment conditions of 18°C, 8 hours light, 48% humidity.
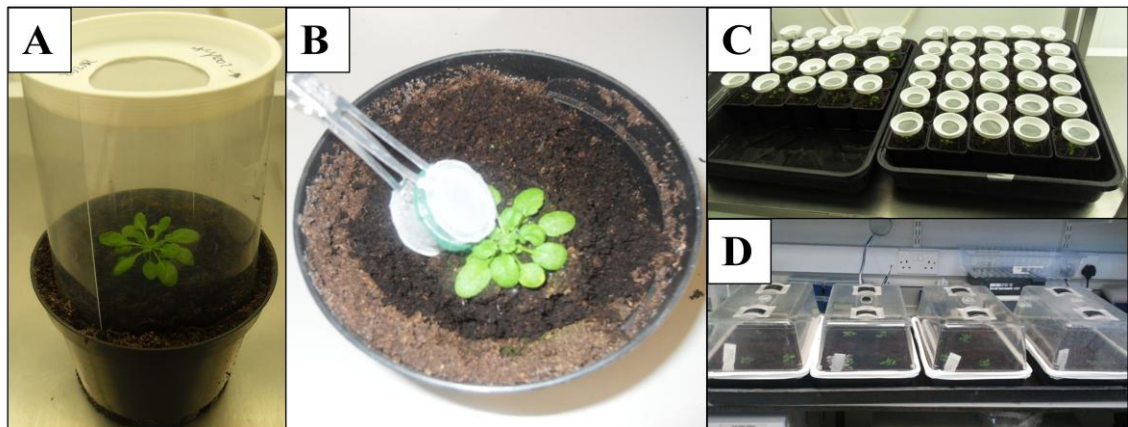
Figure 2.3. | Insectary assays. All cages/containers were custom built and designed to contain insects plus maintain ventilation. *A. thaliana* for whole-plant assays were contained in sealed experimental cages with mesh lids (**A**). GPA were confined on *A. thaliana* leaves using clip-cages (**B**). Seedlings for TuYV transmission efficiency assays were individually contained in smaller, sealed experimental cages with mesh lids (**C**). Long-term GPA population assays were contained in sealed boxes with mesh on top and taped edges (**D**).

2.17. Insect fecundity/survival bioassays on transgenic *A. thaliana*

Five aged GPA nymphs were reared on three independent lines of dsRack1 and dsMpC002 plus control plants expressing dsGFP or wild-type Col-0. The original five insects and their offspring present on the 10th, 14th & 16th day were counted and the counted nymphs were removed at each time point. The experiment was completed three times to create data from three independent biological replicates with four plants per line per replicate. To assay for gene down-regulation by qRT-PCR (Chapter 2.20), three batches of five adult aphids from each dsRNA-expressing line were flash frozen in liquid nitrogen after 16 days exposure to dsRNA-expressing plants.

2.18. Northern blotting to detect siRNAs of the transgene

To assess siRNA accumulation levels by northern blot analyses, *N. benthamiana* leaves were harvested each day for six days after agro-infiltration with the pJawohl8-RNAi constructs and whole, two week-old *A. thaliana* T3 transgenic seedlings were sampled. Approximately 2 g of plant material was flash frozen in liquid nitrogen then total RNA was extracted using the method previously described (Chapter 2.10). 15-30 µg of total RNA per sample was mixed with an equal volume of stopmix buffer (5mM EDTA; 0.1% bromophenol blue; 0.1% xyleno cyanol; 95% formamide) then denatured for five minutes at 65°C. Total RNA was resolved on a 15% polyacrylamide gel (15% acrylamide-bisacrylamide solution 19:1/7 M urea/20 mM MOPS pH 7.0) and blotted to a Hybond-N membrane (Amersham, Little Chalfont, UK) using a Trans-blot™ (Biorad, Hempstead, UK) semi-dry transfer cell. Cross-linking of RNA was performed by incubating the membrane for two hours using a pH 8.0 solution of 0.2 M 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich, Gillingham, UK) containing 0.1 M 1-methylimidazol (Sigma-Aldrich). DNA probes corresponding to the transgene were labeled using Klenow fragment (Life Technologies) with [α-32P] dCTP to generate highly specific probes. Blots were hybridized with a probe corresponding to U6 (snRNA 59 GCTAATCTTCTCTGTATCGTTCC-39) (Lopez-Gomollon & Dalmay, 2011) to control for equal loading of RNA amounts. MicroRNA marker (NEB, Hitchin, UK) was included on blots to determine size of siRNA between 21–23 nucleotides. After 3-5 days exposure to phosphor storage plates (GE Healthcare, Little Chalfont,

UK), screens were scanned with a Typhoon™ 9200 scanner (GE Healthcare) and analyzed using ImageQuant™ (GE Healthcare).

2.19. Design of qRT-PCR primers and reference gene set

Primers were designed for GPA target gene sequences and for a minimum of 8 reference (house-keeping) gene sequences (Table 2.2). Primers were designed to have high transcript specificity with a melting temperature between 58-60°C and an amplicon length between 50-200 nucleotides. The efficiency of each primer set was tested by performing a dilution series of GPA cDNA (1:1; 1:10; 1:100; 1:1000). Each cDNA dilution was represented in 2-4 technical replicates per sample for each primer set. Threshold Cycle (C(t)) values (y axis) were plotted against Log dilution (x axis) and the gradient of line (m) was calculated. The primer efficiency (%) was calculated in Microsoft Excel according to the following formula: =100*POWER(10, 1/m)/2. A primer efficiency of 100% ± 10% was determined for each primer set before further use. Initially, *L27* and *β-Tubulin* were used as GPA reference genes to investigate down-regulation of aphid target genes (Chapter 4). To later improve accuracy, the Genorm software qBASEplus (Biogazelle, Zwijnaarde, Belgium) was used to identify which reference genes are most stable at different GPA ages and dsRNA treatments. Also, the ideal number of reference genes for the experimental system was determined. Subsequently, two reference genes (*L27* & *GAPDH*) were included in qRT-PCRs (Chapter 5). Higher efficiency *Rack1* primers (Rack1 B) were also designed to improve accuracy. *A. thaliana* qRT-PCR primer sequences (Act2) were obtained from Akiko Sugio (Hogenhout lab) for use in TuYV quantification in plant samples.

Primer name	Sequence 5' – 3'
MpC002 F	ACGATGATGAGGGAGGAGTG
MpC002 R	GGGTTGCTAAATGCATCGTT
L27 F	CCGAAAAGCTGTCATAATGAAGAC
L27 R	GGTGAAACCTTGTCTACTGTTACATCTTG
Rack1 F	GGCAAGTGTCTGTCAGTGCT
Rack1 R	ATGCCCATATGCACAAGTCA
Rack1 B F	GGACGTACCACTCGTCGTTT
Rack1 B R	CATGATACCCAATCGCTGTG
ßTubulin F	CCATCTAGTGTCGCTGACCA
ßTubulin R	GTTCTTGGCGTCGAACATTT
MpPIntO2 F *	CGGAAGAAGGAAGAAATTGAAA
MpPIntO2 R *	AGGTCTCCTCCCAATCCAAT
GAPDH F	AGATGAAGTTGTGTCTTCCGACTTT
GAPDH R	GACAAATTGGTCGTTCAATGAAATC
TuYV CP F	AACACAACGCCGACCTAGAC
TuYV CP R	CATGGTAGGCCTTGAGCATT
Act2 F **	GATGAGGCAGGTCCAGGAATC
Act2 R **	GTTTGTCACACACAAGTGCATC

Table 2.2. | GPA primer sequences used in qRT-PCR. * Primer designed by Marco Pitino. ** Primer designed by Akiko Sugio.

2.20. QRT-PCR analyses to investigate down-regulation of aphid target gene

Total RNA or mRNA was extracted from GPA exposed to test plants (Chapter 2.10) and cDNA synthesized (Chapter 2.11). QRT-PCR reactions were laid out in 96-well plate (Thermo Scientific) with each sample represented by the gene of interest and reference genes. Two or three technical replicates were included for each cDNA/primer combination. Individual reactions contained 3 μL of cDNA, 0.5 μL of specific primers (forward and reverse primer at 10 pmol/mL), and 10 μL of 2xSYBR Green (Sigma-

Aldrich) in a final volume of 20 µL. Plates were sealed using adhesive PCR Film (Thermo Scientific). Plates were run in CFX connect™ machine (Bio-Rad) at 90°C for 3 min, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by 10 min at 72°C to end. Quantification of the SYBR-specific fluorophore was read during the reaction by the instrument.

2.21. Statistical analyses

All calculations were performed in Genstat 11-15th Edition (VSNi Ltd, Hemel Hempstead, UK). For replication, 'n = ?' refers to number of technical replicates used for each variable in each biological replicate. For insect bioassays, 'survival' refers to number of adult aphids alive at each measurement point and 'fecundity' refers to either the total number of nymphs or the number of nymphs produced per adult as calculated by Bos *et al.*, 2010.

"Yield at 9% moisture", "seed mass" and "oil content" were used as the response variate in separate models. Un-inoculated and inoculated values were compared for each variety with a Generalized Linear Model (GLM) using *t*-probabilities calculated by pair-wise regression within the GLM. Biological repeat was used as a block and data were checked for approximate normal distribution by visualizing residuals. "Fatty acid profiles" and "glucosinolates" were analyzed similarly but individual metabolites and groups of metabolites were compared between un-inoculated and inoculated.

Classical linear regression analysis using a GLM with Poisson distribution was applied to analyze the GPA fecundity data on *A. thaliana* transgenic lines, with 'total nymphs' as a response variate. The total nymph production on 4 plants per treatment was used as independent data points in statistical analyses in which the biological replicate was used as a variable. Aphid survival/fecundity data on *N. benthamiana* or *B. napus* leaf discs were analyzed using an unbalanced one-way ANOVA design with 'construct' or 'variety' used as the respective treatment and 'biological replicate' as the block. Aphid survival or fecundity was analyzed separately as response variates with values for each leaf disc used as independent data points in statistical analyses. Data were analyzed for significant difference between treatments using a GLM and means

were compared using *t*-probabilities calculated by the GLM. For GPA on *B. napus* leaf discs, a Tukey's multiple comparison test was also used to evaluate all possible mean pairs for the 10 varieties. For both data sets, normal data distribution was checked by visualizing residuals and a Poisson data distribution was used. Leaf discs that dried up because of lack of humidity were excluded giving 4–6 leaf discs per treatment for each biological replicate.

To perform statistical analyses on qRT-PCR data, threshold Cycle (C(t)) values were calculated using CFX manager (Bio-Rad). Relative gene expression was calculated using $2^{-\Delta\Delta C}T$ method as previously described by Livak *et al* (1993); this provided normalized C(t) values for difference in cDNA amount using reference gene C(t) values. Normalized transcript values for three biological replicates were exported into GenStat then analyzed using Student's *t*-test (n=3) to determine whether the mean normalized transcript levels of target genes for GPA fed on transgenic plants expressing dsRNA corresponding to the target gene were significantly different to aphids fed on dsGFP (control) plants. Individual *t*-tests were performed between dsGFP and each other dsRNA treatment for each time period separately. A GLM was also used similar to previous to determine differences between specific RNAi targets or replicates. Means for biological replicates and treatments at each time point were compared using t-probabilities calculated by the GLM.

2.22. Electrical Penetration Graph

Electrical penetration graph experiments were performed as per the protocol described by Tjallingi (1978). Aged GPA nymphs were reared on dsRNA-expressing *A. thaliana* plants for 10-14 days. GPA were removed from plants and starved for one hour prior to start of EPG experiment by placing in a sealed Petri dish. Aphids were attached to the Giga-8 EPG system (EPG systems, Wageningen, Netherlands) as per the manufacturer's instructions. The EPG equipment was contained within a custom-built Faraday cage to minimize electrical interference. At the start of recording, aphids were lowered onto the 4-week old *A. thaliana* expressing dsRack1, dsMpPIntO2, dsMpC002, or dsGFP within the cage. Feeding behavior was recorded for 8 hours using the Stylet+d software (EPG systems). EPG tracks were analyzed manually using the Stylet+a software (EPG systems) to categorize waveform identity and duration according to the

established waveform patterns (EPG systems). A total of 12 insects per treatment were used per treatment (n=12). Data were imported into the Sarria excel workbook as per the operating instructions to generate output parameters (Sarria *et al.*, 2009). Data were analyzed for significant difference between dsGFP or dsRack1 treatment in using an unbalanced one-way ANOVA design. Each parameter (e.g. Time to First E2) was analyzed separately with values for each aphid used as independent data points in statistical analyses. Missing values were excluded from each model (n=≤12 per treatment).

2.23. Development of RNAi in GPA over time on dsRNA plants

A time series experiment was performed to assess level of GPA target-gene down-regulation after exposure to dsRNA-expressing *A. thaliana*. Aged GPA nymphs were reared on dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants for a total of 16 days. Aphids were sampled at days 0, 4, 8, 12 and 16 in three batches consisting of five insects. To assess GPA target gene expression, samples were then subject to qRT-PCR analyses (Chapter 2.20). This experiment was repeated three times to give three biological replicates.

2.24. Recovery of GPA target-gene expression over time after removal from dsRNA plants

A time series experiment was performed to assess the recovery of GPA target-gene expression after exposure to dsRNA-expressing *A. thaliana*. Aged GPA nymphs were reared on dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants for a total of 8 days to give maximal down-regulation of target genes based on the previous experiment (Chapter 2.23; Chapter 5.2.1). Aphids were moved to wild-type Col-0 and sampled at days 0, 2, 4 and 6 days in three batches consisting of five insects. Nymph produced (2nd generation) by these insects were also harvested at 2, 4 and 6 days in three batches consisting of five insects. Excess nymphs were removed to ensure collected nymphs had been produced after previous sample point. To assess GPA target gene expression, samples were then subject to qRT-PCR analyses (Chapter 2.20). This experiment was repeated three times to give three biological replicates.

2.25. GPA target-gene expression in offspring of dsRNA-treated GPA parent insects

Second and third generation nymphs from dsRNA-treated insects were assessed to test for a germline effect of RNAi. Aged GPA nymphs were reared on dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants for a total of 8 days to give maximal down-regulation of target genes based on the previous experiment (Chapter 2.23; Chapter 5.2.1). Aphids were moved to wild-type Col-0 and allowed to produce aged GPA nymphs (Chapter 2.16). Adults were removed from plants leaving only second generation nymphs. Second generation insects were harvested at 0, 4, 8, and 12 days. Nymphs produced by second generation insects (third generation) were also harvested at 12 and 16 days. To assess GPA target gene expression, samples were then subject to qRT-PCR analyses (Chapter 2.20). This experiment was repeated three times to give three biological replicates.

2.26. TuYV transmission efficiency assay

A schematic overview of this assay is shown in **Figure 2.4**. Aged GPA were reared on four-week old dsRack1, dsMpPIntO2 or dsGFP plants for 12 days. GPA were then transferred to dsRack1, dsMpPIntO2 or dsGFP plants infected two weeks previously with approximately 20 viruliferous aphids from TuYV-infected *Capsella* (Chapter 2.2). Insects were contained on single leaves from infected plants using a clip-cage for a two-day acquisition access period (AAP) (**Figure 2.3B**). After the AAP, insects were transferred individually to 24 two-week old *A. thaliana* seedlings (expressing the corresponding dsRNA). Seedlings and insects were sealed in individual experimental cages containing the entire plant (**Figure 2.3C**). Aphids were removed from plants after 7 days. A TAS-ELISA (Chapter 2.4) was performed on seedlings at three-weeks post GPA inoculation to determine TuYV infection ratio of the 24 plants and mean virus titre of infected plants. ELISA was also conducted on plants used for virus acquisition by aphids to determine approximately equal TuYV titer in these plants. This experiment was repeated to give three biological replicates.
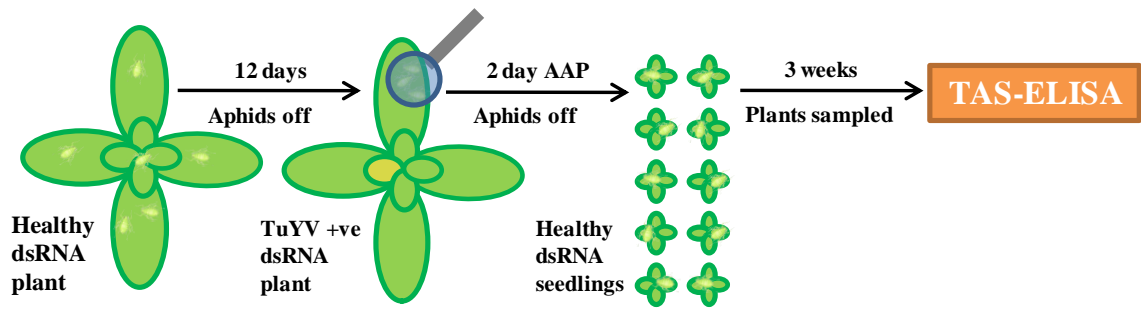
Figure 2.4. | TuYV transmission efficiency assay schematic. Aged GPA nymphs were reared on dsRNA plants for 12 days then transferred to a clip cage on a TuYV +ve dsRNA plant. After a two day AAP, aphids were transferred individually to healthy dsRNA seedlings. At three weeks post GPA inoculation, plants were sampled and tested for TuYV infection by TAS-ELISA.

2.27. Long-term population experiment

A GPA population was established on four-week old dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants and the insects were counted over successive weeks. Four plants per line were potted out in custom-made experimental cages with mesh on top to allow ventilation; all sides were sealed with tape (**Figure 2.3D**). A single, aged GPA nymph was left on each plant to establish a population. Adults and nymphs were counted at two, three and four weeks post introduction of aphids. This experiment was repeated to give three biological replicates.

2.28. TuYV acquisition efficiency assay

A schematic overview of this assay is shown in **Figure 2.5**. Aged GPA nymphs were reared on four-week old dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants for 8 days. GPA were then transferred to dsRack1, dsMpPInt02, dsMpC002 or dsGFP plants infected two weeks previously with viruliferous aphids. Insects were contained on single leaves from the infected plants using a clip-cage for an AAP of 0.5, 1, 2 or 3 days (**Figure 2.3B**). After the AAP, insects were transferred to the corresponding healthy dsRNA plants for two days. GPA were flash frozen in three batches (x5 insects) for analysis of TuYV acquisition and target gene down-regulation by qRT-PCR. This experiment was repeated to give three biological replicates.
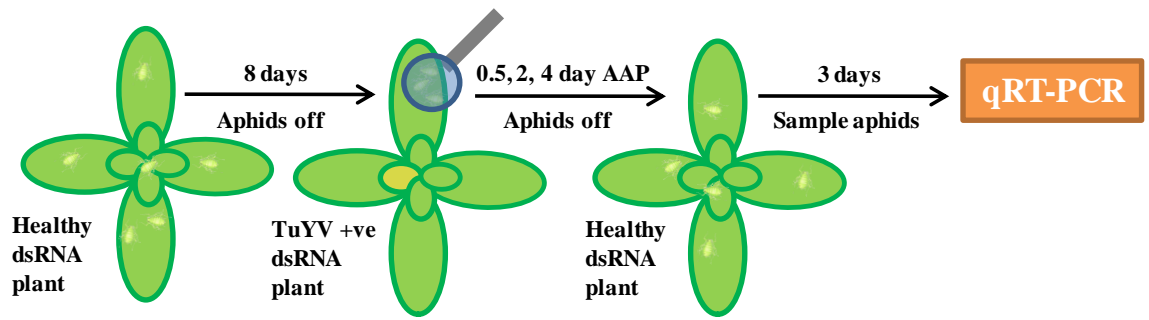
Figure 2.5. | TuYV acquisition efficiency. Aged GPA nymphs were reared on dsRNA plants for 8 days then transferred to a clip cage on a TuYV +ve dsRNA plant. After a 0.5, 2 or 4 day AAP, aphids were transferred to a new dsRNA plant for three days. Aphids were then sampled in batches of 5 insects for qRT-PCR analysis.

3. Impact of *Turnip yellows virus* infection on yield and seed quality traits in commercial oilseed rape

3.1. Introduction

As previously discussed (Chapter 1.1), TuYV has been shown to reduce oilseed rape yield. However, the impact of TuYV on yield in different varieties currently grown in the UK has not been fully investigated. Additionally, although TuYV can reduce overall yield, its effects on oil or chemical composition of seed are not known. For example, there may be a shift towards undesirable fatty acids in seeds or increases in certain harmful glucosinolates. In oilseed rape, glucosinolate and fatty acid profiles have previously been shown to be affected by abiotic factors such as temperature (Aksouh *et al.*, 2001; Baud & Lepiniec, 2010; Baux, *et al.*, 2013) and drought stress (Bouchereau *et al.*, 1996; Aslam *et al.*, 2009). Other physiological factors potentially affecting oil production are: light, oxygen, activation of the RuBisCO bypass pathway and photosynthetic oxygen release (Baud & Lepiniec, 2010). It is therefore likely that virus infection will impact seed physiology. These factors affect the overall quantity of oil and the composition of the oils present which are relevant to downstream nutritional or industrial uses (Kimber & Mcgregor, 1995; Schnurbusch *et al.*, 2000; Schierholt *et al.*, 2001).

The oil biosynthetic process is initiated at the onset of seed maturation, leading to accumulation of oil within the seed (Baud & Lepiniec, 2010). The typical oil content of *Brassica napus* is 40% of the seed dry weight, which is approximately 4mg, and it is stored in the embryo (Baud & Lepiniec, 2010). The typical fatty acid composition is: 16:0 (3.9%) Palmitic acid; 18:0 (1.9%) Stearic acid; 18:1 (64.1%) Oleic acid; 18:2 (18.7%) Linoleic acid; 18:3 (9.2%) α-Linolenic acid; 20:1 (1%) Gadoleic acid (Baud & Lepiniec, 2010).

The nutritional value of rapeseed oil was hugely improved with the development of low erucic acid cultivars, called canola cultivars, which came from the identification of *Brassica napus* and *Brassica rapa* plants with no erucic acid in their seed oil (Kimber & Mcgregor, 1995). Another breeding achievement has been the reduction of linolenic acid improving the storage characteristics of the rapeseed oil. Additionally, a higher content of linoleic acid (vitamin F), an essential fatty acid, would be desirable to improve the nutritional value of the oil (Kimber & Mcgregor, 1995). The production of vegetable oils with increased levels of oleic acid is of interest for nutritional and industrial purposes (Schierholt *et al.*, 2001). Low levels of polyunsaturated fatty acids

result in increased levels of the monounsaturated oleic acid, which have been associated with elevated oxidative stability. Furthermore, an increased content of oleic acid can reduce cholesterol in blood plasma and prevent arteriosclerosis (Schierholt *et al.*, 2001). For nutritional purposes, the content of saturated fatty acids such as palmitic acid and stearic acid should be as reduced as much as possible. However, for margarine production, high levels of saturated fatty acids are desirable so as to avoid the industrial hydrogenation of vegetable oils, which results in the formation of unhealthy *trans* fatty acids (Schnurbusch *et al.*, 2000).

The aim of this research was to provide comprehensive analyses of crop yield and oil quality traits affected by TuYV infection in a variety of commercial oilseed rape lines. Some varieties may accumulate more virus, however, it is not known how this relates to yield or oil quality. This research determined whether changes to yield and seed physiology are directly correlated to TuYV titer or whether individual genotypes respond differently. This is the first investigation of this type, and provides information on the current status of resistance to TuYV in UK oilseed rape varieties to manage the disease in future.

3.2. Results

3.2.1. TuYV infects and reduces yield in all commercial varieties

Forty-nine varieties from the 'HGCA winter oilseed rape recommended list' (HGCA, 2012) were trialed for TuYV susceptibility by ELISA during the 2009-2010 growing season. All varieties tested positive for the virus (**Figure 3.1**). The incidence of TuYV infection varied between 74-94% and background levels of TuYV between 4-14% were recorded in control plots from natural infection. Using ELISA data, 10 varieties were selected from the 49 which represented a range of TuYV susceptibility (**Figure 3.1**).
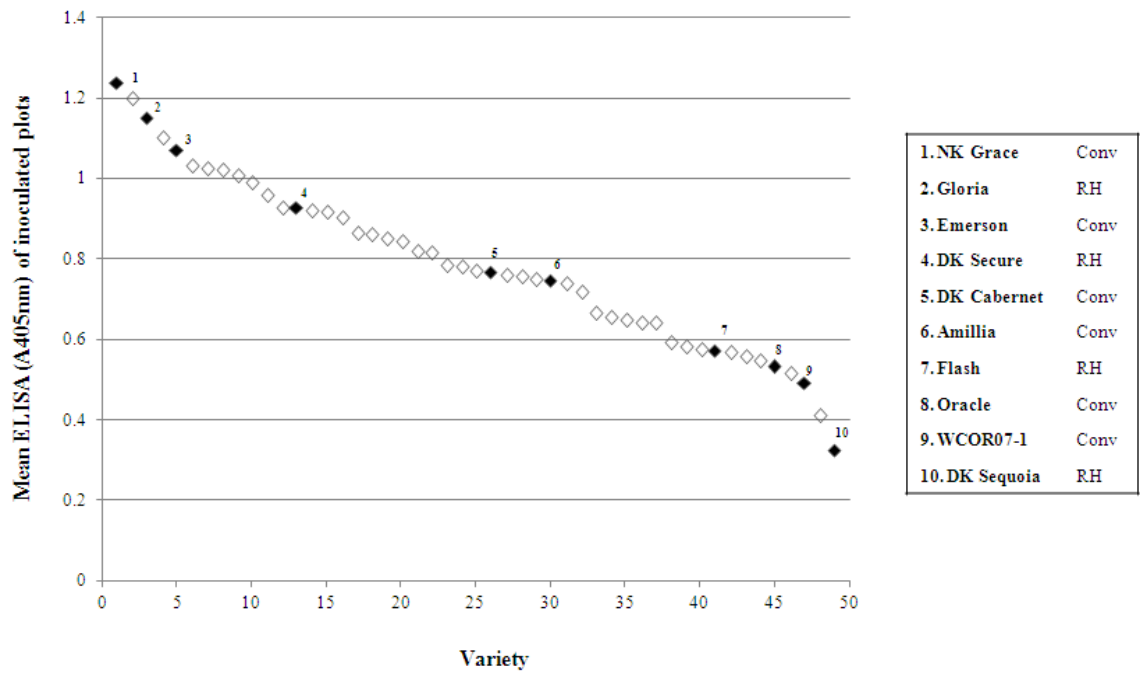
Figure 3.1. | ELISA data plot for 49 varieties. Varieties from 2009-10 field trial ordered according to mean ELISA reading (A405nm) of TuYV infected plots. Each variety is represented by a tile; filled tiles represent varieties chosen for further trial. Names of the ten chosen varieties are indicated with plant type as either conventional (conv) or resynthesized hybrid (RH).

