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

by

Alexander D Coleman[1]

[1]John Innes Centre, Norwich Research Park, Norwich, NR4 7UH

Supervisor: Dr. Christopher Ridout

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CONTENTS

1. Abstract

Turnip yellows virus (TuYV) is one of the most significant viral diseases of oilseed rape and may be one of the 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 study, 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. 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.

2. Introduction

2.1. 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 and Pink, 1990; Graichen, 1996; Stevens *et al.*, 2008). TuYV incidence in oilseed rape crops is 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**) (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 | TuYV symptoms on oilseed rape plant. 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 and 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 transmitted by small, sap-sucking insects called aphids (Chapter 2.2) 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 and 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 and 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.

2.2. 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 recognised by the International Committee on Taxonomy of Viruses are transmitted by insects, predominantly those of the hemipteroid assemblage (ICTV, 2010). 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 and 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, JIC, UK).

Aphids are members of the super family *Aphidoidea* in the suborder *Sternorrhyncha*. Over 4,000 species exist, distributed into 10 families (Blackman and Eastop, 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 and Eastop, 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).

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 colonised 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 and Eastop, 2000).

Hemipteroid insects are characterised by their sap-sucking mouthparts which allow stealthy feeding from the phloem of host plants. This feeding behavior is 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).

2.3. 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 2**) 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 (Vanemden *et al*., 1969). As the majority of insect species feed on one or two different plant species (Schoonhoven *et al*., 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 and October is of particular concern to growers.

Figure 2 | 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).

2.4. 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. 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 chemicals in 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). As a result of concerns that neonicotinoid pesticides pose a risk to bees, 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 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. Resistance (R) genes 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 *et al.*, 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 and 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.

Other ways to prevent aphid colonisation 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 resynthesised 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).

2.5. Circular transmission of TuYV by aphids

Luteovirids are transmitted by aphids in a persistent, circulative and non-propagative manner (Gray and 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 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 3**). 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).

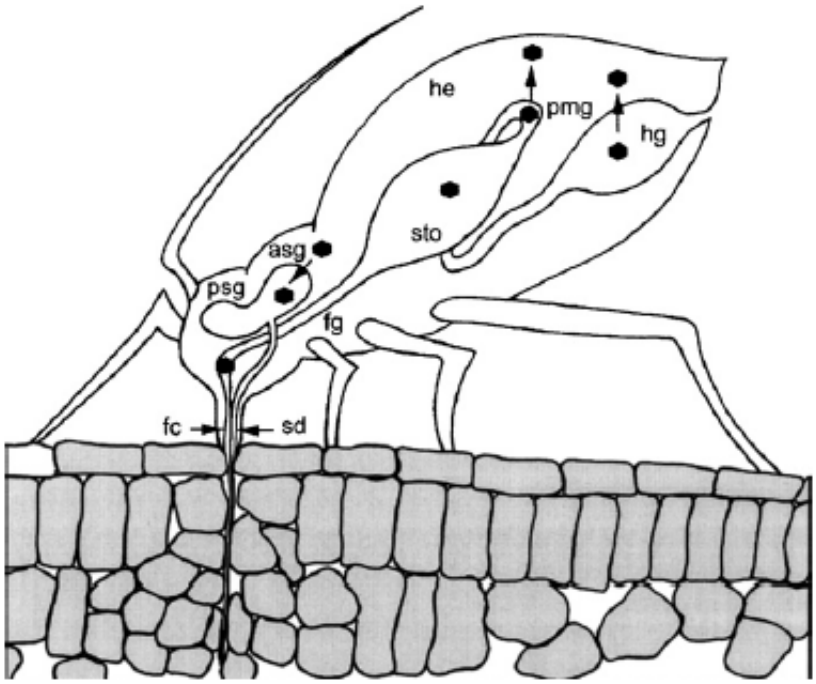

Figure 3 | Virus transmission in the aphid's body. TuYV virions are acquired in the food canal (**fc**), move across the posterior midgut (**pmg**) and/or hindgut (**hg**) to the hemolymph (**he**). TuYV virions cross into the accessory salivary gland (**asg**) for delivery into the plant through the salivary duct (**sd**). Also shown are the **fg**: foregut; **psg**: principal salivary gland; **sto**: stomach. Taken from: Brault *et al* (2007).

Circulative virus particles need to cross a number of physical barriers and endure in several diverse environments within the vector before reaching a new host (Gray and 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 and Gray, 1993). Highly specific protein interactions between virus particles and aphid proteins are therefore critical determinants of insect transmission.

Virus structural proteins have been shown to be important for circulative transmission by aphids, 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. 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). Because

poleroviruses are serologically inter-related (ICTV, 2010), it is likely that TuYV interacts with the GPA Rack1 protein in a similar way to other luteovirids (Seddas *et al.*, 2004; Gray *et al.*, 2013).

2.6. 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 and 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 4**. 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 and Tuschl, 2004). These siRNAs are then incorporated into an RNA-induced silencing complex (RISC). Argonaute proteins (Ago), the catalytic components of RISC, use the siRNA as a template to recognise and degrade the complementary messenger RNA (mRNA) (Meister and Tuschl, 2004). RNAi can therefore be exploited to suppress gene expression through highly specific depletion of target transcripts.

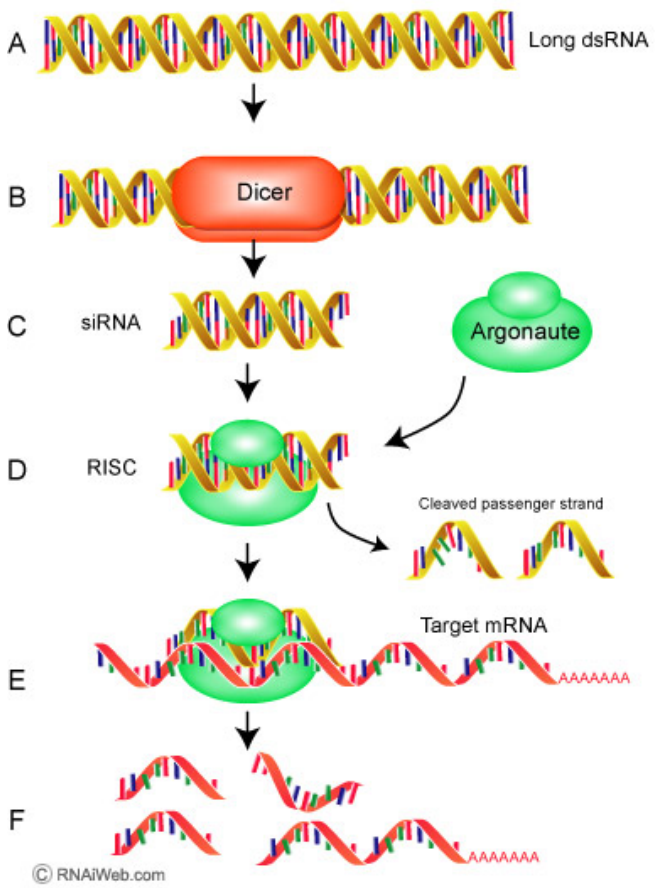

Figure 4 | RNAi process. (A) RNAi in the cell is triggered by dsRNA precursor molecules. **(B)** DsRNA is processed by the RNase III enzyme Dicer in an ATP-dependent reaction. **(C)** Long dsRNA is processed into 21-23nt siRNA with 2nt 3' overhangs. **(D)** The RISC complex consists of siRNA incorporated 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).

2.7. RNAi in insect systems

Some of the earliest RNAi studies in insects include work on the fruit fly, *D. melanogaster* (Elbashir *et al.*, 2001). Since then, RNAi has been successfully utilised 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).

RNAi-mediated gene knockdown can be achieved in aphids through direct injection of dsRNA or small-interfering RNAs (siRNA) into aphid hemolymph (Mutti *et al.*, 2006; Jaubert-Possamai *et al.*, 2007; Mutti *et al.*, 2008). 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). Feeding of dsRNA from an artificial diet can also suppress expression of the corresponding aphid gene (Shakesby *et al*., 2009; 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).

2.8. Focus and aims described in this report

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 4.1). 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 4.2). 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. This approach was used to target GPA Rack1 which may be involved in TuYV transmission by aphids. The potential of PMRi was explored, 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.

3. Materials and methods

3.1. 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 section 3.2). Plants and insects were maintained in custom-built acrylic cages (**Figure 5.A**) located in controlled environment conditions at 18°C under 16 hours of light.

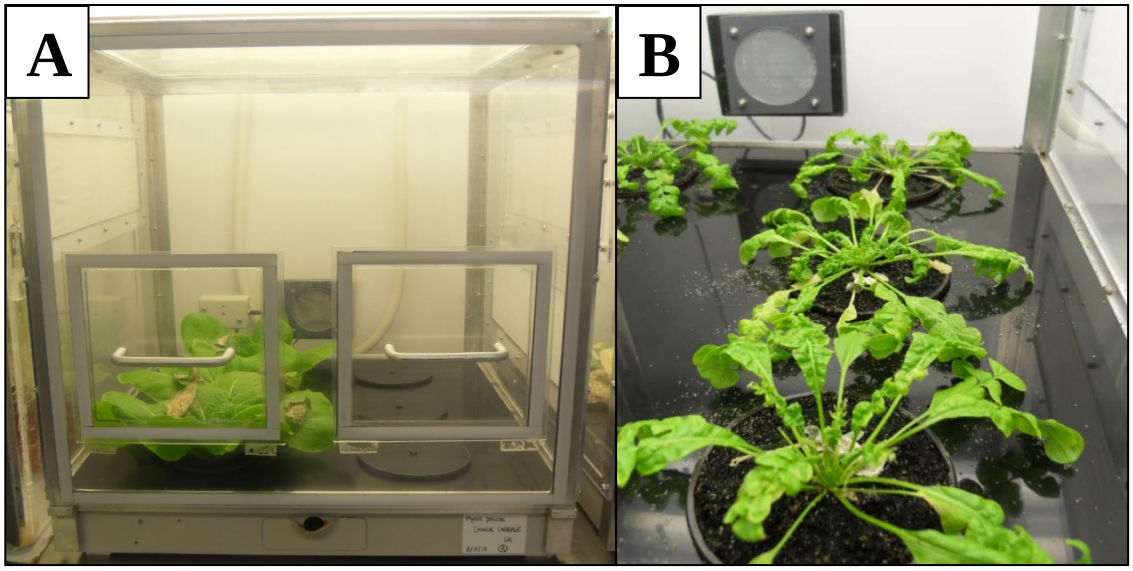

Figure 5 | Insectary stock cages for plants/GPA. GPA were maintained on Chinese cabbage (*Brassica rapa*) **(A)** or TuYV viruliferous insects maintained on Shepherd's Purse (*Capsella bursa-pastoris*) **(B)**.