The 10 varieties selected from the previous trial were grown during the 2010-11 growing season in larger plots which enabled yield data to be obtained. In order to allow direct comparison between virus titer and impact on yield and seed quality traits, the mean ELISA readings for TuYV-inoculated and control plots for each variety were calculated from extensive sampling of plant material (**Figure 3.2**).
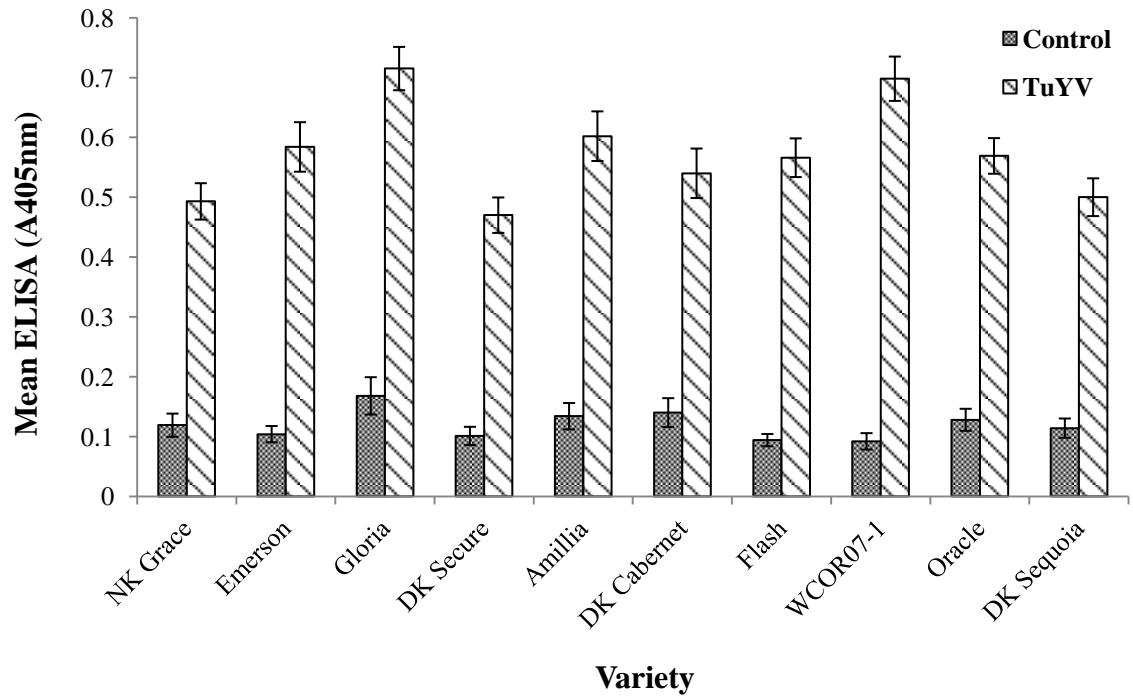
Figure 3.2. | ELISA data for 10 varieties. Mean ELISA readings (A405) for varieties with TuYV inoculation or control treatment. Error bars represent standard error (SE) ± of the mean.

Yield of seed at 9% moisture was determined for each variety under TuYV or control treatment (**Figure 3.3**). TuYV decreased yield in nearly all varieties with significant reductions in NK Grace, Emerson, DK Secure & DK Sequoia (GLM, n=4, p=0.004, 0.023, 0.027, 0.045 respectively).
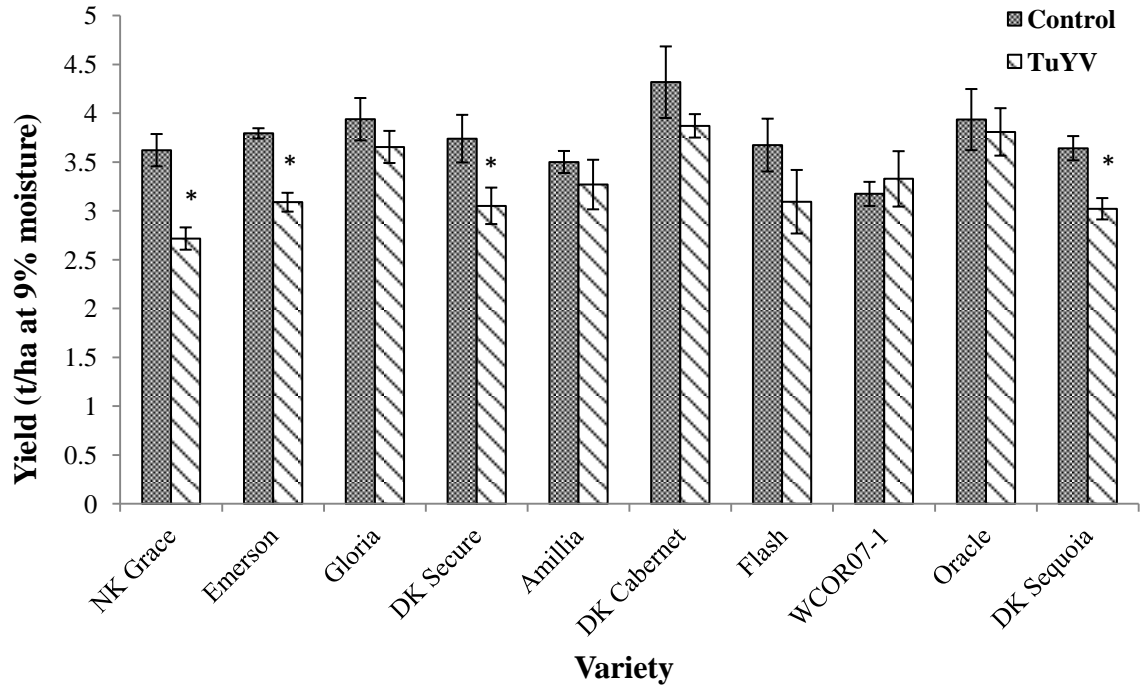
Figure 3.3. | Yield data. Mean yield in tons per hectare adjusted to 9% moisture for varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=4, p=<0.05). Error bars represent standard error (SE) ± of the mean.

3.2.2. TuYV infection increases seed mass and reduces oil content

Seed weight (in grams) showed a general increase with TuYV infection in most varieties. This was borderline significant in some varieties but was only statistically significant in WCOR07-1 (GLM, n=3, p=0.009) (**Figure 3.4**). This trend is consistent with previous literature (Jay *et al.*, 1999).
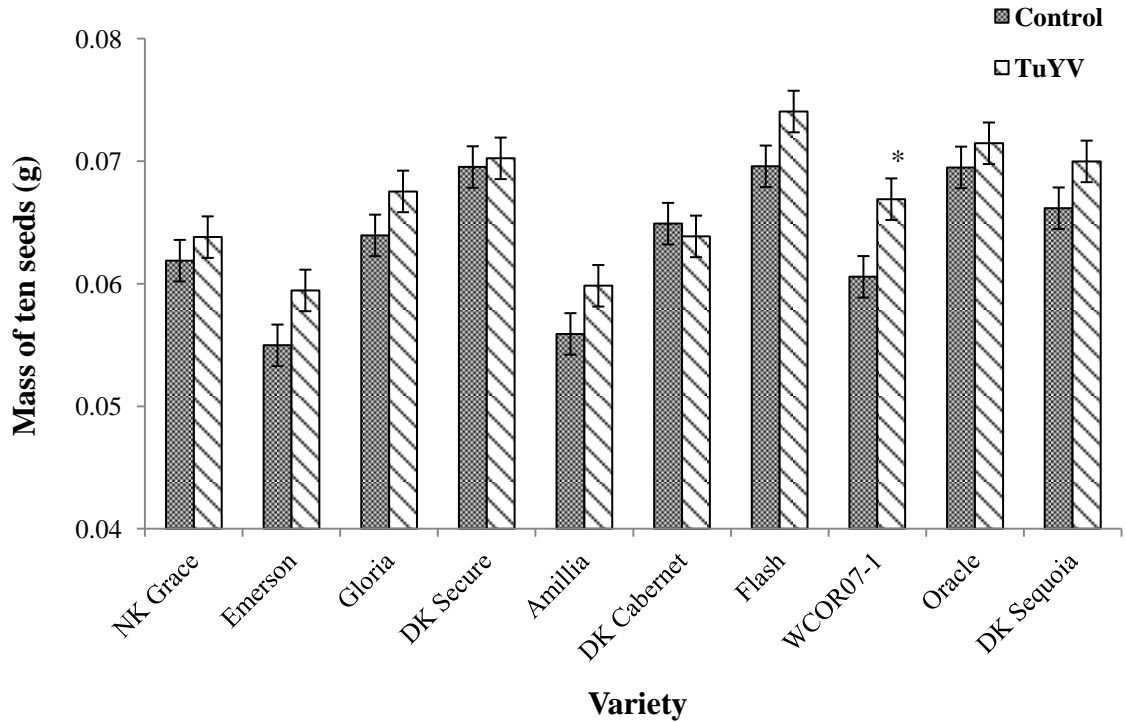
Figure 3.4. | Seed mass. Mass of ten seeds in grams for varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=3, p=<0.05). Error bars represent standard error (SE) ± of the mean.

Although seeds are generally larger, there is a broad trend towards lower oil content in TuYV infected plots (Figure 3.5). Oil content per gram of seed as determined by NMR showed decreases of up to 3% in some varieties. A significant decrease was observed in three varieties (Emerson, Amillia, Flash) (GLM, n=3, p<0.05). Although most varieties showed decreased oil yield after TuYV infection, one variety (DK Cabernet) showed the opposite trend, however this was not statistically significant.
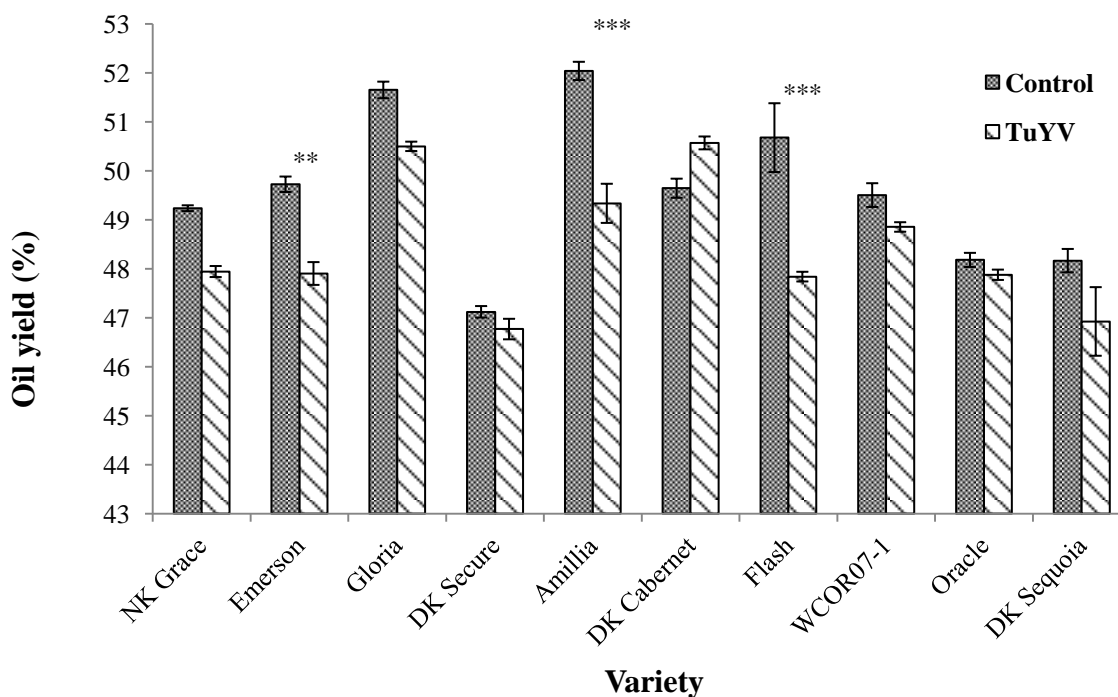
Figure 3.5. | Oil yield. Percentage oil yield as determined by NMR in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=3, p=<0.05). Error bars represent standard error (SE) ± of the mean.

3.2.3. TuYV infection modifies the fatty acid profile of oil

A total of 8 fatty acids were compared between seed from TuYV inoculated and control plots for each variety. Fatty acids profiled include both saturated (16:0 – Palmitic acid, 18:0 – Stearic acid, 20:0 – Arachidic acid, 22:0 – Behenic acid) and unsaturated fatty acids (18:1 – Oleic acid, 18:2 – Linoleic acid. 18:3 - Linolenic acid, 20:1 – Gadoleic acid). Fatty acids 16:0, 18:1 and 18:2 showed the largest number of significant changes between infected and non-infected plants (**Figure 3.6**). Most varieties showed a slight increase in 16:0 under TuYV infection compared to control plots with a significant increase observed in three varieties: Amillia, Flash, and Oracle (**Figure 3.6A**) (GLM, n=3, p=0.014, 0.014, 0.036 respectively). The proportion of 18:1 in seeds from TuYV infected plots compared to control plots showed a general decrease in nearly all varieties. This was significant in six of the ten varieties (GLM, n=3, p<0.05) and highly significant in Emerson and Amillia, where up to 10% reduction was recorded (**Figure 3.6B**). Conversely, the fatty acid 18:2 showed a trend towards increased composition in TuYV infected plants. Significant increases were observed in

four of the trial varieties: Emerson, Gloria, Amillia, and Flash (**Figure 3.6C**) (GLM, n=3, p<0.05).
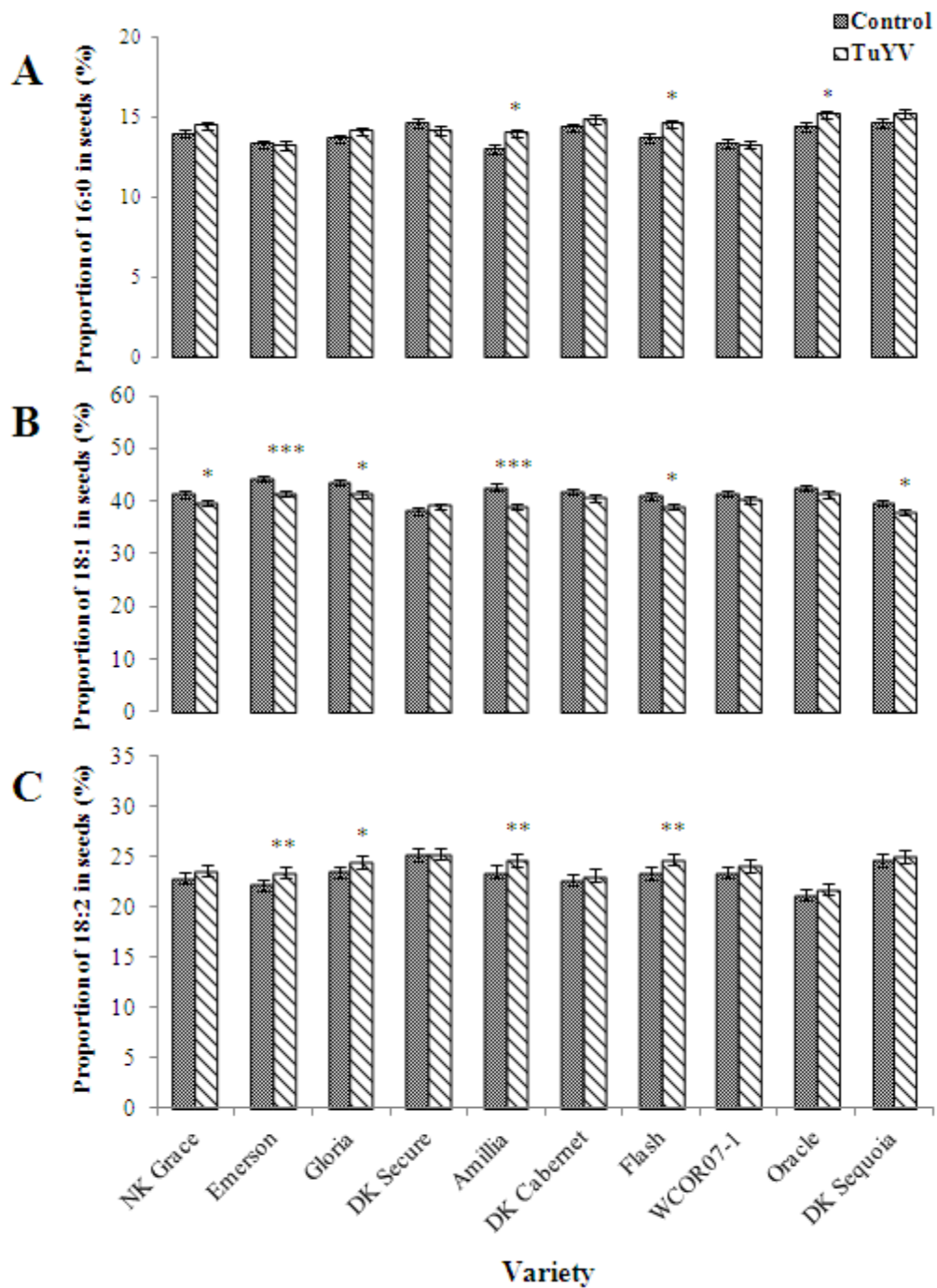
Figure 3.6. | Fatty acid profiles. Percentage proportion of three fatty acids 16:0 (**A**) 18:1 (**B**) or 18:2 (**C**) in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=3, p=<0.05). Error bars represent standard error (SE) ± of the mean.

No significant differences were found in total glucosinolates between control and TuYV infected plants (**Figure 3.7A**). For aliphatic glucosinolates, only the variety DK Sequoia was shown to be significantly affected by the virus as the quantity decreased (**Figure 3.7B**) (GLM, n=2, p=0.45). In contrast, indolic glucosinolates were significantly increased in four varieties: Amillia, DK Cabernet, Oracle, and WCOR07-1 (GLM, n=2, p<0.05) where μmoles per gram of seed more than doubled in some cases (**Figure 3.7C**). There were no changes seen in the aryl class of glucosinolates for any varieties (data not shown).
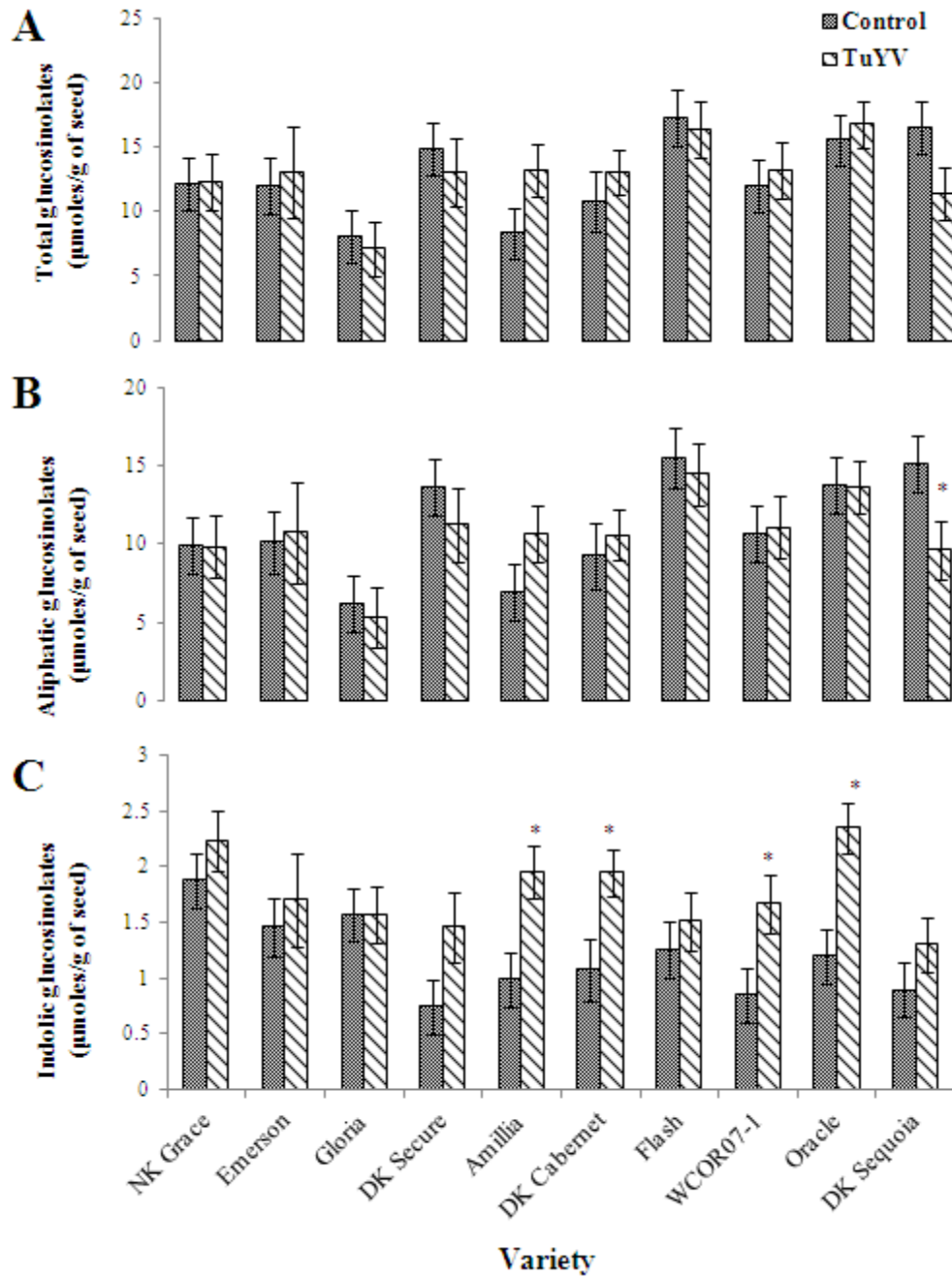
Figure 3.7. | Glucosinolate composition. Percentage of total glucosinolates **(A)** and subclass of aliphatic **(B)** or indolic **(C)** glucosinolates in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=2, p=<0.05). Error bars represent standard error (SE) ± of the mean.

3.2.4. Aphid survival or fecundity was not significantly different between commercial varieties

Varieties were assessed for the level of resistance to GPA in order to determine whether the difference in TuYV titer observed was uncoupled from aphid susceptibility. No significant differences were observed in GPA fecundity or survival on the ten varieties using a GLM, pair-wise regression and Tukey's multiple comparison tests (**Figure 3.8**). The average number of nymphs per adult ranged consistently between 7 and 9 (**Figure 3.8A**), however, none of the varieties showed any significant susceptibility or partial resistance to GPA compared to other varieties. Survival showed a similar trend (**Figure 3.8B**).
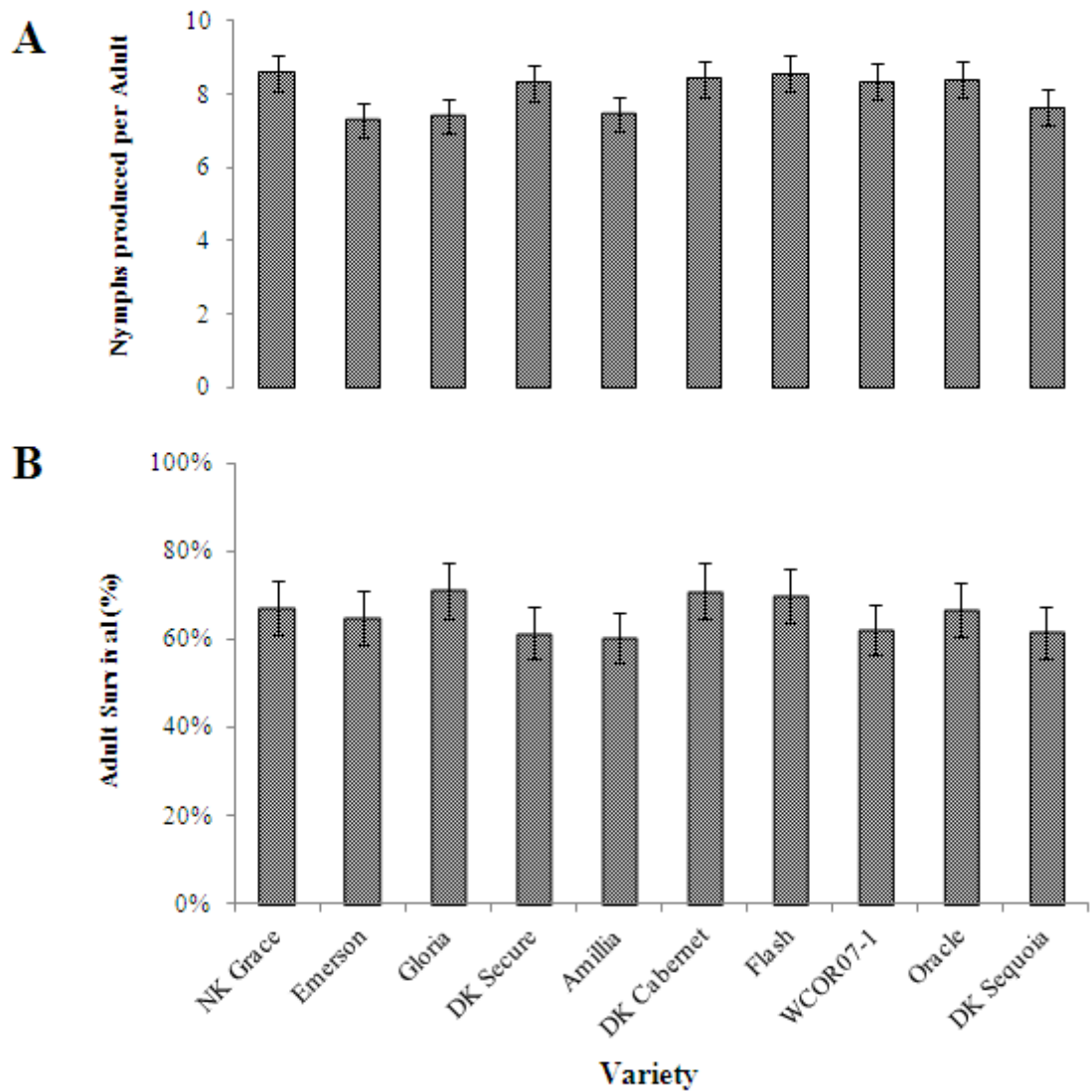
Figure 3.8. | Aphid fitness. Mean number of GPA nymphs produced by adults from six biological replicates **(A)** and mean percentage survival of adult aphids **(B)** on leaf discs cut from 10 selected varieties over 14 days (n=6 per biological replicate). Error bars represent standard error (SE) $\pm$ of the mean.

3.3. Discussion

All 49 tested varieties tested positive for TuYV indicating no complete resistance to TuYV present in these varieties. However, the range of ELISA values suggest variation in the levels of virus accumulation in some commercial varieties. Ten varieties with a range of TuYV titers were chosen to assess yield and seed physiological traits under virus infection.

A general decrease in yield was recorded after TuYV inoculation in all 10 varieties (**Figure 3.3**). This amounted to a 10-15% yield decrease consistent with previous literature (Stevens *et al.*, 2008). This level is not as high as has been previously recorded where up to 26% yield decrease was shown in the UK, and much higher levels shown in Australia for example (Jones *et al.*, 2007). Yield reduction was significant for four varieties: NK Grace, Emerson, DK Secure & DK Sequoia. The yield impact was therefore greater in some varieties. The level of yield impact did not correlate with level of virus titer in the plant, suggesting a genotype-specific interaction (**Figure 3.2**; **Figure 3.3**). For example, DK Sequoia, NK Grace & DK Secure all displayed a costly yield decrease from TuYV despite having the three lowest TuYV titers. Some varieties showed a high virus titer without a significant yield impact e.g. WCOR07-1 and Amillia. Similarly, Gloria had the highest TuYV titer yet displayed negligible impact on yield.

The oils analysis data together suggest that TuYV infection is having an impact on oil characteristics (**Figure 3.4**; **Figure 3.5**; **Figure 3.6**; **Figure 3.7**). Oil content of seeds was significantly decreased in three varieties (**Figure 3.5**) despite a trend towards increased seed mass (**Figure 3.4**). The virus infections changed fatty acid profiles in nearly all varieties with a consistent shift from 18:1 to 18:2 (**Figure 3.6B**; **Figure 3.6C**). This may be an indication of plant stress responses (Upchurch, 2008). There is a slight trend towards more fatty acid profile changes seen in varieties with a higher TuYV titer seen in the field, although this trend was not followed by variety WCOR07-1 which gave high TuYV titers in the field but had no significant fatty acid changes. For glucosinolates, no variety had a change in total amount and one (DK Sequoia) had a decrease in aliphatic glucosinolates (**Figure 3.7B**). Four of ten varieties showed an increase in indolic glucosinolates (**Figure 3.7C**), which may indicate an increased defense response to TuYV. However, there seems to be no direct correlation between virus titer in the plant and changes in glucosinolates.

There was no significant trend between the extents of physiological changes in seed in relation to level of TuYV accumulation in the plant (**Figure 3.2**). Some varieties may build up high levels of the virus, for example Gloria, yet only subtle changes in seed physiology were observed. Conversely, Amillia and Flash both had intermediate virus levels in the field yet showed a number of distinct changes to seed physiology. High virus accumulation therefore may not positively correlate with the severity of

symptoms, including loss of seed quality, again suggesting a variety-dependent effect. The virus could also be impacting the plant in other ways which is not manifested in any of the data shown here, for example through changes to seed fertility, sensitivity towards abiotic stresses, susceptibility to other pathogens or flowering time. For example, TuYV could be decreasing ovule fertility which can result in fewer, though slightly larger, seeds per pod, and consequently yield losses (Bouttier & Morgan, 1992). Also, the results indicate that TuYV could be affecting the activity of enzymes involved in the desaturation of fatty acid biosynthesis (Kimber & Mcgregor, 1995; Bocianowski *et al.*, 2012). It is also interesting to note that even after high TuYV infection, Emerson and Amillia have higher oil content than DK Secure under control treatment, which had the lowest virus count in the field. Also, some varieties such as DK Cabernet are higher yielding after TuYV infection than others which were un-inoculated.

The ten varieties were also assessed for resistance to GPA (**Figure 3.8**). Despite no statistical difference in survival between varieties (**Figure 3.8B**), up to 10% difference could be observed between varieties over 14 days and a range of approximately 50 total nymphs produced between highest and lowest was recorded over this time (**Figure 3.8A**). These differences could therefore be substantial in a field setting. Generally, the aphid fitness assays suggest that the partial resistance to the virus observed in some varieties compared to others is not due to the level of aphid resistance, at least for the predominant vector, GPA.

TuYV can induce a variety of symptoms in oilseed rape which are often inconspicuous (Chapter 1.1). Symptoms were therefore not assessed, as this is more subjective than virus titer quantification. It is possible the host response is linked to the yield impact and changes in seed physiology observed. As oil accumulation is part of the seed maturation process and occurs quite late in seed production, it is likely to be sensitive to factors involved in senescence of leaves and pods (Baud & Lepiniec, 2010). It is possible that loss of green tissue as a result of virus infection may underpin these changes, perhaps through reduced photosynthetic ability. Further investigation is needed to determine why TuYV has a greater impact on some varieties compared to others.

Overall, this data demonstrates oilseed rape yield decreases and subtle yet observable effects on fatty acid profiles, glucosinolates, oil yield and mass of seed in commercial oilseed rape varieties after TuYV infection. The evidence presented in this

study therefore shows that the virus has a clear effect on plant physiology, which is variety-dependent rather than as a result of TuYV accumulation within the plant. Virus titer or infection ratio therefore is not an accurate indicator for predicting TuYV induced changes to yield or oil quality suggesting that each variety needs to be assessed separately. It also seems necessary to look outside of UK commercial lines for sources of complete virus resistance.

4. RNAi of GPA genes by dsRNA feeding from plants

Contributors: Pitino M, Maffei ME, Ridout CJ, Hogenhout SA.

4.1. Introduction

The research aim was to develop tools to investigate aphid genes involved in the transmission of TuYV. To realize this aim, I collaborated with Marco Pitino (Hogenhout lab, JIC, Norwich, UK) to develop an improved method for achieving RNAi in aphids. RNAi, as previously mentioned (Chapter 1.11), is a powerful reverse genetics tool for assessing gene function and has been previously used in several aphid species (Chapter 1.12). GPA Rack1 was chosen as a target as it has been shown to bind luteovirid particles and is linked with endocytosis processes (Chapter 1.10). It is also well characterized in various organisms, and amenable to RNAi-based approaches.

Both micro-injection and artificial diets (Chapter 1.12) are valuable methods for achieving RNAi in aphids. However, dsRNA/siRNA has to be synthesized in both cases and neither treatment is natural for aphids. As RNAi in aphids is indeed feasible, it has the potential to be expanded to include gene knockdown via the delivery of dsRNA from plants (plant-mediated RNAi, or abbreviated to PMRi). This method could allow for studying aphid gene function in the aphid natural habitat and may be useful for controlling aphid pests in crop production. The PMRi method effectively silences genes of Lepidopteran and Coleopteran insect species (Mao *et al.*, 2007; Baum *et al.*, 2007) and the brown planthopper, an hemipteroid species (Zha *et al.*, 2011). However, these insects are larger than aphids and hence consume more plant tissue/sap while feeding. The goal of this study was to determine if the PMRi approach also silences aphid genes.

GPA was selected because it has a broad plant host range, including the model plants *N. benthamiana* and *A. thaliana* for which transgenic materials can be generated relatively quickly. Furthermore, transgenes can be rapidly expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated transient expression providing the possibility to develop a high-throughput system to assess which genes in the aphid genome are essential for survival of aphids on plant hosts. Moreover, this species is the predominant vector of TuYV (Chapter 1.5) so developing tools in this system would be valuable for investigating aphid genes involved in the circulative transmission of TuYV.

To establish the PMRi technique in aphids, it was determined whether silencing was equally effective in different aphid tissues. *C002*, a gene strongly expressed in the salivary glands of the pea aphid was previously silenced by injection (Mutti *et al.*, 2006; Chapter 1.12). C002 has been shown to have an important function in aphid interaction

with the plant host (Mutti *et al.*, 2006; Mutti *et al.*, 2008). The homologue of *C002* from GPA was previously identified and named *MpC002* (Bos *et al.*, 2010). *MpC002* is predominantly expressed in the GPA salivary glands and transient over-expression of *MpC002* in *Nicotiana benthamiana* improved GPA fecundity (Bos *et al.*, 2010). In contrast, *Rack1* is constitutively expressed but strongly expressed in the aphid gut. Both *Rack1* and *MpC002* were therefore selected as gene targets to establish the PMRi tool in GPA.

4.2. Results

4.2.1. Expression profiles of RNAi target genes

C002 and *MpC002* are predominantly expressed in the salivary glands of pea aphids and GPA (Mutti *et al.*, 2008; Mutti *et al.*, 2006; Bos *et al.*, 2010), and *Rack1* in aphid gut tissues (Seddas *et al.*, 2004). To verify this in the GPA colony, RT-PCR was performed on total RNA extracted from different aphid tissues. *MpC002* transcripts were detected in GPA heads and salivary glands, at relatively low abundance in whole aphids but not in dissected aphid guts (**Figure 4.1**). Conversely, *Rack1* transcripts were found in all aphid body parts and at highest abundance in the gut (**Figure 4.1.**). These results confirmed previous findings and provided RNAi targets predominantly expressed in the aphid salivary glands and gut.
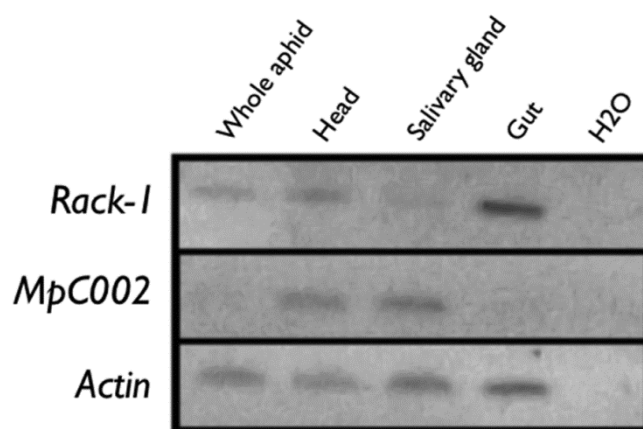
Figure 4.1. | *MpC002* **and** *Rack1* **are differentially expressed in GPA tissues.** RNA isolated from whole aphids and dissected aphid body parts were used for RT-PCR with specific primers for *Rack1*, *MpC002* and *Actin*. The latter showed presence of similar RNA concentrations in the aphid samples.

4.2.2. Detection of *MpC002* and *Rack1* siRNAs in *N. benthamiana* leaves

First, the production and processing of dsRNAs into siRNAs corresponding to GPA *MpC002* (dsMpC002) and *Rack1* (dsRack1) in *N. benthamiana* leaves was investigated. The entire *MpC002* transcript without the region corresponding to the signal peptide (710bp), a fragment corresponding to the 5' coding region of the *Rack1* transcript (309bp) and a fragment corresponding to the majority of the open reading frame (537bp) of the green fluorescent protein (GFP) were cloned into the pJawohl8-RNAi plasmid, which expresses the cloned fragments as inverted repeats under control of a double CaMV (*Cauliflower mosaic virus*) 35S promoter to produce dsRNAs (Chapter 2.12). Double-stranded GFP (dsGFP) was used as a control for the dsRNA treatments as opposed to empty vector in order to assess whether the presence of dsRNA itself would induce some effect in plant response to aphids. The pJawohl8-RNAi constructs were transiently expressed by *Agrobacterium*-mediated infiltration (agro-infiltration) of *N. benthamiana* leaves. *MpC002* and *Rack1* siRNAs were observed starting 2 days post agro-infiltration (Figure 4.2). This indicated that the *MpC002* and *Rack1* dsRNAs are being processed into 21 to 23 nucleotide siRNAs in *N. benthamiana* leaves. The agro-infiltrated leaves did not show obvious phenotypes such as chlorosis or leaf curling/crinkling upon agro-infiltration of the pJawohl8-RNAi constructs.
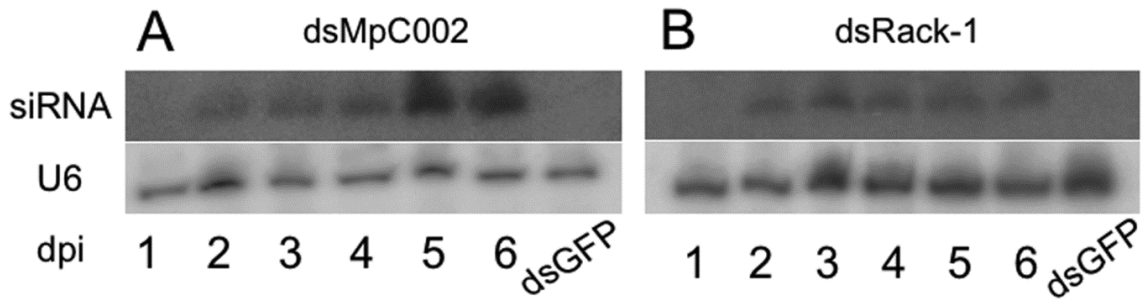
Figure 4.2. | *MpC002* **and** *Rack1* **dsRNAs are processed into siRNAs (21-23nt) in agro-infiltrated** *N.* *benthamiana* **leaves.** *MpC002* and *Rack1* pJawohl8-RNAi constructs were agro-infiltrated in *N.* *benthamiana* leaves, which were harvested 1, 2, 3, 4, 5 or 6 days post-inoculation (dpi) for RNA isolation. Total RNA (15-20 μg) was loaded in each lane. Northern blots were hybridized with probes prepared from *MpC002* (**A**) or *Rack1* (**B**) PCR products. Total RNAs isolated from leaves 6 dpi with GFP pJawohl8-RNAi constructs were included to control for specific hybridization of the *MpC002* and *Rack1* probes (lanes indicated with dsGFP). To control for equal RNA loading, blots were stripped and then hybridized with an snRNA probe corresponding to U6, which is constitutively produced in plants (Hanley & Schuler, 1991).

4.2.3. RNAi of GPA *MpC002* and *Rack1* genes by feeding from transgenic *N.* *benthamiana* leaves

   MpC002 and *Rack1* down-regulation was investigated in GPA after feeding on *N. benthamiana* leaves transiently producing the *MpC002* and *Rack1* RNAs. At one-day post agro-infiltration, 11-mm diameter leaf discs of the infiltrated leaves were placed on top of water agar in wells of 24-well titer plates and exposed to aphids as previously described (Bos *et al.*, 2010; Chapter 2.14). Nymphs born on the leaf discs were transferred every 6 days to newly agro-infiltrated leaf discs to ensure continuous exposure of the aphids to the *MpC002* and *Rack1* RNAs (**Figure 4.2**). At 17 days, the adult aphids were collected to assess *MpC002* and *Rack1* expression levels by quantitative RT-PCR (qRT-PCR). Aphids fed for 17 days on *N. benthamiana* leaf discs infiltrated with dsGFP pJawohl8-RNAi constructs were used as controls. The expression levels of *MpC002* and *Rack1* were reduced by an average 30-40% compared to the controls (**Figure 4.3A**). This down-regulation was consistent and highly significant among three biological replicates for *MpC002* (Student's *t*-test, n=3, p-value = 0.013) and *Rack1* (Student's *t*-test, n=3, p-value = 0.012).

Figure 4.3. | Down-regulation of GPA *MpC002* or *Rack1* by *N. benthamiana*-mediated RNAi reduces aphid fecundity. (**A**) *MpC002* and *Rack1* expression is down-regulated in aphids fed on *N. benthamiana* leaves transiently producing *MpC002* and *Rack1* RNAs. Aphids fed on transgenic *N. benthamiana* leaf discs for 17 days were harvested and analyzed for down-regulation of *MpC002* and *Rack1* by qRT-PCR. Data shown are means ± standard errors of three biological replicates with n=3 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (Student's *t*-test, n=3, p<0.05) (**B**) *MpC002* and *Rack1* RNAi GPA are less fecund. The numbers of nymphs produced by the aphids analyzed for down-regulation of *MpC002* and *Rack1* in A were counted and compared to the nymphs produced from aphids fed on the dsGFP transgenic *N. benthamiana* leaf discs. Data shown are average number of nymphs produced per adult aphid with means ± standard errors of six biological replicates with n=4-6 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (ANOVA, n=4-6, p<0.05).

4.2.4. RNAi of aphid *MpC002* and *Rack1* on stable transgenic *A. thaliana* lines

The down-regulation of GPA genes *MpC002* and *Rack1* upon feeding on stable transgenic *A. thaliana* plants was assessed. The transgenic lines were obtained by floral-dip transformation of Col-0 plants with the *MpC002*, *Rack1* and GFP pJawohl8-RNAi constructs used in the *N. benthamiana* transient assays. Three independent T3 homozygous dsMpC002 and dsRack1 transgenic *A. thaliana* were generated. One T3 homozygous dsGFP transgenic *A. thaliana* line was included as control. All lines contained the transgenes as confirmed by PCR and sequencing. Northern blot analysis of the transgenic *A. thaliana* lines revealed the presence of siRNA for *MpC002* and *Rack1* (**Figure 4.4**). The siRNAs corresponding to GPA *MpC002* were equally abundant in the three independent transgenic lines (**Figure 4.4A**), while the siRNAs

corresponding to *Rack1* were abundant in line 1, less abundant in line 3 and not detected in line 4 (**Figure 4.4B**).
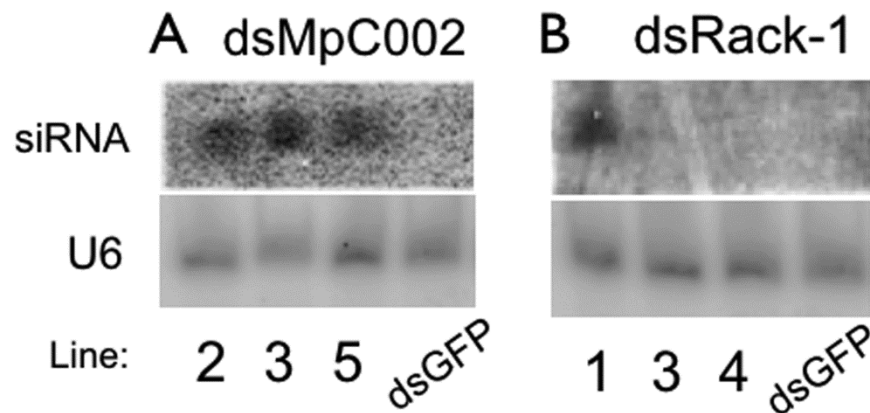
Figure 4.4. | *MpC002* **and** *Rack1* **dsRNAs are processed into siRNAs (21-23nt) in transgenic** *A. thaliana* **lines.** Total RNA was isolated from two-week old seedlings of T3 homozygous stable dsMpC002 (**A**) and dsRack1 (**B**) transgenic lines. Total RNA isolated from two-week old seedlings of a T3 homozygous stable dsGFP line was included to control for specific hybridization (lanes indicated with dsGFP). Each lane contains 15-20 µg of total RNA. Northern blots were hybridized with probes prepared from *MpC002* (**A**) or *Rack1* (**B**) PCR products. To verify equal RNA loading, blots were stripped and then hybridized with an snRNA probe corresponding to U6, which is constitutively produced in plants (Hanley & Schuler, 1991).

To investigate down-regulation of GPA *MpC002* and *Rack1* on the stable transgenic lines, nymphs born on the transgenic plants were kept on these plants for 16 days at which time the adult aphids were collected for RNA extraction and qRT-PCRs. The aphids reared on three independent dsMpC002 lines showed an approximate 60% decrease in *MpC002* expression compared to aphids reared on dsGFP (**Figure 4.5A**). Furthermore, down-regulation of *Rack1* by approximately 50% was demonstrated for aphids reared on dsRack1 line 1 compared to dsGFP but not for aphids fed on dsRack1 lines 3 and 4 (**Figure 4.5A**). *MpC002* down-regulation on the three independent lines was consistent in three replicates (Student's *t*-test, n=3, p<0.05). *Rack1* was also consistently down-regulated on dsRack1 line 1 among three replicates (Student's *t*-test, n=3, p=0.023), while *Rack1* was not significantly down-regulated on dsRack1 lines 3 and 4 (Student's t-test, n=3, p> 0.05). These results are in agreement with the dsMpC002 and dsRack1 expression levels in the transgenic lines in which the expression of the aphid *Rack1* gene was not down-regulated on transgenic lines that have low levels of siRNAs corresponding to *Rack1* (**Figure 4.4B**).
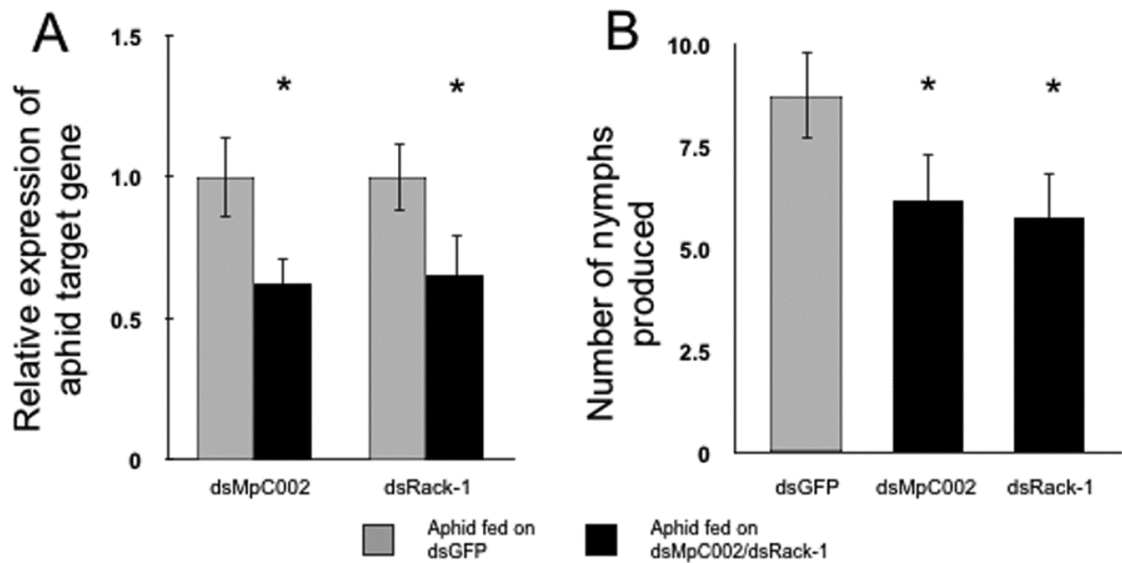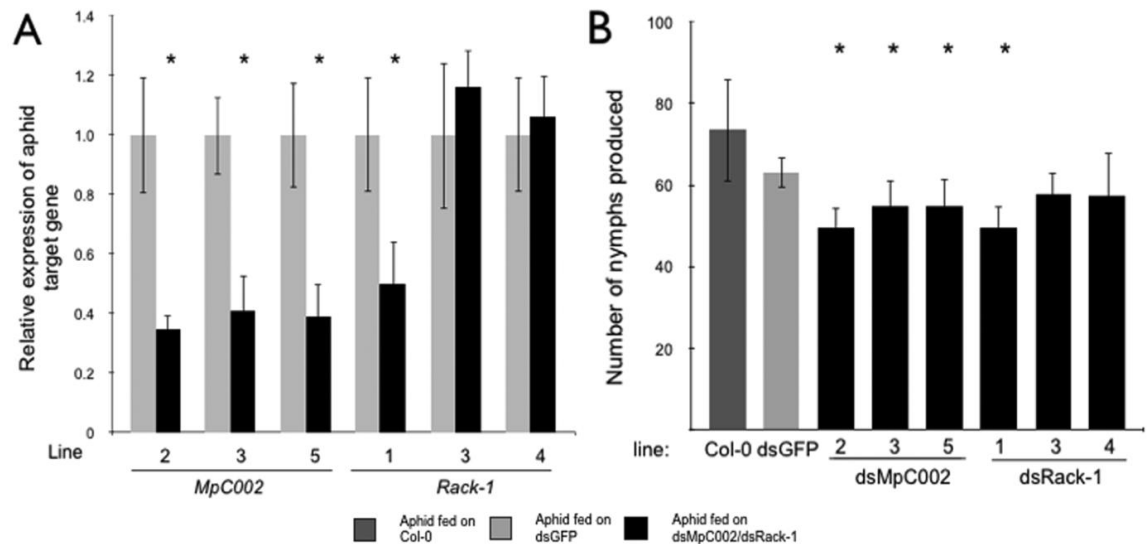
Figure 4.5. | Down-regulation of GPA *MpC002* or *Rack1* by *A. thaliana*-mediated RNAi reduces aphid fecundity. (**A**) *MpC002* and *Rack1* expression is down-regulated in aphids fed on transgenic *A. thaliana* producing *MpC002* and *Rack1* RNAs. Aphids fed on dsMpC002 or dsRack1 producing *A. thaliana* for 16 days were harvested and analyzed for down-regulation of *MpC002* and *Rack1* by qRT-PCR. Data shown are means ± standard errors of three biological replicates with n=3 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (Student's *t*-test, n=3, p<0.05) (**B**) *MpC002* and *Rack1* RNAi GPA are less fecund. The numbers of nymphs produced by the aphids analyzed for down-regulation of *MpC002* and *Rack1* in A were counted and compared to the nymphs produced from aphids fed on Col-0. Data shown are total number of nymphs produced on each line with means ± standard errors of three biological replicates with n=4 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (GLM, n=4, p<0.05).