3.2. 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 (Stevens *et al.*, 1994) 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 5B**) located in controlled environment conditions at 18°C under 16 hours of light.

3.3. 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 randomised 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 randomised block design with four inoculated and four un-inoculated blocks separated by a discard strip similar to the previous trial.

3.4. 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 (4^{th} or 5^{th} leaf) sampled from a plant every meter. These were tested for TuYV infection by TAS-ELISA using TuYV-specific antibodies (Stevens *et al*., 1995). 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.

3.5. 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 standardise 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.* (O'Neill *et al*., 2011)**.** Five separate batches of 10 seeds were weighed per biological sample.

3.6. 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 analysed 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:

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

3.7. 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^{o}C methanol containing internal standard sinigrin. After centrifugation, the supernatant was loaded onto columns containing A25 Sephadex and washed through with water and acetic acid. Glucosinolates were then desulphated overnight with sulphatase, 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.

3.8. 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 oilseed rape plants using an 11 mm diameter cork borer. Six discs per variety were placed in separate wells within 24-well plates on top of 1 mL distilled water agar (1% agarose) with the abaxial (underside) leaf surface facing up (**Figure 6A**). 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 6B**). Plates were then laid with the

lids facing down in controlled environment conditions at 18°C under 16 hours of light (**Figure 6C**). 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.

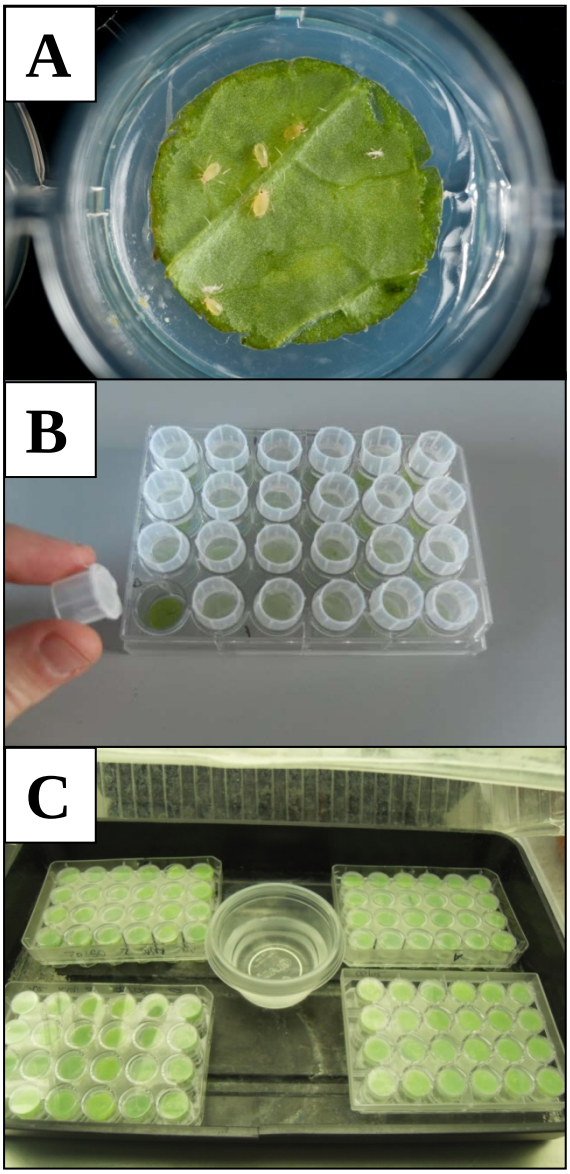

Figure 6 | Leaf disc assays. Leaf discs were laid on top of agar then 1st instar nymphs were added **(A)**. The 24 wells of the plate were individually sealed with mesh-covered lids **(B)**. Plates were laid upside down in controlled environment conditions **(C)**.

3.9. Cloning

Total RNA was extracted using 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 *Rack1* coding sequences were amplified from GPA cDNA by PCR with specific primers containing additional attb1 and attb2 linkers (Pitino *et al.*, 2011) for cloning with gateway system (Invitrogen). The GPA EST dataset was mined for the transcript sequences of both target genes (Ramsey *et al*.,

2007). A 710-bp *MpC002* fragment corresponding to the entire mature MpC002 protein without the signal peptide, a 309-bp *Rack1* fragment starting at nucleotide position +49 (GGGTTAC) and ending at nucleotide position +358 (CGTCAAA) of the Rack1 transcript sequence, and a 537-bp GFP fragment starting at nucleotide position +29 (GAGTGG) and ending at nucleotide position +566 (…TTAGCAG) 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, pJRack1 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*.

3.10. *N. benthamiana* leaf infiltration and leaf disc assays

Single *Agrobacterium* colonies harboring pJMpC002, pJRack1 or pJGFP were inoculated into Luria Broth (LB) containing 25 mg/l Kanamicin, 25 mg/l Gentamicin, 50 mg/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 (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 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 11 mm diameter borer and placed in single wells of a 24-well plate on top of a plug consisting of 1 ml solidified 1% distilled water agar (DWA). Four 1st instar nymphs (1–2 days old) reared on *N. tabacum* were placed 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.

3.11. Generation of transgenic plants

The pJMpC002, pJRack1 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 20 µg 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/Rack1/GFP inserts was confirmed by PCR and sequencing. Three independent lines were chosen for dsMpC002/dsRack1 and one for dsGFP.

3.12. GPA 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 GPA 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.

3.13. 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). 15 µg 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 0.2 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich, Gillingham, UK) and 0.1 M 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 hybridised with a probe to U6 (snRNA 5'-GCTAATCTTCTCTGTATCGTTCC-3') (Lopez-Gomollon and Dalmay, 2011). A microRNA marker (NEB, Hitchin, UK) consisting of three synthetic single-stranded RNA oligonucleotides of 17, 21 and 25 residues was loaded in gels and hybridised on blots with a corresponding microRNA probe to determine size of siRNA between 21-23 nucleotides. The signals were detected after 3 days exposure to phosphor storage plates (GE Healthcare, Little Chalfont, UK) scanned with a Typhoon™ 9200 scanner (GE Healthcare) and analysed using ImageQuant™ (GE Healthcare).

3.14. Quantitative real-time PCR analysis

Total RNA was extracted from adult GPA 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 1 µl 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 value (Mutti *et al.*, 2006; Shakesby *et al.*, 2009). Fold changes were calculated by comparing the normalized transcript levels of *MpC002* and *Rack1* of GPA fed on dsMpC002 and dsRack1 transgenic plants to aphids fed on dsGFP transgenic plants.

3.15. 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 visualising residuals. "Fatty acid profiles" and "glucosinolates" were analysed 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 analyse 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 analysed 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 analysed separately as

response variates with values for each leaf disc used as independent data points in statistical analyses. Data were analysed 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 visualising residuals and a Poisson data distribution was used. Leaf discs that dried up because of lack of a 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 and Schmittgen (2001); this provided normalised C(t) values for difference in cDNA amount using reference gene C(t) values. Normalised transcript values for three biological replicates were exported into GenStat then analysed 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.

4. Results

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

As previously discussed (Chapter 2.1), 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, drought stress, light, oxygen, activation of the RuBisCO bypass pathway and photosynthetic oxygen release (Baud and 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 and 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 and Lepiniec, 2010). The typical oil content of *Brassica napus* is 40% of the seed dry weight, which is approximately 4 mg, and it is stored in the embryo (Baud and 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 and Lepiniec, 2010).

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.

4.1.1. TuYV infects and reduces yield in all commercial varieties

Forty-nine varieties from the HGCA winter oilseed rape Recommended List 2012/13 were trialed for TuYV susceptibility by ELISA during the 2009–2010 growing season. All varieties tested positive for the virus (**Figure 7**). 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 7**).

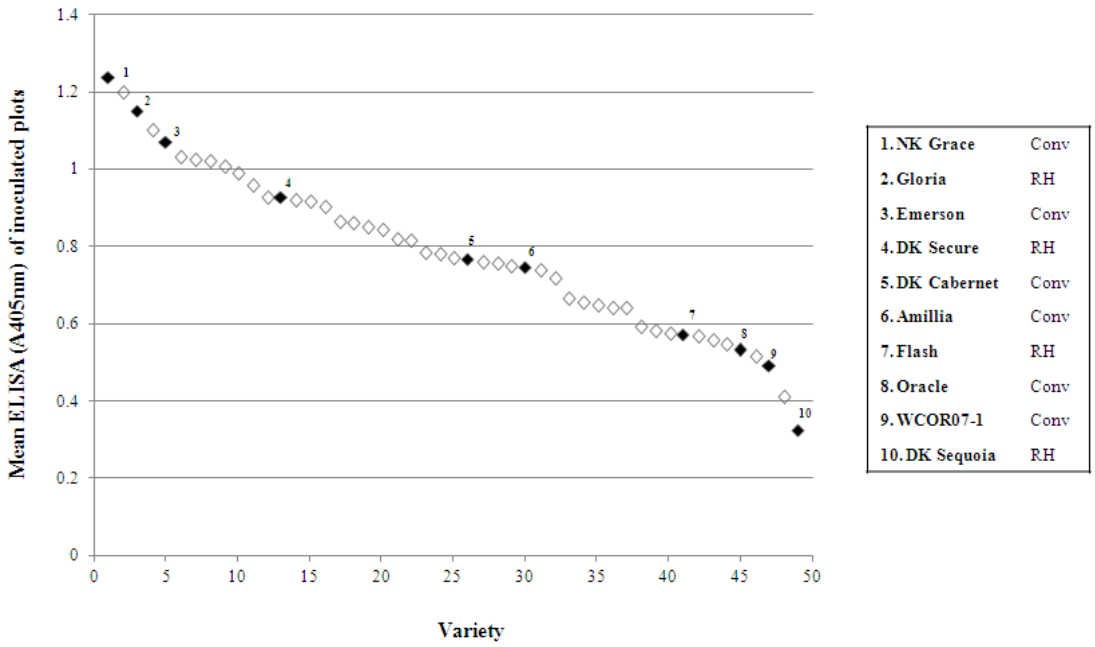

Figure 7 | ELISA data plot for 49 varieties. Varieties from 2009–10 field trial ordered according to mean ELISA reading (A405nm) of TuYV infected plots. Each variety is represented by a tile; filled tiles represent varieties chosen for further trial. Names of the ten chosen varieties are indicated with plant type as either conventional (conv) or resynthesised 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 8**).

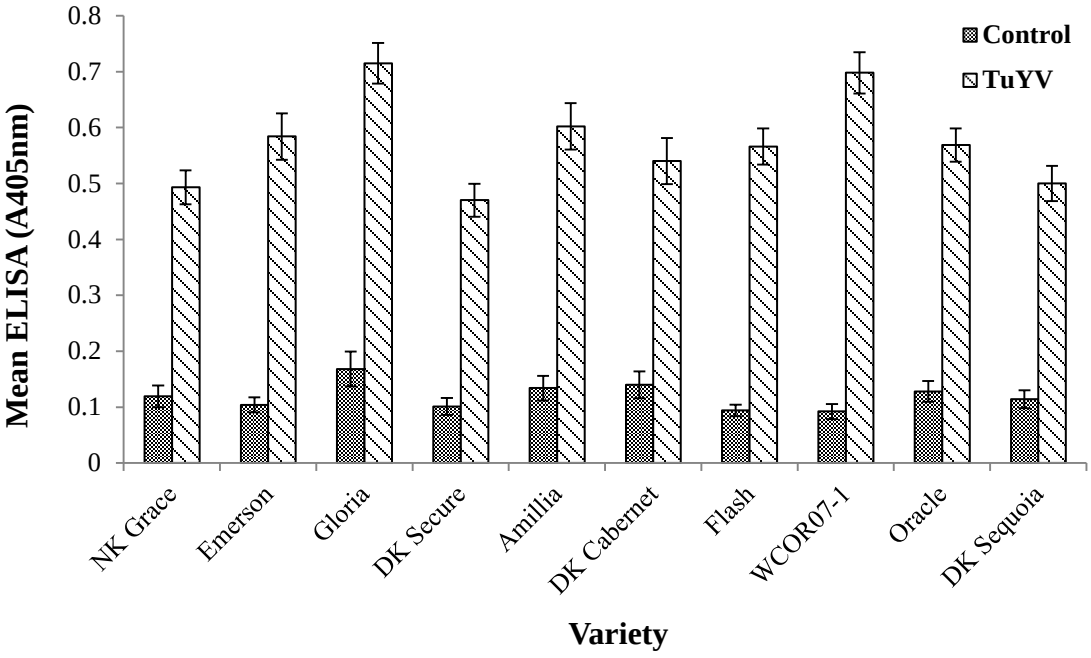

Figure 8 | ELISA data for 10 varieties. Mean ELISA readings (A405) for varieties with TuYV inoculation or control treatment. Error bars represent standard error (SE) ± of the mean.

Yield of seed at 9% moisture was determined for each variety under TuYV or control treatment (**Figure 9**). 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).

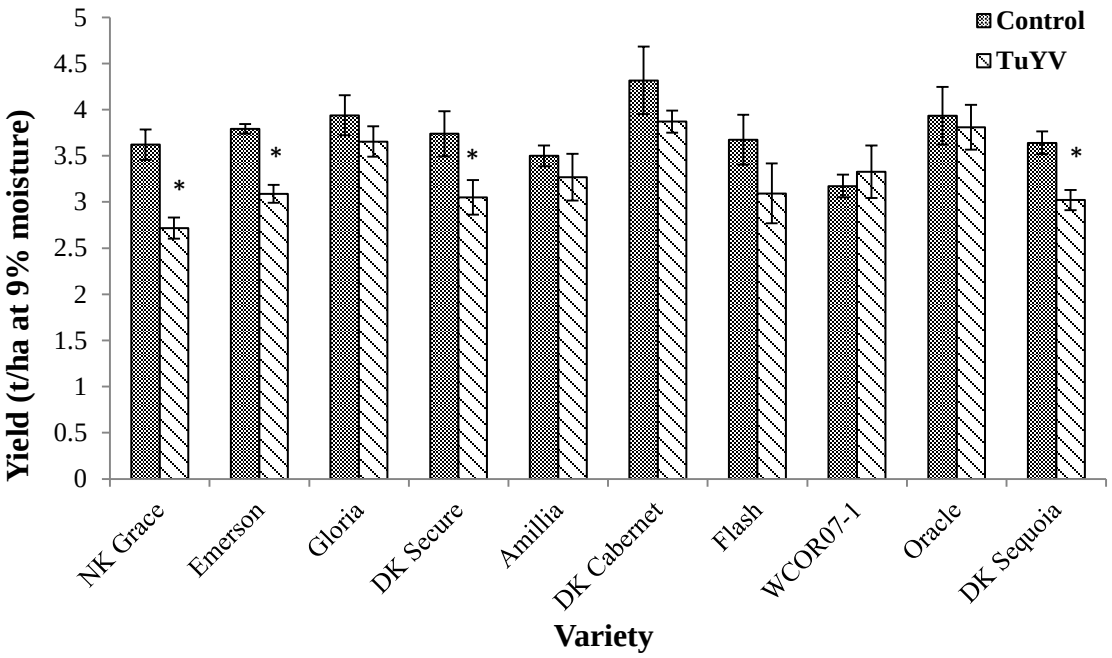

Figure 9 | Yield data. Mean yield in tons per hectare adjusted to 9% moisture for varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=4, p=<0.05). Error bars represent standard error (SE) ± of the mean.

4.1.2. 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 10**). This trend is consistent with previous literature (Jay *et al.*, 1999).

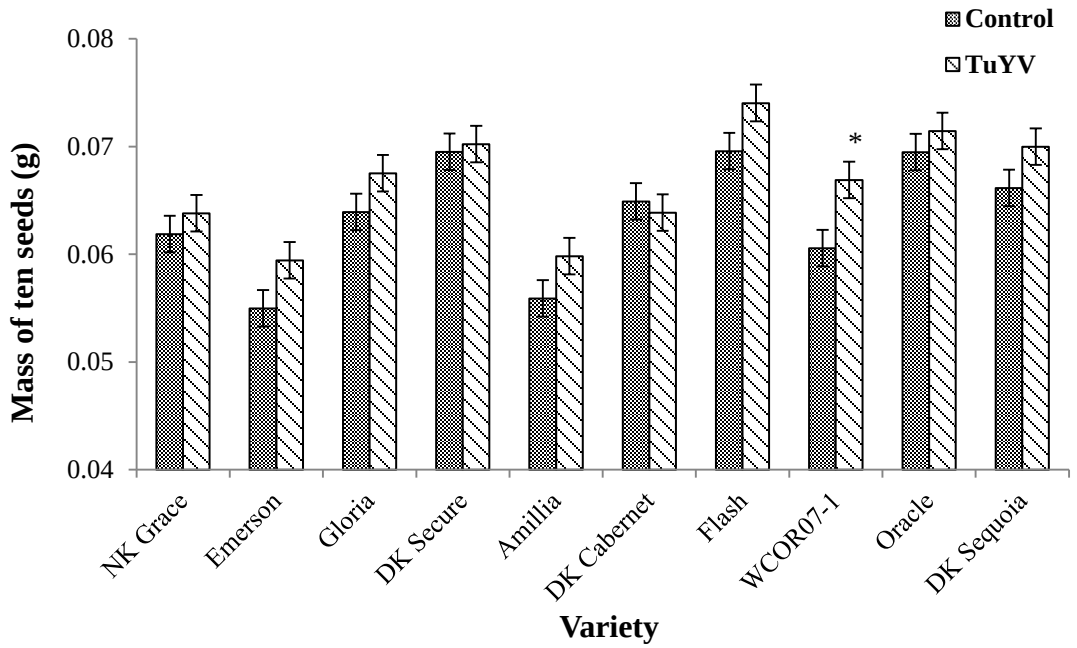

Figure 10 | Seed mass. Mass of ten seeds in grams for varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=3, p=<0.05). Error bars represent standard error (SE) ± of the mean.

Although seeds are generally larger, there is a broad trend towards lower oil content in TuYV infected plots (**Figure 11**). 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.