4.2.5. RNAi of *MpC002* and *Rack1* reduces GPA fecundity

It was previously shown that silencing of *C002* by injection of dsRNAs in the pea aphid increased the lethality of these aphids on plants (Mutti *et al.*, 2008; Mutti *et al.*, 2006). Hence, it was assessed whether silencing of *MpC002* also affected survival of GPA feeding directly on *N. benthamiana* and *A. thaliana*. Nymphs exposed to the *N. benthamiana* leaf discs for 17 days became adults and started to produce their own nymphs after approximately 10 days. The overall survival of the aphids and the production of nymphs on leaf discs transiently producing dsMpC002 were not affected compared to aphids on leaf discs producing dsGFP (**Figure 4.6A**). However, the nymph production by these aphids was significantly lower in six biological replicates (ANOVA, n=4-6, p<0.05) (**Figure 4.3B**). Similarly, on transgenic *A. thaliana* plants the

MpC002 RNAi aphids survived equally well, but produced fewer nymphs in three biological replicates (GLM, n=4, p<0.05) (**Figure 4.6B; Figure 4.5B**).

Survival and nymph production were also investigated for the *Rack1* RNAi aphids. *Rack1* RNAi aphids survived equally well (**Figure 4.6A**), but produced fewer nymphs on *N. benthamiana* leaf discs (ANOVA, n=4-6, p<0.05) (**Figure 4.3B**). Similarly, nymph production was reduced on *Rack1* RNAi aphids feeding on dsRack1 transgenic *A. thaliana* line 1 (GLM, n=4, p<0.05), while survival was not affected (**Figure 4.6B**). GPA fecundity was not reduced on dsRack1 transgenic *A. thaliana* lines 3 and 4 (**Figure 4.5B**) which is consistent with no significant down-regulation of *Rack1* in aphids on these lines (**Figure 4.5A**).
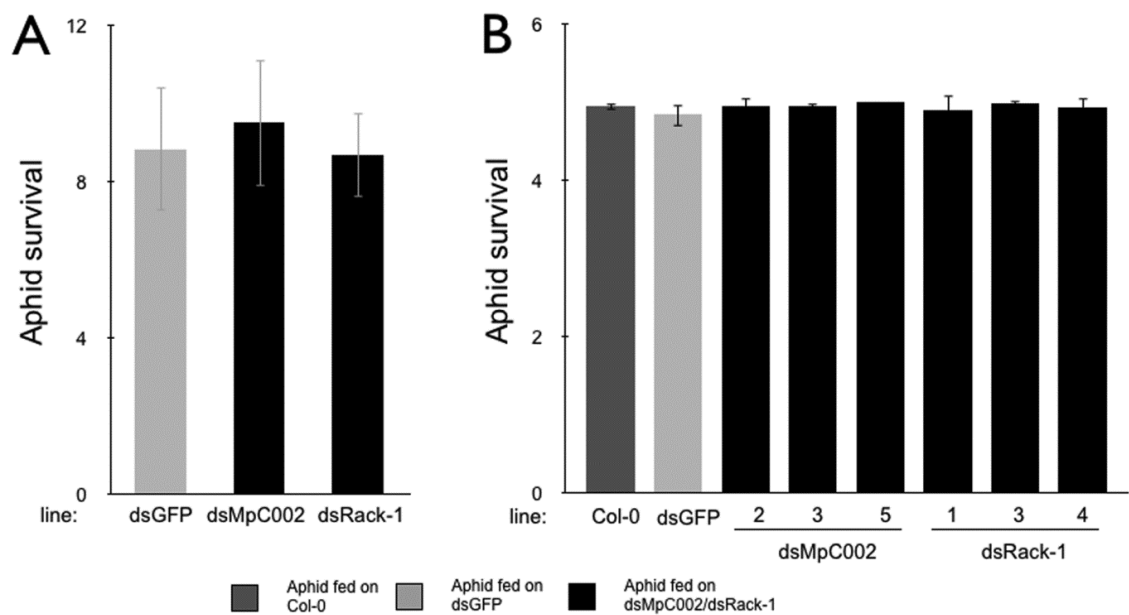
Figure 4.6. | Aphid survival is not affected on dsRack1 and dsMpC002 transgenic plants. (**A**) Aphid survival is not different on dsMpC002, dsRack1 and dsGFP *N. benthamiana* leaf discs. Data shown are means ± standard errors of aphid survival at 16 days for 6 biological replicates with n=4-6 per replicate. The relatively low aphid survival on *N. benthamiana* is likely due to transfer of aphids between leaf discs. (**B**) Aphid survival is not different on stable dsMpC002, dsRack1 and dsGFP transgenic *A. thaliana* lines for 16 days compared to those fed on dsGFP and Col-0 controls. Data shown are means ± standard errors of aphid survival at 16 days for 3 biological replicates with n=4 per replicate.

4.2.6. RNAi of GPA *Rack1* is highly specific

As Rack1 is strongly conserved across different organisms (Adams, 2011), it is possible that dsRNA corresponding to GPA *Rack1* could induce RNAi of *Rack1* in either the plant containing the transgene or other non-target organisms. A nucleotide alignment of the targeted region of Rack1 (Chapter 2.12) in several organisms is shown in **Figure 4.7**. To test for potential off-target effects, an *in silico* analysis was performed using Si-Fi (siRNA finder) software (Institute for Plant Genetics, Gatersleben, Germany). The region of GPA Rack1 used for the dsRack1 construct (**Figure 4.7**; Chapter 2.12) was input in the software and the putative siRNAs from these sequences were tested for hits against the *A. pisum*, *H. sapiens* and *A. thaliana* nucleotide databases. No hits were found against these databases, even for *A. pisum* which shows the closest similarity to GPA Rack1. This suggests that there are no RNAi off-targets in other organisms for the region of GPA Rack1 used and that RNAi constructs can be designed to be highly-specific.

```
M_persicae_(EST)              GGGTTACGCAGATCGCCACCAATCCGATCCACACTGACATGATTCTGTCTTGTTCACGAG 60
A_pisum                       GGGTTACGCAGATCGCCACCAATCCGATCCACACCGACATGATTCTGTCTTGTTCACGAG 60
Homo_sapiens                  GGGTAACCCAGATCGCTACTACCCCGCAGTTCCCGGACATGATCCTCTCCGCCTCTCGAG 60
Arabidopsis_thaliana_(Rack1A) TGGTGACGGCAATCGCCACCCCAATCGATAACGCAGACATCATCGTCTCAGCTTCCCGCG 60
                              *** **  ..***** **  ...  :  :* * ***** **  * **    ** **.*

M_persicae_(EST)              ACAAGACCTTGATTGTTTGGGATCTGACACGTGATGAGCTCAACTATGGTATCCCCAAGA 120
A_pisum                       ACAAGACCCTGATCGTTTGGGACCTGACACGTGACGAACTCAACTACGGAATCCCCAAGA 120
Homo_sapiens                  ATAAGACCATCATCATGTGGAAACTGACCAGGGATGAGACCAACTATGGAATTCCACAGC 120
Arabidopsis_thaliana_(Rack1A) ACAAATCCATCATTTTGTGGAAACTCACCAAGGACGACAAAGCCTACGGTGTAGCTCAGA 120
                              * **.:** * **  * ***.* ** **...  ** **  . ...*** **:.*  * .**.

M_persicae_(EST)              AACGTTTGTACGGACATTCGCACTTCGTCAGCGACGTCGTTCTTTCATCAGATGGTAACT 180
A_pisum                       AACGTTTGTACGGACATTCGCACTTCGTCAGCGACGTCGTTCTTTCGTCAGACGGTAACT 180
Homo_sapiens                  GTGCTCTGCGGGGTCACTCCCACTTTGTTAGTGATGTGGTTATCTCCTCAGATGGCCAGT 180
Arabidopsis_thaliana_(Rack1A) GGCGTCTCACTGGTCACTCTCACTTCGTTGAGGATGTTGTTCTCTCCTCCGATGGACAAT 180
                              .   * *    **:** ** ***** ** .. ** ** ***.* ** **.** ** .* *

M_persicae_(EST)              ACGCTCTTTCCGGTTCTTGGGATAAGACTCTTCGTCTGTGGGATTTGGCTGCTGGACGTA 240
A_pisum                       ACGCGCTTTCCGGTTCTTGGGACAAGACTCTTCGTCTGTGGGATTTGGCTGCTGGACGTA 240
Homo_sapiens                  TTGCCCTCTCAGGCTCCTGGGATGGAACCCTGCGCCTCTGGGATCTCACAACGGGCACCA 240
Arabidopsis_thaliana_(Rack1A) TCGCGCTTTCCGGCAGCTGGGACGGCGAGCTCCGTCTTTGGGATCTTGCTGCTGGTGTCT 240
                              : ** ** **.** :  ***** .. .. ** ** ** ****** * .*:.* **    :

M_persicae_(EST)              CCACTCGTCGTTTTGAAGACCACACCAAGGATGTATTGAGCGTTGCCTTCTCTGCTGACA 300
A_pisum                       CCACTCGTCGTTTTGAAGACCACACCAAGGATGTATTGAGCGTTGCCTTCTCTGCTGACA 300
Homo_sapiens                  CCACGAGGCGATTTGTGGGCCATACCAAGGATGTGCTGAGTGTGGCCTTCTCCTCTGACA 300
Arabidopsis_thaliana_(Rack1A) CCACTCGTAGATTCGTTGGACACACCAAGGACGTGCTCTCCGTCGCCTTCTCACTCGACA 300
                              ****  .*  .*:** **: *..** ******* **.  * :  ** ********    ****

M_persicae_(EST)              ACCGTCAAAT    310
A_pisum                       ACCGTCAAAT    310
Homo_sapiens                  ACCGGCAGAT    310
Arabidopsis_thaliana_(Rack1A) ACCGTCAGAT    310
                              **** **.**
```

Figure 4.7. | Nucleotide alignment of RNAi target region for GPA Rack1 in various organisms. GPA Rack1 mined from the GPA EST dataset (Ramsey *et al.*, 2007) versus Rack1 from *A. pisum* (GI:328711056), *Homo sapiens* (GI:83641897), and from *A. thaliana* (Rack1A) (GI:30685669). Alignment built using CLUSTAL O (1.2.0) by EMBL-EBI.

4.3. Discussion

The results show that GPA gene expression can be down-regulated by feeding GPA dsRNA from plants. This is the first example of RNAi in an aphid system from direct plant feeding and demonstrates that RNAi is possible in GPA, as RNAi was shown previously in pea aphids only. Expression of both *MpC002* and *Rack1* is reduced when GPA are fed from transgenic plants that transiently (*N. benthamiana*) and stably (*A. thaliana*) express dsRNA corresponding to *MpC002* and *Rack1*. Moreover, RNAi aphids have reduced progeny production. Thus, PMRi is feasible, and is a useful tool for studying aphid gene function.

A 30-60% decrease in gene expression was measured, similar to that observed in microinjection and artificial feeding of small RNAs to aphids. The reduction is also similar to that measured in other insects such as *Schistocerca americana* (injection) (Dong & Friedrich, 2005) and *Rhodnius prolixus* (injection and ingestion) (Araujo *et al.*, 2006) but overall lower than the levels found in *Spodoptera litura* (injection) (Rajagopal *et al.*, 2002) or in *Drosophila melanogaster* (injection) (Goto *et al.*, 2003). The method allows the study of gene function during interactions of aphids with plants, which is not possible by feeding of dsRNA and siRNA from diets (Shakesby *et al.*, 2009; Whyard *et al.*, 2009).

Previous studies have demonstrated the silencing signal to be mobile in plants (Mlotshwa *et al.*, 2002), where expressed small RNAs were shown to move within the phloem to where aphids feed. The CaMV 35S promoter enables constitutive expression of dsRNA in transgenic plants tissue, including the leaf phloem (Odell, *et al.*, 1985; Yang & Christou, 1990). The CaMV 35S promoter also allows for transient expression and movement of dsRNAs in *N. benthamiana* phloem (Johansen & Carrington, 2001). The results presented demonstrate that siRNAs can travel from the plant phloem through the aphid stylet and reach the aphid intestinal tissues triggering RNAi of aphid target genes. Given that *MpC002* expression is down-regulated by up to 60% and is predominantly expressed in the salivary glands, the silencing signal appears to spread through the aphid. This is consistent with the finding that small RNA pathways that are highly conserved in animals are also present in aphids (Kim *et al.*, 2009; Jaubert-Possamai *et al.*, 2010; Huvenne & Smagghe, 2010)

Partial knockdown of *Rack1* and *MpC002* reduced aphid fecundity (**Figure 4.3B; Figure 4.5B**) but not survival (**Figure 4.6**). This contrasts with the results obtained by dsRNA injection of pea aphids in which survival was reduced by silencing *C002*. It is possible that the lower pea aphid survival is caused by faster down-regulation of the target gene as a result of the sudden higher presence of the injected dsRNA in the hemolymph. Alternatively, stress caused by the injection could exacerbate the negative impact of *C002* down-regulation. GPA are smaller than pea aphids and hence more difficult to inject without affecting aphid survival rates. Delivery by plant feeding therefore provides a gentle, natural method for studying gene function that is less likely to have indirect effects on aphid behavior. This method is therefore suited to investigating the effects of gene silencing on aphid/plant interactions, and for virus-transmission studies.

GPA produces more progeny on *N. benthamiana* leaves that transiently express *MpC002* (Bos *et al.*, 2010). Thus, the presence of more (*in planta* overexpression) and less (RNAi in aphids) MpC002 leads to, respectively, increased and reduced GPA performance on plants. In addition, silencing of pea aphid *C002* decreases survival of this aphid on plants but not on diet and the C002 protein was detected in plants upon pea aphid feeding (Mutti *et al.*, 2006). Finally, C002 was found in the saliva proteomes of GPA (Harmel *et al.*, 2008) and pea aphids (Carolan *et al.*, 2011). Altogether, this indicates that the *C002* genes of both GPA and pea aphids have essential functions in aphid-plant interactions.

The finding that silencing of *Rack1* in GPA leads to decreased progeny production by this aphid is also in agreement with other findings. Indeed, Rack1 is a scaffold protein that is involved in the regulation of cell proliferation, growth and movement in animals (Albinsson & Kidd, 1999; Liliental & Chang, 1998; Chen *et al.*, 2002). Silencing of *Rack1* in two species of nematodes, *C. elegans* and *H. bacteriophora*, reduces growth of these animals (Simmer *et al.*, 2003; Kamath *et al.*, 2003; Ciche & Sternberg, 2007). GPA Rack1 also interacts with integrins and luteovirids (Seddas *et al.*, 2004), which invade aphid gut cells (Brault *et al.*, 2007), suggesting a role in endocytosis processes, such as nutrient/peptide uptake from the gut lumen. Given that *Rack1* is expressed in multiple tissues of the aphid and particularly in the gut, silencing this gene may affect aphid progeny reproduction indirectly, perhaps

by reducing the growth of gut cells leading to decreased nutrient uptake. Alternatively, silencing may directly reduce the growth of embryo cells.

The GPA genome is not yet sequenced and the functions of the majority of aphid genes are still unknown. Moreover, it is not fully understood how aphids modulate host defenses and mediate the transmission of plant viruses. The *N. benthamiana* leaf disc assay can be developed into a functional genomics screen to assess which aphid genes are essential for aphid survival on plants in the absence or presence of specific plant metabolites or synthetic pesticides. It is also possible to further investigate the role of aphid candidate effector proteins in plant infestation (Bos *et al.*, 2010). Finally, PMRi can be used to identify aphid proteins involved in the non-persistent and persistent transmission of plant viruses.

5. Plant-mediated RNAi to dissect the circulative transmission of TuYV by aphids

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5.1. Introduction

In the previous chapter, the feasibility of using PMRi to initiate down-regulation of gene targets in the aphid was demonstrated. In this chapter, this work was taken further to investigate the circulative transmission of TuYV. As previously mentioned, GPA Rack1 has been shown to bind *in vitro* to luteovirid particles (Seddas *et al.*, 2004; Gray *et al.*, 2013) and is involved in transcytosis mechanisms (Chapter 1.10). Rack1 is a key mediator of various signaling pathways and is involved in diverse physiological processes such as development, cell migration, and circadian rhythm (Adams *et al.*, 2011; see also Chapter 1.10 for further details). *Rack1* is strongly expressed in the gut of GPA ([Figure 4.1](#)) and therefore may be involved in virus movement across the gut barrier. If Rack1 has a positive role in TuYV uptake, *Rack1* down-regulation will reduce Rack1-mediated acquisition of virus particles by GPA i.e. the number of virus particles moving across the gut barrier into the hemolymph. A reduction in virus acquisition by GPA upon feeding could in turn reduce the transmission of the virus to healthy plants.

Subsequently, experiments to measure TuYV acquisition and transmission efficiency of aphids upon down-regulation of *Rack1* using PMRi were performed to determine whether Rack1 is directly involved in luteovirus transmission. To assess these parameters, altered acquisition or inoculation times can be given to test insects. Acquisition access period (AAP) refers to the length of time given for a non-viruliferous insect to acquire virus particles. Inoculation access period (IAP) refers to the length of time given for a viruliferous insect to inoculate virus particles.

Rack1-RNAi may also affect TuYV acquisition and transmission in other ways. In the previous work (Chapter 4) it was found that *Rack1* RNAi aphids produce less progeny. A reduction in the aphid population would result in lower TuYV disease pressures, because aphid population size is positively correlated with percentages of infected plants (Swenson, 1968). Therefore, the dynamics of the RNAi effect upon acquisition of dsRNAs by aphids were investigated, including: i) the time taken to achieve optimal gene down-regulation after exposure to dsRNA; ii) the duration of the gene down-regulation upon removal of aphids from the dsRNA source; iii) the reduction in aphid population when continuously exposed to the dsRNA source over longer periods of time; iv) the level of down-regulation in individual aphids in a

population exposed to the same dsRNA source; and v) whether the RNAi effect is transferred to the aphid progeny. Concerning the latter, as nymphs develop inside the parent insect (Chapter 1.4), it is feasible that genes in nymphs developing in a parent insect exposed to the dsRNA source could also be down-regulated, thus generating a germline effect.

Due to the multiple roles of Rack1, RNAi of *Rack1* may also indirectly affect luteovirid uptake through altered behavior. One of the factors governing the level of virus uptake is aphid feeding behavior, in particular, contact with the phloem tissues where luteovirus particles are limited to. Aphid salivation into the phloem releases polerovirus particles into the host and the virus particles are ingested along with phloem sap (Prado & Tjallingii, 1994; Moreno *et al.*, 2011). Aphids deficient in phloem feeding are likely to ingest fewer virus particles. Thus, if aphids silenced for *Rack1* take longer to reach the phloem or feed less from the phloem overall, they may take up less virus than wild type aphids over time, contributing towards a reduction of TuYV-carrying aphids in the field. Therefore, electrical penetration graph (EPG) studies were conducted to determine whether *Rack1*-silenced aphids are affected in their ability to feed from the plant phloem.

EPG is used to study the interaction of insects such as aphids, leafhoppers and thrips, with plants (Tjallingi, 1978). It has been tailored to closely study virus transmission by these insects as well as host plant selection and plant resistance. EPG has been particularly used for studying aphid feeding behavior and virus transmission. The EPG system consists of a partial circuit (including the aphid and its host plant), which is completed when the aphid inserts its stylet into the plant (Mclean & Kinsey, 1964). Different waveform outputs are produced, indicating different insect activities (e.g. salivation, ingestion, probing) or tissue types that aphid stylets penetrate (e.g. mesophyll, phloem, or xylem) (Tjallingi, 1978). The various insect/plant interaction events correlate with different graphical waveforms (Tjallingii, 1978; Tjallingii & Esch, 1993; Prado & Tjallingii, 1994). Using this system, it is possible to measure whether *Rack1* (or other gene targets) down-regulation alters feeding behavior.

As well as using *Rack1* as a target, alternative RNAi targets that have no known involvement with luteovirus transmission were used as controls. RNAi of both *MpC002* and *MpPInt02* has been successful in GPA making them viable for comparison with

Rack1. *MpC002* has been studied in Chapter 4. MpPInt02 (formerly Mp2) is a salivary protein that is secreted into saliva (Bos *et al.*, 2010; Pitino & Hogenhout, 2013). It has been shown to be important for host colonization as overexpression in the plant increases GPA reproduction, whilst RNAi of *MpPInt02* via the plant resulted in reduced aphid reproduction (Pitino & Hogenhout, 2013).

In summary, experimental work was performed to assess the role of Rack1 in the circulative transmission of TuYV by GPA. *Rack1* RNAi aphids were used to determine whether aphids would have altered TuYV acquisition or transmission capability compared to control aphids or *MpC002/MpPInt02* RNAi aphids. A series of experiments were performed to investigate gene-silencing dynamics and phenotypic effects of RNAi on aphids that may also influence TuYV transmission. Feeding behavior of *Rack1* silenced aphids was assessed using EPG to determine whether *Rack1* silencing indirectly influences TuYV uptake by changing aphid feeding behavior. The role of GPA Rack1 in TuYV transmission is discussed.

5.2. Results

5.2.1. Target gene down-regulation occurs rapidly upon insect feeding on dsRNA plants and remains stable

Previous experiments demonstrated down-regulation of aphid target genes after 16 days feeding on transgenic *A. thaliana* producing dsRNA corresponding to aphid genes (Chapter 4). However, maximal down-regulation of aphid target genes may occur earlier than 16 days and it is not known how long aphid genes remain suppressed after removal of the aphid from the dsRNA source. To investigate this, RNAi of *Rack1*, *MpPIntO2* & *MpC002* were assessed in GPA over time.

'Aged' (0-2 day old) GPA nymphs were reared on transgenic dsRack1, dsMpPIntO2, dsMpC002 or dsGFP plants then three batches of five insects (serving as individual technical replicates) were sampled from these plants at 4-day intervals over 16 days and processed for qRT-PCR analyses to assess the mean level of *Rack1, MpPIntO2* or *MpC002* down-regulation relative to dsGFP fed aphids. As expected, the target genes were not down-regulated in aphids harvested at 0 days (Student's *t*-test, n=3, p=<0.05), whereas up to 60% down-regulation of the target genes was observed

after 4 days and 70% down-regulation at 8 days (Student's *t*-test, n=3, p=<0.05) (**Figure 5.1**). The down-regulation remained at 50-70% up to the end of the experiment at 16 days (Student's *t*-test, n=3, p=<0.05) (**Figure 5.1**). No significant difference in the level of target gene RNAi was found between dsRack1, dsMpPIntO2 and dsMpC002 treatments at each time point indicating that these genes respond similarly to plant-mediated RNAi (GLM, n=3, p=>0.05).
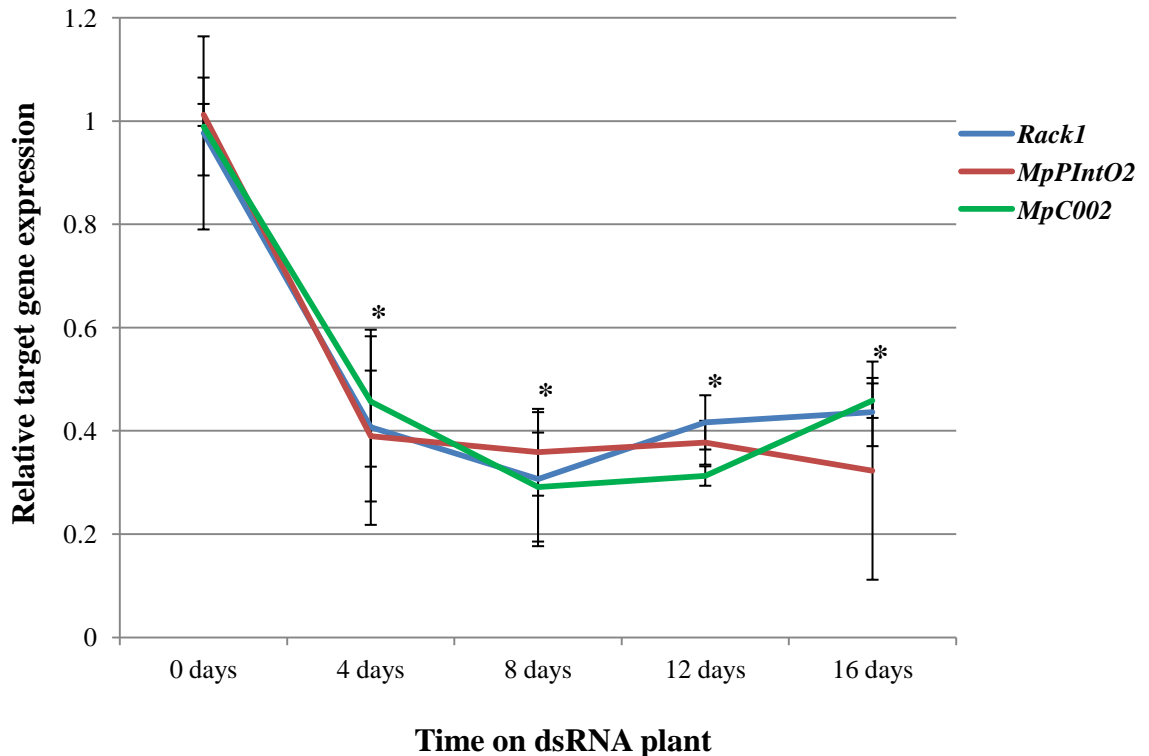
Figure 5.1. | GPA target genes are significantly down-regulated after four days feeding on dsRNA-expressing *A. thaliana* and remain suppressed over 16 days. GPA were reared on dsRNA-expressing plants over a 16-day time series. Aphids were harvested at 0, 4, 8, 12 and 16 days to test for target gene down-regulation by qRT-PCR. Colored lines represent average expression of the corresponding target gene at each time point for aphids reared on dsRack1 (blue), dsMpPIntO2 (red) or dsMpC002 (green) compared to aphids reared on dsGFP. Data represents mean expression levels ± standard deviation for each target gene at each time point for three biological replicates with n=3 per replicate. Asterisk indicates significant difference compared to dsGFP control (Student's *t*-test, n=3, p=<0.05).

5.2.2. Down-regulation of gene targets subsides after removal of aphids from dsRNA source

Next, it was assessed whether target gene down-regulation in GPA reverts to normal levels after removal of aphids from the dsRNA-expressing plants. It was also

determined whether the RNAi effect is transferred to the aphid progeny, because aphid embryos develop in their mothers (Chapter 1.4), they may be either directly exposed to dsRNA/siRNA ingested by the parent or the RNAi effect could be transferred from mother to embryo. Up to three generations of insects were tested in these experiments or in subsequent experiments, RNAi insects (1st generation), the nymphs they produce (2nd generation), plus nymphs produced from 2nd generation insects (3rd generation).