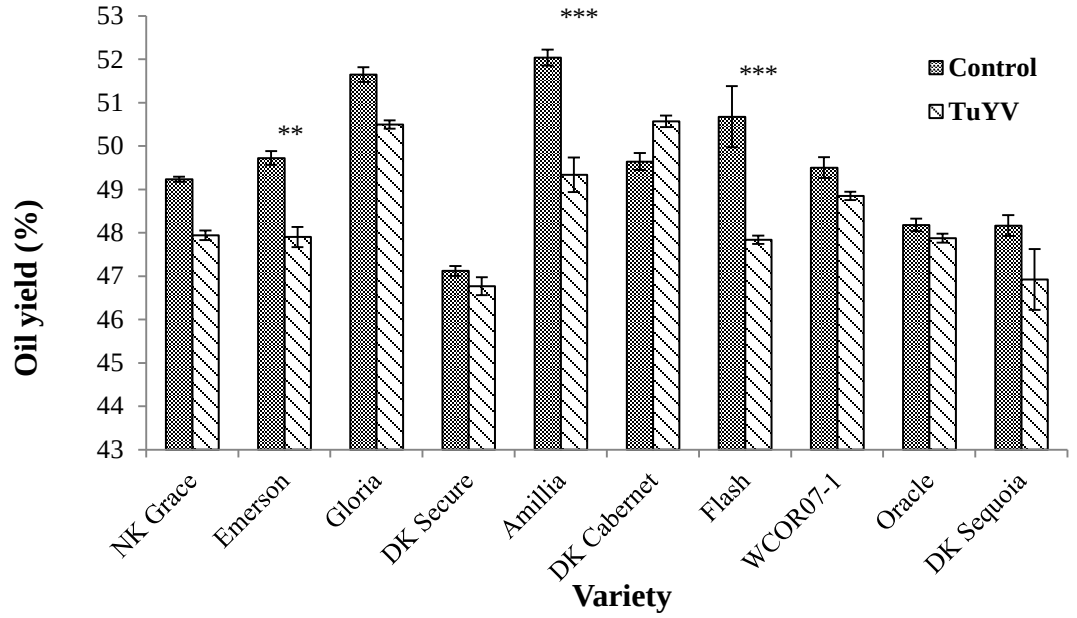

Figure 11 | Oil yield. Percentage oil yield as determined by NMR in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs. un-

inoculated plots for a given variety (GLM, n=3, p=<0.05). Error bars represent standard error (SE) ± of the mean.

4.1.3. 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 12**). 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 12A**) (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 12B**). 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 12C**) (GLM, n=3, p<0.05).

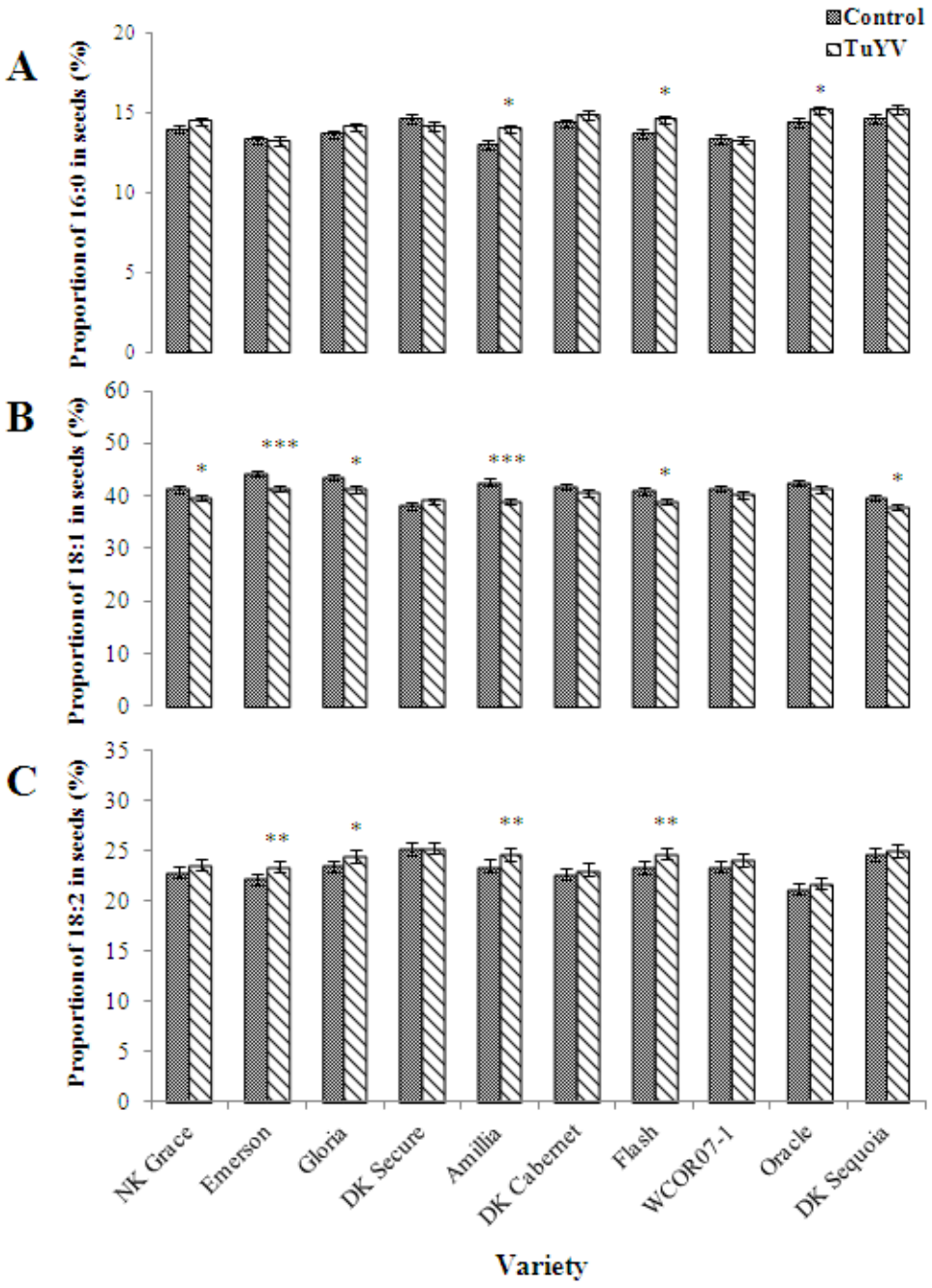

Figure 12 | Fatty acid profiles. Percentage proportion of three fatty acids 16:0 **(A)** 18:1 **(B)** or 18:2 **(C)** in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=3, 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 13A**). For aliphatic glucosinolates, only the variety DK Sequoia was shown to be significantly affected by the virus as the quantity decreased (**Figure 13B**) (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 13C). There were no changes seen in the aryl class of glucosinolates for any varieties (data not shown).

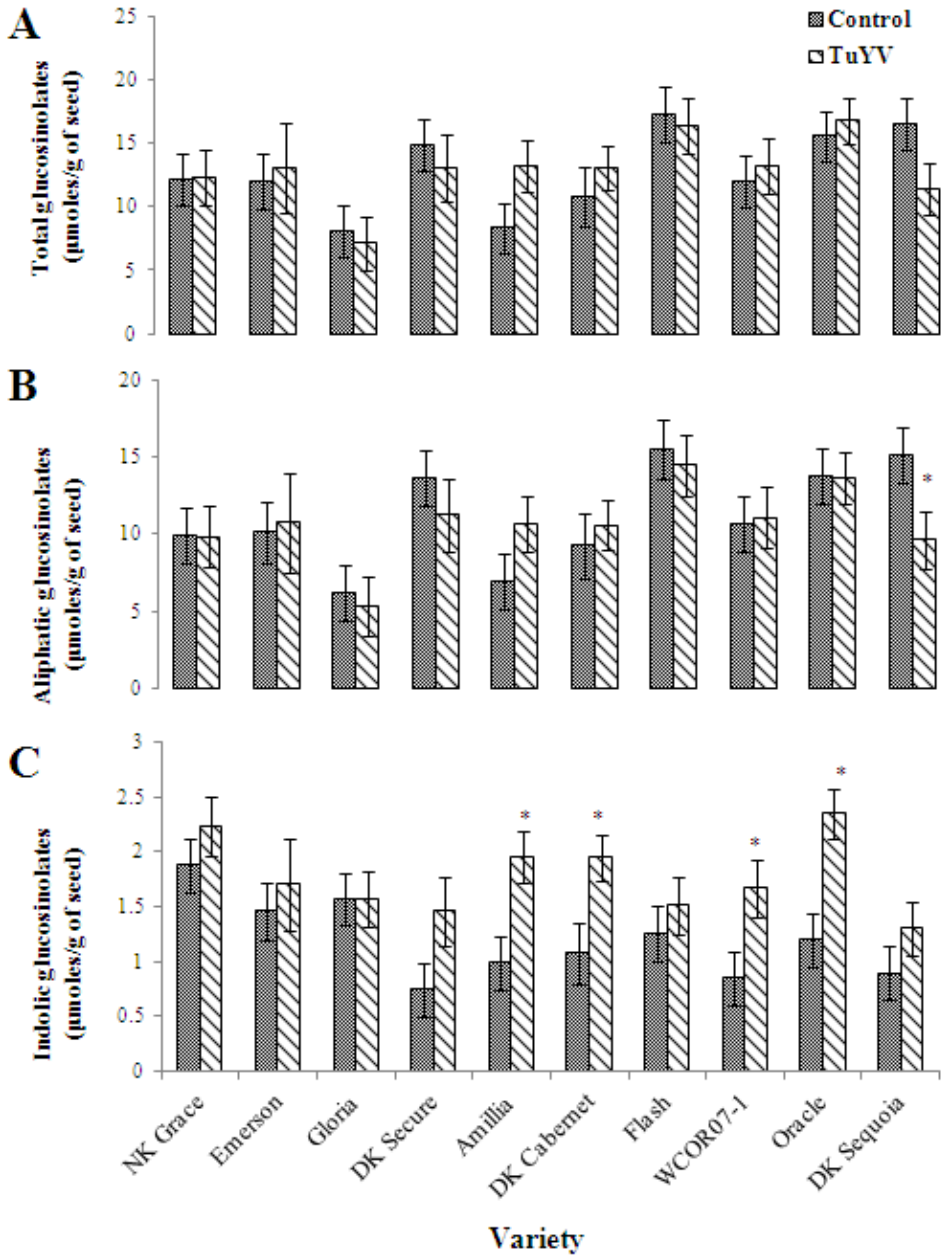

Figure 13 | Glucosinolate composition. Percentage of total glucosinolates **(A)** and subclass of aliphatic **(B)** or indolic **(C)** glucosinolates in seed from varieties with TuYV inoculation or control treatment. Asterisk indicates significant difference in inoculated plots vs un-inoculated plots for a given variety (GLM, n=2, p=<0.05). Error bars represent standard error (SE) ± of the mean.