Aged aphids were removed from dsRNA plants after 8 days (maximally down-regulated based on the previous experiment) and placed on Col-0 plants. Three batches of five insects were sampled immediately and then at two-day intervals for qRT-PCR analyses to determine the mean level of *Rack1, MpPIntO2* or *MpC002* down-regulation relative to dsGFP-exposed aphids. The expression levels of all three target genes were significantly reduced in aphids that were removed from the dsRNA plants at 0, 2 and 4 days (Student's *t*-test, n=3, p=<0.05) (**Figure 5.2A**). The expression levels of target genes slowly increased in a linear fashion and was fully recovered by six days, at which point there was no difference in target genes expression levels compared to aphids exposed to the dsGFP treatment (Student's *t*-test, n=3, p=>0.05) (**Figure 5.2A**). This is consistent with down-regulation in pea aphids which persists for 5 days and is then recovered (Shakesby *et al.*, 2009). No significant difference was found between dsRack1, dsMpPIntO2 and dsMpC002 treatments at each time point (GLM, n=3, p=>0.05) indicating that this recovery of gene expression is a general phenomenon independent of the gene being targeted by RNAi.

Second generation nymphs were sampled similarly at two-day intervals. These nymphs were born after the parent RNAi aphid was transferred to non-transgenic lines, and were therefore not exposed to the dsRNA produced by the transgenic plants. It was found that nymphs born from mothers exposed to the dsRNA source had up to 75% reduced expression of target genes (**Figure 5.2B**). Significant reductions in target gene expression were observed for nymphs produced by adults at two, four and six days after removing the parent insect from dsRNA plants (Student's *t*-test, n=3, p=<0.05). Thus, whilst adults had normal levels of target gene expression at 6 days after removal from the dsRNA source (**Figure 5.2A**), the nymphs produced by these adults still show up to 40% down-regulation (**Figure 5.2B**). Again, no significant difference was found between dsRack1, dsMpPIntO2 and dsMpC002 treatments at each time point (GLM, n=3, p=>0.05).
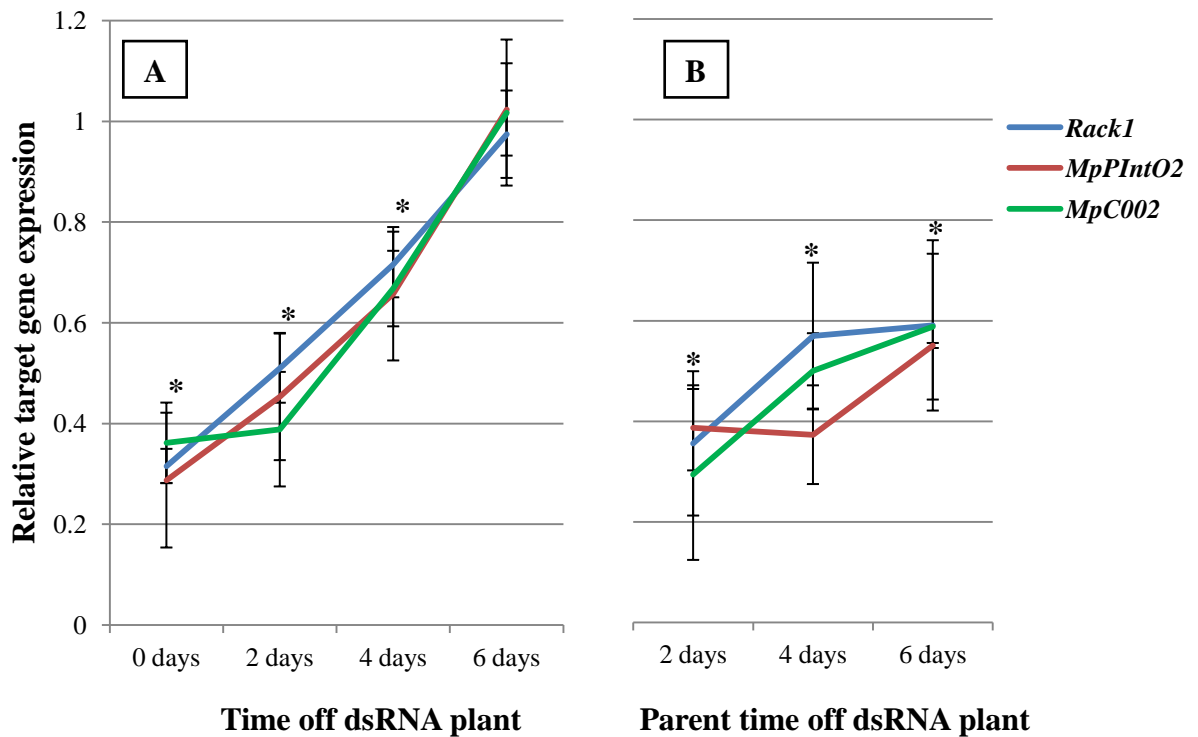
Figure 5.2. | Expression levels of target genes return to 100% at six days after removal from the dsRNA source for adult GPA, but not for nymphs born from these adults. RNAi GPA were transferred to Col-0 plants then harvested at 0, 2, 4 and 6 days to test for target gene down-regulation by qRT-PCR. Nymphs produced by these insects were also collected at 2, 4 and 6 days. Relative expression of *Rack1*, *MpPIntO2* or *MpC002* was determined in adults **(A)** fed on dsRNA(target) and their nymphs **(B)** compared to dsGFP fed equivalents. Colored lines represent average expression of the corresponding target gene at each time point for aphids reared on dsRack1 (blue), dsMpPIntO2 (red) or dsMpC002 (green) compared to aphids reared on dsGFP. Data represents mean expression levels ± standard deviation for each target gene at each time point for three biological replicates with n=3 per replicate. Asterisk indicates significant difference compared to dsGFP control (Student's *t*-test, n=3, p=<0.05).

5.2.3. Down-regulation of target genes persists in progeny of GPA reared on dsRNA plants

To investigate transfer of RNAi to GPA progeny further, the progeny (2nd generation) produced by aphids exposed to dsRNAs (1st generation) for the target genes were assessed in a time series. Aged GPA were removed from the dsRNA transgenic plants at 8 days (at the maximum of 70% down-regulation), placed on non-transgenic Col-0 plants for two days, and then removed. Nymphs produced from these adults were harvested in three batches of five insects at the time adults were removed (day 0) and at four-day intervals thereafter for qRT-PCR to assess the level of *Rack1, MpPIntO2* or

MpC002 down-regulation relative to progeny produced by adult aphids fed on dsGFP transgenic Col-0 plants. The nymphs showed significant reductions in target gene expression levels at 0, 4 and 8 days (Student's *t*-test, n=3, p=<0.05) (**Figure 5.3**). Approximately 50% down-regulation was recorded at days 0 and day 4, partial recovery of gene expression levels was noticed at day 8, whilst gene expression was fully recovered at day 12 (Student's *t*-test, n=3, p=>0.05) (**Figure 5.3**).

It was also investigated whether the RNAi effect was additionally transferred to the progeny (3rd generation) of 12-day and 16-day old (2nd generation) aphids. There appeared to be no significant difference between aphids exposed to dsRack1, dsMpPIntO2, dsMpC002 and dsGFP. However, the sample number was limited and there was a high variability in expression levels.
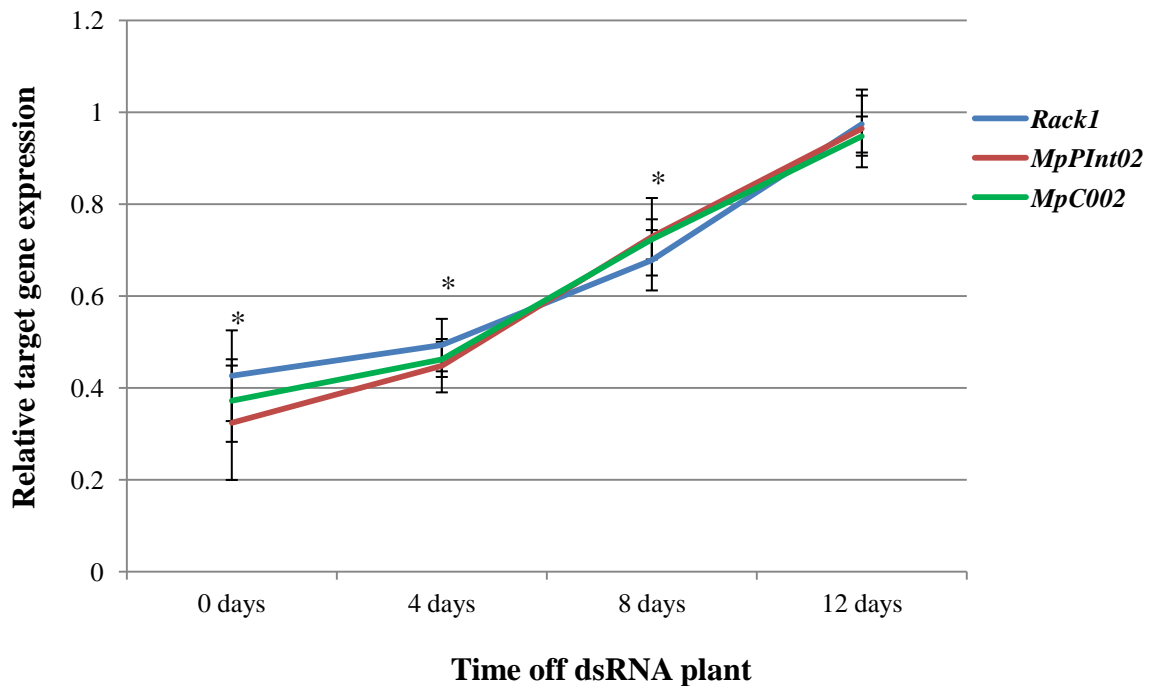
Figure 5.3. | Down-regulation of target genes persists in progeny of GPA reared on dsRNA plants.
The progeny of RNAi aphids were collected at 0, 4, 8 and 12 days feeding on Col-0 to test for target gene down-regulation by qRT-PCR. Relative expression of *Rack1*, *MpPIntO2* or *MpC002* was determined in 2nd generation insects from RNAi aphids compared to corresponding insects produced from 1st generation dsGFP-fed insects. Colored lines represent average expression of the corresponding target gene at each time point for progeny produced by mothers reared on dsRack1 (blue), dsMpPIntO2 (red) or dsMpC002 (green) compared to dsGFP. Data represents mean expression levels ± standard deviation for each target gene at each time point for three biological replicates with n=3 per replicate. Asterisk indicates significant difference compared to corresponding insects from an initial dsGFP treatment (Student's *t*-test, n=3, p=<0.05).

5.2.4. GPA population growth is reduced on dsRNA lines

Previous results showed that RNAi of *Rack1*, *MpPIntO2*, and *MpC002* resulted in decreased aphid fecundity (Chapter 4.2.5; Pitino & Hogenhout, 2013) after 16 days. It was assessed how the decrease in fecundity may affect an aphid population over longer time periods as a proxy to assess if an RNAi approach may be useful for reducing aphid populations in field crops. An aphid population derived from a single 0-2 day old nymph was seeded on dsRack1, dsGFP, dsMpC002, and dsMpPInt2 transgenic *A. thaliana* plants in an enclosed system. The number of adults and progeny was counted at two, three and four weeks post GPA inoculation (constituting about three generations of aphids). Total nymph number at four weeks was significantly

reduced in dsMpPInt02, dsMpC002 and dsRack1 fed aphids compared to dsGFP fed aphids (GLM, n=4, p<0.05). A 25-30% reduction was recorded for aphids fed on dsMpPInt02 or dsRack1 plants and 50% reduction was observed for dsMpC002 fed aphids at four weeks (Figure 5.4). Thus, RNAi of MpC002 seems to be the most effective at reducing the GPA population in the long term.
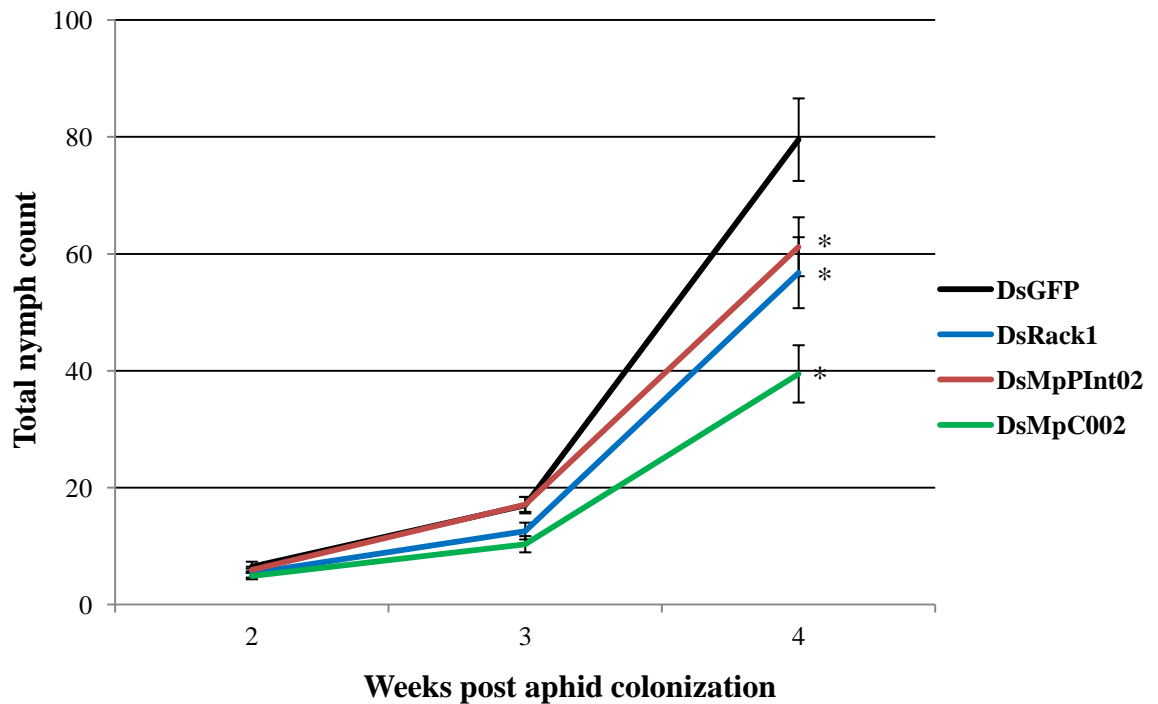
Figure 5.4. | Aphid population growth is significantly reduced on dsRack1, dsMpPInt02, and dsMpC002 transgenic A. *thaliana*. Aphid populations were established on dsRack1, dsMpPInt02, dsMpC002 or dsGFP expressing *A. thaliana* over four weeks. Data shown are total number of nymphs counted on each line at 2, 3 and 4 weeks post aphid colonization with means ± standard errors of four biological replicates with n=4 per replicate. Asterisk indicates significant difference in treatments at 4 weeks compared to dsGFP (GLM, n=4, p<0.05).

5.2.5. *Rack1* RNAi reduces TuYV acquisition by the aphid

A series of experiments were performed to determine whether *Rack1*, *MpPIntO2* or *MpC002* RNAi aphids would internalize different quantities of virus particles after three alternative acquisition access periods (AAP) on infected plants. Aged GPA nymphs were reared on dsRack1, dsMpC002, dsMpPIntO2 or dsGFP transgenic *A. thaliana* for 8 days then confined on leaves of these plants infected with TuYV for a 0.5, 2, or 4 days AAP. Aphids were then transferred to non-infected dsRNA transgenic plants for 72 hours to clear gut contents from TuYV-containing plant sap. TuYV titers

in TuYV-containing plants were measured by qRT-PCR to ensure that TuYV titers were similar amongst the different plant lines so that test aphids were exposed to equal levels of inoculum.

Aphids were sampled to measure down-regulation of aphid target genes and TuYV abundance by qRT-PCR (**Figure 5.5**). In all experiments, target genes were down-regulated by 25-50% compared to dsGFP-fed aphids (Student's *t*-test, n=3, p=<0.05) (**Figure 5.5A; Figure 5.5C; Figure 5.5E**). The TuYV quantity in *Rack1* RNAi aphids was reduced by approximately 40-50% after 12-hour and 2-day AAPs compared to dsGFP aphids (Student's *t*-test, n=3, p=<0.05) (**Figure 5.5B; Figure 5.5D**), whilst no reduction of TuYV was noticed in *MpPInt02* or *MpC002* RNAi aphids at these AAPs (**Figure 5.5B; Figure 5.5D**). However, no difference in TuYV quantity was observed in *Rack1, MpPInt02* or *MpC002* RNAi aphids at a 4-day AAP (Student's *t*-test, n=3, p=>0.05) (**Figure 5.5F**).
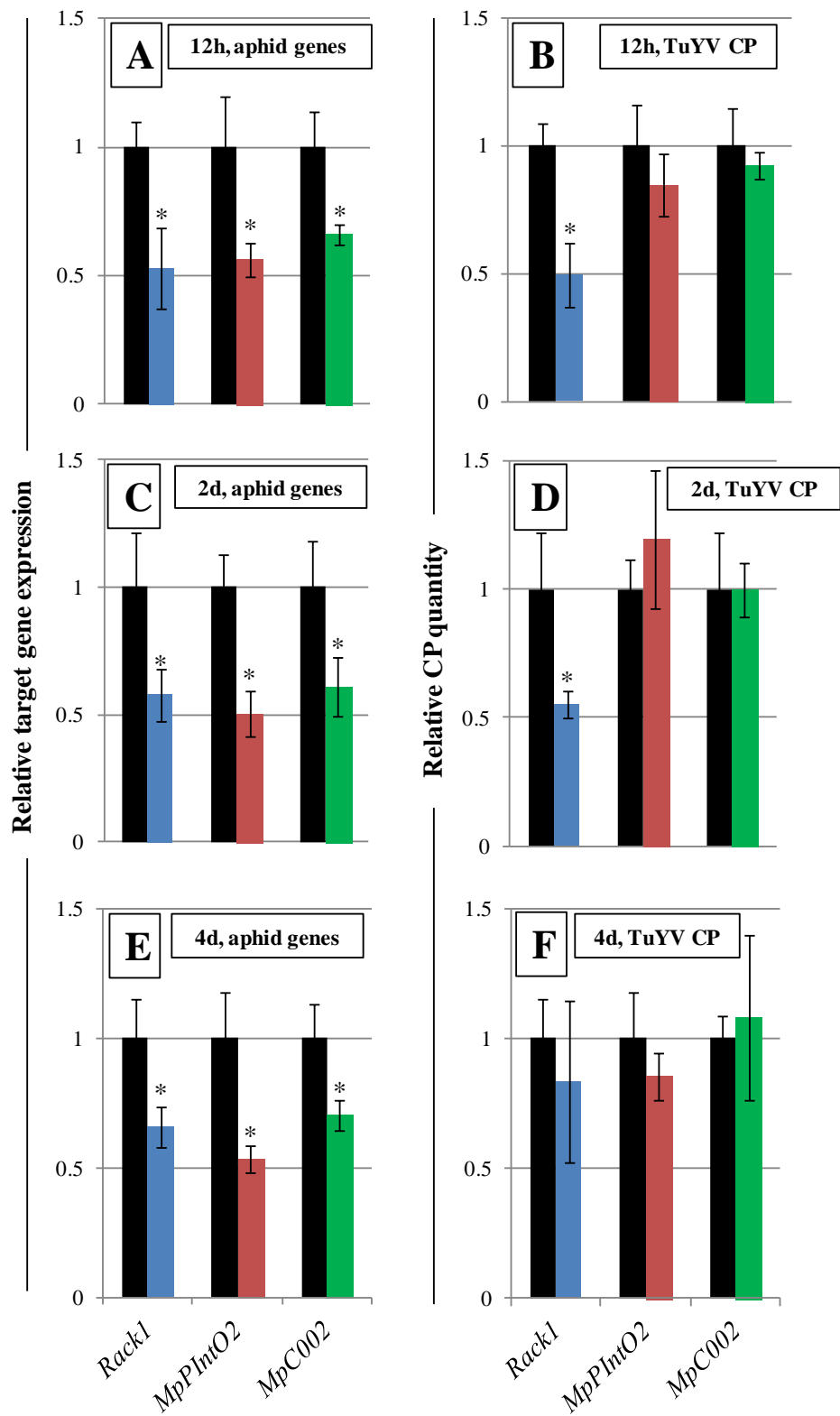
Figure 5.5. | *Rack1* **RNAi aphids acquire fewer TuYV particles after 12 hours and 2 days feeding on infected plants.** GPA were initially reared on dsRack1, dsMPIntO2, dsMpC002 or dsGFP expressing *A. thaliana* for 8 days to ensure target gene down-regulation. RNAi aphids were given a 12 hour, 2 day or 4 day AAP on TuYV-infected plants. Aphids were harvested to determine relative target gene expression

(A, C, E) and TuYV CP quantity **(B, D, F)** in RNAi aphids compared to dsGFP aphids by qRT-PCR. Columns indicate relative expression of *Rack1, MpPIntO2* or *MpC002* in GPA reared on dsRNA transgenic plants for these genes compared to GPA on dsGFP transgenic *A. thaliana*. Data represents mean expression levels ± standard deviation for each target gene or TuYV CP from three biological replicates with n=3 per replicate. Asterisk indicates significant difference (Student's *t*-test, n=3, p=<0.05).

5.2.6. Silencing of *Rack1* or *MpPIntO2* in GPA does not alter TuYV transmission capability by aphids after a 2-day AAP

Experiments were performed to determine whether *Rack1* or *MpPIntO2* RNAi would affect transmission efficiency of TuYV by the aphid. Aged GPA nymphs were reared on dsRack1, dsMpPIntO2 or dsGFP transgenic *A. thaliana* for 12 days to ensure target gene down-regulation. On average, approximately 30% down-regulation of target genes was observed over three biological replicates (Student's *t*-test, n=3, p=<0.05) (**Figure 5.6A**). The RNAi aphids were confined on TuYV-infected plants using clip cages. After a two-day acquisition access period (AAP) on infected plants, aphids were transferred individually to healthy seedlings. The percentage of infected to healthy seedlings was determined by TAS-ELISA and the mean virus titer of infected plants was also calculated. *Rack1* RNAi GPA infected slightly fewer plants with TuYV but no significant difference was observed in the number of infected plants (**Figure 5.6B**). The infected plants did not show differences in TuYV titers (**Figure 5.6C**).
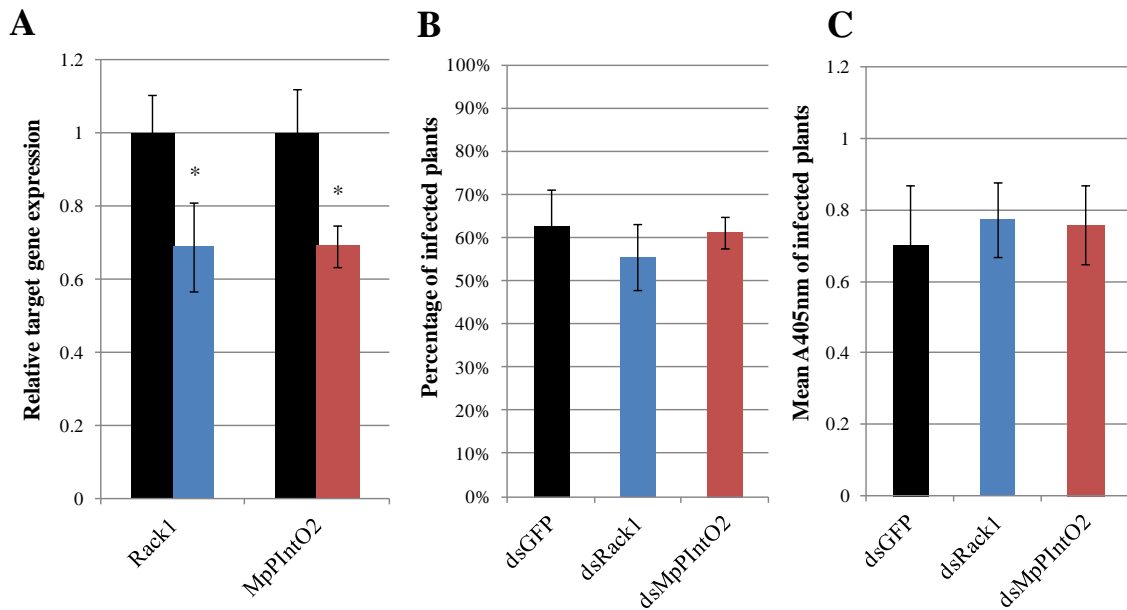
Figure 5.6. | *Rack1* or *MpPInt02* **RNAi aphids do not have altered TuYV transmission capability after a 2-day AAP on TuYV-infected plants.** GPA were initially reared on dsRack1, dsMPIntO2 or dsGFP expressing *A. thaliana* for 12 days to ensure target gene RNAi. Target gene expression in RNAi aphids compared to dsGFP aphids was determined by qRT-PCR **(A)**. Columns indicate relative expression of *Rack1* or *MpPIntO2* in GPA reared on dsRNA(target) for 12 days compared to GPA on dsGFP expressing transgenic *A. thaliana*. Data represents mean expression levels ± standard deviation for each target gene from three biological replicates with n=3 per replicate. Asterisk indicates significant difference (Student's *t*-test, n=3, p=<0.05). Silenced aphids were given a 2-day AAP on TuYV-infected plants then transferred individually to *A. thaliana* seedlings (n=24 per biological replicate). Percentage of infected plants **(B)** and mean A405nm **(C)** of infected plants was determined by TAS-ELISA at 3 weeks post inoculation. Data represent means from three biological replicates ± standard error.