4.1.4. 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 14**). The average number of nymphs per adult ranged consistently between 7 and 9 (**Figure 14A**), however, none of the varieties showed any significant susceptibility or partial resistance to GPA compared to other varieties. Survival showed a similar trend (**Figure 14B**).

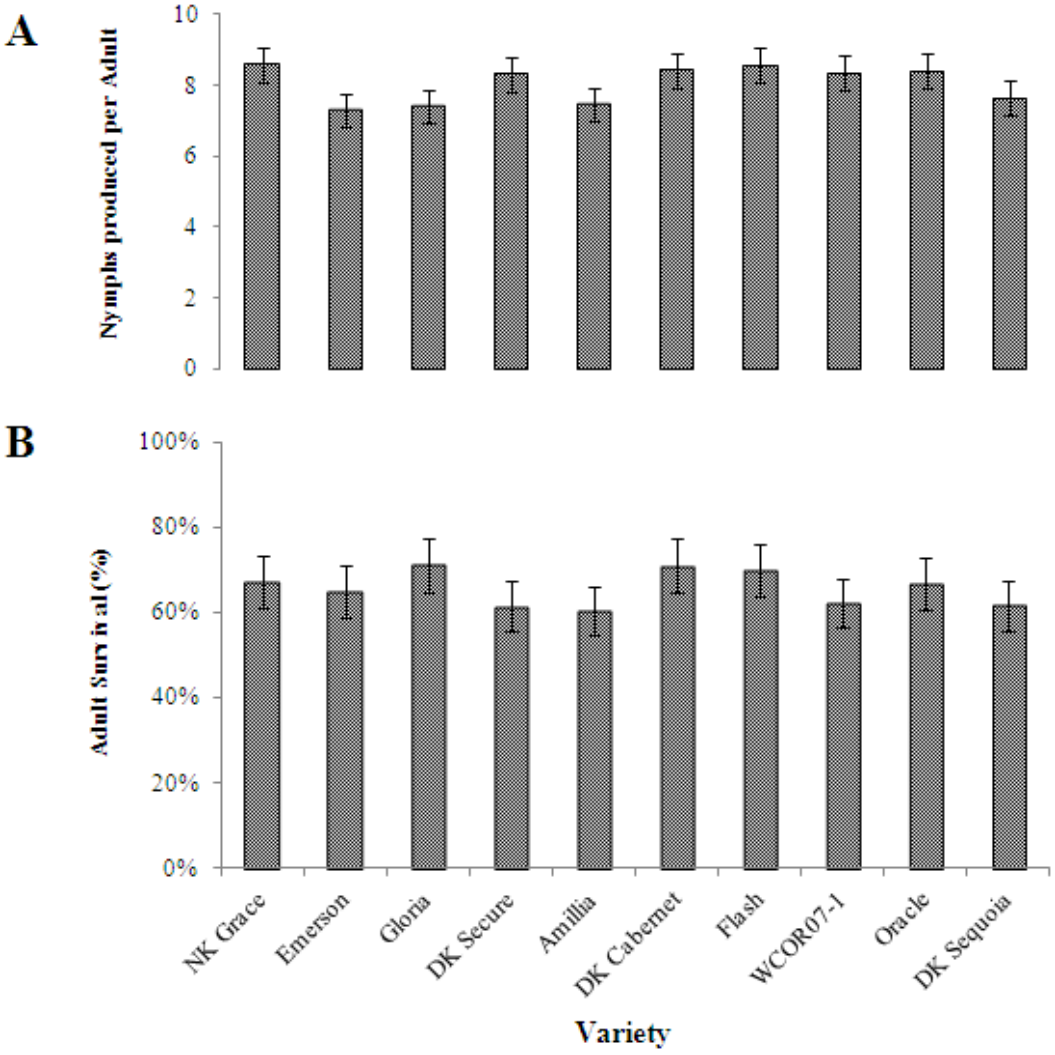

Figure 14 | Aphid fitness. Mean number of GPA nymphs produced by adults from six biological replicates **(A)** and mean percentage survival of adult aphids **(B)** on leaf discs cut from 10 selected varieties over 14 days (n=6 per biological replicate). Error bars represent standard error (SE) ± of the mean.

4.2. RNAi of GPA genes by dsRNA feeding from plants

[N.B. The following work was published in Pitino & Coleman, *et al*., 2011.]

The research aim was to develop tools to investigate aphid genes involved in the transmission of TuYV. To realise 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 2.6), is a powerful reverse-genetics tool for assessing gene function and has been previously used in several aphid species (Chapter 2.7). GPA Rack1 was chosen as a target as it has been shown to bind luteovirid particles and is linked with endocytosis processes (Chapter 2.5). It is also well characterised in various organisms, and amenable to RNAi-based approaches.

Both micro-injection and artificial diets (Chapter 2.7) are valuable methods for achieving RNAi in aphids. However, dsRNA/siRNA has to be synthesised 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 2.3) 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 2.7). *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.

4.2.1. Expression profiles of RNAi target genes

C002 and *MpC002* are predominantly expressed in the salivary glands of pea aphids, GPA (Mutti *et al.*, 2006; Mutti *et al*., 2008; 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 15**). Conversely, *Rack1* transcripts were found in all aphid body parts and at highest abundance in the gut (**Figure 15**). These results confirmed previous findings and provided RNAi targets predominantly expressed in the aphid salivary glands and gut.

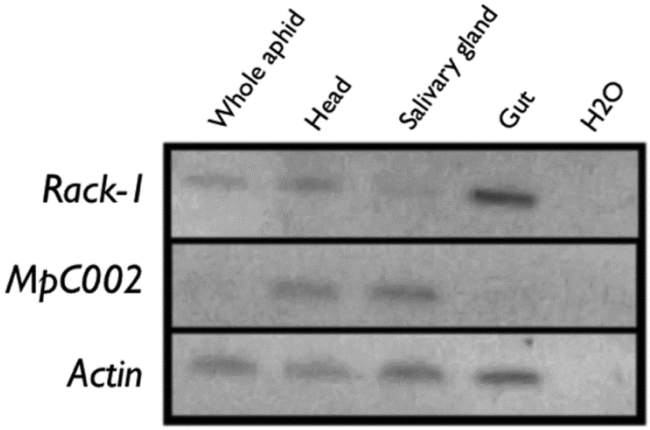

Figure 15* | MpC002 *and* Rack1 *are differentially expressed in GPA tissues. RNA isolated from whole aphids and dissected aphid body parts were used for RT-PCR with specific primers for *Rack1*, *MpC002* and *Actin*. The latter showed presence of similar RNA concentrations in the aphid samples.

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 3.9). 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 16**). 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.

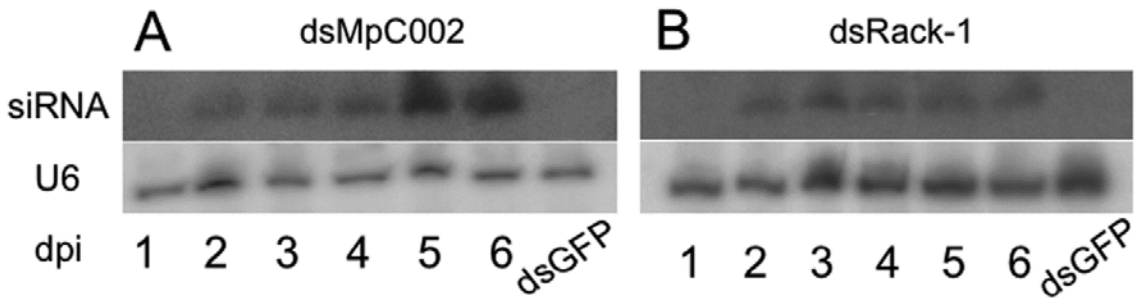

Figure 16 | MpC002 and Rack1 dsRNAs are processed into siRNAs (21-23 nt) in agro-infiltrated N. benthamiana leaves. *MpC002* and *Rack1* pJawohl8-RNAi constructs were agro-infiltrated in *N. benthamiana* leaves, which were harvested 1, 2, 3, 4, 5 or 6 days post-inoculation (dpi) for RNA isolation. Total RNA (15-20 µg) was loaded in each lane. Northern blots were hybridised 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 hybridisation of the *MpC002* and *Rack1* probes (lanes indicated with dsGFP). To control for equal RNA loading, blots were stripped and then hybridised with an snRNA probe corresponding to U6, which is constitutively produced in plants (Hanley and 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 3.10). 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 16**). 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 17A**). 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).