5.2.7. Down-regulation of target genes varies between individual aphids

Analysis of gene down-regulation after PMRi has previously been performed on batches of GPA (Chapter 4). It is unknown how this would vary for individual insects. In addition, single insects are examined in EPG experiments. Therefore, variation in target gene down-regulation among individual aphids exposed to the same *Rack1* dsRNA (and dsGFP as control) source was analyzed. Aged GPA nymphs were reared on dsRNA plants for 16 days before harvesting. A large variation in *Rack1* down-regulation was observed between individual insects, ranging from 5% to 80% down-regulation compared to dsGFP aphids (**Figure 5.7**). The mean *Rack1* expression of six insects was approximately 50% that of dsGFP fed aphids (Student's *t*-test, n=6,

p=0.0026) and is in agreement with earlier results. However, this experiment was performed only once, more repetition is necessary to give a more robust analysis.
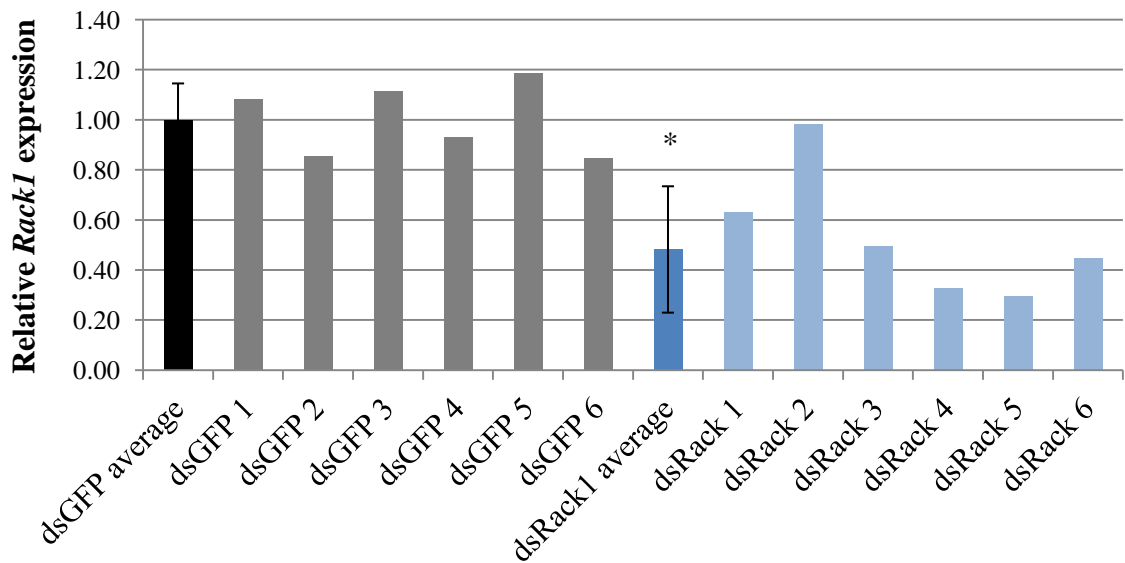
Figure 5.7. | *Rack1* **down-regulation is variable in individual GPA fed on dsRack1 expressing** *A.* *thaliana***.** GPA were reared on dsRack1 or dsGFP expressing plants for 16 days before harvesting individually. *Rack1* expression in dsRack1 fed aphids compared to dsGFP aphids was determined by qRT-PCR. Columns indicate expression of *Rack1* in six individual GPA reared on dsRack1 or dsGFP expressing transgenic *A. thaliana*. Mean of six insects per treatment ± standard deviation is also included (n=6). Asterisk indicates significant difference (Student's *t*-test, n=6, p=<0.0026).

5.2.8. Silencing of *Rack1* in GPA alters phloem feeding behavior

EPG experiments were performed to assess the impact of *Rack1* RNAi on aphid feeding behavior. Aged GPA nymphs were reared on dsRack1 or dsGFP transgenic *A. thaliana* for 8-12 days. Aphid batches were sampled to confirm down-regulation by qRT-PCR prior to EPG experiments. Aphid feeding behavior was recorded by EPG for 8 hours (Mutti *et al.*, 2008) then recordings manually analyzed (EPG systems, 2012). A total of 12 recordings per treatment (n=12) were imported into the Sarria Excel workbook (Sarria *et al.*, 2009) which automatically calculates a large number of EPG parameters related to insect probing and ingestion behavior. The workbook summarized the results and generated an output sheet for further statistical analyses.

Because poleroviruses are phloem-limited, aphid phloem-feeding behavior was analyzed. The percentage of the total recording length (8 hours) spent in phloem contact (termed 'E', which includes both E1 [salivation] and E2 [ingestion] behaviors) was

calculated for the *Rack1* RNAi aphids compared to dsGFP controls (**Figure 5.8**). *Rack1* RNAi aphids may spend up to 30% less time in contact with the phloem compared to dsGFP aphids. However, results were highly variable so that no significant differences were found between treatments (ANOVA, n=12, p=0.572) (**Figure 5.8**). Nonetheless, *Rack1* RNAi aphids showed clear differences in other EPG parameters, such as longer periods of non-probing and less activity upon probing, revealed by fewer cell penetrations (**Table 5.1**). Prior to feeding, aphids typically penetrate multiple cells and explore numerous routes in the plant tissue before reaching the phloem sieve elements (Chapter 1.3). This probing behavior is essential for insects to quickly establish a feeding site. The EPG data indicates that *Rack1* RNAi aphids are sluggish, taking longer to reach the phloem and initiate feeding.
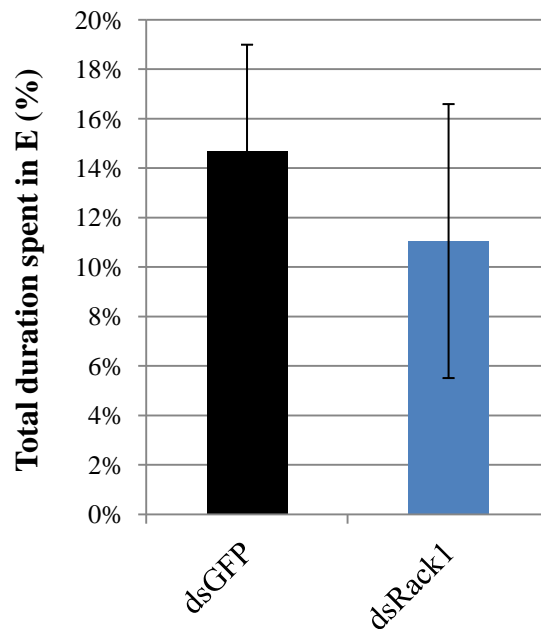
Figure 5.8. | *Rack1* **RNAi aphids have reduced phloem contact.** The total duration of E was calculated for each recording using the Sarria Excel workbook (Sarria *et al.*, 2009). Percentage of total recording length (8 hours) spent in E was calculated for aphids under dsGFP or dsRack1treament. Data represent means from 12 aphid recordings per treatment ± standard error. No significant difference was found between treatments (ANOVA, n=12, p=0.572).

	dsGFP		dsRack1		
	Mean	**S.E.**	**Mean**	**S.E.**	*P*-value
Duration of NP before first E1	3577	1048	10911	1655	0.0039
Mean duration of each NP	297	75	589	91	0.0426
Number PD	190	10	120	19	0.0101
Time from first probe to first E	9020	2578	18651	2355	0.0143
Time from start of EPG to first E	9490	2504	19227	2441	0.0146
Total duration of C	14687	720	10398	1331	0.0208
Total duration NP	7460	1232	12603	1393	0.0244
Total duration of PD	3446	916	1518	598	0.0161
Total probing time	21339	1232	16196	1393	0.0038

Table 5.1. | *Rack1* **down-regulation affects GPA feeding behavior.** Multiple feeding parameters were calculated for each recording using the Sarria excel workbook (Sarria *et al.*, 2009). Miscellaneous parameters showing a statistically significant difference between treatments are shown (ANOVA, n=12, p<0.05). Numbers in columns represent mean values and standard errors (in seconds) for each parameter for dsGFP and dsRack1 fed aphids.

KEY: E1 = phloem salivation. **E2** = phloem ingestion. **E** = sum of E1 and E2. **PD** = potential drop (or cell penetrations). **C** = probing in epidermis/mesophyll. **NP** = non-probing. **Total probing time** = sum of E1, E2, C, and PD.

5.3. Discussion

The data presented suggests that the GPA Rack1 protein may be directly involved in TuYV uptake. *Rack1* RNAi aphids (approximately 50% target gene down-regulation) acquire significantly less TuYV particles after a short acquisition access period of 12 hours or two days compared to control aphids (dsGFP-fed) (**Figure 5.5B; Figure 5.5D**). Furthermore, RNAi of alternative targets *MpC002* or *MpPInt02* did not

result in reduced TuYV acquisition compared to control aphids at any of the three acquisition access times (**Figure 5.5B; Figure 5.5D; Figure 5.5F**).

However, the EPG data does not support a direct role of Rack1 in TuYV uptake as reduced acquisition may be an indirect result of altered feeding behavior. EPG data shows that *Rack1* RNAi aphids exhibit a number of feeding behavior changes relevant to circulative virus uptake. Phloem-contact time by *Rack1* RNAi aphids (**Figure 5.8**) is reduced by approximately 30% compared to control aphids. Additionally, *Rack1* RNAi aphids take significantly longer to reach the phloem (**Table 5.1**), which could have an impact on both acquisition and inoculation of TuYV particles. Furthermore, *Rack1* RNAi aphids display a 'sluggish' phenotype consistent with *Rack1* RNAi phenotypes in the nematode *C. elegans* (Simmer *et al.*, 2003; Kamath *et al.*, 2003), as shown by less time spent probing, and fewer cell penetrations (**Table 5.1**). As a result of this, metabolic processes may be slower and reduce the rate of virus uptake.

Despite altered feeding behavior, *Rack1* RNAi aphids do still reach the phloem and no differential feeding is observed upon finding a feeding site. Results from *MpC002* RNAi aphids suggest that reduced TuYV uptake by *Rack1* RNAi aphids is independent of the altered feeding behavior observed. *C002*-silenced pea aphids have severe phloem-feeding impairment (Mutti *et al.*, 2008), therefore RNAi of this homolog in GPA should result in a reduced virus acquisition by these aphids. However, *MpC002* RNAi aphids do not have reduced TuYV acquisition compared to control aphids at any of the three acquisition access times (**Figure 5.5B; Figure 5.5D; Figure 5.5F**). As the method of RNAi was different and in a different aphid species, preliminary EPG experiments were completed on *MpC002* RNAi aphids to confirm whether the feeding behavior of *MpC002* RNAi GPA was consistent with *C002*-silenced pea aphids (Mutti *et al.*, 2008). These appear to be in agreement; however, a more thorough investigation with a larger cohort of test insects is necessary. Other RNAi targets affecting feeding behavior in various ways but not involved in luteovirid transmission could also be used to independently verify a direct role of *Rack1* on TuYV uptake.

Given that the variation in phloem contact time was higher in *Rack1* RNAi aphids compared to dsGFP aphids (**Figure 5.8**), part of the variation may be caused by the range in *Rack1* down-regulation levels amongst individual aphids (**Figure 5.7**). The variation may be derived from differences in RNAi potential amongst individual aphids

or because of differences in the amount of dsRNA acquisition that may depend on unequal dsRNA presence in various areas of the plant which the aphids are feeding on. Variation in RNAi potential amongst aphids is unlikely given that the aphids are genetically identical (derived from the same mother). Nonetheless, a large number of individuals may need to be analyzed to assess differences in feeding behavior for EPG experiments.

No significant difference was observed in TuYV acquisition by *Rack1* RNAi aphids after a four day acquisition period, (**Figure 5.5F**). This could indicate that *Rack1*-mediated uptake of TuYV particles is not affected after longer feeding times, perhaps because virus internalization has reached a saturation point. Alternatively, feeding behavior may be impaired over short time periods but not over longer periods. Measuring honeydew production could be used to determine whether feeding in *Rack1* RNAi aphids is reduced over longer time periods (Paguia *et al.*, 1980).

Taken together, the data presented supports a direct role of *Rack1* in luteovirid uptake; however more experiments are needed to prove this assertion. PMRi of another target gene(s) strongly expressed in the gut could be performed to determine whether this also affects TuYV acquisition by aphids similar to *Rack1*. Also, as there is difficulty ensuring the different dsRNA plants or individual leaves used for acquisition have similar TuYV titers, GPA could be fed on artificial diets containing a quantified amount of virus to ensure even levels of inoculum. However, this would require further handling of insects and would abate the RNAi effect through removal from the dsRNA source.

Although *Rack1* RNAi aphids acquire fewer virus particles, this does not alter the transmission efficiency as the number of plants infected after a 2-day AAP was not significantly reduced by these aphids (**Figure 5.6B**). However, *Rack1* down-regulation was not as high in this experiment as has previously been recorded (**Figure 5.6A**); the TuYV titer in these aphids may therefore not be significantly different from control aphids. This indicates that aphids only require a minimum quantity of internalized virus for efficient transmission. Transmission efficiency experiments may be completed at the other time points used in acquisition efficiency experiments; using the same aphids for both experiments would be the ideal scenario as this would allow a direct correlation.

One of the most interesting findings from these experiments is the observation that nymphs acquire the silencing signal from silenced parent insects (**Figure 5.2B**; **Figure 5.3**). Furthermore, these silenced nymphs require up to 12 days to recover normal expression levels (**Figure 5.3**), compared to 6 days for adults removed from the dsRNA source (**Figure 5.2A**). This may indicate that PMRi has a germline effect which would have various implications in the application of this technique. These results suggest that the RNAi effect is transferred to the embryos within the mother. As insects apparently lack an RdRP-dependent RNAi amplification mechanism (Chapter 1.12) it is difficult to explain how this has a sustained effect on nymphs. As little is understood about RNAi mechanism in insect systems, future work should try to elucidate the mechanism behind this, perhaps by measuring progression of RNAi signal through GPA tissues over time. Gene down-regulation over several generations can be assessed in dsRNA-fed populations to confirm the generational effect and also whether RNAi efficiency is cumulative.

The long term population experiment (**Figure 5.4**) showed that GPA populations on dsRack1, dsMpPInt02 and dsMpC002 develop significantly slower. It needs to be determined whether long-term virus titer alters in dsRNA-expressing transgenic plant populations over time. Another important experiment to conduct could be choice assays on dsRNA plants. In all experiments performed, GPA were confined on the plants in individual plant cages or on individual leaves using clip cages. DsRack1 plants may deter aphids, hence feeding on these plants may cause them to move and actually increase virus spread.

In summary, the PMRi technique was applied in GPA to investigate the role of Rack1 in the circulative transmission of TuYV by aphids. *Rack1* RNAi aphids acquire fewer virus particles than control aphids after a 12 hour or 2 day AAP, however the mechanism behind this is unknown as phenotypic effects on aphid feeding behavior were recorded. As part of this work, the way in which GPA are affected by PMRi was demonstrated. Intriguingly, nymphs developing inside silenced parent insects are also silenced. This is a novel and potentially significant discovery for future application of this technique. Further work should be completed to increase target gene down-regulation by PMRi and explore other practical applications of the technology such as protection against insect pests in agriculture.

6. General discussion

6.1. Summary of research

In this thesis, the impact of TuYV on UK commercial oilseed rape was established and sources of partial resistance to TuYV and aphids were investigated. This research confirmed that TuYV reduces oilseed rape yield and may have a subtle impact on seed physiology. These effects on the plant appear to be variety-dependent. Molecular techniques were utilized to improve understanding of virus acquisition and transmission by aphids. The PMRi tool was developed in two separate plant model-systems and successful down-regulation of two GPA target genes, *Rack1 & MpC002*, which are predominantly expressed in different aphid tissues, was demonstrated. PMRi was then applied to determine the function of *Rack1* in TuYV transmission by GPA. *Rack1* RNAi in GPA reduces aphid progeny, negatively affects feeding behavior, and reduces TuYV acquisition. Collectively, this suggests that *Rack1* would be a good target for GM approaches to aphid/virus control. Industry links have been developed to ensure the science will have impact and can potentially be used by breeders and farmers. Ultimately, this may provide renewed strategies towards TuYV control.

6.2. Impact of TuYV on yield and seed quality traits

The data presented show that TuYV impact on yield and seed quality traits is variety-dependent and not related to the amount of virus accumulation in the plant. This has implications for selecting the best varieties to grow. As previously discussed, yield reduction is the most important concern as virus-induced changes to seed physiology are unlikely to greatly affect quality. Each variety should therefore be individually assessed to identify those that have minimal yield impact from TuYV infection. Ideally, multiple years should be assessed similarly as there may be yearly differences between varieties. As composition of oils can be affected by a variety of factors (Baud & Lipiniec, 2010), TuYV may exacerbate the impact of certain abiotic factors like drought stress. A similar trial could be conducted elsewhere in the UK under different environmental conditions (e.g. climate, soil, etc) to test whether the TuYV tolerance observed in certain varieties is consistent.

TuYV epidemiology is also extremely environment-dependent with different yearly patterns of its aphid vectors. This yearly variation is important as it affects the

timing of TuYV inoculation in oilseed rape crops. It should be assessed how TuYV infection affects crop yield after inoculation with TuYV at different times throughout the growing season as this is not known. Autumn infection may be more damaging as the virus has more time to establish and spread in the plant. Conversely, resistance responses to early inoculation may not have much impact on seed production later in the plant lifecycle. Later infection at the time of flowering, diverting resources towards plant defense, could influence seed maturation. By establishing when oilseed rape crops are most vulnerable to TuYV inoculation, pest control strategies can be tailored accordingly. Estimates of how the crop could be affected in future climate scenarios can also be assessed. UK climate predictions (Semenov, 2007) suggest extended aphid seasons in the future and thus an increased range of inoculation timing, e.g. earlier spring inoculations.

Even in the narrow gene pool of UK commercial varieties, certain varieties appear more tolerant to virus-induced changes to yield and seed physiology. However, it may be necessary to look outside of elite germplasm for sources of resistance. Some preliminary work was conducted on varieties from the Oilseed Rape Genetic Improvement Network (OREGIN) *B. napus* diversity set (OREGIN, 2013). Varieties within this set were included based on various traits judged in need of improvement by the private sector plant breeding community, including nutrient efficiency, early vigor, premature seed loss and pest resistance (OREGIN, 2013). Double haploid populations were generated by crossing parents with potentially useful traits with a single contrasting parent line (Temple) known for its good agronomy (OREGIN, 2013). Within these parent lines, one variety ('POSH MC169') showed a consistently lower TuYV titer and rate of infection than other varieties tested, including Temple. Furthermore, there may be some partial GPA resistance segregating in 'Tapidor' & 'Ningyou7' (TN) crosses. As the mapping resources are available for these parent genotypes, there is potential to map aphid or TuYV resistance traits and potentially generate quantitative trait loci (QTLs).

Finally, further work may explore the mechanism behind TuYV-related changes to oilseed rape yield and quality. For example, the respective roles of host defense responses to TuYV or virus-induced host-modulation as discussed previously. Virus-induced changes to similar *Brassica* crops could be assessed to determine whether different host species are similarly affected. Ultimately, understanding the mechanisms

underlying these changes could help reduce loss of yield or seed quality to oilseed rape crops. It may also inform work on other economically significant luteovirids such as BYDV, which is a huge problem in cereal crops (Lister & Ranieri, 1995).

6.3. Role of Rack1 in TuYV transmission

Molecular interactions between TuYV and its aphid vector were investigated to determine the role of aphid proteins in the transmission process. Collectively, the data indicate that Rack1 may have a direct role in TuYV acquisition by GPA. However, future work is necessary to determine this.

Results from this investigation will enable further experimental work to investigate the *Rack1* RNAi effect on TuYV transmission by GPA. In a research collaboration with the group of Véronique Brault, INRA, France, *Rack1* RNAi aphids will be generated using dsRack1-expressing plants using similar methods to those developed in this study. Aphids will be fed on an artificial diet containing purified virus and several virus concentrations and acquisition times will be tested. Subsequently, aphid acquisition and transmission of TuYV to healthy plants will be examined. *Rack1* RNAi affects GPA feeding behavior on plants causing delayed phloem feeding, however, on an artificial diet this should be less pronounced due to easier feeding on these diets. Honeydew accumulation measurements will be used to assess equal levels of feeding.

Overall, targeting *Rack1* for these studies has been proven to be a good choice. Not only was is it potentially significant in luteovirid transmission but it was a good target for establishing the PMRi in aphids as *Rack1* is fairly well studied in other organisms plus silencing phenotypes are available. The phenotypes observed in *Rack1* RNAi aphids are in agreement with the known roles of *Rack1* (Chapter 1.10), proving the effectiveness of the PMRi tool.

6.4. Potential of plant-mediated RNAi in aphid functional genomics

RNAi is a powerful tool to characterize gene function and is particularly useful in insect systems as the functions of most insect genes are poorly understood (Huvenne

& Smagghe, 2010). A lack of genomics data can provide a barrier to potential RNAi-based post-genomic research. However, there is a growing amount of genomics data becoming available. The costs of genome sequencing have fallen greatly in recent years making it possible to cheaply sequence an organism of interest (Mardis, 2011). Genomics information is currently being gathered for a multitude of insect species, for example the i5k initiative is a five year project aiming to sequence the genomes of 5,000 insect and related arthropod species by 2016 (i5k, 2011). This opens up RNAi-based tools to an increasing number of insect systems. Moreover, exploiting the function of known orthologs in model insect systems will increase the speed of this process in less well-studied insects. PMRi may therefore be a valuable tool to use alongside the growing wealth of sequence data.

Vast amounts of genomics data are also being generated for aphid systems. The pea aphid genome has been published (Richards *et al.*, 2010), and an initiative between the John Innes Centre (JIC), and The Genome Analysis Centre (TGAC) (both Norwich, Norfolk, UK) has been working towards sequencing the genome of GPA clone O (Hogenhout SA, Clavijo B, Fenton B, Field LM, Swarbreck D, *et al.,* unpublished). The genome of another GPA clone (G006) is also being sequenced (Wilson A, Jander G, Legeai F, Tagu D, *et al.,* unpublished) by groups in France and the USA. PMRi could have multiple applications in diverse areas of aphid research including development, metabolism, insecticide resistance, as well interactions with hosts, viruses and endosymbionts. PMRi could be used to investigate aphid genes involved in insecticide resistance e.g. detoxifying enzymes such as cytochrome p450s (Ramsey *et al.*, 2010), to understand how insects quickly develop pesticide resistance. From this, it would be easier to develop novel, environmentally friendly pesticides. This also could aid the search for suitable compounds to use as highly specific pesticides, i.e. pesticides which kill a specific pest, but leave beneficial insects unharmed.

As aphids subjected to PMRi are reared on host plants, this makes it particularly amenable to study plant-insect interactions. It could therefore contribute towards understanding how insects overcome plant defenses and adapt to their hosts. Aphid species differ in their life strategies and host range. The pea aphid for example, is a specialist legume feeder whereas GPA can feed on over 40+ plant families. One of the reasons for this may be due to successful exploitation of host plants by means of

effectors which were discussed earlier (Chapter 1.6). RNAi may be used to understand the function of aphid salivary proteins involved in colonization of host plants (Bos *et al.*, 2010; Pitino & Hogenhout, 2013). From this, novel strategies to counter aphid infestations can be discovered.

The PMRi technique may be applied to investigate other gene targets putatively involved in TuYV transmission. Targets could be screened using transient *N. benthamiana* and transgenic *A. thaliana* produced. Subsequently, acquisition and transmission of RNAi aphids assessed as in the *Rack1* investigation. Putative targets could include proteins from the literature shown to interact with luteovirus particles. Alternatively, other targets can be uncovered experimentally. One approach to realize this aim would be to utilize the yeast two-hybrid system to determine interaction between the TuYV CP and RTD with GPA proteins. Additionally, using a co-immunoprecipitation technique as described in Yang *et al* (2008), novel GPA proteins that bind TuYV *in vivo* could be identified. Microarray or Illumina-based transcriptome approaches could also be used to assess TuYV-induced changes in GPA gene expression (as in Brault *et al.*, 2009), identifying gene targets for investigation by RNAi. Ultimately, this approach could be expanded to investigate vector-borne transmission of various virus species in diverse insect systems.

Future work on PMRi for aphids should focus on achieving close to 100% silencing of target genes. There may be various ways to achieve this. As previously discussed (Chapter 1.12), several factors have been shown to affect the efficacy of RNAi in insects (Huvenne & Smagghe, 2010). DsRNA constructs for PMRi can be designed to produce the optimal length of dsRNA at the desired concentration which will correspond to the most effective region of the target transcript, thus providing maximal silencing.

In this work, dsRNA expression in transgenic plant material was driven by a double CaMV 35S promoter which provides constitutive expression across all tissue types (Odell *et al.*, 1985; Yang & Christou, 1990). However, it is not known how much dsRNA/siRNA is present in the phloem where aphids feed. In order to optimize the expression of dsRNA in phloem tissues, a phloem specific promoter could be used such as the promoter region from the *A. thaliana sucrose-H+ symporter gene-2* (*AtSuc2*) (Truernit & Sauer, 1995). It may also be possible to further increase dsRNA expression

and thus aphid uptake by utilizing a more powerful promoter. Detection of plant-derived dsRNA/siRNA in aphids would demonstrate uptake of these RNAs by the insects. This was previously attempted but was unsuccessful, presumably due to the low levels of plant-derived siRNAs. Additionally, *Rack1* transcript down-regulation in dsRack1-fed insects is not necessarily proportional to Rack1 protein levels. Using a Rack1 antibody, Rack1 protein levels in silenced insects should be determined to provide further verification of the PMRi technique.

In order to improve target gene down-regulation by PMRi, better molecular understanding of insect RNAi such as modes of uptake, spread, and phenotypic effects, needs to be investigated. Turner *et al* (2006) demonstrated systemic spread of the RNAi signal from the gut to the antennae of the light brown apple moth (*Epiphyas postvittana*) after dsRNA feeding, suggesting that RNAi is systemic in insects after dsRNA ingestion. This should be investigated similarly for aphids. As gut cells are directly exposed to dsRNA/siRNA after PMRi, target gene down-regulation could be expected to be higher in gut tissues. In this work however, target genes expressed predominantly in the gut and salivary glands showed similar levels of down-regulation after PMRi. To determine the full range of RNAi spread in aphids, genes expressed in other tissues of GPA could be targeted by PMRi and assessed for down-regulation. By measuring how long RNAi takes to initiate in distal tissues, for example the antennae, this could improve understanding of RNAi spread in aphids. Furthermore, as dsRNA/siRNA injection methods introduce these RNAs directly in to insect hemolymph, the silencing signal may reach distal cells quicker. It would be useful to compare and contrast differences in RNAi spread between the different RNAi techniques to evaluate both methods.

In all experiments described in this thesis, RNAi aphids were generated by feeding developing insects on dsRNA plants. Younger stages often show larger silencing effects in other insects (Araujo *et al.*, 2006; Griebler *et al.*, 2008). It is not known if PMRi works effectively on adult aphids; it should be determined whether different growth stages are differentially affected by RNAi. It may be that silencing is more effective in aphid nymphs and other developing tissues. This may explain why nymphs show high levels of silencing and take longer to revert to normal transcript levels. As whole insects were sampled in these experiments, the silencing recorded by qRT-PCR may be coming from the nymphs inside the parent insect. Moreover, parent

insects may lose overall silencing after producing nymphs. Therefore it would be logical to assess how dsRNA ingestion affects different aphid tissues (e.g. gut, salivary gland, and developing nymphs) by dissecting insects after PMRi.