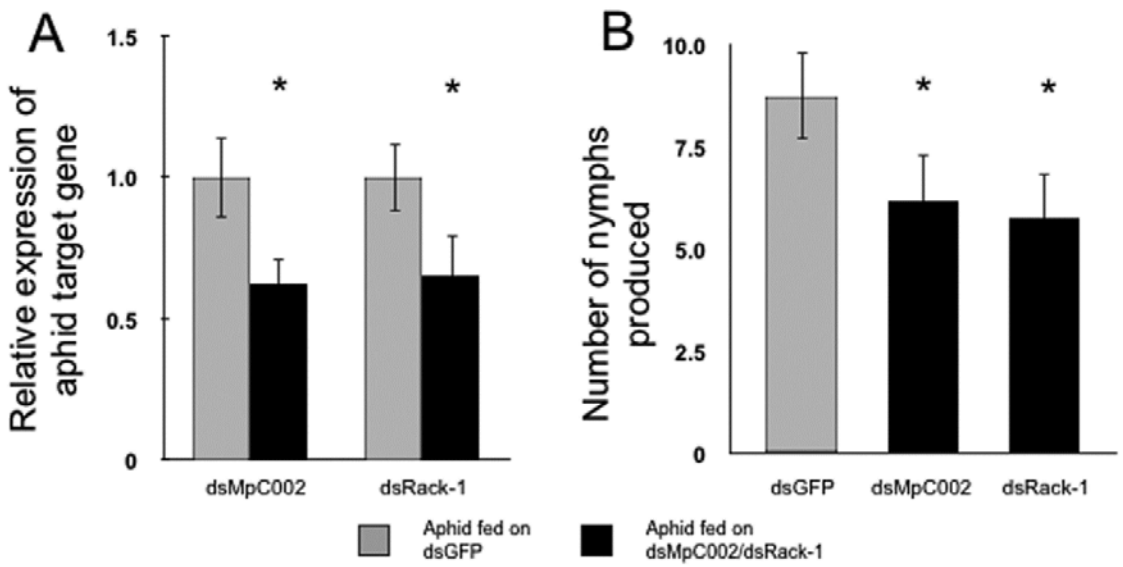

Figure 17 | Down-regulation of GPA *MpC002* or *Rack1* by *N. benthamiana*-mediated RNAi reduces aphid fecundity. (**A**) *MpC002* and *Rack1* expression is down-regulated in aphids fed on *N. benthamiana* leaves transiently producing *MpC002* and *Rack1* RNAs. Aphids fed on transgenic *N. benthamiana* leaf discs for 17 days were harvested and analysed 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 analysed 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 F3 homozygous dsMpC002 and dsRack1 transgenic *A. thaliana* were generated. One F3 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 18**). The siRNAs corresponding to GPA *MpC002* were equally abundant in the three independent transgenic lines (**Figure 18A**), while the siRNAs corresponding to *Rack1* were abundant in line 1, less abundant in line 3 and not detected in line 4 (**Figure 18B**).

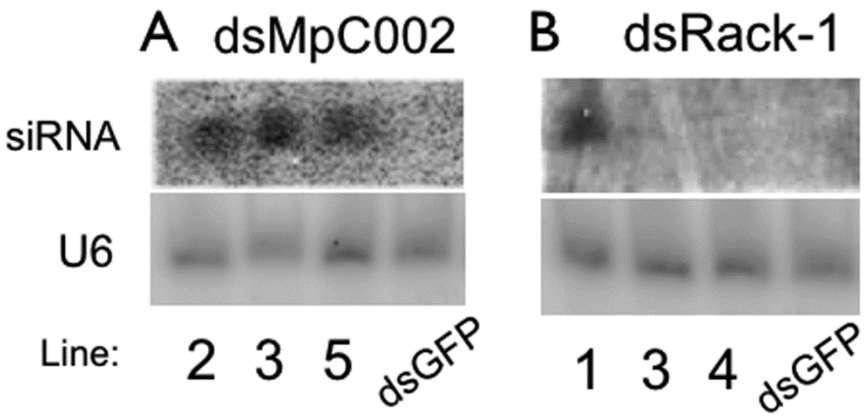

Figure 18 | *MpC002* and *Rack1* dsRNAs are processed into siRNAs (21-23 nt) in transgenic *A. thaliana* lines. Total RNA was isolated from two-week old seedlings of F3 homozygous stable dsMpC002 (**A**) and dsRack1 (**B**) transgenic lines. Total RNA isolated from two-week old seedlings of a F3 homozygous stable dsGFP line was included to control for specific hybridisation (lanes indicated with dsGFP). Each lane contains 15–20 µg of total RNA. Northern blots were hybridised with probes prepared from *MpC002* (**A**) or *Rack1* (**B**) PCR products. To verify equal RNA loading, blots were stripped and then hybridised with an snRNA probe corresponding to U6, which is constitutively produced in plants (Hanley and 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 19A**). 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 19A**). *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 18B**).

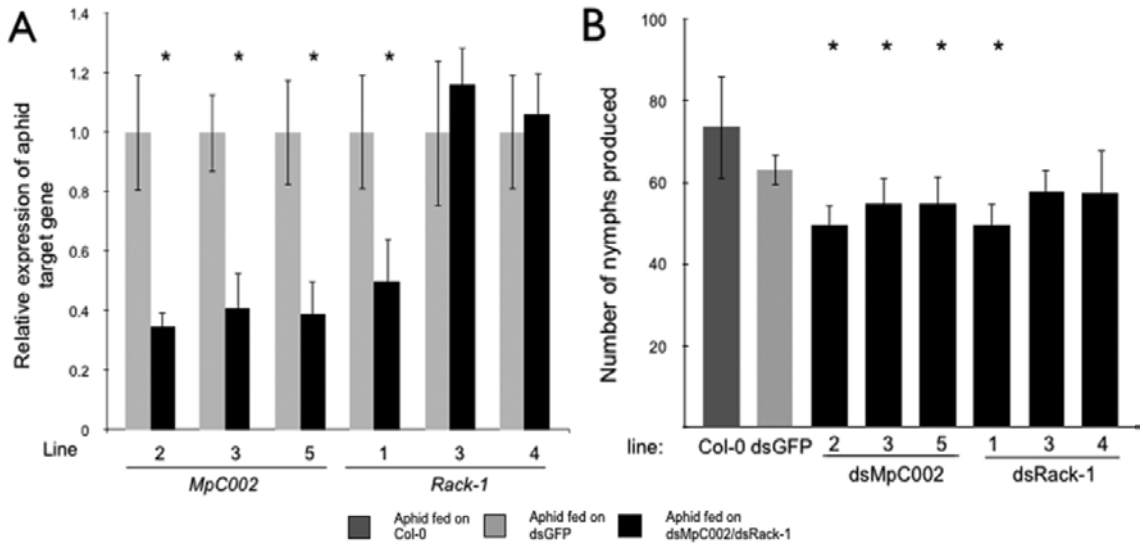

Figure 19 | Down-regulation of GPA *MpC002* or *Rack1* by *A. thaliana*-mediated RNAi reduces aphid fecundity. (**A**) *MpC002* and *Rack1* expression is down-regulated in aphids fed on transgenic *A. thaliana* producing *MpC002* and *Rack1* RNAs. Aphids fed on dsMpC002 or dsRack1 producing *A. thaliana* for 16 days were harvested and analysed 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 analysed 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 RNAi of *C002* by injection of dsRNAs in the pea aphid increased the lethality of these aphids on plants (Mutti *et al.,* 2006; Mutti *et al*., 2008). Hence, it was assessed whether RNAi 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 20A**). However, the nymph production by these aphids was significantly lower in six biological replicates (ANOVA, n=4-6, p<0.05) (**Figure 17B**). 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 20B**; **Figure 19B**).

Survival and nymph production were also investigated for the *Rack1* RNAi aphids. *Rack1* RNAi aphids survived equally well (**Figure 20A**), but produced fewer nymphs on *N. benthamiana* leaf discs (ANOVA, n=4-6, p<0.05) (**Figure 17B**). 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 20B**). GPA fecundity was not reduced on dsRack1 transgenic *A. thaliana* lines 3 and 4 (**Figure 19B**) which is consistent with no significant down-regulation of *Rack1* in aphids on these lines (**Figure 19A**).

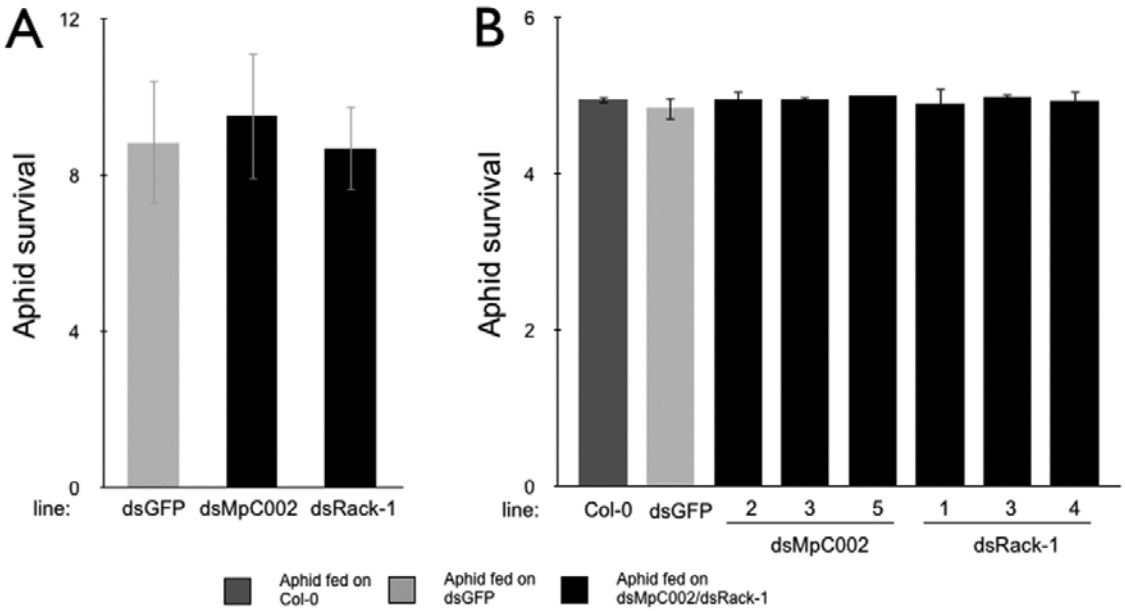

Figure 20 | Aphid survival is not affected on dsRack1 and dsMpC002 transgenic plants. (**A**) Aphid survival is not different on dsMpC002, dsRack1 and dsGFP *N. benthamiana* leaf discs. Data shown are means ± standard errors of aphid survival at 16 days for 6 biological replicates with n=4-6 per replicate. 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.

5. Discussion

5.1. Summary of research

In this project, 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 utilised 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* and *MpC002*, which are predominantly expressed in different aphid tissues, was demonstrated.

5.2. Impact of TuYV on yield and seed quality traits

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. 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 and 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. For example, DK Sequoia, NK Grace and 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. Oil content of seeds was significantly decreased in three varieties despite a trend towards increased seed mass. The virus infections changed fatty acid profiles in nearly all varieties with a consistent shift from 18:1 to 18:2. 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. Four of ten varieties showed an increase in indolic glucosinolates, 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. 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 has 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 and Morgan, 1992). Also, the results indicate that TuYV could be affecting the activity of enzymes involved in the desaturation of fatty acid biosynthesis (Kimber and 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. Despite no statistical difference in survival between varieties, 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. 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 2.1). 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 and 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.

5.3. Development of the plant-mediated RNAi tool

The results shown in chapter 4.2 demonstrate 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 and 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).

RNAi of *Rack1* and *MpC002* reduced aphid fecundity but not survival. 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 RNAi 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 (Adams *et al.*, 2011). 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 and 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.

5.4. Potential of plant-mediated RNAi in aphid functional genomics and crop protection

RNAi is a powerful tool to characterise gene function and is particularly useful in insect systems as the functions of most insect genes are poorly understood (Huvenne and Smagghe, 2010). PMRi may therefore be a valuable tool to use alongside the growing wealth of sequence data and can be expanded to include many other similar insects.

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.

PMRi can be used to investigate components of the aphid involved in transmission of viruses, so that these may be targeted to control viruses. Further work can be completed on Rack1 to determine its precise role in circulative transmission of TuYV. Other gene targets can be found from the literature or uncovered experimentally.

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. From this, novel strategies to counter aphid infestations can be discovered.

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. 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.

Before PMRi could realistically be used as a crop protection measure, it needs to be optimised so that high levels of gene silencing can be achieved. 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.

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.

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 (Gassmann *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.

5.5. Future TuYV crop protection strategies

As outlined in Chapter 2.4, 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. 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). However, should public attitudes and legislation against GM become more moderate in future, there could be multiple applications of the technology for aphid or virus control.

The most practical outcome of the research presented is 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.

6. References

1) Adams DR, Ron D, Kiely PA, 2011. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell communication and signaling : CCS* **9**, 22.
2) Araujo RN, Santos A, Pinto FS, Gontijo NF, Lehane MJ, Pereira MH, 2006. RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixu*s (Hemiptera : *Reduviidae*) by dsRNA ingestion or injection. *Insect Biochemistry and Molecular Biology* **36**, 683-93.
3) Baud S, Lepiniec L, 2010. Physiological and developmental regulation of seed oil production. *Progress in Lipid Research* **49**, 235-49.
4) Baum JA, Bogaert T, Clinton W*, et al.*, 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* **25**, 1322-6.
5) Bechtold N, Ellis J, Pelletier G, 1993. In-Planta Agrobacterium-Mediated Gene-Transfer by Infiltration of Adult Arabidopsis-thaliana Plants. *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences* **316**, 1194-9.
6) Bhatia V, Bhattacharya R, Uniyal P, Singh R, Niranjan R, 2012. Host Generated siRNAs Attenuate Expression of Serine Protease Gene in Myzus persicae. *Plos One*.
7) Bhatia V, Uniyal PL, Bhattacharya R, 2011. Aphid resistance in Brassica crops: challenges, biotechnological progress and emerging possibilities. *Biotechnology Advances* **29**, 879-88.
8) Blackman RL, Eastop VF, 2000. *Aphids on the world's crops: an identification and information guide*. Wiley-Blackwell; 2nd Edition.
9) Bocianowski J, Mikolajczyk K, Bartkowiak-Broda I, 2012. Determination of fatty acid composition in seed oil of rapeseed (Brassica napus L.) by mutated alleles of the FAD3 desaturase genes. *Journal of Applied Genetics* **53**, 27-30.
10) Bolognesi R, Ramaseshadri P, Anderson J*, et al.*, 2012. Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (Diabrotica virgifera virgifera LeConte). *Plos One* **7**.
11) Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA, 2010. A Functional Genomics Approach Identifies Candidate Effectors from the Aphid Species *Myzus persicae* (Green Peach Aphid). *Plos Genetics* **6**, e1001216.
12) Bouttier C, Morgan DG, 1992. Ovule Development and Determination of Seed Number Per Pod in Oilseed rape (Brassica-napus L.). *Journal of Experimental Botany* **43**, 709-14.
13) Brault V, Herrbach E, Reinbold C, 2007. Electron microscopy studies on luteovirid transmission by aphids. *Micron* **38**, 302-12.
14) Brault V, Uzest M, Monsion B, Jacquot E, Blanc S, 2010. Aphids as transport devices for plant viruses. *Comptes Rendus Biologies* **333**, 524-38.

15) Brault W, Perigon S, Reinbold C*, et al.*, 2005. The polerovirus minor capsid protein determines vector specificity and intestinal tropism in the aphid. *Journal of Virology* **79**, 9685-93.
16) Bruce TJA, 2012. GM as a route for delivery of sustainable crop protection. *Journal of Experimental Botany* **63**, 537-41.
17) Carolan JC, Caragea D, Reardon KT*, et al.*, 2011. Predicted Effector Molecules in the Salivary Secretome of the Pea Aphid (*Acyrthosiphon pisum*): A Dual Transcriptomic/Proteomic Approach. *Journal of Proteome Research* **10**, 1505-18.
18) Ciche TA, Sternberg PW, 2007. Postembryonic RNAi in *Heterorhabditis bacteriophora*: a nematode insect parasite and host for insect pathogenic symbionts. *Bmc Developmental Biology* **7**.
19) Davison J, 2010. GM plants: Science, politics and EC regulations. *Plant Science* **178**, 94-8.
20) Dedryver CA, Le Ralec A, Fabre F, 2010. The conflicting relationships between aphids and men: A review of aphid damage and control strategies. *Comptes Rendus Biologies* **333**, 539-53.
21) Defra, 2012. Agriculture in the United Kingdom. In.: Statistics Hub of Department of Environment, Food & Rural Affairs.
22) Diepenbrock W, 2000. Yield analysis of winter oilseed rape (*Brassica napus* L.): a review. *Field Crops Research* **67**, 35-49.
23) Dogimont C, Bendahmane A, Chovelon V, Boissot N, 2010. Host plant resistance to aphids in cultivated crops: Genetic and molecular bases, and interactions with aphid populations. *Comptes Rendus Biologies* **333**, 566-73.
24) Dong Y, Friedrich M, 2005. Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. *Bmc Biotechnology* **5**.
25) Dpv, 2013. Descriptions of plant viruses. In.: Association of Applied Biologists.
26) Dreyer F, Graichen K, Jung C, 2001. A major quantitative trait locus for resistance to Turnip Yellows Virus (TuYV, syn. beet western yellows virus, BWYV) in rapeseed. *Plant Breeding* **120**, 457-62.
27) El-Beltagi HE-DS, Mohamed AA, 2010. Variations in fatty acid composition, glucosinolate profile and some phytochemical contents in selected oil seed rape (Brassica napus L.) cultivars. *Grasas Y Aceites* **61**, 143-50.
28) Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T, 2001. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *Embo Journal* **20**, 6877-88.
29) European-Commission, 2013. Commission Implementing Regulation (EU) No 485/2013. *Official Journal of the European Union* **L 139/12**.

30) Fenton B, Margaritopoulos JT, Malloch GL, Foster SP, 2010. Micro-evolutionary change in relation to insecticide resistance in the peach-potato aphid, Myzus persicae. *Ecological Entomology* **35**, 131-46.
31) Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC, 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-11.
32) Fresco LO, 2013. The GMO Stalemate in Europe. *Science* **339**, 883-.
33) Gassmann AJ, Petzold-Maxwell JL, Keweshan RS, Dunbar MW, 2011. Field-Evolved Resistance to Bt Maize by Western Corn Rootworm. *Plos One* **6**.
34) Gervais JA, Luukinen B, Buhl K, Stone D, 2010. Imidacloprid Technical Fact Sheet. In.: National Pesticide Information Center. (2013.)
35) Gildow FE, Gray SM, 1993. The aphid salivary-gland basal lamina as a selective barrier associated with vector-specific transmission of *Barley yellow dwarf* luteoviruses. *Phytopathology* **83**, 1293-302.
36) Goggin FL, Williamson VM, Ullman DE, 2001. Variability in the response of Macrosiphum euphorbiae and Myzus persicae (Hemiptera : Aphididae) to the tomato resistance gene Mi. *Environmental Entomology* **30**, 101-6.
37) Goto A, Blandin S, Royet J, Reichhart JM, Levashina EA, 2003. Silencing of Toll pathway components by direct injection of double-stranded RNA into *Drosophila* adult flies. *Nucleic Acids Research* **31**, 6619-23.
38) Graichen K, 1994. Nachweis von Resistenz gegenu¨ber dem Turnip yellows luteovirus (TuYV) in Winterraps und verwandten Arten. *Vortr Pflanzenzuchtung* **30**, 132–43.
39) Graichen K, 1996. Infestation by turnip yellows virus on winter oilseed rape as cause of yield losses. *[Communications from the Federal Biological Institute for Agriculture and Forestry Berlin-Dahlem]|Mitteilungen aus der Biologischen Bundesanstalt fuer Land- und Forstwirtschaft Berlin-Dahlem*, 179.
40) Graichen K, Schliephake E, 1999. Infestation of winter oilseed rape by Turnip yellows luteovirus and its effect on yield in Germany. In. *10th International Rapeseed Congress - New Horizons from an Old Crop.* 131.
41) Gray S, Cilia M, Bereman M*, et al.* Circulative, nonpropagative transmission: a carefully tuned orchestra of virus, vector and host proteins. *Proceedings of the International Congress of Plant Pathology*, *2013*. Beijing, China.
42) Gray S, Gildow FE, 2003. Luteovirus-aphid interactions. *Annual Review of Phytopathology* **41**, 539-66.
43) Hamilton AJ, Baulcombe DC, 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-2.
44) Hanley BA, Schuler MA, 1991. Developmental expression of plant snRNAs. *Nucleic Acids Research* **19**, 6319-25.