Interestingly, expression of dsRNA in an *A. thaliana* dicer mutant (*A. thaliana* dicer genes DCL2, DCL3 and DCL4 knockout) (Henderson *et al.*, 2006) results in the production of longer dsRNAs in the plant and subsequently improves the efficiency of target-gene silencing in the cotton bollworm (Mao *et al.*, 2007). Similarly, up to 95% silencing was achieved in Tobacco hornworms (*Manduca sexta*) when fed on dsRNA-expressing transgenic *N. attenuata* after transient silencing of DCLs in various combinations (Kumar *et al.*, 2012). This indicates that optimal silencing efficiency of targeted genes in insects might require stabilization of dsRNAs into longer (>70bp), un-diced fragments. Future work in aphids should explore this further. Crosses of *dcl234* mutant lines with dsRack1- and dsGFP-expressing lines were performed. However, due to each dcl mutation location present on independent loci, obtaining homozygous plants was extremely problematic. These issues along with time constraints meant these plants were not able to be used; however using the *dcl234* plants for *Agrobacterium*-mediated transformation with pJawohl8:dsRack1 construct should be relatively straightforward. This could be an aim of future studies as this may help to improve our understanding of RNAi pathways in Hemipteroids & other insects.

There are also alternative ways to achieve genetic manipulation of aphids through non-transgenic means. Emerging technologies such as transcription activator-like effector nuclease (TALEN) make introduction of single base mutations into genes of interest easily achievable and have been used to generate highly specific modifications in the *D. melanogaster* (Liu *et al.*, 2012) and *Aedes aegypti* (Aryan *et al.*, 2013) genomes. Additionally, genome modifications using both TALENs and zinc-finger nucleases in hemimetabolous insects have also recently been demonstrated (Watanabe *et al.*, 2012). Clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated endonuclease Cas9 has been used to modify genomic sequences in *D. melanogaster* (Bassett *et al.* 2013). There are various opportunities to expand these emerging techniques for genetic manipulation of aphids. For example, aphid components potentially involved in transmission of viruses could be targeted.

6.5. Potential of plant-mediated RNAi as a means of aphid control

As well as having a role in assessing gene function, RNAi can be used agriculturally to control aphids; *in planta* expression of dsRNA can be used as a form of insecticide (Baum *et al.*, 2007; Mao *et al.*, 2007; Chen *et al.*, 2010). As previously mentioned, aphids are significant pests in agriculture causing direct damage to crops as well as transmission of multiple plant viruses. Targeting the vector could therefore be very beneficial to not only reduce damage caused by aphid feeding but also to limit virus spread. Reduced aphid populations could also lead to less insect overcrowding and fewer alate insects which facilitate virus spread.

PMRi occurs rapidly in GPA, suggesting that populations establishing on transgenic plants would quickly be affected. Also, there are long-term effects on the dsRNA-exposed population. As RNAi appears to have a germline effect in aphids, this would further increase the effectiveness in agriculture as successive generations can be targeted through continual exposure to PMRi. GPA populations were significantly reduced after PMRi of *Rack1*, *MpC002* & *MpPInt02* over four weeks. Collectively, this indicates PMRi is a viable option for aphid control.

Before PMRi could realistically be used as a crop protection measure, it needs to be optimized so that high levels of gene knockdown can be achieved, as previously discussed (Chapter 6.4). Subsequently, PMRi can be adapted for a variety of uses in agriculture. The effectiveness of PMRi as an insect control mechanism may be improved by targeting key aphid genes e.g. essential housekeeping genes (Bhatia *et al.*, 2012) or insect detoxification mechanisms against plant secondary metabolites (Mao *et al.*, 2007). This could also be a difficult resistance to breakdown by the insect as it cannot lose an essential gene or modify the conserved RNAi pathway. *MpC002* RNAi was most effective at reducing GPA population growth, hence may be an effective target to use for aphid control. Silencing this gene in the pea aphid reduced phloem contact (Mutti *et al.*, 2008) so could also be a good target in GPA to prevent transmission of non-persistent viruses. However, *MpC002* RNAi GPA did not have reduced TuYV uptake (**Figure 5.5**).

Rack1 could be an effective target for many reasons. *Rack1* silencing in GPA has been shown to reduce aphid fecundity, negatively affect feeding behavior and reduce virus uptake by GPA. *Rack1* silencing may therefore provide the double benefit

of reducing aphid numbers as well as reducing the efficiency of which the virus gains uptake and disseminates to new host plants. It could therefore be a good target for translating into crops e.g. dsRack1 transgenic oilseed rape. Furthermore, sluggishness in *Rack1* RNAi aphids may have various fitness costs to the aphid, such as reduced response to predators. It may also reduce the propensity for aphid to move to a neighboring plant and thus limit virus spread. Moreover, reduced probing by these insects could also have an impact on transmission of non-persistent viruses which are dependent on insect probing (Martin *et al.*, 1997).

One of the major issues with insecticides is that they can kill non-target species. To address this issue, Whyard *et al* (2009) harnessed the sequence specificity of RNAi to design orally-delivered dsRNAs that selectively killed target species. RNAi can therefore be used for species-specific insecticides. Alternatively, constructs can be designed generically to exploit conserved regions in genes to silence multiple insect species at once. Targeting genes belonging to large families with high sequence similarity could lead to broad-spectrum resistance against insect pests e.g. all Hemipteroids. As *Rack1* is conserved across different insect species (Adams *et al.*, 2011), it could be a good target for this.

In order to remain effective, gene targets need to be carefully chosen to ensure that that loss of function is not compensated for by another untargeted gene. One way to overcome this would be to build constructs which can target multiple target genes in parallel i.e. multiple housekeeping genes and/or genes associated with virus transmission. Stacking of different gene targets would make for durable resistance. The feasibility of stacking multiple targets by RNAi has been demonstrated in *D. melanogaster* (Schmid *et al.*, 2002) but the effectiveness in PMRi against insect herbivores has not been explored. Crops may ultimately be engineered to express a deadly cocktail of dsRNAs that are highly effective against target insect pest species.

Companies like Monsanto are expanding work on RNAi for pest management; recently they have moved four RNAi-based products through their research and development pipeline (Monsanto, 2013). These include approaches for control of the western corn rootworm (Gassman *et al.*, 2011). Monsanto researchers have recently published the use of orally delivered dsRNAs targeting the Snf7 ortholog (encodes a protein essential for intracellular trafficking), to kill rootworms (Bolognesi *et al.*, 2012).

Subsequently, a strain of corn ('Corn Rootworm III'), that uses RNAi to create resistance to rootworm is in advanced development as well as topical sprays to deliver RNA that impairs the metabolic functions of target insects (Monsanto, 2013). This strongly indicates that RNA-based products will become available in the future.

6.6. Future TuYV crop protection strategies

As outlined in Chapter 1.7, pesticide use is likely to decline in the future. In order to continue to control TuYV effectively, alternative strategies need to be introduced. This could include an expansion of conventional strategies as well as novel approaches. One approach could include substitution of neonicotinoid seed treatments with treatments incorporating Jasmonic acid (JA) and/or β-aminobutyric acid (BABA) to prime plants for defense (Worrall *et al.*, 2012). Entry into a primed state can enhance plant resistance to future pest attack with minimal costs to growth and development (Worrall *et al.*, 2012).

TuYV resistance in oilseed rape germplasm can be screened and conventional breeding methods employed to introduce TuYV tolerance or resistance into commercial varieties. However, traditional crop-breeding programs are limited by the time taken to move resistance traits into elite crop genetic backgrounds and the narrow germplasm in which to search for novel resistance. Furthermore, monogenic resistance does not protect against the full spectrum of pests and diseases, and is more likely to break down as pests evolve counter-resistance.

Genetic modification (GM) of plants is one of the most powerful tools for improvements in agriculture as genes can be precisely and conveniently moved into mainstream crop cultivars. GM has the potential to improve plant resistance to pests or pathogens, resistance to particular herbicides, increase yield and crop quality, vitamin fortification to improve human/animal health, resistant to abiotic stresses such as drought and increased temperature due to climate change (Bruce, 2012). GM may also reduce environmental impact through reduced agrochemical, nitrogen, and water input, as well as decreased CO_2 emissions and reduced strain on land, soil and energy usage (Bruce, 2012). GM is not a universal solution to issues of food security but is nevertheless a powerful tool for crop improvement.

As discussed, PMRi could be a good approach to TuYV control. However, this and other GM approaches are likely to meet significant opposition in various parts of the world, especially in the EU which has possibly the strictest GM regulations (Davison, 2010). Only two GM crops have been approved for use in the EU, 'MON810', maize resistant to the European corn borer, and 'Amflora', a potato variety modified for industrial uses (Fresco, 2013). Strict legislation and expensive GM licensing mean that only large corporations can afford it, consequently public stigma has been associated with companies such as Monsanto who require returns on their investment (Davison, 2010). Due to these issues, it's likely that greatest potential to be reached from PMRi technology within the UK for the immediate future is as a laboratory tool. Other non-transgenic methods of achieving RNAi effect in aphids could be applicable for use in UK agriculture e.g. dsRNA pesticide sprays (Wang *et al.*, 2011). Should public attitudes and legislation against GM become more moderate in future however, there could be multiple applications of the technology for aphid or virus control.

Effective insect control can be achieved with transgenic crops expressing insecticidal toxins such as those derived from the bacterial species *Bacillus thuringiensis* (Bt) (Gatehouse, 2008). Bt-derived toxins have provided effective suppression of lepidopteran (moth) and coleopteran (beetle) pests but hemipteroid pests are not particularly susceptible (Porcar *et al.*, 2009; Li *et al.*, 2011; Chougule & Bonning, 2012). Binding of Bt toxins to the gut of the target insect is an important step for toxicity, therefore modification of the Bt toxin Cyt2Aa with an additional aphid gut-binding peptide has recently been developed, providing enhanced binding and toxicity against pea aphids and GPA (Chougule *et al.*, 2013).

Another GM strategy takes advantage of the aphid alarm signal (E)-β-farnesene (Eβf), which is secreted by aphids upon predator attack. Eβf is the primary constituent (Du *et al.*, 1998) of the alarm signal and exposure to Eβf causes other aphids nearby to drop off the host plant or to disperse to distant tissues. The pheromone also functions to attract aphid enemies (Beale *et al.*, 2006; De Vos & Jander, 2010). Therefore, transgenic production of Eβf may protect plants by both deterring aphids and increasing the rates of parasitism on aphid colonies. The potential of producing Eβf in transgenic wheat crops is being trialed at Rothamsted Reseach, Hertfordshire, UK (Rothamsted wheat trial, unpublished).

Development of ways to block virus transmission by aphids could lead to novel and broad-spectrum means of controlling plant viruses. Liu *et al.* (2010) described a peptide that binds the pea aphid gut and impedes entry of PEMV into the aphid hemocoel (Liu *et al.*, 2010). Plants could be engineered to produce these blocking factors which could directly impede the uptake of multiple virus species. Furthermore, GM approaches could directly engineer TuYV resistance into crops plants. A study by Wang *et al* (Wang *et al.*, 2000) showed that a single copy of a virus-derived transgene encoding hairpin RNA gave immunity to BYDV in barley. A similar approach could be used for TuYV in oilseed rape.

The most practical outcome of the research presented is the finding that oilseed rape varieties can tolerate virus accumulation better than others. So screening in the recommended list may enable the most useful varieties to be developed. It's likely that all available tools will be necessary to improve agriculture sustainably in the future; therefore the GM approaches described may be integrated into future control strategies.

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Appendix A – published works

I. **Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA.** (2011) Silencing of aphid genes by dsRNA feeding from plants. Plos One **6**: e25709 **[See the following pages for this publication]**.

II. **Coleman AD, Pitino M, Hogenhout SA.** (2014) Silencing of aphid genes by feeding on stable transgenic *Arabidopsis thaliana*. In: Plant-Pathogen Interactions: Methods and Protocols, **2**(1127). *In press*.

III. **Coleman AD, Gannon L, Vives-Garcia P, Hogenhout SA, Ridout CJ, Stevens M.** (2013) Impact of *Turnip yellows virus* (TuYV) infection on yield and seed quality traits in elite oilseed rape is variety-dependent. *In revision*.

Silencing of Aphid Genes by dsRNA Feeding from Plants

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Abstract

Background*:* RNA interference (RNAi) is a valuable reverse genetics tool to study gene function in various organisms, including hemipteran insects such as aphids. Previous work has shown that RNAi-mediated knockdown of pea aphid (*Acyrthosiphon pisum*) genes can be achieved through direct injection of double-stranded RNA (dsRNA) or small-interfering RNAs (siRNA) into the pea aphid hemolymph or by feeding these insects on artificial diets containing the small RNAs.

Methodology/Principal Findings*:* In this study, we have developed the plant-mediated RNAi technology for aphids to allow for gene silencing in the aphid natural environment and minimize handling of these insects during experiments. The green peach aphid *M. persicae* was selected because it has a broad plant host range that includes the model plants *Nicotiana benthamiana* and *Arabidopsis thaliana* for which transgenic materials can relatively quickly be generated. We targeted *M. persicae Rack1*, which is predominantly expressed in the gut, and *M. persicae C002* (*MpC002*), which is predominantly expressed in the salivary glands. The aphids were fed on *N. benthamiana* leaf disks transiently producing dsRNA corresponding to these genes and on *A. thaliana* plants stably producing the dsRNAs. *MpC002* and *Rack-1* expression were knocked down by up to 60% on transgenic *N. benthamiana* and *A. thaliana*. Moreover, silenced *M. persicae* produced less progeny consistent with these genes having essential functions.

Conclusions/Significance*:* Similar levels of gene silencing were achieved in our plant-mediated RNAi approach and published silencing methods for aphids. Furthermore, the *N. benthamiana* leaf disk assay can be developed into a screen to assess which genes are essential for aphid survival on plants. Our results also demonstrate the feasibility of the plant-mediated RNAi approach for aphid control.

Introduction

RNA interference (RNAi) is a valuable reverse genetics tool to study gene function in various organisms [1]. The process of RNAi was described as 'post-transcriptional gene silencing' (PTGS) in plant systems [2] and is a technique well established in numerous eukaryotic systems across kingdoms, e.g. *Caenorhabditis elegans* [3], *Arabidopsis thaliana* [4] and *Drosophila melanogaster* [5].

With the RNAi method, double-stranded RNA (dsRNA) can specifically lower the transcript abundance of a target gene when injected into an organism or introduced into cultured cells [3]. RNAi involves the cleavage of dsRNA precursors into small-interfering RNA (siRNA) of approximately 21 to 23 nucleotides by the enzyme Dicer [6]. These siRNAs are then incorporated into a RNA-induced silencing complex (RISC). Argonaute proteins, the catalytic components of RISC, use the siRNA as a template to recognize and degrade the complementary messenger RNA (mRNA) [6]. RNAi can therefore be exploited to suppress gene expression through highly specific depletion of target transcripts.

Aphids are sap-sucking insects of the order Hemiptera and are important crop pests in terms of direct feeding damage and also transmission of plant viruses [7]. RNAi has been successfully used to investigate gene function in the pea aphid *Acyrthosiphon pisum*, a relatively large aphid that can be injected with dsRNA. Nonetheless, the *A. pisum* host range is predominantly restricted to leguminous species. On the other hand, the green peach aphid *Myzus persicae* can feed on over 40 different plant families [8] and is capable of efficiently transmitting over 100 types of plant viruses [9]. Hence, *M. persicae* is one of the most important aphid pests in agricultural crops. However, RNAi has not previously been documented in this species.

RNAi-mediated gene knockdown can be achieved in aphids through direct injection of dsRNA or small-interfering RNAs (siRNA) into aphid hemolymph [10,11]. This approach was used to silence *C002*, a gene strongly expressed in the salivary glands of *A. pisum* [10]. Silencing the gene resulted in lethality of the aphids on plants, but not on artificial diet, indicating that C002 has a function in aphid interaction with the plant host [10,12]. We identified the homologue of *C002* from *M. persicae* and named it *MpC002* [13]. *MpC002* is predominantly expressed in the *M. persicae* salivary glands and transient over-expression of *MpC002* in *Nicotiana benthamiana* improved *M. persicae*

fecundity [13]. Microinjection of long dsRNA into *A. pisum* also leads to silencing of genes encoding calreticulin and cathepsin by 30-40% [11]. Calreticulin is a calcium-binding protein that is produced in most aphid tissues, while cathepsin is specifically produced in the pea aphid gut. Thus, gene silencing appears to occur in different aphid tissues [11].

Aphids can be fed on artificial diet, which is sandwiched between thin parafilm membranes. *A. pisum* fed on an artificial diet containing dsRNA corresponding to the aquaporin transcript lead to downregulation by more than 2-fold within 24 hours [14]. Since aquaporin is involved in osmoregulation, this resulted in elevated osmotic pressure in the hemolymph [14]. Feeding of dsRNA targeting vATPase transcripts from an artificial diet achieved a 30% decrease in transcript levels in *A. pisum* and a significant increase in aphid mortality [15].

Both micro-injection and artificial diets are valuable methods for achieving RNAi in aphids. However, dsRNA/siRNA has to be synthesized in both cases and neither treatment is natural for aphids. As RNAi in aphids is indeed feasible, it has the potential to be expanded to include gene knockdown via the delivery of dsRNA from plants (plant-mediated RNAi). This method could allow for studying aphid gene function in the aphid natural habitat and may be useful for control aphid pests in crop production. The plant-mediated RNAi method effectively silences genes of lepidopteran and coleopteran insect species [16,17] and the brown planthopper, a hemipteran species [18]. However, these insects are larger than aphids and hence consume more plant tissue/sap while feeding. Our goal was to determine if the plant-mediated RNAi approach also silences aphid genes. The green peach aphid *M. persicae* was selected because it has a broad plant host range, including the model plants *N. benthamiana* and *Arabidopsis thaliana* for which transgenic materials can relatively quickly be generated. Furthermore, transgenes can be rapidly expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated transient expression providing the possibility to develop a high-throughput system to assess which genes in the aphid genome are essential for survival of aphids on plant hosts. To test the plant-mediated RNAi approach, we selected two *M. persicae* genes, *MpC002* and *Receptor of Activated Kinase C (Rack-1)* as targets. As discussed above, *MpC002* is predominantly expressed in the aphid salivary gland. In contrast, *Rack-1* is predominantly expressed in the aphid gut.

Rack1 is an intracellular receptor that binds activated protein kinase C (PKC), an enzyme primarily involved in signal transduction cascades [19]. Rack-1 is conserved amongst plants and animals and is an essential multifunctional scaffold protein which physically connects diverse signal transduction components into stable complexes [20]. *Rack-1* binds to integrins [21], has a function in actin organisation [22] and is an integral component of the mammalian circadian clock [23]. Rack-1 from *M. persicae* was identified as a luteovirus-binding protein [19] as it was found to bind in vitro to purified wild type or mutant particles of *Beet Mild Yellows Virus* (BMYV). *Rack-1* is a good candidate for RNAi in aphids as *Rack-1* knockdown has been demonstrated in the nematodes *Caenorhabditis elegans* [24,25] and *Heterorhabditis bacteriophora,* [26]. Knockdown of *Rack-1* resulted in developmentally defective phenotypes in *C. elegans* including slow growth, embryonic lethality, egg laying defectiveness and sluggishness [24,25] as well as sterility and abnormal gonad development [26]. *Rack-1* in *Drosophila* functions during oogenesis [27] and is required in early oocyte polarity [28].

We found that the expression of both *MpC002* and *Rack-1* is knocked down when *M. persicae* are fed from transgenic plants that transiently (*N. benthamiana*) and stably (*A. thaliana*) express dsRNA corresponding to *MpC002* and *Rack-1*. Moreover, silenced aphids have reduced progeny production. Thus, plant-mediated RNAi is feasible, and is a useful tool for studying aphid gene function.

Results

Expression profiles of RNAi target genes

C002 and *MpC002* are predominantly expressed in the salivary glands of *A. pisum* and *M. persicae* [10,12,13], and *Rack-1* in aphid gut tissues [19]. To verify this in our colony of *M. persicae*, RT-PCR was performed on total RNA extracted from different aphid tissues. *MpC002* transcripts were detected in *M. persicae* heads and salivary glands, at relatively low abundance in whole aphids but not in dissected aphid guts (Figure 1). Conversely, *Rack-1* transcripts were found in all aphid body parts and at highest abundance in the gut (Figure 1). These results confirmed previous findings and provided RNAi targets predominantly expressed in the aphid salivary glands and gut.
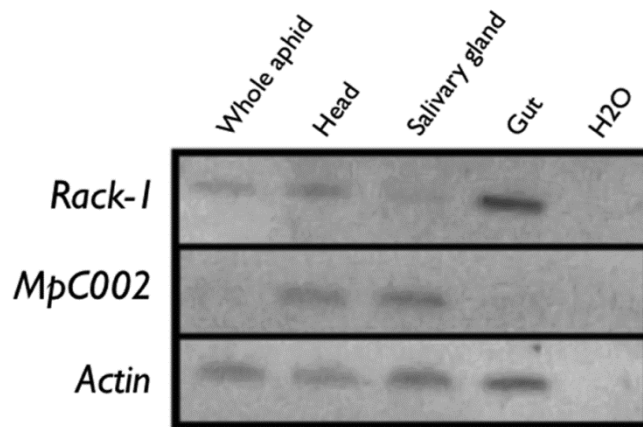

Figure 1. *MpC002* and *Rack-1* are differentially expressed in *M. persicae* tissues. RNA isolated from whole aphids and dissected aphid body parts were used for RT-PCR with specific primers for *Rack-1*, *MpC002* and *Actin*. The latter showed presence of similar RNA concentrations in the aphid samples.

Detection of *MpC002* and *Rack-1* siRNAs in *N. benthamiana* leaves

First, we investigated if dsRNAs corresponding to *M. persicae MpC002* (dsMpC002) and *Rack-1* (dsRack-1) were produced and processed into siRNAs in *N. benthamiana* leaves. The entire *MpC002* transcript without the region corresponding to the signal peptide (710bp), a fragment corresponding to the 5' coding region of the *Rack-1* transcript (309bp) and a fragment corresponding to the majority of the open reading frame (537bp) of the green fluorescent protein (GFP) were cloned into the pJawohl8-RNAi plasmid, which expresses the cloned fragments as inverted repeats under control of a double CaMV 35S promoter to produce dsRNAs (I.E. Sommsich, see

acknowledgments). Double-stranded GFP (dsGFP) was used as a control for the dsRNA treatments as opposed to empty vector in order to assess whether the presence of dsRNA itself would induce some effect in plant response to aphids. The pJawohl8-RNAi constructs were transiently expressed by *Agrobacterium*-mediated infiltration (agro-infiltration) of *N. benthamiana* leaves. *MpC002* and *Rack-1* siRNAs were observed starting 2 days post agro-infiltration (Figure 2). This indicated that the *MpC002* and *Rack-1* dsRNAs are being processed into 21 to 23 nucleotide siRNAs in *N. benthamiana* leaves. The agro-infiltrated leaves did not show obvious phenotypes such as chlorosis or leaf curling/crinkling upon agro-infiltration of the pJawohl8-RNAi constructs.
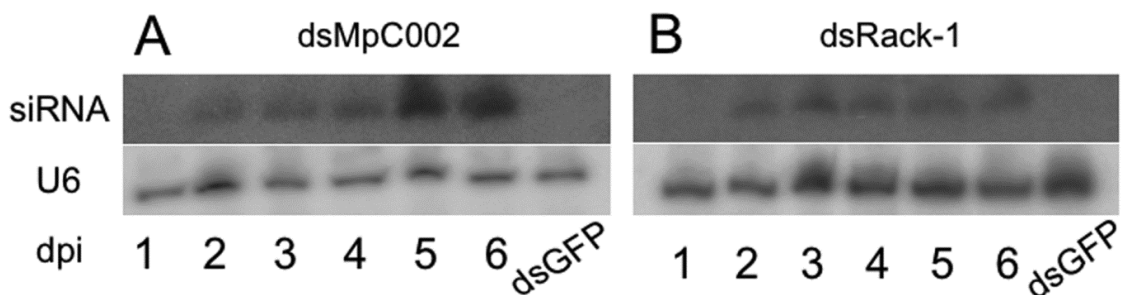

Figure 2. *MpC002* **and** *Rack-1* **dsRNAs are processed into siRNAs (21-23nt) in agro-infiltrated** *N. benthamiana* **leaves.** *MpC002* and *Rack-1* pJawohl8-RNAi constructs were agro-infiltrated in *N. benthamiana* leaves, which were harvested 1, 2, 3, 4, 5 or 6 days post-inoculation (dpi) for RNA isolation. Total RNA (15-20 μg) was loaded in each lane. Northern blots were hybridized with probes prepared from *MpC002* (**A**) or *Rack-1* (**B**) PCR products. Total RNAs isolated from leaves 6 dpi with GFP pJawohl8-RNAi constructs were included to control for specific hybridization of the *MpC002* and *Rack-1* probes (lanes indicated with dsGFP). To control for equal RNA loading, blots were stripped and then hybridized with an snRNA probe corresponding to U6, which is constitutively produced in plants [45].