45) Hannon GJ, 2002. RNA interference. *Nature* **418**, 244-51.
46) Harmel N, Letocart E, Cherqui A*, et al.*, 2008. Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. *Insect Molecular Biology* **17**, 165-74.
47) HGCA, 2012. Home Grown Cereals Authority Recommended lists.
48) Hillocks RJ, 2012. Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. *Crop Protection* **31**, 85-93.
49) Hogenhout SA, Ammar ED, Whitfield AE, Redinbaugh MG, 2008. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology* **46**, 327-59.
50) Huvenne H, Smagghe G, 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal of Insect Physiology* **56**, 227-35.
51) ICTV, 2010. Virus taxonomy. In. International Committee on Taxonomy of Viruses online database.
52) Ingwell LL, Eigenbrode SD, Bosque-Perez NA, 2012. Plant viruses alter insect behavior to enhance their spread. *Scientific Reports* **2**.
53) Jaubert-Possamai S, Le Trionnaire G, Bonhomme J, Christophides GK, Rispe C, Tagu D, 2007. Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. *Bmc Biotechnology* **7**, 8.
54) Jay CN, Rossall S, Smith HG, 1999. Effects of beet western yellows virus on growth and yield of oilseed rape (*Brassica napus*). *Journal of Agricultural Science* **133**, 131-9.
55) Jones RaC, Coutts BA, Hawkes J, 2007. Yield-limiting potential of *Beet western yellows virus* in *Brassica napus*. *Australian Journal of Agricultural Research* **58**, 788-801.
56) Juergens M, Paetsch C, Kraemer I*, et al.*, 2010. Genetic analyses of the host-pathogen system *Turnip yellows virus* (TuYV)-rapeseed (Brassica napus L.) and development of molecular markers for TuYV-resistance. *Theoretical and Applied Genetics* **120**, 735-44.
57) Kamath RS, Fraser AG, Dong Y*, et al.*, 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231-7.
58) Ketting RF, 2011. The Many Faces of RNAi. *Developmental Cell* **20**, 148-61.
59) Kimber D, Mcgregor DI, 1995. *Brassica Oilseeds - Production and Utilization*. CABI Publishing.
60) Klingler J, Creasy R, Gao LL*, et al.*, 2005. Aphid resistance in *Medicago truncatula* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. *Plant Physiology* **137**, 1445-55.
61) Livak KJ, Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**, 402-8.
62) Lopez-Gomollon S, Dalmay T, 2011. Detecting sRNAs by Northern Blotting. *MicroRNAs in Development: Methods and Protocols*, 25-38.

63) Mao YB, Cai WJ, Wang JW*, et al.*, 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* **25**, 1307-13.
64) Mcdonald BA, Linde C, 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**, 349-+.
65) Meister G, Tuschl T, 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343-9.
66) Monsanto, 2013. Research and Development Pipeline. In.: Monsanto Company.
67) Mugford SG, Matthewman CA, Hill L, Kopriva S, 2010. Adenosine-5 '-phosphosulfate kinase is essential for *Arabidopsis* viability. *Febs Letters* **584**, 119-23.
68) Mutti NS, Louis J, Pappan LK*, et al.*, 2008. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 9965-9.
69) Mutti NS, Park Y, Reese JC, Reeck GR, 2006. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Science* **6**.
70) Ng JCK, Perry KL, 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5**, 505-11.
71) O'Neill CM, Morgan C, Hattori C*, et al.*, 2011. Towards the genetic architecture of seed lipid biosynthesis and accumulation in Arabidopsis thaliana. *Heredity* **108**, 115-23.
72) Oldroyd BP, 2007. What's killing American honey Bees? *Plos Biology* **5**, 1195-9.
73) Onstad DW, 2008. *Insecticide Resistance Management: Biology, Economics and Prediction*. UK: Academic Press.
74) Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA, 2011. Silencing of Aphid Genes by dsRNA Feeding from Plants. *Plos One* **6**, e25709.
75) Rajagopal R, Sivakumar S, Agrawal N, Malhotra P, Bhatnagar RK, 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as bacillus thuringiensis toxin receptor. *Journal of Biological Chemistry* **277**, 46849-51.
76) Ramsey JS, Rider DS, Walsh TK*, et al.*, 2010. Comparative analysis of detoxification enzymes in *Acyrthosiphon pisum* and *Myzus persicae*. *Insect Molecular Biology* **19**, 155-64.
77) Ramsey JS, Wilson ACC, De Vos M*, et al.*, 2007. Genomic resources for *Myzus persicae*: EST sequencing, SNP identification, and microarray design. *Bmc Genomics* **8**.
78) Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM, 1998. The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9750-4.
79) Schierholt A, Rucker B, Becker HC, 2001. Inheritance of high oleic acid mutations in winter oilseed rape (Brassica napus L.). *Crop Science* **41**, 1444-9.

80) Schliephake E, Graichen K, Rabenstein F, 2000. Investigations on the vector transmission of the *Beet mild yellowing virus* (BMYV) and the *Turnip yellows virus* (TuYV). *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection* **107**, 81-7.
81) Schnurbusch T, Mollers C, Becker HC, 2000. A mutant of *Brassica napus* with increased palmitic acid content. *Plant Breeding* **119**, 141-4.
82) Schoonhoven LM, Loon JJaV, Dicke M, 2005. *Insect-plant biology*. Oxford University Press.
83) Seddas P, Boissinot S, Strub JM, Van Dorsselaer A, Van Regenmortel MHV, Pattus F, 2004. Rack-1, GAPDH3, and actin: proteins of *Myzus persicae* potentially involved in the transcytosis of *beet western yellows virus* particles in the aphid. *Virology* **325**, 399-412.
84) Semenov MA, 2007. Development of high-resolution UKCIP02-based climate change scenarios in the UK. *Agricultural and Forest Meteorology* **144**, 127-38.
85) Shakesby AJ, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE, 2009. A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology* **39**, 1-10.
86) Silva AX, Jander G, Samaniego H, Ramsey JS, Figueroa CC, 2012. Insecticide Resistance Mechanisms in the Green Peach Aphid *Myzus persicae* (Hemiptera: Aphididae) I: A Transcriptomic Survey. *Plos One* **7**.
87) Simmer F, Moorman C, Van Der Linden AM*, et al.*, 2003. Genome-wide RNAi of *C-elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *Plos Biology* **1**, 77-84.
88) Stevens M, Mcgrann G, Clark B, 2008. *Turnip yellows virus* (syn *Beet western yellows virus*): an emerging threat to European oilseed rape production? *HGCA Research Review*, 36 pp.
89) Stevens M, Patron NJ, Dolby CA*, et al.*, 2005. Distribution and properties of geographically distinct isolates of sugar beet yellowing viruses. *Plant Pathology* **54**, 100-7.
90) Stevens M, Smith HG, Hallsworth PB, 1994. The host-range of beet yellowing viruses among common arable weed species. *Plant Pathology* **43**, 579-88.
91) Stevens M, Smith HG, Hallsworth PB, 1995. Detection of the luteoviruses, beet mild yellowing virus and *beet western yellows virus*, in aphids caught in sugar-beet and oilseed rape crops, 1990-1993. *Annals of Applied Biology* **127**, 309-20.
92) Upchurch RG, 2008. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters* **30**, 967-77.
93) Vanemden HF, Eastop VF, Hughes RD, Way MJ, 1969. Ecology of *Myzus persicae*. *Annual Review of Entomology* **14**, 197-&.
94) Walkey DGA, Pink DaC, 1990. Studies on Resistance to Beet Western Yellows Virus in Lettuce (Lactuca-sativa) and the Occurrence of Field Sources of the Virus. *Plant Pathology* **39**, 141-55.

95) Wang YB, Zhang H, Li HC, Miao XX, 2011. Second-Generation Sequencing Supply an Effective Way to Screen RNAi Targets in Large Scale for Potential Application in Pest Insect Control. *Plos One* **6**, 10.
96) Whalon M, Mota-Sanchez D, Hollingworth R, 2008. *Global Pesticide Resistance in Arthropods*. UK: Oxford University Press.
97) Whyard S, Singh AD, Wong S, 2009. Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochemistry and Molecular Biology* **39**, 824-32.
98) Xie ZX, Johansen LK, Gustafson AM*, et al.*, 2004. Genetic and functional diversification of small RNA pathways in plants. *Plos Biology* **2**, 642-52.
99) Yu N, Christiaens O, Liu J*, et al.*, 2013. Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Science* **20**, 4-14.
100) Zha W, Peng X, Chen R, Du B, Zhu L, He G, 2011. Knockdown of Midgut Genes by dsRNA-Transgenic Plant-Mediated RNA Interference in the Hemipteran Insect *Nilaparvata lugens*. *Plos One* **6**.

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