Silencing of *M. persicae MpC002* **and** *Rack-1* **genes by feeding from transgenic** *N. benthamiana* **leaves**

Next we investigated if *MpC002* and *Rack-1* are down-regulated in *M. persicae* after feeding on *N. benthamiana* leaves transiently producing the *MpC002* and *Rack-1* RNAs. At one-day post agro-infiltration, 11-mm diameter leaf discs of the infiltrated leaves were placed on top of water agar in wells of 24-well titre plates and exposed to aphids as previously described [13]. Nymphs born on the leaf discs were transferred every 6 days to newly agro-infiltrated leaf discs to ensure continuous exposure of the aphids to the *MpC002* and *Rack-1* RNAs (Figure 2). At 17 days, the adult aphids were collected

to assess *MpC002* and *Rack-1* expression levels by quantitative RT-PCR (qRT-PCR). Aphids fed for 17 days on *N. benthamiana* leaf discs infiltrated with dsGFP pJawohl8-RNAi constructs were used as controls. The expression levels of *MpC002* and *Rack-1* were reduced by an average 30-40% compared to the controls (Figure 3A). This downregulation was consistent and highly significant among three biological replicates for *MpC002* (Student's *t*-test, n=3, p-value = 0.013) and *Rack-1* (Student's *t*-test, n=3, p-value = 0.012).
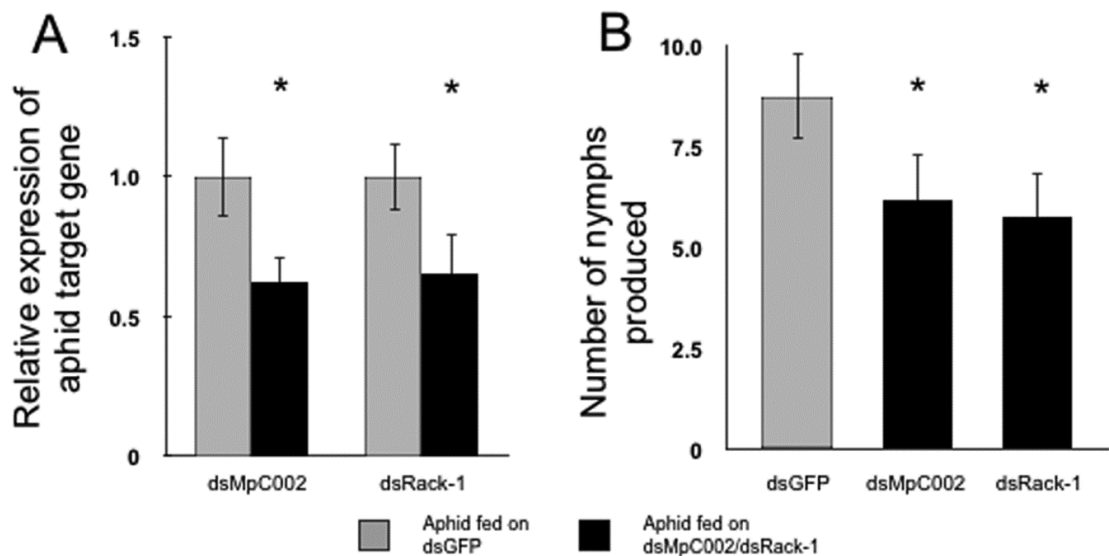

Figure 3. Silencing of *M. persicae MpC002* or *Rack-1* by *N. benthamiana*-mediated RNAi reduces aphid fecundity.

(**A**) *MpC002* and *Rack-1* expression is down-regulated in aphids fed on *N. benthamiana* leaves transiently producing *MpC002* and *Rack-1* RNAs. Aphids fed on transgenic *N. benthamiana* leaf discs for 17 days were harvested and analyzed for down-regulation of *MpC002* and *Rack-1* by qRT-PCR. Data shown are means ± standard errors of three biological replicates with n=3 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (Student's *t*-test, n=3, p<0.05) (**B**) *MpC002* and *Rack-1*-silenced *M. persicae* are less fecund. The numbers of nymphs produced by the aphids analyzed for down-regulation of *MpC002* and *Rack-1* in A were counted and compared to the nymphs produced from aphids fed on the dsGFP transgenic *N. benthamiana* leaf discs. Data shown are average number of nymphs produced per adult aphid with means ± standard errors of six biological replicates with n=4-6 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (ANOVA, n=4-6, p<0.05).

Silencing of aphid *MpC002* and *Rack-1* on stable transgenic *Arabidopsis* lines

We also investigated the downregulation of *M. persicae* genes *MpC002* and *Rack-1* upon feeding on stable transgenic *A. thaliana* plants. The transgenic lines were obtained by floral-dip transformation of Col-0 plants with the *MpC002*, *Rack-1* and GFP

pJawohl8-RNAi constructs used in the *N. benthamiana* transient assays. Three independent F3 homozygous dsMpC002 and dsRack-1 transgenic *A. thaliana* were generated. One F3 homozygous dsGFP transgenic *Arabidopsis* line was included as control. All lines contained the transgenes as confirmed by PCR and sequencing. Northern blot analysis of the transgenic *Arabidopsis* lines revealed the presence of siRNA for *MpC002* and *Rack-1* (Figure 4). The siRNAs corresponding to *M. persicae MpC002* were equally abundant in the three independent transgenic lines (Figure 4A), while the siRNAs corresponding to *Rack-1* were abundant in line 1, less abundant in line 3 and not detected in line 4 (Figure 4B).
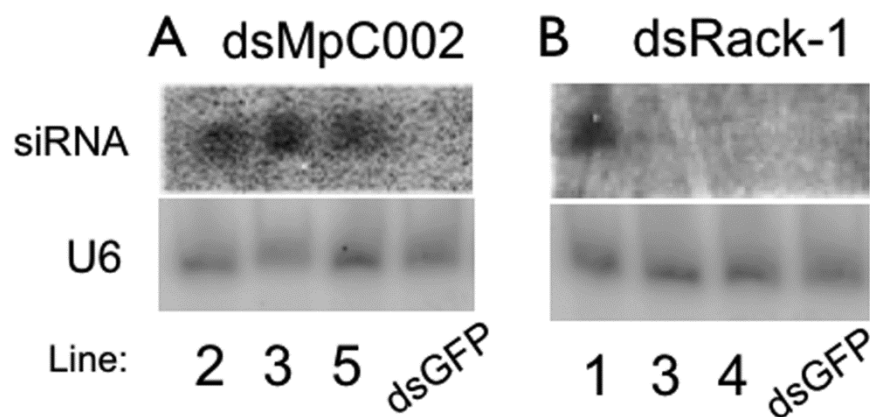

Figure 4. *MpC002* **and** *Rack-1* **dsRNAs are processed into siRNAs (21-23nt) in transgenic** *A. thaliana* **lines.** Total RNA was isolated from two-week old seedlings of F3 homozygous stable dsMpC002 (**A**) and dsRack-1 (**B**) transgenic lines. Total RNA isolated from two-week old seedlings of a F3 homozygous stable dsGFP line was included to control for specific hybridization (lanes indicated with dsGFP). Each lane contains 15-20 μg of total RNA. Northern blots were hybridized with probes prepared from *MpC002* (**A**) or *Rack-1* (**B**) PCR products. To verify equal RNA loading, blots were stripped and then hybridized with an snRNA probe corresponding to U6, which is constitutively produced in plants [45].

To investigate down-regulation of *M. persicae MpC002* and *Rack-1* on the stable transgenic lines, nymphs born on the transgenic plants were kept on these plants for 16 days at which time the adult aphids were collected for RNA extraction and qRT-PCRs. The aphids reared on three independent dsMpC002 lines showed an approximate 60% decrease in *MpC002* expression compared to aphids reared on dsGFP (Figure 5A). Furthermore, down-regulation of *Rack-1* by approximately 50% was demonstrated for aphids reared on dsRack-1 line 1 compared to dsGFP but not for aphids fed on dsRack-1 lines 3 and 4 (Figure 5A). *MpC002* down-regulation on the three independent lines was consistent in three replicates (Student's *t*-test, n=3, p<0.05). *Rack-1* was also

consistently down-regulated on dsRack-1 line 1 among three replicates (Student's *t*-test, n=3, p=0.023), while *Rack-1* was not significantly down-regulated on dsRack-1 lines 3 and 4 (Student's t-test, n=3, p> 0.05). These results are in agreement with the dsMpC002 and dsRack-1 expression levels in the transgenic lines in which the expression of the aphid *Rack-1* gene was not down-regulated on transgenic lines that have low levels of siRNAs corresponding to *Rack-1* (Figure 4B).
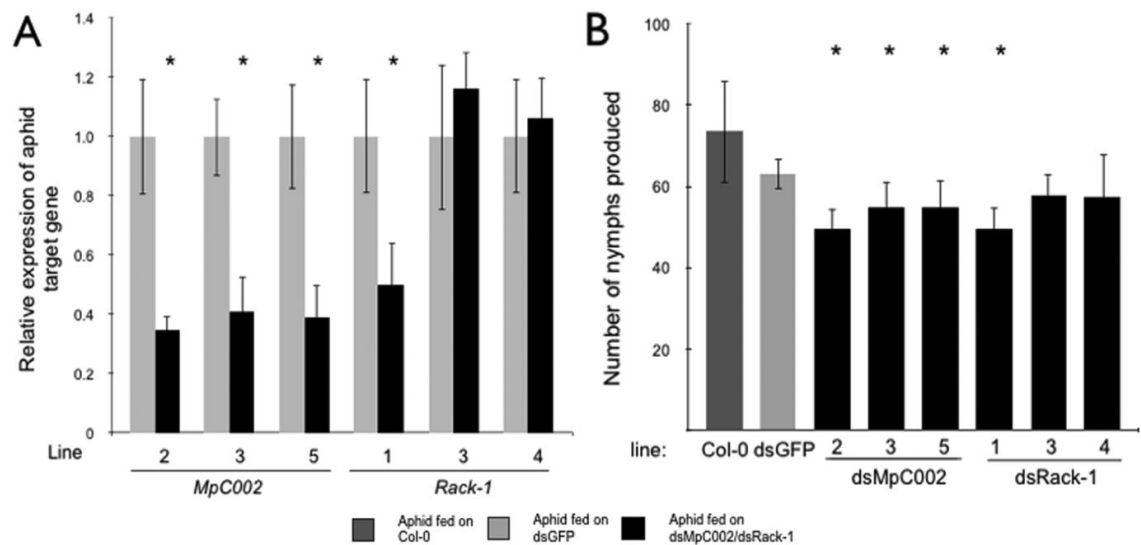

Figure 5. Silencing of *M. persicae MpC002* or *Rack-1* by Arabidopsis-mediated RNAi reduces aphid fecundity.

(**A**) *MpC002* and *Rack-1* expression is down-regulated in aphids fed on transgenic Arabidopsis producing *MpC002* and *Rack-1* RNAs. Aphids fed on dsMpC002 or dsRack-1 producing *Arabidopsis* for 16 days were harvested and analyzed for downregulation of *MpC002* and *Rack-1* by qRT-PCR. Data shown are means ± standard errors of three biological replicates with n=3 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (Student's *t*-test, n=3, p<0.05) (**B**) *MpC002* and *Rack-1*-silenced *M. persicae* are less fecund. The numbers of nymphs produced by the aphids analyzed for downregulation of *MpC002* and *Rack-1* in A were counted and compared to the nymphs produced from aphids fed on Col-0. Data shown are total number of nymphs produced on each line with means ± standard errors of three biological replicates with n=4 per replicate. Asterisk indicates significant difference in treatments compared to dsGFP (GLM, n=4, p<0.05).

Silencing of *MpC002* and *Rack-1* reduces *M. persicae* fecundity

It was previously shown that silencing of *C002* by injection of dsRNAs in the pea aphid increased the lethality of these aphids on plants [10,12]. Hence, we assessed if silencing of *MpC002* also affected survival of *M. persicae* feeding directly on *N. benthamiana* and *A. thaliana*. Nymphs exposed to the *N. benthamiana* leaf discs for 17 days became

adults and started to produce their own nymphs after approximately 10 days. The overall survival of the aphids and the production of nymphs on leaf discs transiently producing dsMpC002 were not affected compared to aphids on leaf discs producing dsGFP (Figure S1A). However, the nymph production by these aphids was significantly lower in six biological replicates (ANOVA, n=4-6, p<0.05) (Figure 3B). Similarly, on transgenic *Arabidopsis* plants the *MpC002*-silenced aphids survived equally well, but produced less nymphs in three biological replicates (GLM, n=4, p<0.05) (Figure S1B, Figure 5B).

Survival and nymph production were also investigated for the *Rack-1*-silenced aphids. *Rack-1*-silenced aphids survived equally well (Figure S1A), but produced fewer nymphs on *N. benthamiana* leaf discs (ANOVA, n=4-6, p<0.05) (Figure 3B). Similarly, nymph production was reduced on *Rack-1*-silenced aphids feeding on dsRack-1 transgenic *Arabidopsis* line 1 (GLM, n=4, p<0.05), while survival was not affected (Figure S1B). *M. persicae* fecundity was not reduced on dsRack-1 transgenic *Arabidopsis* lines 3 and 4 (Figure 5B) which is consistent with no significant down-regulation of *Rack-1* in aphids on these lines (Figure 5A).

Discussion

We have shown that it is possible to down-regulate *M. persicae* gene expression by feeding the aphids dsRNA from plants. As far as we are aware, this is the first example of RNAi in an aphid system from direct plant feeding. We also show that RNAi is possible in *M. persicae*, as RNAi was shown previously in *A. pisum* only.

We measured a 30-60% decrease in gene expression, similar to that observed in microinjection and artificial feeding of small RNAs to aphids. The reduction is also similar to that measured in other insects such as *Schistocerca americana* (injection) [29] and *Rhodnius prolixus* (injection and ingestion) [30] but overall lower than the levels found in *Spodoptera litura* (injection) [31] or in *Drosophila melanogaster* (injection) [32]. Our method allows the study of gene function during interactions of aphids with plants, which is not possible by feeding of dsRNA and siRNA from diets [14,15].

Previous studies have demonstrated the silencing signal to be mobile in plants [33], where expressed small RNAs to move within the phloem to where aphids feed. The CaMV 35S promoter enables constitutive expression of dsRNA in transgenic plants tissue, including the leaf phloem [34]. The CaMV 35S promoter also allows for transient expression and movement of dsRNAs in *N. benthamiana* phloem [35]. Our results demonstrate that siRNAs can travel from the plant phloem through the aphid stylet and reach the aphid intestinal tissues triggering the silencing of aphid target genes. Given that *MpC002* expression is knocked down by up to 60% and is predominantly expressed in the salivary glands, the silencing signal appears to spread through the aphid. This is consistent with the finding that small RNA pathways that are highly conserved in animals are also present in aphids [36,37,38]

Knockdown of *Rack-1* and *MpC002* reduced aphid fecundity (Figure 3B, Figure 5B) but not survival (Figure S1). This contrasts with the results obtained by dsRNA injection of *A. pisum* in which survival was reduced by silencing *C002*. It is possible that the lower *A. pisum* survival is caused by faster downregulation of the target gene as a result of the sudden higher presence of the injected dsRNA in the hemolymph. Alternatively, stress caused by the injection could exacerbate the negative impact of *C002* downregulation. *M. persicae* are smaller than pea aphids and hence more difficult to inject without affecting aphid survival rates. Delivery by plant feeding therefore provides a gentle, natural method for studying gene function that is less likely to have

indirect effects on aphid behaviour. Our method is therefore suited to investigating the effects of gene silencing on aphid/plant interactions, and for virus-transmission studies.

M. persicae produces more progeny on *N. benthamiana* leaves that transiently express *MpC002* [13]. Thus, the presence of more (*in planta* overexpression) and less (RNAi in aphids) MpC002 leads to, respectively, increased and reduced *M. persicae* performance on plants. In addition, silencing of *A. pisum C002* decreases survival of this aphid on plants but not on diet and the C002 protein was detected in plants upon *A. pisum* feeding [10]. Finally, C002 was found in the saliva proteomes of *M. persicae* [39] and *A. pisum* [40]. Altogether, this indicates that the *C002* genes of both *M. persicae* and *A. pisum* have essential functions in aphid-plant interactions.

Our finding that silencing of *Rack-1* in *M. persicae* leads to decreased progeny production by this aphid is also in agreement with other findings. Indeed, Rack-1 is a scaffold protein that is involved in the regulation of cell proliferation, growth and movement in animals [20,21,22]. Silencing of *Rack-1* in two species of nematodes, *C. elegans* and *H. bacteriophora*, reduces growth of these animals [24,25,26]. *M. persicae* Rack-1 also interacts with integrins and luteoviruses [19], which invade aphid gut cells [41], suggesting a role in endocytosis processes, such as nutrient/peptide uptake from the gut lumen. Given that *Rack-1* is expressed in multiple tissues of the aphid and particularly in the gut, silencing this gene may affect aphid progeny reproduction indirectly, perhaps by reducing the growth of gut cells leading to decreased nutrient uptake. Alternatively, silencing may directly reduce the growth of embryo cells.

The *M. persicae* genome is being sequenced, but the functions of the majority of aphid genes are still unknown. Moreover, it is not fully understood how aphids modulate host defenses and mediate the transmission of plant viruses. The *N. benthamiana* leaf disc assay can be developed into a functional genomics screen to assess which aphid genes are essential for aphid survival on plants in the absence or presence of specific plant metabolites or synthetic pesticides. It is also possible to further investigate the role of aphid candidate effector proteins in plant infestation [13]. Finally, we can use plant-mediated RNAi to identify aphid proteins involved in the non-persistent and persistent transmission of plant viruses.

Materials and Methods

Insect rearing

The aphid lineage used in this study is *Myzus persicae,* lineage of RRes (genotype O) [13]. *M. persicae* were reared on *Nicotiana tabacum* plants for *Nicotiana benthamiana* leaf disc assays and on Chinese cabbage (*Brassica rapa*) for the fecundity assays on *Arabidopsis thaliana*. The insects were maintained in custom-built acrylic cages located in controlled environment conditions at 18°C under 16 hours of light.

Cloning

Total RNA was extracted using the TRIzol Reagent (Invitrogen, Paisley, UK) and the synthesis of cDNA was performed with poly-T primers using the M-MLV reverse transcriptase system (Promega, Southampton, UK) according to the manufacturer's instructions. *MpC002* and *Rack-1* coding sequences were amplified from *M. persicae* cDNA by PCR with specific primers containing additional attb1 and attb2 linkers (Table S1) for cloning with gateway system (Invitrogen). The *Myzus persicae* EST dataset was mined for the transcript sequences of both target genes [42]. A 710-bp *MpC002* fragment corresponding to the entire mature MpC002 protein without the signal peptide, a 309-bp *Rack-1* fragment starting at nucleotide position +49 (<u>G</u>GGTTAC) and ending at nucleotide position +358 (CGTCAA<u>A</u>) of the Rack1 transcript sequence, and a 537-bp GFP fragment starting at nucleotide position +29 (<u>G</u>AGTGG) and ending at nucleotide position +566 (…TTAGCA<u>G</u>) of the GFP open reading frame were introduced into pDONR^{TM}207 (Invitrogen) plasmid using Gateway BP reaction and transformed into DH5α. Subsequent clones were sequenced to verify correct size and sequence of inserts. Subsequently, the inserts were introduced into the pJawohl8-RNAi binary silencing vector (kindly provided by I.E. Somssich, Max Planck Institute for Plant Breeding Research, Germany) using Gateway LB reaction generating plasmids pJMpC002, pJRack-1 and pJGFP, which were introduced into *A. tumefaciens* strain GV3101 containing pMP90RK plasmid and used for transient assays in *N. benthamiana* leaves and transformation of *A. thaliana*.

N. benthamiana leaf infiltration and leaf disc assays

Single *Agrobacterium* colonies harboring pJMpC002, pJRack-1 or pJGFP were

inoculated into Luria Broth (LB) containing 25mg/l Kanamicin, 25mg/l Gentamicin, 50mg/l Rifampicin and 25mg/l Carbenicillin and grown (28ºC at 225 rpm) until an Optical Density (OD_{600nm}) of 0.3 was reached (Eppendorf® BioPhotometer™, Eppendorf, Cambridge, UK). Cultures were resuspended in infiltration medium (10mM $MgCl_2$, 10mM MES 2-(*N*-morpholino)ethanesulfonic acid, pH 5.6) with 150µM Acetosyringone to initiate expression. Each construct was infiltrated into the youngest fully expanded leaves of 4-6-week old *N. benthamiana* plants. The plants were grown in a growth chamber with daily temperatures ranging between 22˚- 25˚C under a short day regime. One day after infiltration, leaves were harvested and used in leaf disc assays. The leaf discs were cut from the infiltrated areas using an 11mm diameter borer and placed in single wells of a 24-well plate on top of a plug consisting of 1ml solidified 1% distilled water agar (DWA). Four 1st instar nymphs (1-2 days old) reared on *N. tabacum* were places onto the leaf discs for a total of 6 leaves per construct. The wells were individually sealed with mesh and put upside down in controlled environment conditions at temperature 18˚C under 16 hours of light. The 24-well plate was replaced with freshly infiltrated (one day post infiltration) leaf discs after 6 and 12 days. Aphid survival by counting was assessed at 6, 12, 14 and 17 days after the day of transfer of aphids to the first 24-well plate and the numbers of nymphs produced by these aphids at 12, 14 and 17 days were also counted. The nymphs were removed after counting. This experiment was repeated 6 times to generate 6 independent biological replicates each containing 6 leaf discs per construct.

Generation of transgenic plants

The pJMpC002, pJRack-1 or pJGFP constructs were transformed into *A. thaliana* ecotype Col-0 using the floral dip method (Bechtold et al., 1993). Seeds were sown and seedlings were sprayed with phosphinothricin (BASTA) to select for transformants. F2 seeds were germinated on Murashige and Skoog (MS) medium supplemented with 20µg ml BASTA for selection. Plant ratio of 3:1 dead/alive (evidence of single insertion) segregation, were taken forward to the F3. Seed from F3 were sown on MS + BASTA and lines with 100% survival ratio (homozygous) were selected. The presence of MpC002/Rack-1/GFP inserts was confirmed by PCR and sequencing. Three independent lines were chosen for dsMpC002/dsRack-1 and one for dsGFP.

M. persicae survival and fecundity assay on *Arabidopsis* transgenic lines

F3 seed were sown and seedlings were transferred to single pots (10cm diameter) and transferred to an environmental growth room at temperature 18ºC day/16ºC night under 8 hours of light. Five *M. persicae* adults were confined to single four-week-old *Arabidopsis* lines in sealed experimental cages containing the entire plant. Two days later adults were removed and five nymphs remained on the plants. The number of offspring produced on the 10th, 14th, 16th day of the experiment were counted and removed. This experiment was repeated three times to create data from three independent biological replicates with four plants per line per replicate.

Northern blot analysis

To assess siRNA accumulation levels by northern blot analyses, *N. benthamiana* leaves were harvested each day for 6 days after agro-infiltration with the pJawohl8-RNAi constructs and whole two-week-old *A. thaliana* F3 transgenic seedlings were used.

Total RNA was extracted from leaves/seedlings using TRIzol reagent (Invitrogen). 15µg of total RNA was resolved on a 15% polyacrylamide gel (15% acrylamide-bisacrylamide solution 19:1/7M urea/20mM MOPS pH 7.0) and blotted to a Hybond-N membrane (Amersham, Little Chalfont, UK) by a Trans-blot™ (Biorad, Hempstead, UK) semi-dry transfer cell. Cross-linking of RNA was performed by incubating the membrane for two hours using a pH 8.0 solution of 0.2M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich, Gillingham, UK) and 0.1M 1-methlyimidazol (Sigma-Aldrich). DNA probes were labeled using Klenow fragment (Ambion, Lingley House, UK) with [α-32P] dCTP to generate highly specific probes. To control for equal loading of RNA amounts, blots were hybridized with a probe to U6 (snRNA 5'-GCTAATCTTCTCTGTATCGTTCC-3') [43]. MicroRNA marker (NEB, Hitchin, UK) consisting of three synthetic single-stranded RNA oligonucleotides of 17, 21 and 25 residues was loaded in gels and hybridized on blots with corresponding microRNA probe to determine size of siRNA between 21-23 nucleotides. The signals were detected after 3-day exposure to phosphor storage plates (GE Healthcare, Little Chalfont, UK) scanned with a Typhoon™ 9200 scanner (GE Healthcare) and analyzed using ImageQuant™ (GE Healthcare).

Quantitative real-time PCR analysis

Total RNA was extracted from adult *Myzus persicae* after *A. thaliana* and *N. benthamiana* fecundity assays using TRIzol reagent. DNA contaminations were removed by treating RNA extraction with RNase-free DNase (QIAGEN, West Sussex, UK) and purified with QIAamp columns (QIAGEN). First-strand cDNA was synthesized at 37°C from total RNA using M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions.

Each reaction contained 1μl of cDNA, 0.5μl of each specific primers (10pmol/ μl) (Table S1), and 10μl of 2x SYBR Green Super-mix reagent (Bio-Rad) in a final volume of 20μl. The following PCR program was used for all PCR reactions: 90°C for 3m, followed by 40 cycles of 95°C for 30s, 60°C for 30s, 72°C for 30s followed by 10m at 72°C at the end. Threshold cycle (CT) values were calculated using Bio-Rad CFX Manager™ software (Bio-Rad).

The CT values were normalized for difference in cDNA amount using ßTubulin and L27 CT values [10,14]. Fold changes were calculated by comparing the normalized transcript levels of *MpC002* and *Rack-1* of *M. persicae* fed on dsMpC002 and dsRack-1 transgenic plants to aphids fed on dsGFP transgenic plants.

Statistical analyses

All statistical analyses were conducted using GenStat 11 statistical package (VSNi Ltd, Hemel Hempstead, UK) (Table S2, Table S3). Data were checked for approximate normal distribution by visualising residuals. Classical linear regression analysis using a generalized linear model (GLM) with Poisson distributions was applied to analyse the *M. persicae* fecundity data on *A. thaliana* transgenic lines, with "nymphs" as a response variate. The aphid nymph production on 4 plants per treatment was used as independent data points in statistical analyses in which the biological replicate was used as a variable.

N. benthamiana leaf disc assay fecundity data were analyzed using an unbalanced one-way ANOVA design with ''construct'' as the treatment and ''repeat'' as the block. In the *N. benthamiana* leaf disc assay, aphid fecundity was monitored on individual leaf discs at 6 discs per treatment. Numbers of aphid nymph produced on each leaf disc were used as independent data points in statistical analyses in which the biological replicate was used as a variable. Leaf discs that dried up because of lack of humidity were

excluded giving 4-6 leaf discs per construct for each biological replicate. The relative gene expression data were analyzed using $2^{-\Delta\Delta C_T}$ method as previously described [44]. The results were analyzed for significant difference with Student's *t*-test. For replication, 'n=?' refers to number of technical replicates used for each variable in each biological replicate i.e. n=4 *Arabidopsis* plants per line per biological replicate, n=4-6 *N. benthamiana* leaf discs per construct per biological replicate, n=3 technical replicates per qRT-PCR biological replicate.

Acknowledgments

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Supporting Information (see online version)

Figure S1. Aphid survival is not affected on *dsRack-1* and *dsMpC002* transgenic plants.

Table S1. Primer sequences.

Table S2. Statistical analysis data for aphid gene silencing and fecundity experiments on *N. benthamiana*.

Table S3. Statistical analysis data for aphid gene silencing and fecundity experiments on *Arabidopsis*.